Thailand – Japan Joint Conference on Animal Health 2012:

The 25th Year Anniversary

of National Institute of Animal Health

Organized by
National Institute of Animal Health, Department of Livestock Development
Rama Gardens Hotel, Bangkok, Thailand
May 30 – 31, 2012
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Thailand-Japan Joint Conference on Animal Health 2012 : The 25th Year Anniversary of National Institute of Animal Health is organized to commemorate of good relationship between NIAH, Thailand and NIAH, Japan on 30 May to 2 June 2012. This event includes the celebration of the 25th Year Anniversary of our institute, NIAH, Thailand. The theme of the conference is “Healthy animals, healthy people.” The aim of the conference is to gather scientists in the area of animal health and public health to exchange new idea and better understand the challenge and opportunities that each group faces and to develop solutions for future research and network that fulfill the needs of both people and animal science.

This book is published in order to note the significant opinions, guidelines for future international co-operation, and remarkable speeches delivered at the conference.

I take this opportunity to extend my sincere appreciation to the organizing committee, working groups, speakers, participants in the preparation of, participation in, and achievement of the conference and especially to Department of Livestock Development for financial support to the conference’s arrangement.

Editor
CONFERENCE PROGRAM
Thailand-Japan Joint Conference on Animal Health 2012:
The 25th Year Anniversary of National Institute of Animal Health
Rama Gardens Hotel, Bangkok, Thailand
May 30 – 31, 2012

Wednesday May 30, 2012
08:30 – 09:00 AM Registration
09:00 – 10:15 AM - Opening Ceremony
   Welcoming Addresses by Dr. Tritsadee Chaosuancharoen
   Director General, DLD
   and Dr. Takafulmi Hamaoka
   Director General, NIAH/NARO
   Opening Addresses by Mr. Seiji Kojima
   Ambassador of Japan to Thailand
   and Mr. Theera Wongsamut
   Minister, MOAC
   - Signing of MOU (DG, DLD & DG, NIAH/NARO)
   - Group photo
   - VDO Presentation
10:15 – 10:30 AM Coffee/Tea Break
10:30 – 11:00 AM Keynote Speaker I (Dr. Hiroshi Kida, Hokkaido University): Influenza
11:00 – 11:30 AM Keynote Speaker II (Dr. Bruno Garin-Bastuji, ANSES): Brucellosis & OIE Twinning Program
11:30 – 12:00 AM Poster Presentation I
12:00 – 01:00 PM Lunch
01:00 – 01:30 PM Keynote Speaker III (Dr. Masato Akiba, NIAH/NARO): Bacterial Foodborne Diseases (Emergence of an extended spectrum cephalosporin resistant Salmonella enterica serovar Typhimurium clone harboring a multidrug resistance genomic island among cattle population in Japan)
01:30 – 03:20 PM Oral Presentation I (5 papers; 20 min/paper)
1. Jamras Lerdsri: Simple method to regenerate the strong cation exchange cartridge for oxytetracycline and chlortetracycline analysis
2. Daisuke Takamatsu: Diversity of Melissococcus plutonius isolates from honeybee larvae in Japan
3. Patchara Vitoorakool: Parasitic infection of honeybees (Apis spp.) in upper northern region of Thailand
4. Makoto Matsubayashi: Transcriptome analysis on the asexual second-generation development of Eimeria tenella in the chicken ceca using specific DNA microarray
5. Wilai Linchongsubongkoch: Vaccine matching strain characterization of foot and mouth disease virus in South East Asia during 2010-2012

03:20 – 03:40 PM Coffee/Tea Break
03:40 – 05:00 PM Oral Presentation II (4 papers; 20 min/paper)
   1. Hiroaki Shirafuji: Recent efforts toward the establishment of surveillance and diagnosis systems for arboviral diseases in Thailand
   2. Aroonpan Doongsoongnern: A study of gross lesions in bovine tuberculosis from single intradermal test reactor in swamp buffaloes
   3. Kenji Kawashima: Lesion development and its relation to pathogenicity of variant porcine reproductive and respiratory syndrome virus
   4. Jadsada Ratthanophart: Tibial dyschondroplasia (TD) in ducks: case reports
05:00 – 05:30 PM Poster Presentation II
06:00 – 09:00 PM Welcome Party

Thursday May 31, 2012
09:00 – 09:30 AM Keynote Speaker IV (Dr. Pascal Boireau, ANSES): Parasitic Immunology
09:30 – 10:00 AM Keynote Speaker V (Dr. Suk-Chan Jung, OIE): Brucellosis
10:00 – 10:10 AM Coffee/Tea Break
10:20 – 12:00 AM Oral Presentation III (5 papers; 20 min/paper)
2. Hirokazu Hikono: Adjuvant effect of CpG oligodeoxynucleotides on antibody responses to avian influenza vaccine in chickens
4. Takeshi Hatta: RNA-Seq analysis of embryo of the tick Haemaphysalis longicornis
5. Yuko Uchida: Pathogenicity analysis of highly pathogenic avian influenza virus by profiling host gene expression

12:00 – 01:00 PM Lunch
01:00 – 01:30 PM Keynote Speaker VI (Dr. John Allen, AAHL): Emerging Infectious Diseases
01:30 – 03:20 PM Oral Presentation IV (5 papers; 20 min/paper)
1. Kingkarn Boonsuya Seeyo: FMD antigenic profiling ELISA
2. Ayako Miyazaki: Repeated and intermittent shedding of rotavirus A during the lives of farm-raised pigs
3. Bandit Nuansrichay: Molecular characterization and phylogenetic study of caprine arthritis-encephalitis viral sequences from goats in Northern Thailand
4. Nobuhiro Takemae: Reactivity of monoclonal antibodies raised against an A(H1N1)pdm2009 virus with swine influenza viruses of different lineages
5. Michihiro Takagi: Genetic analysis of Japanese porcine reproductive and respiratory syndrome virus strains from 1992 to 2010

03:20 – 03:30 PM Coffee/Tea Break
03:30 – 04:00 PM Closing Ceremony
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OPENING ADDRESSES
WELCOME ADDRESS

by

Dr. Tritsadee Chaosuancharoen

Director General, Department of Livestock Development

at Thailand-Japan Joint Conference on Animal Health 2012:
The 25th Year Anniversary of National Institute of Animal Health

Rama Gardens Hotel, Bangkok

May 30, 2012

His Excellency Mr. Theera Wongsamut, Minister of Agriculture and Cooperatives,
His Excellency Mr. Seiji Kojima, Ambassador of Japan to Thailand,
Dr. Tokio Inbe, Vice President, National Agriculture and Food Research Organization, Japan
Dr. Takaufumi Hamaoka, Director General, National Institute of Animal Health, National Agriculture and Food Research Organization, Japan

Distinguished delegates, honorable guests, ladies and gentlemen,

On behalf of the Department of Livestock Development and the organizing committee, I would like to express my sincere gratitude to His Excellency Mr. Theera Wongsamut, Minister of Agriculture and Cooperatives, and His Excellency Mr. Seiji Kojima, Ambassador of Japan to Thailand, for kindly presiding over the Opening Ceremony of Thailand-Japan Joint Conference on Animal Health 2012: The 25th Year Anniversary of National Institute of Animal Health. I also would like to extend a warm and cordial welcome to all of you, in particular to our distinguished overseas guests, participants and speakers.

This conference is specially organized to commemorate the 25th Anniversary of the establishment of Thailand’s NIAH in connection with the Silver Jubilee celebration of successful Thailand-Japan Technical Cooperation in Animal Health.

It has been my great pleasure to witness that NIAH has been dramatically growing since its founding in 1986 and became one of the most leading institute for animal health in Southeast Asia. NIAH has achieved to this stage with kind assistance, great collaboration and generous support from various partners and funding agencies, especially from the Japanese Government. Through the Japan International Cooperatives Agency or JICA, the Japanese Government has played a major role in providing funding for the building, infrastructure and equipment as well as the technical support, which laid the firm and strong foundation for NIAH’s success.

Within a quarter of century of joint effort between NIAH and its counterparts, it is evident that the collaboration has expanded to a broader perspective and networking in the region covering the diagnostic, reference, research, and training activities on the control of major diseases of livestock such as Avian Influenza, Brucellosis and Foot and Mouth Disease. At present, NIAH is taking a leading role in laboratory diagnosis of these diseases for Thailand and the region.

The aim of the conference is to gather scientists in the area of animal health and public health to exchange new idea and better understand the challenges and opportunities that each group faces and to develop solutions for future research and network that fulfill the needs of both human and animal sciences. “Healthy animals, healthy people” is the theme of the conference.
The conference will cover invited lectures by distinguished keynote speakers on Influenza, Brucellosis and Twining Program, Bacterial Food-borne Diseases, Parasitic Immunology, and Emerging Infectious Diseases, oral and poster presentations by researchers and scientists on various topics of animal health.

There are altogether approximately 250 participants attending this conference, consisting of representatives from various organizations such as JICA, FAO, OIE, AAHL, ANSES, retired and present Thai officers from Department of Livestock Development and Japanese experts involved in founding of NIAH and collaborative project with NIAH. Academics from the Faculty of Veterinary Medicine, guests from Ministry of Agriculture and Cooperatives, Ministry of Public Health, and research funding agencies are also present.

Finally, I would like to take this opportunity to express my sincere appreciation to the Japanese Government and JICA for their kind contributions to Thailand and NIAH. The precious friendship bonding the institutions and individuals has been a major driver of success and I am confident that this conference will help to further strengthen friendship and cooperation between our two nations and peoples.

Thank you.
WELCOME ADDRESS

by

Dr. Takafumi Hamaoka

Director General, National Institute of Animal Health,
National Agriculture and Food Research Organization, Japan

at Thailand-Japan Joint Conference on Animal Health 2012:
The 25th Year Anniversary of National Institute of Animal Health
Rama Gardens Hotel, Bangkok
May 30, 2012

His Excellency Mr. Theera Wongsamut, Minister of Agriculture and Cooperatives, Thailand,
Dr. Triotsadee Chaosuancharoen, Director General, Department of Livestock Development, Thailand,
Dr. Vimol Jirathanawat, Director, National Institute of Animal Health, Thailand,
His Excellency Mr. Seiji Kojima, Ambassador of Japan to Thailand,
Dr. Tokio Inbe, Vice President, National Agriculture and Food Research Organization, Japan,
Distinguished delegates, honorable guests, ladies and gentlemen,

On behalf of the National Institute of Animal Health, National Agriculture and Food Research Organization, Japan, and the organizing committee, I would like to cordially welcome you all with warmest greetings to Thailand-Japan Joint Conference on Animal Health 2012: The 25th Year Anniversary of National Institute of Animal Health Thailand. We feel very much honored that His Excellency Mr. Theera Wongsamut, Minister of Agriculture and Cooperatives, and His Excellency Mr. Seiji Kojima, Ambassador of Japan to Thailand kindly presides over this Opening Ceremony.

This conference is specially organized to commemorate the 25th Anniversary of the establishment of Thailand's NIAH in connection with the Silver Jubilee celebration of successful Thailand-Japan Technical Cooperation in Animal Health.

Tracking back the history of the relationship between NIAH-NARO-Japan and DLD Thailand, a technical cooperation had started at 1958 which was historical Colombo Plan. NIAH-NARO, Japan joined this global scheme and dispatched staffs to Thailand to transfer the technology of control and prevention of rinderpest and the vaccine production.

At 1967, Tropical Agriculture Research Center, MAFF-Japan was started a technical cooperation program at FMD center, Packchong. At 1977, JICA project, the Animal Health Improvement Project at FMD center and Diagnostic Laboratory Center, Tung Song, was started. At 1986, the technical cooperation project, "National Animal Health and Production Institute (NAHPI) Project" was started and was continued until 1999. During the course of the project, National institute of Animal Health (NIAH-Thailand) was established and started the service of functional national central Laboratory in Thailand.

From 2001, JICA project had shifted from bilateral to regional scheme and a project, "Animal Disease Control in Thailand and neighboring countries", called ADC project, had started. The project had included Cambodia, Lao, Malaysia, Myanmar, Thailand and Vietnam. NIAH-Thailand had played the key role of the project as resource institution and established the status of leading veterinary laboratory in the region.
At 2005, an oversea office of Zoonotic Disease Collaboration Center (ZDCC) was opened at NIAH-Thailand under Japan Global Research Network program, J-GRID, which is to cope with emerging and re-emerging diseases. The office is operating joint research projects especially on avian and swine influenza since 2005.

And now, we can find highly developed NIAH-Thailand which contributes to the improvement of the animal health condition of Thailand and the region.

The aim of this joint conference is not only to exchange new scientific topics in animal health and public health, but also to stimulate the relationship between Thai and Japanese scientists, especially younger scientists who are going to bear the next networking and cooperation in the region. I believe from the bottom of my heart that the joint conference is going to be successful and to be a great start of young generation toward the next era of cooperation under our common theme, “Healthy animals, healthy people”.

Finally, I would like to take this opportunity to express my sincere appreciation to Thai Government, DLD and NIAH-Thailand for the warmest hospitality you had given and the greatest achievement of the development of close relationship between NIAH-Thailand and NIAH-NARO-Japan. And also, I would like to offer the highest praise to the Japanese experts and Thai counterparts who had been worked.

Thank you.
OPENING ADDRESS
by
H.E. Mr. Theera Wongsamut
Minister of Agriculture and Cooperatives
at Thailand-Japan Joint Conference on Animal Health 2012:
The 25th Year Anniversary of National Institute of Animal Health
Rama Gardens Hotel, Bangkok
May 30, 2012

His Excellency Mr. Seiji Kojima, Ambassador of Japan to Thailand,
Dr. Tokio Inbe, Vice President, National Agriculture and Food Research Organization, Japan
Dr. Takafuli Hamaoka, Director General, National Institute of Animal Health, National Agriculture
and Food Research Organization, Japan
Dr. Tritsadee Chaosuancharoen, Director General, Department of Livestock Development,
Distinguished delegates, honorable guests, ladies and gentlemen,

It is indeed an honor and privilege for me to preside over the Opening Ceremony of the Thailand-Japan Joint Conference on Animal Health 2012: The 25th Year Anniversary of National Institute of Animal Health. On this occasion, I would like to express my heartfelt congratulations to the Department of Livestock Development on the 25th Anniversary of the establishment of the National Institute of Animal Health. I am pleased to see various representatives present today which clearly reflect collaborative efforts between NIAH and its counterparts towards strengthening cooperation on animal health. I also would like to extend my warm welcome to the honorable guests and distinguished delegates attending this conference.

It is my great pleasure to learn that the technical cooperation in animal health between JICA and Thailand’s NIAH has successfully developed for more than 25 years. In the area of livestock development, the establishment of NIAH is a testimony of success of the cooperation between the two agencies. With extensive and strong support from the Japanese Government and JICA, NIAH has developed diagnostic laboratories to meet with the international standard to serve veterinary services and public health which benefit not only our farmers and Thai people but also the people in the neighboring countries. I would like to take this opportunity to extend my most sincere appreciation to the Japanese Government and JICA for their kind contributions and guidance to NIAH. I do hope that the success in cooperation between Thailand and Japan will be more than just another success story. I am confident that the friendship behind the long-lasting relations and successes over a quarter of century will continue to serve as solid foundations that will lead to even stronger and closer future cooperation between our two countries.

I also appreciate hard work carried out by NIAH during the past years especially during the outbreak of Avian Influenza and the Thailand’s Great Flood Crisis in 2011. I am confident that NIAH will keep its high performance level and will always be ready to face with future challenges. I believe that this conference will provide a great opportunity for exchanging of cutting edge knowledge and innovative ideas and fostering networking, collaboration and joint efforts among the conference participants. Because of our diverse strengths, expertise and experiences,
we have so much to learn from and share with each other. In the rapidly changing world, we are facing common challenges in such areas as environment and climate changes, natural disasters and infectious diseases, in particular zoonotic and emerging/re-emerging diseases. Therefore, it is more urgent than ever to further strengthen our cooperation in order to prepare for future challenges in animal health and public health.

Excellency,
Ladies and gentlemen,

At this auspicious moment, may I declare the opening of the Thailand-Japan Joint Conference on Animal Health 2012: The 25th Anniversary of Nation Institute of Animal Health. I wish you all successful deliberations as well as a pleasant, enjoyable and memorable stay in Thailand and hope that our hospitality will make you come back again in the very near future.

Thank you.
Salmonella enterica, enterohemorrhagic Escherichia coli, and Campylobacter jejuni/coli are leading causes of human gastroenteritis worldwide. Origins of these pathogens are farm animals including cattle, swine, and chickens. Contaminated meats, milk, eggs, and vegetables are major sources of infections. To control food poisoning, researches should focus on many directions. To identify an epidemic clone, molecular epidemiological studies should be carried out continuously. Development of novel molecular typing methods would contribute to the investigations. In postharvest steps, development of rapid and highly-sensitive methods to identify specific pathogens has been desired. To reduce the prevalence of these pathogens in farms, basic researches leading to a development of anti-colonization factors should also be implemented. In this lecture, two topics of our recent achievements will be presented. First topic is about a molecular epidemiological study of S. enterica serovar Typhimurium (ST) from bovine origin. Since 2004, extended-spectrum cephalosporin (ESC)-resistant ST isolates have been detected from cattle in the northern major island of Japan, Hokkaido. We demonstrated that a particular clone harboring antimicrobial resistance genomic island conferring ESC resistance has disseminated in this area. Second topic is on the development of rapid identification methods of S. enterica. We constructed multiplex PCR assays to identify seven major serovars of S. enterica and demonstrated that these assays had sufficient specificity as rapid screening methods.
Bacterial Foodborne Diseases

Masato Akiba
National Institute of Animal Health, Japan

Genus *Salmonella*

<table>
<thead>
<tr>
<th>Species</th>
<th>Subspecies</th>
<th>Number of variants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella enterica</em></td>
<td>enterica</td>
<td>1,531</td>
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<tr>
<td></td>
<td></td>
<td>30%</td>
</tr>
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<td></td>
<td>9%</td>
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<tr>
<td></td>
<td></td>
<td>336</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13%</td>
</tr>
<tr>
<td><em>Salmonella bongori</em></td>
<td></td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2007)</td>
</tr>
</tbody>
</table>

- Serovars are defined by a combination of somatic (O) and flagellar (H) antigens.
- Pathogenic nature of *Salmonella* is based on invasion of the host cells.
- Several serovars are host-restricted and highly pathogenic to the specific host.
- *Salmonella Enteritidis* (SE) is the most prevalent serovar in food poisons.
- Eggs contaminated by SE are the most common source of human infections.
- *Salmonella Typhimurium* is one of the most common sources of gastroenteritis for both humans and animals.

Genus *Campylobacter*

- Gram-negative, microaerophilic, spiral rods.
- One flagellum on both ends of body, cork-screw like movement.
- 25 species, 13 sub-species have been reported to date (http://www.bacteria.info.fr/)
- *C. jejuni* and *C. coli* are the most common causes of human gastroenteritis.
- Pathogenic nature of *Campylobacter* to humans is not clear to date.
- Cattle, swine, sheep, chickens, and wild birds can be hosts of *Campylobacter*.
- Undercooked chicken meats are the most common sources of *C. jejuni* infections.
- Swine are reservoirs of *C. coli*, rather than *C. jejuni*.

How to control food-poisonings?

- Reduce prevalence in terms
- Antimicrobial treatments
- Emergence of resistant bacteria
- Phage therapy
- Vaccines etc.
- Study phase or limited effects
- Identification of epidemic phases
- Molecular epidemiological studies
- Rapid and sensitive detection of pathogens
- Development of identification methods
- Identification of transmission routes
- Development of typing methods

Emergence of an extended-spectrum cephalosporin resistant *Salmonella enterica serovar Typhimurium* clone harboring a multidrug resistance genomic island among cattle population in Japan.
**Options of first-line therapy for Salmonella infections**

- Ampicillin
- Sulfamethoxazole-trimethoprim
- Fluoroquinolones

**Resistant is common**

- Contraindicated in children

**Extended-spectrum cephalosporins (ESCs)**

Plasmid-mediated *β*-lactamases → Transferable to other bacteria

| Classical class A extended-spectrum *β*-lactamases (ESBLs) |
| Classical class C cephamycinsases (AmpC *β*-lactamases) |

**ST-VII-6 Characteristics**

- Indistinguishable Pulse-field gel electrophoresis pattern
  → Dissemination of a specific clone?

- Extended-spectrum *β*-lactamase (ESBL) or AmpC *β*-lactamase?
  - Resistance to CFZ, FOX, CAZ, CTX
  - No inhibition by cephalaxin and Cephalotinase positive
  - *blaCTX-M*-1, *blaTEM* detected by PCR

- Location of *blaCTX-M-1*?

**bio2MVNC gene is not located on plasmids**

**Schematic view of full-length GI-VII-6 of S. Typhimurium l-3553**

- DNA A (125 kb)
- DNA B (3.4 kb)
- DNA C (6.9 kb)

**GI-VII-6 has originated from IncA/C plasmid**

- pAR06302 (148,630 bp)
- pCH1 (126,843 bp)

- *GI-VII-6 shows a high sequence similarity with E. coli IncA/C plasmid pAR06302.
- *GI-VII-6 does not contain sequences of ori and repA.*
Conclusion

A particular clone of S. Typhimurium harboring GI-VII-6 has spread among the cattle population in Hokkaido, Japan.

Rapid identification of Salmonella enterica serovars, Typhimurium, Choleraesuis, Infantis, Hadar, Enteritidis, Dublin, and Gallinarum, by multiplex PCR

Selection of serovar specific genomic regions

Location of serovar-specific genomic regions

Results of multiplex PCR (m-PCR) assays

Amplification results of each SSGR by m-PCR assays
Conclusion

These assays had sufficient specificity to identify the seven Salmonella serovars, and therefore, have the potential for use as rapid screening methods.

Albà et al., Microbial Methods, 86: 9-18, 2011
The nematode Trichinella, real parasite of a cell.

Pascal Boireau1 and Liu Mingyuan2  
1 Laboratory for Animal Health, ANSES, Maisons Alfort, France  
2 Key Laboratory of Zoonoses, Ministry of Education, Institute of Zoonoses, Jilin University, 5333 Xian Road, 130062 Changchun, P. R. China

Trichinella spiralis is a unique intracellular parasitic nematode that is distributed worldwide and can infect almost all mammals, including humans. The life cycle of T. spiralis is completed within a single host species and infection starts with the consumption of infective muscle larvae (ML) and digestion of the protective capsule within the host stomach. Larvae undergo four fast molts in intestinal epithelial cells and eventually develop into sexually mature adults (Ad) approximately 2-3 days post infection (pi). Freshly released newborn larvae (NBL) are carried to host tissues by blood flow and invade new host cells. The NBL penetrate striated muscle cells and undergo developmental changes to induce the development of a nurse cell surrounding the parasite. This nurse cell may remain viable for the entire life span of the host and the parasite that control all the cell metabolism.

To date, little is known about the molecular mechanisms that are involved in parasite development and survival within the cytoplasm of host cell. Identification of stage-specific genes will be important for elucidation of these mechanisms. ML, Ad and NBL are three major stages in the life cycle of T. spiralis that exhibit distinct antigenicity, indicating differential regulation of many parasite proteins (Boireau et al., 1997). Previously, very few developmentally regulated antigens have been characterized, except the ML stage-specific TSL-1 antigens identified by monoclonal antibodies. Several genes expressed during the ML stage were identified recently (review Boireau et al., 2004) and one of these genes has been speculated to be involved in nurse cell formation. Proteins synthesized in T. spiralis and secreted in the host cell are suspected to be involved in the process of the nurse cell in the previous studies, however, up to now, only one of them, the 43 kDa polypeptide (P43), has a helix-loop-helix (HLH) motif that have been suggestive of a function that might be relevant to the Nurse cell formation, this cDNA has been identified to encode a homologue of DNase II now. Further more, the DNase activity has also been proved to be present in the ES of Trichinella spiralis ML but not in it from Trichinella pseudospiralis ML (a non-encapsulated Trichinella species having an “ancestral” nurse cell). Mitreva et al. generated a T. spiralis expression sequence tags (ESTs) database containing 3262 unique genes from cDNA libraries of immature L1 larvae (also known as NBL), mature ML and adults (3-day-old adults, Ad3). However, the majority of the identified genes have been clustered in only one developmental stage.

In an attempt to identify stage-specific genes of T. spiralis, subtracted cDNA libraries of NBL, Ad3 and Ad5 were constructed respectively, using a suppression subtractive hybridization (SSH) technique (Liu et al., 2007). Differential immunoscreening of cDNA libraries was also applied (Ozcovic et al 2011). A number of stage-specific cDNAs derived from NBL, Ad3 and Ad5 were identified and analyzed. Six genes were identified as NBL stage-specific (Boireau et al., 2006; Fu et al, 2009; Niu et al 2005), including one member of the T. spiralis gene family encoding glutamic acid rich proteins, two genes encoding novel serine proteases, two closely related genes encoding proteins that are members of a deoxyribonuclease II (DNase II)-like family (Liu et al 2008) and one nucleotidic sequence with no similarity to known genes. The twin genes encoding...
DNaseII (DnaseII1Ts/NBL and DnaseII2Ts/NBL) have a high percentage of identity in their amino acid (aa) sequence (89.6%), and their predicted aa sequences exhibited a N-terminal signal peptide, a potential helix-loop-helix motif and the conserved domains of DNase II. The DNase activity have been obtained with the purified recombinant proteins DnaseII1Ts/NBL. Considering that NBL directly promote the nurse cell forming of infected muscle cell the importance of DNase II in the nurse cell formation of T. spiralis will be discussed. Four stage-specific clones encoding homologues of retinoid X receptor, caveolin, C2H2 type zinc finger protein and a putative protein with no homology to known sequences were obtained from 3-day-old adult worms. The caveolin-1 gene (CavTs) was characterized and identified as an adult-specific antigen (Hernandez-Bello R. 2008). CavTs is gradually accumulated only on the ova surface reaching a maximum at 3 days pi, and decreasing during newborn larva (NBL) development. Reverse transcriptase polymerase chain reaction (RT-PCR) assays of parasites from 1 to 4 days pi showed a similar gene expression profile that observed for CavTs, which suggests a specific developmental regulation. One gene specifically up-regulated in the 5-day-old adult worms (Wu et al, 2005) encoding a putative cuticle collagen was also identified.

During the analysis of the subtractive cDNA library additional clones were selected that could be relevant in the biology of Trichinella. But all these clones are expressed during all the Trichinella developmental stages some of them having more intense transcription during a period of Trichinella life. So, nucleotidic sequence of the GST24 gene of various Trichinella species was obtained. The open reading frame coding for GST24Ts was expressed in a prokaryotic system and purified by glutathione-affinity columns. GST24Ts enzymatic activity was proved to be different from the activity of mammalian pi class GSTs, and other pi class related nematode GSTs. With the universal substrate 1-chloro-2, 4-dinitrobenzene (CDNB), the specific activity of GST24Ts was 3.79 µmole.min-1(mg protein)-1. The tissue distribution of GST24Ts was examined by immunohistochemistry in muscular larvae (ML) of T. spiralis and the nurse cell. GST24Ts was located exclusively in the stichocytes and the genital primordium without any export in the cytoplasm of the nurse cell.

Recent sequencing of T pseudopsiralis transcriptome was achieved and compared with T spiralis one (Liu Mingyuan and P Boireau submitted). We identified a core of shared and constitutively expressed ‘housekeeping’ genes that evidently are essential to basic Trichinella parasite metabolism, as well as elucidated genes and gene families that are differentially expressed in particular parasite species and life history stages.

In brief the Trichinella developmentally regulated genes that has been described, can be divided into two main functions: i) the development of the parasite (CavTs AdTs1) ii) the interaction with the host cell (proteases, Dnasell) to allow the generation of the nurse cell. The data generated from this study have provided new knowledge to our understanding of T. spiralis gene expression mechanisms during various developmental stages particularly in the nurse cell. These findings have provided information of Trichinella parasite biology and pathogenesis, which will pave the way for the development of more specific and sensitive immunodiagnostic technologies and for the prevention of trichinellosis.

Part of this work was supported by the EU contracts TRICHIPORSE QLRT-2000-01156 MedVetNet (Food-CT-2004-506122) WP11 Trichinet and WP27 Trichimed and the grant of China 863 program 2006AA02Z451. Sequencing project was supported by INRA grant (genomic program 2005)


National Surveillance and Control Measures of Brucellosis in South Korea

Suk Chan Jung*, Moon Her, Ji-Yeon Kim, Kichan Lee, Kiyoon Chang

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Brucellosis is a worldwide zoonotic infectious disease caused by Brucella species. Bovine brucellosis is characterized by symptoms such as abortion, infertility and reduced milk yield. Even though the infected cows give birth to weak or even healthy calves, the cows continue to harbor and discharge the causative agents, and will be a dangerous source of disease transmission.

The Korean government has implemented stamping-out policy for control of bovine brucellosis since the 1960s. Control measures for national eradication program also include pre-marketing test, strict movement restriction and quarantine of infected herds, epidemiological trace-backs and testing of cattle herds that had contact with infected herds, and testing of herds in areas surrounding infected herds. The tests for bovine brucellosis include both identification of causative agents and serological diagnosis, including milk ring test (MRT), serum agglutination test (SAT), Rose Bengal test (RBT) and ELISA. Regional offices in 9-provinces of Korea usually conduct the required tests for regular survey of bovine brucellosis.

The first outbreak of bovine brucellosis in Korea was reported in 1955. Of the 124 dairy cattle imported from the USA, 30 cattle (24%) were identified as reactors. Since then, the disease occurred sporadically until 1983 and most of the outbreaks were reported in dairy cattle until 1999. However, the annual number of infected cattle began to increase to over 100 from 1984, and the prevalence of the disease has increased continuously since then. Especially the detection rate of brucellosis in Korean native cattle (Hanwoo) has been sharply increasing since 2003, due to the adoption of ‘brucellosis-free certification system’ in livestock to enhance the detection of infected animals. After the peak of an outbreak (about 2.2%) in 2006, the prevalence of bovine brucellosis decreased gradually and as of 2011, the incidence rate is estimated to be around 0.26%.

Human brucellosis is closely related to certain occupations, such as animal husbandry. A single case of B. abortus infection in humans in Korea was officially reported in 2002. However, the number of human cases has continued to rise since then, and 215 such cases were reported in 2006. Serological survey for three different high risk groups of brucellosis found that the seropositive rates were 0.23% (15/6,721) in livestock workers, 1.78% (6/338) in veterinarians and 0.27% (1/377) in artificial insemination technicians.

According to epidemiological survey for cattle farms, the causes of infection are as follows: It appears that among the total number of infected farms, 46.2% were infected by bringing cattle with latent infection into the herd and 12.2%, from neighbors. About 68% of the affected farms became brucellosis-free, however, 32% experienced the recurrence of the disease. In particular, farms that had aborted cattle showed significantly higher rate of recurrence than those that didn’t have them. Therefore, the period of retesting for brucellosis should be extended to more than 6 months from the initial testing and the movement restriction of animals also should be maintained more than 6 months.
In Korea, *Brucella (B.) abortus* were isolated from Korean native and dairy cattle, and most of the isolates fall into biovar 1. *B. abortus* was found in domestic elk in 2008, wild water deer in 2010 and goral (wild goat) in 2011. With possible transmission between domestic and wild animals, continuous monitoring system with a great vigilance is necessary. In order to survey epidemiological relations using molecular tools, we analyzed a total of 177 isolates from 105 cattle farms of nine Korean provinces from 1996 to 2008 using MLVA (multi-locus variable-number-tandem-repeats analysis) assay. In clustering analysis, *Brucella* isolates were classified into nine clusters and 23 genotypes. In parsimony analysis with foreign *Brucella* isolates, domestic isolates were clustered distinctively, and located near isolates found in Central and South America. We proved that MLVA assay can be utilized as a tool for epidemiological trace back in a restricted area and also can be applied to determine the relationship between *Brucella* isolates from animals and from humans.

With respect to microbiological isolation and identification, it takes 7 to 10 days to complete the procedure. In the case of chronic infection, the isolation rate is low. *Brucella* spp. can be isolated on a variety of plain media (e.g. Brucella agar, trycase soy agar) or selective media (e.g. Farrell’s medium, Thayer-Martin’s modified medium). We developed a new selective medium, modified brucella selective (MBS) medium. The MBS medium, which contains improved antibiotic mixtures, erythritol as the only carbon source, and neutral red as a pH indicator, showed good selectivity for the *B. abortus* strains, including the RB51 vaccine strain. The *Brucella* colonies, shown to be pinkish in their central parts, were easily differentiated from other organisms. The MBS medium also allows the isolation of the *Brucella* strains even in contaminated specimens and/or in specimens containing small numbers of viable organisms. The newly developed MBS medium was as sensitive as or even more sensitive than the Farrell’s medium, particularly in contaminated samples and in samples containing very small numbers of viable strains.

The diagnosis of brucellosis conducted along with regular test in Korea is currently based on serological tests. However, classical serological methods have been reported to cross-react with antigens, other than those from *Brucella* spp. Therefore, a quick, sensitive and specific diagnostics of animal brucellosis will be necessary. Recently we have developed and tried out the fluorescence polarization assay (FPA) and Dip-stick kit. In order to detect brucellosis in its early stages, following measures can be taken: introduction of methods with a high sensitivity and specificity such as competitive-ELISA as well as methods that can be applied in farms, slaughter houses and livestock markets such as FPA and Dip-stick.

In detecting a causative agent, the microbiological isolation and identification are inappropriate since they require lots of time and effort. Therefore, diagnosis methods such as PCR assays with high sensitivity and specificity are recently used for rapid results. The various genes used for the PCR assay include IS711, *omp* (2a, 2b), 16S rRNA, and genes encoding 31 kDa. To diagnose brucellosis, we implemented rapid and precise PCR assays using BCSP (*Brucella* 31 kDa cell surface protein) and OMPB (*Brucella* 36 kDa outer membrane protein) genes. Multiplex PCR assays have also been developed to differentiate among *Brucella* species and/or biovars. The AMOS-PCR assay can identify and differentiate among *B. abortus* biovars 1, 2 and 4; *B. melitensis*, *B. ovis* and *B. suis* biovar 1, and we applied a modified version of the AMOS-PCR assay which included strain-specific primers for the two commonly used vaccine strains, S19 and RB51. A new single-step multiplex PCR assay (Bruce-ladder) can identify and differentiate
most of the *Brucella* species including marine mammal strains as well as vaccine strains. The inconvenience of Bruce-ladder PCR is that some *B. canis* strains can be identified erroneously as *B. suis* or *B. microti*. To resolve this problem, we introduced two new primer sets of a 766- and a 344-bp fragment into the conventional Bruce-ladder PCR assay. This novel multiplex PCR assay can rapidly and concisely discriminate *B. canis* from *B. microti* and *B. suis* strains and also can differentiate all of the 10 *Brucella* species. Recently, SNP based real-time PCR assay for 5 classical *Brucella* species has been developed and it is being evaluated against a variety of specimens. Now, we are also developing specific loop-mediated isothermal amplification (LAMP) assay for *B. abortus* and *B. canis*.

*Brucella*, an intracellular bacterium, has generally latent period of 6 months to 1 year, and it tends to reoccur in once infected cattle farms, which can lead to abortion, stillbirth and infertility among cattle populations, incurring huge economic losses. Furthermore, if the disease is prevalent and many people avoid consuming livestock products, it can be a huge blow to the domestic livestock industry. Brucellosis continues to be a big threat to animal and human health in Korea. The control and prevention programs against brucellosis in cattle have always been based on the stamping-out policy. For the efficient prevention and early eradication of brucellosis, first and foremost, epidemiological analysis which identifies the precise causes of outbreaks should be made and efforts to develop more sensitive diagnosis methods should be followed. Furthermore, surveillance systems need to be enhanced. First, epidemiological trace-back should be strengthened. Second, movement restrictions need to be imposed on brucellosis-positive farms. And the cohabited animals must be subject to inspection. Third, continuous monitoring system is also needed, which includes both farmed and wild animals. Lastly, well-planned research needs to be conducted and research findings need to be utilized for better programs in the fight against brucellosis.
Simple method to regenerate the strong cation exchange cartridge for oxytetracycline and chlortetracycline analysis

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Abstract

Background: Oxytetracycline (OTC) and chlortetracycline (CTC) are broad-spectrum antibiotics which have been extensively used for controlling bacterial infections. Since 1950s, OTC and CTC were legally added in animal feed as growth enhancers and for bacterial control. HPLC was performed to quantify these antibiotics in animal feed. In the clean-up step, the strong cation exchange (SCX) cartridge is commonly used to remove interfering substances by cation exchange interaction. However, SCX cartridge is costly; therefore, the method to regenerate cartridges for OTC and CTC analysis was studied.

Method: To regenerate the cartridges, exhausted SCX cartridges were washed by 5.0 ml of 0.1M Na$_2$CO$_3$, 1.0M HCl, phosphate buffer and water, respectively, with a flow rate of 5 ml/min. The percent of recovery and relative standard deviation (RSD) were evaluated for the accuracy and precision of regenerated SCX cartridges with the fortified OTC and CTC at the concentrations of 10, 100 and 300 mg/kg.

Results: SCX cartridges could be regenerated 20 times for analysis of OTC and CTC ranging 10-300 mg/kg in animal feed. The OTC had average recovery and RSD of 93.0-101.3% and 2.8-6.2%, respectively whereas CTC had 93.8-100.8% and 4.5-6.5%, respectively.

Conclusion: The regenerated SCX cartridge can be repeatedly used with cost-effectiveness for OTC and CTC analysis.

Keywords: SCX cartridge, regenerated

Background

Oxytetracycline (OTC) and chlortetracycline (CTC) are broad-spectrum antibiotics which have been extensively used for controlling bacterial infections. Since 1950s, OTC and CTC were legally added in animal feed as growth enhancers and for bacterial control (Paige et al., 1997; Joint FAO/WHO Expert Committee on Food Additive, 1999). The OTC and CTC levels were used in animal feeds varying from 5-15 mg/kg; however, the antibiotic level tendency has been increased. Used as feed additive, they were reported at 100-200 mg/kg or even higher, which was therapeutic dose, (WHO, 1963). In Thailand, OTC and CTC in animal feed for growth-promoters have been prohibited but they are allowed for treatment under veterinary prescription (Tuantumkaew et al., 2011).

OTC and CTC are derived from a system of four six-member rings arranged linearly with characteristic double bonds. They are soluble in aqueous, polar, organic solvents, and several functional groups resulting in strong complex properties. In strong acidic pH below 2.5, the molecules exist in the full protons as a singly charged cation (Knox and Jurand, 1975), which is favorable for the successful separation of the OTC and CTC by cation exchange ion chromatography (Ding and Mou, 2000).
The HPLC method has been employed successfully for monitoring and confirmation of OTC and CTC in animal feeds. In sample preparation for HPLC, a simple clean up step, solid phase extraction (SPE) was used to concentrate and purify OTC and CTC via strong cation exchange cartridge (SCX). The SCX cartridges are polymeric bonded, benzenesulfonic acid functional group, covalently bonded to the surface of a silica particle. Counter ion is hydrogen ion (H$^+$). Typical applications include positively charged basic compounds (Vertical, 2011). SCX cartridge is costly; therefore, the objective of this study is to regenerate SCX cartridges for analysis of OTC and CTC in animal feeds based on ion exchange, in order to reduce laboratory cost.

**Materials and methods**

**Apparatus**

The HPLC system consisting of Water alliance separations 2998e module was obtained from Water Corporation (USA). Photodiode array detector (DAD) was set at the wavelength multi scan mode between 200-400 nm and fixable at 365 nm for identifying of OTC and CTC. The analytical column was used a reversed phase Sunfire C18 (150x4.6 mm I.D., 5µm, Water, USA). Data acquisition was controlled by an Empower2 software LC 3D® (USA).

**Reagents**

All chemicals and solvents were of analytical grade except solvents in mobile phase that were of liquid chromatographic grade. Pure standards of OTC and CTC as their hydrochloride were obtained from Sigma-Aldrich (Switzerland). Ultrapure water was purified using a Mill-Q system (Millipore, USA). Sodium carbonate (Na$\text{$_2$CO}_3$) was obtained from Ajax Finechem (Australia). Oxalic acid dihydrate was obtained from Merck (USA). Acetonitrile and methanol were obtained from Labscan (Thailand). Hydrochloric acid (HCl) was obtained from J.T. Baker (USA). Phosphate buffer was obtained from Calbiochem (USA). SCX cartridges (500mg/6mL) were obtained from Vertical (Thailand).

**Standard solution**

A mixed stock standard solution of OTC and CTC (free base) (1,000 mg/l) was prepared by dissolving in methanol and stored at -20ºC. Calibration standard was prepared prior to use by diluting of the stock standard solutions with 0.4 M HCl in methanol and ultrapure water (1:1, v/v) to 0.5, 1.0, 5.0, 10, 20 and 40 mg/l.

**Fortification sample**

Animal feed samples known not to contain OTC and CTC residues were used to fortified samples. Samples were spiked by adding the required amount of OTC and CTC with a mixed stock standard solution (1,000 mg/l). The bulk of homogenized samples were spiked with OTC and CTC at 10, 100 and 300 mg/kg.

**SCX cartridge regeneration**

To regenerate the cartridges, exhausted SCX cartridges were washed by 5.0 ml of 0.1M Na$_2$CO$_3$, 1.0M HCl, phosphate buffer and ultrapure water, respectively. Each solution was siphoned using vacuum through SCX cartridges at a flow rate of 5 ml/min. The regenerated cartridges were allowed to dry after the final wash.
**Evaluation of regenerated cartridge**

HPLC was used to quantify concentration of OTC and CTC after SCX cartridges regeneration in order to evaluate the accuracy and precision. Extraction and simultaneous analysis of both OTC and CTC were performed according to Tuantumkaew, et al. (2011) and AOAC (2009) with some modifications. Briefly, 5.0 g of fortified samples were extracted with 25 ml of 0.1 M HCl in methanol. The supernatants were collected 10 ml and diluted with 10 ml of ultrapure water, filtered through glass microfiber GF/A. 5.0 ml of filtrated solution were loaded onto a SCX cartridge previously conditioned with 2.5 ml of methanol and 2.5 ml of ultrapure water, respectively. OTC and CTC were eluted from SCX cartridge by using 2.5 ml of 0.4 M HCl in methanol and ultrapure water, respectively. Then 20 µL of the aliquot were injected into the HPLC system.

Accuracy and precision were evaluated by six replicate analyses of fortified samples which the concentrations of each OTC and CTC were 10, 100 and 300 mg/kg (EURACHEM, 1998). The accuracy was presented by recovery percentages within 85-110% whereas the precision was indicated by relative standard deviation (RSD) of less than 8% (AOAC, 2002).

The ability of repeated use was assessed by performing twenty times analyses from fortified samples where the concentrations of each OTC and CTC were 10, 100 and 300 mg/kg. The recoveries (%) were calculated

**Results and Discussion**

The evaluation of regenerated cartridge was presented by the accuracy and precision. The accuracies from six replicate analyses were shown as recoveries (%) ranging between 93.0-101.3% and 93.8-100.8% for OTC and CTC, respectively. The precisions as relative standard deviations (RSD) were ranging between 2.8-6.2% and 4.5-6.5% for OTC and CTC, respectively (Table 1).

<table>
<thead>
<tr>
<th>Regeneration of SCX cartridges (times)</th>
<th>Recovery of OTC (mg/kg)</th>
<th>Recovery of CTC (mg/kg)</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
<td>100</td>
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<td>1</td>
<td>9.4</td>
<td>102.3</td>
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<tr>
<td>2</td>
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<td>104.4</td>
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<tr>
<td>3</td>
<td>8.9</td>
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<td>101.6</td>
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<tr>
<td>Recovery (%)</td>
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<td>101.6</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>2.8</td>
<td>4.0</td>
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The ability of SCX cartridge could be regenerated at least twenty times. The recoveries (%) were ranging between 86.1-108.4 which is in the accepted ranges of both OTC and CTC (Table 2).
Table 2 The recovery (%) of OTC and CTC from fortified animal feed samples at 10, 100 and 300 mg/kg

<table>
<thead>
<tr>
<th>SCX cartridges</th>
<th>Recovery (%) of OTC</th>
<th>Recovery (%) of CTC</th>
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<tr>
<td></td>
<td>10 (mg/kg)</td>
<td>100 (mg/kg)</td>
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<tr>
<td>Initial</td>
<td>93.8</td>
<td>95.3</td>
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<tr>
<td>1st regenerated</td>
<td>92.3</td>
<td>98.4</td>
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<tr>
<td>2nd regenerated</td>
<td>94.3</td>
<td>103.4</td>
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<tr>
<td>3rd regenerated</td>
<td>94.3</td>
<td>99.5</td>
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<td>4th regenerated</td>
<td>95.3</td>
<td>105.5</td>
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<td>5th regenerated</td>
<td>102.5</td>
<td>87.8</td>
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<td>6th regenerated</td>
<td>95.4</td>
<td>104.2</td>
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<tr>
<td>7th regenerated</td>
<td>92.4</td>
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<td>10th regenerated</td>
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<td>11th regenerated</td>
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<td>12th regenerated</td>
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<td>16th regenerated</td>
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<td>96.2</td>
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<td>91.3</td>
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<td>19th regenerated</td>
<td>91.1</td>
<td>94.3</td>
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<td>20th regenerated</td>
<td>88.4</td>
<td>89.8</td>
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SCX cartridge has positively charged mobile ions available for cation exchange. In order to clean-up this cartridge, the retained impurity was replaced by stronger ions. In this study, 0.1M Na$_2$CO$_3$ and 1.0M HCl were used to remove strongly bound substances, then phosphate buffer was used for neutralization and re-equilibration.

Similar to the study of Cai and Katsumura (1998), 0.1M Na$_2$CO$_3$ and 0.05M H$_2$SO$_4$ were used for SCX cartridge regeneration in formate and oxalate analysis. However, in this study the divalent ions in animal feed could be bound to sulfate ions and form precipitated substances, therefore, 1.0M HCl was used.

From twenty times repetition, the recovery percentages were in the acceptable range. Compare to the study of Tuantumkaew et al. (2011) which reported recovery (%) and RSD of single use of SCX cartridges as 84-105% and 2-11% for OTC, whereas 83-106% and 0.3-11% for CTC. However, there was a report of successful fifty times SCX-regeneration (Cai and Katsumura, 1998). Therefore, in a further study, more repetition could be trialed until unacceptable recovery (%) will present. As can be seen, this process could be performed with no detrimental effect. However, many laboratories are not recommended to reuse due to lack of understanding of interference clean-up and regeneration procedure.

References


Diversity of Melissococcus plutonius Isolates from Honeybee Larvae in Japan

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Abstract

European foulbrood (EFB) is an important infectious and contagious bacterial disease of honeybee larvae, but our understanding of the pathogenesis of EFB remains very limited. The causative agent, Melissococcus plutonius, is a fastidious organism, and microaerophilic to anaerobic conditions and the addition of potassium phosphate to culture media are required for growth. Although M. plutonius is believed to be remarkably homologous, in addition to M. plutonius isolates with typical cultural characteristics, M. plutonius-like organisms, with characteristics seemingly different from those of typical M. plutonius, have often been isolated from diseased larvae in Japan. Phenotypic characterization of 14 M. plutonius and 19 M. plutonius-like strain/isolates revealed that, unlike typical M. plutonius strain/isolates, M. plutonius-like isolates were not fastidious, and the addition of potassium phosphate was not required for normal growth. Moreover, only M. plutonius-like isolates, but not typical M. plutonius strain/isolates, grew anaerobically on sodium phosphate-supplemented medium and aerobically on some potassium salt-supplemented media, were positive for β-glucosidase activity, hydrolyzed esculin, and produced acid from L-arabinose, D-cellobiose, and salicin. Despite the phenotypic differences, 16S rRNA gene sequence analysis and DNA-DNA hybridization demonstrated that M. plutonius-like organisms were taxonomically identical to M. plutonius. However, by pulsed-field gel electrophoresis analysis, these typical and atypical (M. plutonius-like) isolates were separately grouped into two genetically distinct clusters. Although M. plutonius is considered to lose virulence quickly when cultured artificially, experimental infection of representative isolates showed that atypical M. plutonius maintained the ability to cause EFB in honeybee larvae even after repeated subculture in vitro in laboratory media. Because the rapid decrease of virulence in cultured M. plutonius was a major impediment to elucidation of the pathogenesis of EFB, atypical M. plutonius discovered in this study will be a breakthrough in EFB research.

Background

European foulbrood (EFB) is an infectious bacterial disease of honeybee larvae. It affects mainly unsealed larvae and kills them at the age of 4 to 5 days. The dead larvae turn yellowish, then brown, decompose, become watery, and often give off a foul or sour smell. EFB occurs in most areas in the world where apiculture is practiced, and is recognized as an economically important disease for apiculture.
The causative agent of EFB is a Gram-positive lanceolate coccus, *Melissococcus plutonius*. This bacterium was originally described in 1912 by White (1912), and was first cultured and characterized in detail by Bailey (1957b). *M. plutonius* is a fastidious organism, requiring microaerophilic to anaerobic conditions and carbon dioxide for growth. In addition, the Na:K ratio required for growth is described to be 1 or less (Bailey and Collins, 1982), and thus, the addition of potassium phosphate to the culture media is usually required for isolation and maintenance of this bacterium. Despite the long history of *M. plutonius* research, our understanding of the pathogenesis of EFB remains very limited. For elucidation, experimental infections of honeybee larvae with well-characterized *M. plutonius* strains are needed. However, because *M. plutonius* is reported to lose its virulence quickly when subcultured in vitro, reproduction of EFB in honeybee larvae by artificially cultured *M. plutonius* is extremely difficult (Bailey, 1957a; McKee et al., 2004). This largely hampers studies of the etiology of EFB.

*M. plutonius* is a one genus-one species bacterium and is thought to be remarkably homogeneous based on morphological, physiological, immunological, and genetic studies (Bailey and Gibbs, 1962; Allen and Ball, 1993; Djordjevic et al., 1999). For instance, Djordjevic et al. (1999) reported that Australian *M. plutonius* isolates originating from geographically diverse regions were markedly similar in their whole cell proteins, immunoreactive antigens, and DNA restriction endonuclease profiles. Moreover, they showed genetic homogeneity among Australian and British isolates and suggested that this species may be clonal (Djordjevic et al., 1999). However, the geographical locations of the isolates characterized so far have been mainly Europe, North and South America, and Australia. In contrast, information on *M. plutonius* isolates in other areas, including Asian countries remains limited.

In Japan, EFB has been occurring sporadically since suspected cases were recognized in the 1980s. In Japanese cases, however, in addition to *M. plutonius* isolates with typical cultural characteristics, *M. plutonius*-like organisms have often been isolated from diseased larvae with clinical signs of EFB. These *M. plutonius*-like isolates are morphologically similar to *M. plutonius* and positive for *M. plutonius*-specific PCR (Govan et al., 1998). However, unlike typical *M. plutonius* strains, they can grow independently of the Na:K ratio in the medium, implying that *M. plutonius* is a more heterogeneous species than reported previously. However, the taxonomic position of *M. plutonius*-like organisms was unclear. In addition, it has not been experimentally demonstrated yet whether the organisms can really cause EFB in honeybee larvae. In this study, we therefore investigated in detail the phenotypic characteristics of 33 *M. plutonius* and *M. plutonius*-like strain/isolates and determined their taxonomic position by molecular approaches. Moreover, we analyzed the genetic diversity of the isolates by pulsed-field gel electrophoresis (PFGE) and investigated the virulence of representative isolates by experimental infections of artificially reared honeybee larvae.

**Materials and methods**

**Bacterial strain/isolates, culture media, growth conditions, and biochemical tests.** Thirty-three *M. plutonius* and *M. plutonius*-like strain/isolates were used in this study. Thirty-one isolates (12 *M. plutonius* and 19 *M. plutonius*-like) were isolated from diseased larvae of *Apis mellifera* in Japan and two were isolated in the UK (type strain ATCC 35311) and Paraguay. The formulas of the culture media are shown in Table 1. For cultural characterization, bacteria were cultured on various media at 37°C for a week under aerobic, air plus 5 % CO₂ and anaerobic conditions. Unless otherwise stated, bacteria were cultured on KSBHI at 37°C under anaerobic conditions.
for other analyses. Biochemical tests were performed using API 20A (bioMerieux), Rapid ID 32A (bioMerieux) and ID-Test HN-20 (Nissui Pharmaceutical Co., Ltd.).

**Genomic DNA extraction.** Bacterial cells were harvested from KSBHI, suspended in 500 µl 5 x TE [50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0)] containing 5 mg/ml lysozyme and 40 U/ml mutanolysin, and incubated for 1 h at 37°C. After the addition of 20 µl of 10% sodium dodecyl sulfate, the mixtures were extracted with phenol, phenol-chloroform-isoamyl alcohol (25:24:1) (PCI) and chloroform at least once, three times and once, respectively. Nucleic acid was then precipitated by ethanol, rinsed with 70% ethanol and suspended in 100 µl TE. For DNA-DNA hybridization, the extracted DNA was further treated with 50 µg/ml RNase at 37°C for 1 hour and then 200 µg/ml proteinase K at 37°C for 1 hour, extracted with PCI and chloroform, and precipitated by ethanol.

**16S rRNA gene sequence analysis and DNA-DNA hybridization.** The 16S rRNA genes were amplified using primers F1 (5'-GAGTTTGATCCTGGCTCAG-3') and R13 (5'-AGAAAGGAGGTGATCCAGGC-3'). The amplified fragments were sequenced by a BigDye terminator v3.1 cycle sequencing kit using a 3130xl Genetic Analyzer (Applied Biosystems). DNA-DNA hybridization was carried out at 37°C according to the microplate method (Ezaki et. al., 1989; Kusunoki et. al., 1991).

**PFGE.** For genomic DNA extraction, bacterial cells on KSBHI were harvested, washed with Tris-saline buffer [10 mM Tris-HCl (pH 8.0), 1 M NaCl], and suspended in EDTA–sarcosine buffer (6 mM Tris-HCl, 1 mM NaCl, 100 mM EDTA, 1 % sodium N-lauroylsarcosine; pH 7.6). The suspension was mixed with an equal volume of 1.0 % SeaKem Gold agarose (Lonza Rockland) in EDTA–sarcosine buffer and allowed to solidify in a 0.7-mm sample plug caster (Bio-Rad). The sample plugs were incubated in lysis buffer [0.5 M EDTA (pH 8.0), 2.5 mg/ml lysozyme, 10 U/ml mutanolysin] for 3 h at 37 °C, followed by incubation in proteinase K solution [0.5 M EDTA (pH 8.0), 1 % sodium N-lauroylsarcosine, 1 mg/ml proteinase K] for 18 h at 50 °C. The samples were then treated twice with 1 mM Pefabloc SC (Roche) in TE for 30 min at 50 °C and washed three times with TE at 4 °C. Genomic DNA contained in each plug was then incubated in restriction enzyme buffer for 30 min at 4 °C, digested with 20 U Smal (Takara Bio) for 18 h at 30 °C and separated on a 1.0 % SeaKem Gold agarose gel in TBE buffer supplemented with 50 µM thioreua using a CHEF-DR II System (Bio-Rad) (6 V/cm with pulse times of 2.9–17.3 s for 20 h at 15 °C). A dendrogram was produced by BioNumerics software, version 5.1 (Applied Maths) using the unweighted pair group method with the average linkage algorithm.

**Experimental infection of honeybee larvae.** Honeybee larvae (<24 h of age) were collected from A. mellifera colonies at National Institute of Livestock and Grassland Science and reared with artificial diet (50% royal jelly, 37% sterile distilled water, 6% D-glucose, 6% D-fructose and 1% yeast extract) at 35°C. Larvae were fed for first 24 h with artificial diet containing M. plutonius (approximately 5 × 10⁴ CFU/larva) and the mortality of larvae was observed under a stereomicroscope every day. Thirty-five larvae were used to calculate the survival rate in each group and the experiment was performed for 5 days. Differences in the survival rate of larvae were statistically analyzed by the log-rank test. For comparison, P <0.05 was considered significant.
Results

Phenotypic differences between *M. plutonius* and *M. plutonius*-like organisms. As reported previously (Bailey, 1957b; Bailey and Gibbs, 1962; Bailey and Collins, 1982; Allen and Ball, 1993), *M. plutonius* type strain ATCC 35311 and all typical *M. plutonius* isolates were fastidious in their culture requirements. On media supplemented with potassium phosphate (Medium 1, KSBHI), they grew well under anaerobic conditions (Table 1). However, in the absence of potassium phosphate (Medium 2, BHI) or when the potassium phosphate in Medium 1 was replaced with sodium phosphate (Medium 6), growth became weak or was inhibited completely (Table 1). In addition, under aerobic conditions, these strain/isolates did not grow in any of the media tested (Table 1). In contrast, the culture requirements of *M. plutonius*-like isolates were not fastidious. Under anaerobic condition, they grew well not only on media supplemented with potassium phosphate (Medium 1, KSBHI), but also on media not supplemented with potassium phosphate (Medium 2, BHI) or supplemented with sodium phosphate (Medium 6). Moreover, the *M. plutonius*-like isolates grew on various media even under aerobic and air plus 5% CO₂ conditions (Table 1) (Arai et al., 2012).

Biochemical characteristics were also different between *M. plutonius* and *M. plutonius*-like organisms. Although *M. plutonius* strain/isolates produced acid from glucose, fructose, and D-mannose, they did not utilize the other carbohydrates tested. In contrast, in addition to the three sugars, all *M. plutonius*-like isolates produced acids from L-arabinose, D-cellobiose, and salicin. Moreover, only *M. plutonius*-like isolates, but not *M. plutonius* strain/isolates, were positive for β-glucosidase activity and hydrolyzed esculin (Arai et al., 2012).

Table 1. Culture characteristics of *M. plutonius* and *M. plutonius*-like strain/isolates used in this study.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Culture conditions</th>
<th>ATCC 35311</th>
<th><em>M. plutonius</em> isolates</th>
<th><em>M. plutonius</em>-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 1 (1% Yeast extract, 1% glucose, 1% soluble starch, 0.1 M KH₂PO₄, 1.5% agar, pH 6.6)</td>
<td>anaerobic air + 5% CO₂ aerobic</td>
<td>+⁵</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Medium 2 (Medium 1 without KH₂PO₄)</td>
<td>anaerobic air + 5% CO₂ aerobic</td>
<td>-</td>
<td>-</td>
<td>+ or +⁵</td>
</tr>
<tr>
<td>Medium 6 (Medium 1, in which KH₂PO₄ is replaced with 0.1 M NaH₂PO₄•2H₂O)</td>
<td>anaerobic air + 5% CO₂ aerobic</td>
<td>-</td>
<td>-</td>
<td>+ or +⁵</td>
</tr>
<tr>
<td>KSBHI</td>
<td>Brain Heart Infusion agar supplemented with 0.15 M KHPO₄ and 1% soluble starch</td>
<td>anaerobic air + 5% CO₂ aerobic</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion agar</td>
<td>anaerobic air + 5% CO₂ aerobic</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The growth of ATCC 35311 cultured on KSBHI agar under anaerobic conditions was scored as +. Compared to this growth, more vigorous and weaker growth was scored as +⁵ and +⁵w, respectively. No growth or only trace levels of growth was scored as –.

Taxonomic position of *M. plutonius*-like organisms. The 16S rRNA gene sequences of both *M. plutonius* and *M. plutonius*-like isolates showed more than 99.8% homology with that of ATCC 35311, suggesting that the *M. plutonius*-like organisms also belong to *M. plutonius*. To confirm this result, we selected representative strain/isolates and further analyzed the levels of DNA relatedness by DNA-DNA hybridization. Reciprocal hybridization between ATCC 35311 and *M. plutonius*-like
isolates, as well as between \textit{M. plutonius} and \textit{M. plutonius}-like isolates, showed more than 80% DNA relatedness, whereas DNA relatedness between \textit{M. plutonius}-like isolates and \textit{E. faecalis} type strain NCTC 775 was only approximately 10% (Arai et. al., 2012). These results confirmed that \textit{M. plutonius}-like organisms were taxonomically identical to \textit{M. plutonius}. We hereafter refer to \textit{M. plutonius} and \textit{M. plutonius}-like organisms as typical and atypical \textit{M. plutonius}, respectively.

**Genetic diversity of \textit{M. plutonius} isolates.** PFGE analysis showed that typical \textit{M. plutonius} strain/isolates had similar PFGE profiles (more than 83.77% similarity) and formed a single PFGE cluster (Fig. 1). Atypical \textit{M. plutonius} isolates also had similar PFGE profiles (more than 78.81% similarity) and formed another single cluster (Fig. 1). However, the profiles were clearly different from those of typical \textit{M. plutonius}, demonstrating that phenotypically distinct \textit{M. plutonius} isolates have distinct genetic backgrounds (Arai et. al., 2012).

![Fig. 1. Dendrogram of Smal-digested PFGE profiles of typical and atypical \textit{M. plutonius} strain/isolates. Phenotypically distinct strain/isolates were also grouped separately into two distinct genetic clusters.](image)

**Experimental reproduction of EFB by atypical \textit{M. plutonius} cultured in vitro.** Although \textit{M. plutonius} is well proven to be the causative agent of EFB (Bailey, 1983), it has not been demonstrated experimentally that atypical \textit{M. plutonius} found in this study can also cause this disease. However, because \textit{M. plutonius} is considered to lose its virulence quickly when cultured artificially in laboratory media \textit{in vitro}, experimental reproduction of EFB by well-characterized isolates is extremely difficult. To investigate whether \textit{M. plutonius} isolates characterized in this study still show virulence in honeybee larvae, we performed experimental infections of honeybee larvae using representative \textit{M. plutonius} isolates subcultured approximately six times.

Artificially cultured typical \textit{M. plutonius} isolate DAT606 did not cause EFB at all. All survived larvae were well grown, and the mortality was comparable to that in the control group (log-rank test, \(P = 1\)) (Table 2). On the other hand, when larvae were fed with a diet containing artificially cultured atypical \textit{M. plutonius} isolate DAT561, all larvae stopped growing at day 2 or 3, and 94.3% of larvae died within 5 days (Table 2). Mortality was significantly higher than in control and DAT606-inoculated groups (log-rank test, \(P < 0.0001\)) (Arai et. al., 2012). Similar results were also observed when other typical and atypical isolates were used (data not shown). To confirm \textit{M. plutonius} infection in larvae,
we randomly selected more than 11 larvae from each group and isolated *M. plutonius* on KSBHI agar. As expected, no bacteria were isolated from larvae in the control group. In contrast, a large number of atypical *M. plutonius* were isolated from dead larvae in the DAT561-inoculated group (1.00 x 10^6 to 2.93 x 10^7 CFU/larva). Interestingly, numerous typical *M. plutonius* were also isolated from all well-grown larvae in the DAT606-inoculated group tested (5.88 x 10^6 to 1.65 x 10^8 CFU/larva) (Arai et. al., 2012). These results demonstrated that atypical *M. plutonius* has the ability to cause EFB in honeybee larvae and that, unlike typical *M. plutonius*, virulence can be maintained even after repeated subculture in laboratory media.

**Table.2. Experimental Infection of Honeybee Larvae.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of surviving larvae</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>Control</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Typical isolate (DAT606)-inoculated</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Atypical isolate (DAT561)-inoculated</td>
<td>35</td>
<td>35</td>
</tr>
</tbody>
</table>

**Summary and Conclusions**

In this study, we demonstrated that *M. plutonius* is a more heterogeneous species than believed so far and that artificially cultured atypical *M. plutonius* isolates can cause EFB in honeybee larvae. To our knowledge, the atypical isolates found in this study are the first *M. plutonius* that can maintain virulence even after repeated subculture *in vitro*, so our discovery is a major breakthrough for future research on the pathogenesis of this important honeybee disease.

**Acknowledgements**

We thank S. Kobayashi for suggestions on statistical analysis. Parts of this study were supported by a Grant-in-Aid for Scientific Research (C) (22580345) from the Japan Society for the Promotion of Science to D. Takamatsu and a grant from Promotion of Basic Research Activities for Innovative Biosciences (PRO-BRAIN) to M. Yoshiyama. All data presented in this study have been reported in our previous publication (Arai et. al., 2012).

**References**


Bailey, L. (1957b). The isolation and cultural characteristics of *Streptococcus pluton* and further observations on *Bacterium eurydice*. Journal of General Microbiology, 17, 39-48.


Parasitic infection of honeybees (Apis spp.) in upper northern region of Thailand

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¹Veterinary Research and Development Center (Upper Northern Region), Lampang 52190, Thailand

Abstract

Background: The greatest amounts of honey as well as honeybee products are produced from northern region of Thailand for both domestic consumption and exports. Honeybees are affected by a number of diseases, four of which are notifiable diseases according to World Organisation of Animal Health; those are nosemosis, varroosis, tropilaelaps infestation and acarapisosis. For the internal parasites, Nosema spores which germinate in the midgut of adult honeybees cause the shortened lifespan and losses in honey production whereas Acarapis woodi induces obstruction of air ducts. The external parasites, both Varroa and Tropilaelaps mites cause larval malformation and wing deformities. Therefore, the improving of general health of honeybee stocks and subsequent could be considered in order to amend quality and maximize honey yields. This study revealed the parasitic situation of honeybees in upper northern region of Thailand.

Method: A hundred of adult honeybees per sample were collected from apiary and submitted to Veterinary Research and Development center (Upper Northern Region). They were submersed in 70% alcohol for 60 minutes to separate mites. Varroa and Tropilaelaps mites were inspected under microscope. About 50 adult honeybees were dissected in order to tracheal inspection for Acarapis spp. Moreover, 20 honeybee abdomens were separated and ground in 3 ml of sterile water, smeared on glass slide and stained with Giemsa’s for Nosema spores examination.

Results: From total of 274 honeybee samples, Varroa, Tropilaelaps and Nosema were found at 25.18, 15.33 and 53.28%, respectively. Furthermore, Acarapis woodi was not found in any sample.

Conclusions: Nosema spores were found in highest percentage, however, it can also be found in normal honeybees. Interestingly, both Varroa and Tropilaelaps mites which are more harmful to bee broods were found in the lower percentage. Therefore, these results should be concerned for honeybee health control.

Keywords: honeybees, nosemosis, varroosis, tropilaelaps infestation, acarapisosis

Background

Honeybees (Apis mellifera) are social insects; they live in family groups called colonies. In Thailand, 200,000 beehives are kept in 2,500 apiaries, mostly in the upper northern region since it is suitable for beekeeping due to succession of floral sources such as longan, lychee and wild flowers (Ratananakorn et al., 2008). The registered bee apiaries are mostly located in Chiang Mai, Chiang Rai, Lamphun and Nan. Estimate amount of honey produced in the upper northern region is account for more than 90% of country grand total (Bureau of Livestock Standards and Certification, 2012). Despite of honey, other honeybee products such as royal jelly, bee pollen, propolis and beeswax make a lot of profit for beekeepers.

Honeybees are affected by a number of diseases, four of which are notifiable diseases according to World Organisation of Animal Health (OIE); those are nosemosis, varroosis, tropilaelaps infestation and acarapisosis. Nosemosis cause by microsporidian Nosema spp., its spore is 5 to 7 micrometers
in length and 3 to 4 micrometers in width, germinate in the midgut of adult honeybees cause diarrhea in mild case to shorten lifespan and losses in honey production in severely infection. Infection occurred via contaminated water and food ingestion, food exchange with other bees or contaminated comb cleaning (Lotfi et al., 2009). However they may be found in normal honeybee, especially inadequate nutrition supporting (Bailey, 1968). Acarapis woodi or tracheal mite is approximately 150 micrometers in length and 70 micrometers in width (Sammataro et al., 2000). It causes acarapisosis, in respiratory system, mainly in large prothoracic trachea. In heavy infection, mites induce obstruction of air ducts and tracheal walls become opaque or darken due to mite infestation. There is no report of this mite in Thailand (Vongpakorn and Neramitmansook, 2003). The external parasites, both Varroa and Tropilaelaps mites, enter the honeybee brood cells shortly before sealed, cause larval malformation and wing deformities. Varroa mites have reddish brown color with oval and flat in shape approximately 1.1 millimeters length and 1.5 millimeters width (Sammataro et al., 2000). Varroa mites that affect honeybee worldwide were identified as V. jacobsoni and V. destructor (OIE, 2008). Tropilaelaps disease, caused by Tropilaelaps spp. is elongated light brown mite approximately 1.0x0.5 millimeters in size (Delfinado and Baker, 1961).

The government realizes the important of food safety and food standard for consumer protection and trade competition. The committee on agricultural commodity and food standards announced Good Agricultural Practices for bee farm involving bee health management (The National Bureau of Agricultural Commodity and Food Standards, 2003). According to regulations, general health of honeybee stocks and subsequent are improved in order to amend quality and maximize honey yields and bee product. This study revealed the parasitic situation of honeybees in upper northern region of Thailand.

**Materials and methods**

**Study area and Samples**

Two hundred and seventy-four samples were collected from 7 provinces in upper northern region; Chiang Mai, Chiang Rai, Lampang, Lamphun, Nan, Phare and Phayao, during September 2008 to August 2011.

A hundred of adult honeybees per sample were packed, tightly sealed in a plastic container and submitted to Veterinary Research and Development center in cool condition.

**Parasitic detection**

For Varroa and Tropilaelaps mite examination, honeybees were submersed in 70% alcohol for 60 minutes, after that bees were removed, dried on paper towel and brushed to separate remaining mites. Alcohol submersed and separated mites were examined under stereomicroscope.

For Acarapis woodi, about 50 honeybees were dissected and their tracheas were observed under stereomicroscope. Nosema spores were examined by homogeneous grinding of 20 honeybee abdomens in 3 milliliters sterile water. This suspension was circular smeared two circles per sample on glass slide and air-dried overnight. The smear was fixed with absolute methanol, stained with 5% Giemsa’s and examined under oil immersion.

**Results**

From the total of 274 samples, Varroa, Tropilaelaps mites and Nosema spores were found at 25.18, 15.33 and 53.28%, respectively. A. woodi was not found in any sample. Varroa was presented mostly in Nan and Lamphun of 40.91 and 37.93%, respectively whereas Tropilaelaps
was highly revealed in Lampang 57.14%. Nosema was remarkably detected in Phrae, Chiang Mai and Chiang Rai of 78.57, 69.41 and 54.26%, respectively (Table I).

### Table I  Honeybee parasitic infection in upper northern region during September 2008 to August 2011

<table>
<thead>
<tr>
<th>Provinces</th>
<th>Samples</th>
<th>Varroa (%)</th>
<th>Tropilaelaps (%)</th>
<th>Nosema (%)</th>
<th>Acarapis woodi (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiang Mai</td>
<td>85</td>
<td>17 (20.00)</td>
<td>15 (17.65)</td>
<td>59 (69.41)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Chiang Rai</td>
<td>94</td>
<td>17 (18.09)</td>
<td>17 (18.09)</td>
<td>51 (54.26)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lampang</td>
<td>7</td>
<td>2 (28.57)</td>
<td>4 (57.14)</td>
<td>2 (28.57)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lamphun</td>
<td>29</td>
<td>11 (37.93)</td>
<td>0 (0)</td>
<td>9 (31.03)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nan</td>
<td>44</td>
<td>18 (40.91)</td>
<td>3 (6.82)</td>
<td>13 (29.55)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Phare</td>
<td>14</td>
<td>4 (28.57)</td>
<td>3 (21.43)</td>
<td>11 (78.57)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Phayao</td>
<td>1</td>
<td>0 (0.00)</td>
<td>0 (0)</td>
<td>1 (100.00)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>274</td>
<td>69 (25.18)</td>
<td>42 (15.33)</td>
<td>146 (53.28)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

This study also revealed mixed infection of Varroa and Tropilaelaps (2.92%), Varroa and Nosema (8.39%), Tropilaelaps and Nosema (5.11%), and Varroa, Tropilaelaps and Nosema (1.82%) (Table II).

### Table II  Mixed parasitic infection of honeybees in upper northern region during September 2008 to August 2011

<table>
<thead>
<tr>
<th>Provinces</th>
<th>Samples</th>
<th>Varroa + Tropilaelaps (%)</th>
<th>Varroa + Nosema (%)</th>
<th>Tropilaelaps + Nosema (%)</th>
<th>Varroa + Tropilaelaps + Nosema (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiang Mai</td>
<td>85</td>
<td>3 (3.53)</td>
<td>9 (10.59)</td>
<td>6 (7.06)</td>
<td>1 (1.18)</td>
</tr>
<tr>
<td>Chiang Rai</td>
<td>94</td>
<td>1 (1.06)</td>
<td>4 (4.26)</td>
<td>6 (6.38)</td>
<td>3 (3.19)</td>
</tr>
<tr>
<td>Lampang</td>
<td>7</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lamphun</td>
<td>29</td>
<td>0 (0)</td>
<td>4 (13.79)</td>
<td>0 (0)</td>
<td>1 (14.28)</td>
</tr>
<tr>
<td>Nan</td>
<td>44</td>
<td>3 (6.82)</td>
<td>4 (9.09)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Phare</td>
<td>14</td>
<td>1 (7.14)</td>
<td>2 (14.28)</td>
<td>2 (14.28)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Phayao</td>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>274</td>
<td>8 (2.92)</td>
<td>23 (8.39)</td>
<td>14 (5.11)</td>
<td>5 (1.82)</td>
</tr>
</tbody>
</table>

### Discussion

In this study, all parasites were found in higher percentage than previous study of Vongpakorn and Neramitmansook (2003) due to longer period of data collection (three years versus 4 months). Nosema is a distinct infection with highest percentage may because of persistent of spore in fluctuated environment. There is a report that Nosema spore can remain viable in the colonies for several months (Coffey, 2007). Nosema spreading relies on climate, humidity, population density and colony vulnerability (Lotfi et al., 2009).

Varroa and Tropilaelaps were found in lower percentages. Varroa was more detected since it could live on adult bee for several months. There were reported that V. destructor was identified in all apiaries in western Romania, 72% of adult bee samples and 96% of the samples of bees brood (Balint et al., 2011). The same result was reported from Ontario, Canada that 75.7% of the colonies were infested (Guzmán-Novoa et al., 2010). Whereas, Tropilaelaps mites are mobile and can readily move between bees within a colony, thus, it is quite difficult to examine the mites on adult bee (Coffey, 2007). Then, colony, brood and debris were considered to collect for further study.

Fortunately, there was no report of A. woodi in Thailand as same as this study.
The mixed infection was observed in this study as same as the previous report that found two mixed infection only in northern Thailand (Vongpakorn and Neramitmansook, 2003). In addition, this study found three mixed infection from apiaries in Chiang Rai, Chiang Mai and Lampang. However, the severity of the mixed infection remains doubtful.

Nosema can detect from all provinces with higher than 50% in Phrae, Chiang Mai and Chiang Rai. Varroa was mainly reported from Lamphun and Nan, while Tropilaelaps was mostly found in Lampang. The result of Phayao could not be discussed because only one sample was submitted. This is the primary data for beekeepers and responsible authorities for monitor and control of parasitic infection. However, this qualitative study could not indicate the severity of infection, quantitative testing should be done in further study.

References


Transcriptome analysis on the asexual second-generation development of Eimeria tenella in the chicken ceca using specific DNA microarray

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Abstract

Avian Eimeria spp. belong to the phylum Apicomplexa which includes many important pathogenic protozoa such as Plasmodium, Toxoplasma, and Cryptosporidium. Coccidiosis caused by Eimeria spp. is characterized by watery or bloody diarrhea. It is a fatal disease and thus, has a major impact on the poultry industry worldwide. Resistance to all of the anticoccidial drugs designed for commercial use so far has stimulated the search for new control methods. Previously, we showed synchronous development of E. tenella during homoxenous life cycle, consisting of three asexual and sexual stages in chicken ceca. We describe the use of laser microdissection (LMD) for isolation of second-generation schizonts from tissues during the most pathogenic stage of infection. We successfully synthesized cDNA from purified mRNA and constructed an E. tenella-specific DNA microarray using EST sequences registered in the database. We used probe hybridization to show that genes of the energy metabolism-, transporter- and microneme-proteins as well as heat shock proteins were up-regulated. Some proteases and protease inhibitors were also up-regulated in the later development stages to mature schizonts or formation of merozoites. Taken together, the LMD technique could be useful for gene expression analyses of the intracellular stages of Eimeria spp. Identification of changes in gene expression may improve the understanding of the developmental processes of E. tenella and reveal new targets for controlling coccidiosis. To our knowledge, this is the first report of transcriptome analysis of developing schizonts.

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Background

Eimeria spp. are enteropathogens that can infect a variety of mammals and birds. Coccidiosis in poultry is caused by infection with parasites of one or more Eimeria species. Currently there are seven Eimeria spp. that can infect chickens, namely E. tenella which is parasitized in the ceca, E. maxima in the entire small intestine, E. mitis in the duodenum and jejunum, E. acervulina in the anterior region of the small intestine, E. necatrix in the small intestine and ceca, and E. praecox and E. brunetti in the ileum and colon. The parasites have their own characteristics with respect to prevalence, pathogenicity, immunogenicity as well as site of infection. These infectious diseases cause diarrhea, morbidity and
mortality leading to major economic losses in the poultry industry worldwide (Shirley et al., 2005).

Among the seven species of *Eimeria*, *E. tenella* has the highest virulence, depending on its intracellular development. After ingestion of sporulated oocysts by chickens, four sporocysts containing two sporozoites are released into the intestine. After excystation, motile infective sporozoites actively enter the epithelial cells of the ceca. As three rounds of asexual multiplication, the first generation of schizonts following trophozoites develop in crypt epithelial cells. The development of second-generation schizonts occurs in the lamina propria where rapid maturation leads to more than 100 large-sized merozoites (>40 µm). Release of these merozoites causes severe tissue destruction and bloody feces. This second-generation stage is thought to be associated with the induction of pathogenicity of *E. tenella* (Fernando, 1982; McDonald and Shirley, 1987). Differentiation to sexual stages of micro- and macrogametocytes occurs in the epithelium or submucosa, followed by the third asexual multiplication (McDonald and Rose, 1987). Finally, oocysts followed by zygotes are formed resulting in release in the feces.

To date, few studies focusing on the second-generation schizont stage of *E. tenella* infection have been performed. One possible reason is that methods to isolate and purify schizonts at this stage are not available. Thus, to understand the pathogenicity of *E. tenella*, we applied laser microdissection (LMD) to isolate the schizonts from the lamina propria. Following cDNA synthesis reverse transcription polymerase chain reaction (RT-PCR) was evaluated and we analyzed gene expression profiles during the development of the second-generation schizonts.

**Materials and methods**

**Parasites**

The *E. tenella* NIAH strain, which is virulent and maintained at the Laboratory of Parasitic Diseases, National Institute of Animal Health (Tsukuba, Ibaraki, Japan), was used. *E. tenella* cells were purified by the sugar flotation method, sporulated at 28 °C in 2.5% potassium dichromate, and stored at 4 °C for up to one month before use.

**Histological examination**

Twenty-four two-week-old chicks were orally inoculated with $2 \times 10^4$ sporulated oocysts in 0.1 ml of distilled water, anesthetized and killed by cervical dislocation from 48 h to 132 h at 12-h intervals. The ceca were removed and fixed in 10% buffered formalin at room temperature for one week. The medial region of the ceca was embedded in paraffin, cut as cross sections at a thickness of 4 µm and stained with hematoxylin and eosin. Chicks were treated in accordance with protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 10-009, 11-026).

**LMD and RT-PCR**

For isolation of the second-generation schizonts using LMD, ceca were removed at 84 h and 96 h after inoculation, and washed with cold phosphate-buffered saline (PBS). The medial regions of these samples were embedded in OCT (Sakura Finetek), frozen, sectioned at 10 to 20 µm using a cryostat, and mounted on membrane slides for LMD (LMD6000, Leica Microsystems). Sections were fixed using ethanol-acetic acid (19:1; v/v) for 3 min on ice, washed with RNase-free water for 1 min, and stained with 0.1% toluidine blue for 30 s. All steps were carried out under RNase-free conditions on ice. We morphologically differentiated four types of second-generation schizonts under a microscope, 1, <10 µm early stage; 2, 10-25 µm small immature schizonts; 3, 25-30 µm large immature schizonts; 4, >30 µm mature schizonts. These four types of schizonts in lamina propria were cut out into each tube by LMD, and mRNAs from each sample were collected.
For RT-PCR, total RNA was extracted from four types of collected schizonts using an RNeasy Mini kit (QIAGEN Sciences). RNA concentration and degradation were assessed from the band densities in the bioanalyzer gel-like image (Agilent Technologies) (Roberts et al., 2009). Total RNA was converted into cDNA with a Takara RNA PCR kit (AMV) (Takara Bio Inc.). First strand reactions were used in PCR amplifications with oligonucleotides specific for the *E. tenella* actin gene (Ryan et al., 2000) and *E. tenella* microneme protein gene (Ding et al. 2005), of which the predicted sizes were 350 bp and 1,100 bp, respectively.

**Microarray analysis**

We constructed an *E. tenella* specific DNA microarray using 3,192 EST sequences registered in the NCBI database, and array hybridization was performed according to the manufacturer's instructions (Filgen, Inc.). Up-regulated or down-regulated genes were detected by the fluorescence signal intensity of hybridized microarrays using GenePix® 4000B (Molecular Devices).

**Results**

Various sizes of schizonts were observed with small second-generation schizonts observed in the lamina propria from 60 h, increasing in size and number from 84 h to 108 h post infection (Fig. 1). Small third-generation schizonts were present at 96 h and gametocytes appeared in the superficial areas of the villi around crypts at 108 h.

Four types of second-generation schizonts were successfully excised from the lamina propria using LMD at 84 h and 96 h after inoculation. Extracted total RNA was estimated to contain about 1 µg of RNA with an RNA integrity score around 6 (from 10; highest integrity to 2; lowest integrity). Two distinct peaks indicated that the RNA was not degraded. Using RT-PCR, mRNA specific for the *E. tenella* actin and microneme 2 genes were successfully amplified.

**Figure 1.** The second-generation schizonts developing in chicken ceca. 1, <10 µm early stage schizonts (60 h); 2, 10-25 µm small immature schizonts (84 h); 3, 25-30 µm large immature schizonts (84 h); 4, >30 µm mature schizonts (108 h).

Up- or down-regulated genes were determined using microarray analysis of cDNAs of four types of schizonts. Consequently, 71 genes were found to be up-regulated during schizont development from early immature to mature schizonts. Up-regulated genes in the early development of schizonts were found to be involved in energy metabolism, transporter, microneme proteins, and heat shock proteins. Some proteases and protease inhibitors were up-regulated during later development, and more than 20 surface antigens of *E. tenella* expressed at each developmental stage were identified.
Discussion

Analysis of the second-generation schizont stage has rarely been conducted to date. In this study, we clarified the synchronous development of sexual and asexual stages of *E. tenella*. We applied the LMD method to isolate only the second-generation schizonts from the lamina propria in the ceca. The purified RNA was not degraded, and *E. tenella* specific genes were successfully amplified by RT-PCR. Therefore, this application is useful for transcriptome analysis, including microarray.

Using microarray analysis, we identified genes that are up-regulated during schizont development. Most up-regulated genes were related to cell division, and genes such as microneme proteins which are associated with cell-invasion of merozoites, were also identified. The results suggest that protozoa specific proteases could play an important role in promoting cell development. These proteases often have low homology with mammalian, including human, proteases. Although our data is preliminary, these detected genes or molecules might be useful targets for controlling coccidiosis in chickens. These results are also useful for understanding the developmental process of *E. tenella*.

Acknowledgement

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References


Vaccine matching strain characterization of foot and mouth disease virus in South East Asia during 2010-2012

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Abstract

The vaccine matching strain characterization of foot and mouth disease virus type O and A causing outbreak in Thailand, Cambodia, Lao PDR, Vietnam, Myanmar and Sri Lanka during 2010 to 2012 was investigated. The result was expressed as r-value by determining the serological relationship between field virus isolate and reference virus vaccine strain. The r-value of FMDV type O from Thailand, Cambodia, Lao PDR and Vietnam demonstrated very close antigenic relationship to vaccine strains with O189/87. It is therefore suggested that the existing seed vaccine strains for type O need not to be changed. In contrast, the r-value of type A causing outbreak in Thailand demonstrated a close antigenic relationship to A/Sakolnakorn/97 than A118/87 current vaccine strain. This study indicated that recent type A virus gave antigenic diverse from A118/87 vaccine strain, therefore, it was recommended to put an additional of A/Sakolnakorn/97 vaccine in the existing trivalent vaccine production in order to give a wide broader protection of type A outbreak in the field. In conclusion, the recent field outbreak of type O in South East Asia (SEA) indicated that no antigenic variation has been found, while the antigenic variation of type A has been found only in Thailand, due to less FMD sample of type A from SEA country was submitted to the laboratory at that period. However this study would be useful in supporting the selection of appropriate virus vaccine strain for production of high efficacy vaccine and enhancing the efficiency of the strategic planning for FMD control in SEA region.

Background

Foot and mouth disease, a highly contagious disease of cloven-hoofed animals, is important in South East Asia region. Serotypes O, A and Asia1 are considered endemic in Thailand and South East Asia (SEA), causing significant economic losses primarily due to lower production of affected animals and subsequent constrain of international trading. Rapid and accurate diagnosis plays an important role in the prevention of disease spread and can ensure that appropriate vaccines are selected for use against circulating filed strains. Standard ELISA typing test for type identification of field samples (Roeder and Le Blance Smith, 1987) and other serological tests including virus neutralization (VN) test (Rweyemamu,1978), liquid phase blocking ELISA (LP ELISA) (Hamblin et al.,1986) in parallel with ELISA-non structural protein (ELISA-NSP) (Linchongsubongkoch et al., 2008), can be used to support disease surveillance and sero-monitoring of vaccinated and infected animals. In addition, strain differentiation investigation by vaccine matching study of field viruses and reference vaccine
strains become a very important in supporting a scientific information of the antigenic variation in
FMD field outbreak strains. The vaccine matching investigation was carried out by determination
of the serological relationship (r-value) between filed virus strains and the reference vaccine strains
and is useful for selecting the appropriate virus strain for vaccine production and enhancing the
vaccination strategic of FMD control at the national and regional level. Linchongsubongkoch et al.
(2008) reported that the r-value of type O causing outbreak during 1997-2007 and 2008-2009 (data
not published) indicated that the serological relationship was close to O189/87 vaccine strain, while
type A showed antigenically changed from time to time, it was indicated that the type A field viruses
causing outbreak during 2001-2007 and during 2008-2009 were closely related to A118/87 vaccine
strain. Therefore, in this study, a number of field isolate viruses type O and A causing outbreak in SEA
region during 2010-2012 were studied for updating the epidemiological information and recent FMD
antigenic variation by determining the serological relationship (r-value) between field virus strains and
the reference virus vaccine which would be useful to support the selection of appropriate virus strain
for vaccine production and control of FMD in SEA region.

Materials and methods

1. Reference viruses and field viruses

Reference viruses vaccine strain O189/87, A118/87 and A/Sakolnakom/97 were obtained from
current seed vaccine strains. Field samples from FMD infected animals submitted for laboratory diagnosis
which were from Cambodia, Lao PDR, Myanmar, Vietnam, Sri Lanka and Thailand during 2010-2012
which subjected for serotype identification using standard ELISA typing and the virus isolation test by
inoculating to primary lamb kidney cell for 2-3 passages and further 4 or 5 passages in BHK-21 cell line.
Then cell culture supernatant fluid was again confirmed by antigen typing test as described by Roeder
and Le Blanc Smith (1987). The reference vaccine strain and field isolate viruses were titrated by indirect
sandwich ELISA method (Kitching et al., 1988) and selected the working dilution for virus to use in the
liquid phase blocking ELISA (LP ELISA) (Linchongsubongkoch et al., 2000).

2. Bovine antiserum

The reference sera used in the LP ELISA, including bovine anti FMD type O189/87, A118/87
and A/Sakolnakom/97 were prepared from experimental cattle that have been vaccinated with reference
homologous vaccine strain. Blood from vaccinated animals were taken at 21 days post vaccination for
immune sera.

3. Liquid phase blocking ELISA (LP ELISA)

Bovine antiserum against homologous vaccine strain was used to determine antibodies to
FMD virus by LP ELISA. The bovine serum was diluted into two fold dilution series, and then a fixed
concentration of reference vaccine strain and field isolate viruses giving an optical density (OD) in the
range of 1.0 –1.5 were reacted with bovine post vaccination serum with homologous virus of each
serotype. The antibody titer to FMD virus was determined as described by Hamblin et al. (1986), Kitching
et al.(1988) and Linchongsubongkoch et al. (2000).

4. The serological relationship (r-value)

The LP ELISA method has been used to examine the serological relationship between field
isolate viruses and the reference virus vaccine strains which was expressed as r-value.
r-value = Serum titer against heterologous field strain
Serum titer against homologous vaccine strain

The guideline suggestion for r-value obtained by LP ELISA and criteria of interpretation were described by Samuel et al. (1990) as this follows.

- \( r = 0.0-0.19 \) highly significant serological variation from the reference strain
- \( r = 0.20-0.39 \) significant difference from the reference strain, but protection may be satisfactory if using a sufficiently potent vaccine.
- \( r = 0.40-1.0 \) not significantly difference from vaccine strain.

Results

FMDV samples from SEA country causing outbreak during 2010-2012 were submitted to the Regional Reference Laboratory, Pakchong, Thailand for FMD diagnosis, the diagnostic results were shown in table 1. Then, some of them were used for further investigation by vaccine matching test, the result of r-value was shown in table 2, 3 and figure 1.

Table 1. Situation of FMD outbreak in SEA during 2010-2012, diagnostic assay using standard ELISA typing test and virus isolation.

<table>
<thead>
<tr>
<th>Year</th>
<th>Country</th>
<th>No. of sample</th>
<th>Type identification by ELISA typing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>O</td>
</tr>
<tr>
<td>2010</td>
<td>Thailand</td>
<td>48</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Cambodia</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Myanmar</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Vietnam</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>2011</td>
<td>Cambodia</td>
<td>31</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Lao PDR</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Sri Lanka</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Thailand</td>
<td>64</td>
<td>13</td>
</tr>
<tr>
<td>2012 (Feb)</td>
<td>Thailand</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>221</td>
<td>108</td>
</tr>
</tbody>
</table>

NVD* = no virus detected

Table 2. Result of r-value of FMD type O field viruses in Thailand, Cambodia, Lao PDR and Vietnam during 2010-2012, using O189/87 as a homologous system.

<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
<th>Total sample</th>
<th>% r-value range of type O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0-0.19</td>
</tr>
<tr>
<td>Cambodia</td>
<td>2010</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Vietnam</td>
<td>2010</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>Lao PDR</td>
<td>2011</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Thailand</td>
<td>2010</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39</td>
<td>-</td>
</tr>
</tbody>
</table>

* no sample of type O was received in February, 2012
Table 3. Result of r-value of FMDV type A field viruses in Thailand and Myanmar during 2010-2012, using A118/87 and A/ Sakolnakorn/97 as homologous system.

<table>
<thead>
<tr>
<th>Year/Country</th>
<th>Total sample</th>
<th>r-value range by A118/87</th>
<th>r-value range by A/Sakolnakorn/97</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-0.19</td>
<td>0.20-0.39</td>
</tr>
<tr>
<td>2010-Thailand</td>
<td>3</td>
<td>no binding reaction by titration</td>
<td>-</td>
</tr>
<tr>
<td>Vietnam</td>
<td>1</td>
<td>&quot;--------&quot;</td>
<td>-</td>
</tr>
<tr>
<td>Myanmar</td>
<td>2*</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>2011-Thailand</td>
<td>43</td>
<td>no binding reaction by titration</td>
<td>4</td>
</tr>
<tr>
<td>2012-Thailand</td>
<td>2</td>
<td>no binding reaction by titration</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td></td>
<td>4(8.1%) 45(91.9%)</td>
</tr>
</tbody>
</table>

Remark: *FMD type A of Myanmar was not investigated for r-value, due to no virus could be adapted in cell culture.

Figure 1. Result of mean r-value of field isolate virus of type O and A, r-value greater than 0.40 defined as good matching.

Conclusion and discussion

The vaccine matching strain characterization or antigenic variation of FMDV in SEA during 2010-2012 was investigated by determining the serological relationship between the reference vaccine viruses and field viruses, the result was expressed as r-value. Table 2 showed that 100% of field viruses type O from Cambodia, Lao PDR, Vietnam and Thailand gave the r-value greater than 0.40 indicated that the serological relationship was very close to virus vaccine strain O189/87, therefore the current vaccine of O189/87 could give a protection to the circulating viruses in Thailand and the region. While the situation of type A was different, the r-value in table 3 showed the 91.9% of field viruses from Thailand gave the r-value greater that 0.40, and the rest of 8.1% gave the r-value in range 0.20-0.39 to A/Sakolnakorn/97 but not A118/87 system. Similarity result was found in the FMD type A antigenic profiling test demonstrated that no binding reaction to the A118/87 system but distinguished in high binding reaction with A/ Sakolnakorn/97 system (data not published). Therefore, the recent situation of type A field viruses during 2010-2012 have antigenic changed from current vaccine of A118/87 to A/Sakolnakorn/97 that might be resulting from disease outbreak from time to time (Doughty et al.,1995). By the history of type A seed vaccine selection, A/ Sakolnakorn/97 was selected as a new seed vaccine strain and being used from 1997-2001, then again a new seed vaccine strain of A118/87 was selected in 2001 and being used up to the present (Linchongsombongkoch et al., 2008). Interestingly, the vaccine matching result of type A causing outbreak during 2010-2012 was demonstrated the antigenically changed from A118/87 to A/Sakolnakorn/97. In this regard, it was necessary to select a new vaccine strain again, therefore, it was recommended to use the monovalent vaccine of A/Sakolnakorn/97 as an additional vaccine in the existing trivalent vaccine in order to give a wide broader protection to the field outbreak viruses. However,
the nucleotide sequencing of FMD field outbreak was also investigated and analyzed as phylogenetic tree by World Reference Laboratory, (WRL) (data not published). The lineage of FMD type O in SEA region was majority defined as South East Asia (SEA) topotype, Mya98 strain and some was defined as PanAsia strain. It was interesting that FMD sample from Myanmar was submitted to Regional Reference Laboratory at Pakchong, Thailand in October 2010, the initial ELISA typing resulted as type A and unfortunately this virus was not able to adapted in cell culture for further investigation of vaccine matching test. Hence this viral fluid and RNA sample was sent to World Reference Laboratory for FMD, Pirbright Laboratory, UK for sequencing analysis, the phylogenetic tree indicated that type A from Myanmar/2010 was close related to the virus originally from A/India/2000 (IND/2000) (report from WRL, data not published). However FMD type A in Thailand and Vietnam was defined as only one topotype of ASIA (Asia97 strain). This studied would be useful for tracing back to the original virus causing outbreak in the field and to give a molecular epidemiological information in supporting the seed virus selection for vaccine production in enhancing the action plan for FMD control in the SEA region.

Keywords:  FMDV, vaccine matching,  r-value,  LP ELISA

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References


Our efforts toward the establishment of surveillance and diagnosis systems for arboviral diseases in Thailand

A collaborative work among young researchers at NIAH-Thailand and NIAH-Japan

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Abstract

**Background:** Various arthropod-borne viruses (arboviruses) are known to cause damages to human health and/or livestock industry. In Southeast and East Asia, the numbers of livestock have increased in many countries in this decade; however, systems are not well established for surveillance and diagnosis of arbovirus infection in livestock, although many species of arboviruses are thought to be actively circulating among the livestock there. Hence, we agreed to set up a collaborative study on arboviral diseases in Thailand, in order to pursue the goal of establishing a comprehensive system for surveillance and diagnosis of arbovirus infection.

**Method:** We shared information about arboviruses and their vectors through a seminar and discussion at NIAH-Thailand in Aug. 2011. After that, we continued communication by using e-mail and a social networking service run by Japan Science and Technology Agency (JST), “Friends of SATREPS”, and worked together to set up the study.

**Results:** We acquired a budget from JST for a preliminary study (Special Project Formation Investigation for Science and Technology Research Partnership for Sustainable Development: SATREPS). In Feb. 2012, Thai participants of the study were invited to Kyushu research station, NIAH-Japan and several techniques for arbovirus survey, such as serological tests for Akabane and Japanese encephalitis viruses and identification of *Culicoides* biting midges (vectors for bluetongue and Akabane viruses), were transferred to them. In Mar. 2012, we collected *Culicoides* biting midges in cattle farms in Thailand.

**Summary/Conclusions:** A collaborative study on arboviral diseases has just started by young researchers at NIAH-Thailand and NIAH-Japan with the supports of many other staffs. Our next step will include isolation of arboviruses and pathological investigation of field cases in Thailand. We will continue the efforts together toward our goal.

**Keywords:** arthropod-borne virus, bluetongue virus, *Culicoides* biting midges, Japanese encephalitis virus, Akabane virus

**Background**

Various arthropod-borne viruses (arboviruses), such as Japanese encephalitis virus (JEV) Akabane virus (AKAV), and bluetongue virus (BTV), are known to cause damages to human health and/or livestock industry (Forman et al., 2008; Hateley, 2009; Mackenzie et al., 2004; OIE, 2008). In Japan, JEV,
AKAV, BTV and some other arboviruses become epidemic year after year (Arai et al., 2008; Forman et al., 2008; Goto et al., 2004; Kono et al., 2008; Shirafuji et al., 2012). Former reports have indicated that these viruses are also distributed in other Asian countries of tropical, subtropical and temperate zones (Forman et al., 2008; Lee et al., 2010; Ting et al., 2005). We speculate that the viruses have widely spread in these areas and sometimes have been introduced from neighboring countries with the infected vectors. In fact, our preliminary studies suggested that several arboviral epidemics widely spread across national borders (Kato et al., 2009). Therefore, the impact of arboviral infections in livestock should be recognized as a common issue among the affected Asian countries. Consistent with economical development and population growth, the size of livestock production has dramatically increased in tropical and subtropical Asia in this decade. Although the prevalence of many species of arboviruses implies the potential losses in livestock industry, little is known about the epidemiology of arboviral infections and the biology of their vectors in these areas. Also, systems are not well established for surveillance and diagnosis of arboviral infections in many countries. Therefore, we agreed to set up a collaborative study on arboviral diseases in Thailand, in order to pursue the goal of establishing a comprehensive system for surveillance and diagnosis of arboviral infection. Our hope is that our efforts will provide valuable information for reducing economic losses in Asian livestock industry.

Materials and methods

We shared information about arboviruses and their vectors through a seminar and discussion at NIAH-Thailand in Aug. 2011. At that time, a long-term vision was also come up for establishing a comprehensive system for surveillance and diagnosis of arboviral infection in Thailand (Fig. 1). After that, in order to form the project, we continued communication by using e-mail and a social networking service on “Friends of SATREPS” organized by Japan Science and Technology Agency (JST).

Results

We acquired a budget from JST for a preliminary study (Special Project Formation Investigation for Science and Technology Research Partnership for Sustainable Development: SATREPS), and the budget period was from Nov. 2011 to Mar. 2012. In Feb. 2012, Thai participants of the study were invited to Kyushu research station, NIAH-Japan, and several techniques for arbovirus survey were transferred to them. The participants were divided into two groups; some serological and virological techniques were transferred to the first group of 3 participants (Fig. 2), and some techniques for identification of Culicoides biting midges (vectors for AKAV and BTV) and detection of viral genes were transferred to the second group of 4 participants (Fig. 3). In addition, all the invited Thai participants had discussion with Japanese researchers, such as detailed schedule and sites for collection of Culicoides biting midges in Thailand, required equipments and reagents for arbovirus survey, and concerns about arboviral diseases of livestock in Thailand. In Mar. 2012, we collected Culicoides biting midges in farms in Thailand. Here, we describe the results of the collection in goat farms in Ayutthaya Province, which locates about 50 kilometers north of Bangkok. The collection was carried out in 2 goat farms that were surrounded by paddy fields (Fig. 4). The midges were collected by using light traps, and then they were sorted under a stereoscopic microscope according to their wind patterns and other morphological keys (Wada et al., 1999). Although the collected number of the midges was small, Culicoides oxystoma, C. imicola and C. arakawae were included in the collected midges.
Discussion

In the present study, *C. oxystoma*, *C. imicola* and *C. arakawae*, which have been considered to be vectors of arthropod-borne diseases, were collected in Ayutthaya Province, Thailand. *Culicoides oxystoma* has been found to harbor AKAV, epizootic hemorrhagic disease virus and several other arboviruses in endemic areas (Yanase et al., 2005). *Culicoides imicola* is known to transmit not only BTV but also African horse sickness virus, causing a fatal disease in equids. (Mellor et al., 2000). *Culicoides arakawae* is an incriminated vector of a species of protozoan parasite, *Leucocytozoon caulleryi*, as an etiological agent of chicken anemia (Morii et al., 1981). It was suggested that there were some favorable habitats for larvae of *Culicoides* biting midges in the collected sites, since many *Culicoides* species are known to grow in marshes including paddy fields. However, the flood in 2011 probably destroyed the larval habitats of *Culicoides* biting midges and removed the blood source animals around the trapping sites, and therefore the number of collected midges was quite small in the present study.

A collaborative study on arboviral diseases has just started by young researchers at NIAH-Thailand and NIAH-Japan with the supports of many other staffs. Both the Thai and Japanese researchers have shared common awareness and a goal on arboviral diseases in this feasibility study. Our next step will include the isolation of arboviruses from livestock animals and vector insects and the pathological investigation of field cases in Thailand. Especially, the isolation of arboviruses will need to be focused on, because it would provide us valuable materials for molecular epidemiology and establishment of a diagnostic system. We will continue the efforts together toward our goal.

References


**Fig. 1.** A long-term vision for establishing a comprehensive system for surveillance and diagnosis of arboviral infection in Thailand.

**Fig. 2.** A scene of training for serological tests held in Kyushu Research Station, NIAH-Japan.

**Fig. 3.** A scene of training for identification of Culicoides biting midges held in Kyushu Research Station, NIAH-Japan.

**Fig. 4.** The goat farm where Culicoides biting midges were collected (Ayutthaya, Thailand).
A study of gross lesions in bovine tuberculosis from single intradermal test reactor in swamp buffaloes

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Abstract

Granulomatous tubercle is the primary lesion of tuberculosis in cattle caused by Mycobacterium bovis. Two-hundred sixty-seven buffaloes were sacrificed and examined by visual and by palpation; and also by means of Single Intradermal Test (SIDt). Out of the 267 buffaloes, granulomatous tubercles were found in 45% of the animals (120/267), whereas non visible lesions and other non-tubercles lesions were found in 33% (88/267) and 22% (59/267) of the animals, respectively. Most tubercle lesions were localized in lung, mediastinal lymph node, and liver. The other diseases were fibrinous deposition on the surface of liver, mediastinal lymph node, and liver. The other diseases were fibrinous adhesion (included pleura and pericardium adhesion) and peritoneum adhesion. Most of the swamp buffaloes that were 1-3 years old showing no sign of tubercles or any other lesions were approximately 65% (95/147). For confirmation, the samples of this latter group were tested with polymerase chain reaction (PCR) method and 90% (9/10) were found to contain M.tuberculosis complex. The results indicate that the infected buffaloes that aged 1-3 years may have no tubercles or other lesions from post mortem examination.

Key words : granulomatous tubercle, Bovine tuberculosis, lesions, SIDt

Background

Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by Mycobacterium bovis. In many countries, bovine tuberculosis is a major infectious disease among cattle, other domesticated animals, and certain wildlife populations (CSFPH, 2009). Transmission to humans constitutes a public health problem. Bovine tuberculosis infection in cattle is usually diagnosed in the live animal on the basis of delayed hypersensitivity reactions such as Single Intradermal Test (SIDt), Stormont Test and Comparative Intradermal Test. These tests are standard methods for detection of bovine tuberculosis due to their higher specificity and easier standardization. Infection is often subclinical; when present, clinical signs are not specifically distinctive of this disease and might include weakness, anorexia, emaciation, dyspnea, enlargement of lymph nodes (LN), and cough, particularly with advanced tuberculosis. After death, infection is diagnosed by necropsy and histopathological and bacteriological techniques (OIE, 2008). Inhalation of infected droplets is the usual route of infection. Inhaled bacilli are phagocytosed by alveolar macrophages that may either clear the infection or allow the mycobacteria to proliferate. Immune response by hypersensitivity reaction that consists of dead and degenerate macrophages surrounded by epithelioid cells, granulocytes, lymphocytes and later, giant cells. The purulent to caseous, necrotic center may calcify and the lesion may become surrounded by granulation tissue and fibrous capsule to form the nodular granulomas known as “tubercle” (The Merck veterinary manual, 2005) and tubercle is the primary lesions of tuberculosis that the inspector will expect
to find in the carcasses by post-mortem examination. Certainly the granulomatous inflammation could be found in others chronic disease; fungal infection or non tuberculous Mycobacteria (Anucha, 2006). The inspector can find tubercles by post-mortem examination. In advanced cases the lesions were visible on the surface of organs but in many cases the inspector had to palpate inside the organs to get the correct result. Sometimes the owners were not confident in the accuracy of the SID test because the lesions were not visible. This leads the owners to believe the SID test might produce a false positive result. This certainly complicates the contact with farmers for the next SID test. The purpose of this report was to determine the distribution of sites with visible lesions of tuberculosis from post mortem in SIDt reactor buffaloes that the lesions may change from our expectations and to confirm \( M.tuberculosis \) complex infected that do not have visible tubercle lesion for ensures the SIDt is also useful for live animals. The result can be useful for the inspector.

**Materials and methods**

**Animals:**

The 267 buffaloes of Single Intradermal test (caudal fold part) reactor were culled, euthanized, necropsied and examined post mortem during April 2009 to September 2010.

**Necropsy and Post mortem examinations:**

Necropsy and post mortem examinations were carried out in field conditions and buried immediately after the examination. The gross lesions were recorded under these conditions and the tubercles had a creamy or yellowish appearance color and were caseous or calcified in consistency (Fig. 1B). Occasionally, its appearance may be purulent. Tubercles were either visible or palpable and may be found only 1 tubercle per carcass. In some cases tubercles were not found in any part of the organs; but instead, other lesions such as pleura and/or pericardium adhesion (Fig. 2), fibrinous deposition on the surface of the liver (Fig.4) and abdominal adhesion (Fig. 3), were found and recorded.

**Diagnostic test:**

Ten samples from the “no tubercles found” category, SIDt reactor buffaloes were confirmed by Polymerase Chain Reaction (PCR) for the detection of \( M. tuberculosis \) complex.

![Fig. 1](image)

(A) The pleural thickening up make non-visible lesions on the lung surface should be detect by palpation and found (B) caseous and calcified nodules within the lung.

**Results**

The gross lesions of 267 SIDt reactor buffaloes showed granulomatous tubercles, non-visible diseases, and other lesions with no tubercles, 45% (120/267), 33% (88/267) and 22% (59/267), respectively (Table 1). Most tubercle lesions were localized within the lungs of 84 buffaloes, the mediastinal lymph nodes of 43 buffaloes, and the livers of 36 buffaloes (Table 2). The other remarkable lesions were pleural and/or pericardium adhesion, fibrinous deposition on the surface of liver and abdominal adhesion (Table 3).
**Table 1** Gross lesion from 267 buffalo.

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Buffaloes (heads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulomatous tubercles</td>
<td>120 (45%)</td>
</tr>
<tr>
<td>non visible diseases</td>
<td>88 (33%)</td>
</tr>
<tr>
<td>other lesions without tubercles</td>
<td>59 (22%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>267</strong></td>
</tr>
</tbody>
</table>

**Table 2** Distribution of tubercles*.

<table>
<thead>
<tr>
<th>Organs that found tubercles</th>
<th>Buffaloes (heads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>84</td>
</tr>
<tr>
<td>Mediastinum LN</td>
<td>43</td>
</tr>
<tr>
<td>Liver</td>
<td>36</td>
</tr>
<tr>
<td>Mesenteric LN</td>
<td>16</td>
</tr>
<tr>
<td>Tracheobronchial LN</td>
<td>8</td>
</tr>
<tr>
<td>Hepatic LN</td>
<td>7</td>
</tr>
<tr>
<td>Abdominal viscera/Omentum</td>
<td>7</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>3</td>
</tr>
<tr>
<td>Heart</td>
<td>3</td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
</tr>
<tr>
<td>Thoracic wall</td>
<td>2</td>
</tr>
<tr>
<td>Kidney</td>
<td>2</td>
</tr>
<tr>
<td>Axillary LN</td>
<td>2</td>
</tr>
<tr>
<td>Retropharyngeal LN</td>
<td>1</td>
</tr>
</tbody>
</table>

*Tubercles may be found in more than one organ in each animal.

**Table 3** Other lesions in the “no tubercles found” category.

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Buffaloes (heads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinous deposition on liver surface</td>
<td>36</td>
</tr>
<tr>
<td>Pleura and/or Pericardial adhesion</td>
<td>16</td>
</tr>
<tr>
<td>Abdominal adhesion</td>
<td>7</td>
</tr>
</tbody>
</table>

* In each carcass, more than one kind of lesions other than tubercle can be found.

**Fig. 2**. Pericardial adhesion.  
**Fig. 3**. Abdominal adhesion.  
**Fig. 4**. Fibrinous deposition on the surface of liver (A, B) with tubercle nodules (C).
When analyzed by age: 1-3 years old, 4-6 years, 7-9 years and 10-14 years, tubercles were found in 52 (35%), 32 (52%), 22 (38%) and 14 (70%) of buffaloes respectively. (Table 4).

**Table 4 Tuberculosis lesions by age.**

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Tubercles</th>
<th>No Tubercles Found</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Other lesions (A)</td>
<td>Non visible lesions (B)</td>
<td>(A+B)</td>
</tr>
<tr>
<td>1-3</td>
<td>52/147 (35%)</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>4-6</td>
<td>32/62 (52%)</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>7-9</td>
<td>22/38 (58%)</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>10-14</td>
<td>14/20 (70%)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>120/267 (45%)</td>
<td>59</td>
<td>88</td>
</tr>
</tbody>
</table>

The ten samples from “no tubercles found” category were confirmed by PCR showed 9 positive of *Mycobacterium tuberculosis* complex.

**Table 5 Laboratory confirmation by PCR.**

<table>
<thead>
<tr>
<th>Number</th>
<th>Age (years)</th>
<th>Lesions</th>
<th>SIDt</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Pericardium adhesion</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>NVLs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>NVLs</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

NVLs: Non Visible Lesions

**Discussion**

A total of 120 (45%) SIDt reactor buffaloes were examined post mortem and were found to have tubercle lesions which is different from Bongkarn (2008) who studied in 1996-2006 and found lesions up to 94.84% (184/194), but close to Udom (2010) who found 40.3% (20/72). Granulomatous is the chronic inflammation that requires at least 2-4 weeks to form nodules. The Eradication program for removal of infected animals changed by increasing the frequency of SID testing from every 6 months to every 4 months and all the reactor buffaloes have been culled from the herd, reducing the contact time from infected animals in the herd, this can cause the lesions decreased. NVLs may be due to early infection, poor necropsy technique or infection with mycobacteria other than *M. bovis*.

The tubercle lesions are mostly localized in the thoracic cavity which relate to Kanameda (1997) and Whipple DL (1996), including the lungs, the mediastinal LN and the tracheobronchial LN. Post mortem examination for detecting lesions in the thoracic cavity should be a thorough examination by visual inspection and palpation. Lung lesions were visible on the surface and often palpable inside the lung lobes (Kanameda, 1997) (Fig. 1A), frequently found only 1 tubercle per head. The failure to detect lesions during inspection has its greatest significance in an animal with a single lesion. In addition, the lymph nodes are also important. Because we found caseous or calcified lymph nodes in the chest, the most were found in the mediastinal LN were 36% (43/120) and a 42% (18/43) of this group found lesions in mediastinum LN but not found lesions in lungs. A study by Whipple DL
(1996) detected tubercle lesions in subiliac lymph nodes where sight is not used to check the meat in the slaughterhouse and certainly it is not checked for in the chest. It shows that the body’s immune system response to infection is by macrophage and the infection spreads to the lymph nodes, and moreover, the respiratory system.

The other notable lesions were 22% (59/267), including pleural and/or pericardial adhesion, fibrinous deposition on the surface of the liver and abdominal adhesion found in the process of opening the necropsy. After opening and peeling off the abdominal skin, typically the abdominal muscles and peritoneum can be easily opened into the abdomen. Thereafter look at the omentum to examine the organs in the abdomen such as stomach, intestine, kidney or uterus. But if they are adhered together in the abdomen, that can be seen clearly. This lesion can make post mortem inspection difficult so the adhesion of peritoneum and the omentum will have to be cleared before the inspection can proceed. Fibrin on the surface of the liver can be found explicitly, as it looks like white powder on the surface of the liver, which is not found in normal livers. As with the adhesion of the organs in the thoracic cavity the thorax and lungs may be adhered to the heart or the pericardium and can be seen clearly. This is a barrier for examination of the lung and heart. All these lesions are most common in ages 1-3 years. A study by Amanda H. (1986) found that the pleural mesothelium cells are sensitive to foreign matter such as air, water, saline and abnormal proteins. This response was found in the thoracic cavity (effusion) and was followed by a fibrous healing resulting in retention of organs in the chest and Kanameda (1997) found that the formation of fibrinous deposits on the surface of the liver can be often found, and the incidence of pleural adhesion (adhesive pleurisy) can be found without tubercles. It should not be overlooked that the tubercles were found because these lesions are indicative of the condition in which the body responds to foreign substances that may be caused by TB in the first period. It was found that correlated with age, the lesions and tubercles were found less in lower aged buffaloes. The rates were found to be 35% for 1-3 years, 52% for 4-6 years, 58% for 7-9 years and the most common age is 10-14 years at 70%. This indicated the amount of time that animals were exposed to infection and inflammatory processes and that the lesions and tubercles are related to the chronic inflammation allowing a longer period to form the tubercle. It also found the animals aged 1-3 years did have non visible lesions included with other lesions and had up to 65% (95/147) more than the other age ranges. The 10 samples from the no tubercles found group were confirmed in laboratory testing by PCR, which is the only way to confirm the results of each method (validated procedures, OIE 2008) to have 9 samples positive to Mycobacterium tuberculosis complex. The 9 positives were 1-3 years of age. Although these studies have a number of samples that were confirmed by PCR, only 10 samples, were confirmed in ages 1-3. Also the result of this study indicate that not all infected buffaloes have visible lesions of tuberculosis which are caused by an infection in early stage.

The most aged of reactor buffaloes were 1-6 years had a relative with Udom (2553) who found that the SIDt reactor most are aged 3-6 years, too. The young buffaloes showed minimal tubercle lesions and tubercles were more variable with age. The same could not find any lesions and other lesions in the animals as well. Due to the nature of TB, chronic infection and the gradually forming tubercle. The tubercle is not detected in younger animals that have a positive on SIDt. Therefore, the TB skin test (SIDt) is used in live animals, and the standard test is used as a measure to eliminate tuberculosis. This method is simple and takes less time than the other skin test, such as the Stormont test (Chitt, 1992), although the results of the necropsy of SIDt reactor
will not find any tubercles. But there are limitations to some groups such as new born animals, and animals with chronic infection. This may give a false negative, which requires a combination of other tests. Necropsy and dissection of the tubercles lesions involves risks and dangers. Those who have served with the animals should always protect themselves by wearing their protective equipment. At least two layers of gloves, masks, apron, boots, hats and glasses are needed to prevent contact with a spill to prevent bacteria from entering the body. Including those who work with animals, including people involved with the remains of slaughtered animals or veterinarians.

Acknowledgment

We are thank you to farmers that allows the study to be conducted smoothly. Dr. Udom Chauchan, Dr. Bongkarn Boontarat, involved all staffs of Veterinary Research and Development Center (Lower Northeastern Region) in the operation. And thanks to Sue and Val Klemm for help to finish editing a document.

References

Lesion development and its relation to pathogenicity of variant porcine reproductive and respiratory syndrome virus

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Abstract

Background: Highly pathogenic porcine reproductive and respiratory syndrome (PRRS) is a fatal viral disease which has recently emerged among swine populations in Asian countries. While a variant of PRRS virus (PRRSV) was isolated in the affected pigs, its pathogenicity has not yet been fully determined. Here, we aimed to clarify the sequential development of lesions due to variant PRRSV infection under experimental conditions.

Methods: Variant PRRSV was isolated from an infected pig in Vietnam and propagated using porcine alveolar macrophages. Thirteen 4-week-old specific pathogen-free pigs were intranasally inoculated with 10⁵.5 TCID₅₀ PRRSV, while 4 separate animals were kept as uninfected controls. All pigs were monitored daily for clinical signs. Three or four infected pigs each were necropsied on 3, 7, 14, and 21 days post-inoculation (dpi), and any pathological alterations and amounts of PRRSV RNA were assessed in the collected tissues.

Results: All infected pigs exhibited high fever and depression with anorexia, edema, and dyspnea. In addition, necropsy examination revealed consolidated pneumonia and lymphadenopathy in all infected animals. Renal petechiae and swelling of knee joints were observed in some pigs. One pig died at 10 dpi with severe hemorrhagic pneumonia and ileocecal ulcer. Microscopically, severe interstitial pneumonia characterized by marked alveolar exudates due to apoptotic or necrotic cells was observed at 7 and 14 dpi. In lymphoid tissues, focal necrosis or abundant single cell necrosis were prominent at 3 and 7 dpi. PRRSV antigen was detected in necrotic cell debris and monocyte/macrophage lineage cells in multiple organs, while PRRSV RNA was detected in serum and multiple organs as well. No clinical signs or lesions were observed in control animals.

Conclusions: Striking necrotic lesions in lung and lymph nodes were detected in infected pigs, strongly supporting the high pathogenicity of variant PRRSV.

Keywords: Highly pathogenic PRRS, lymphoid necrosis, pneumonia

Background

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure of sows and respiratory symptoms in piglets and growing pigs. The etiologic agent of PRRS, PRRS virus (PRRSV) is an enveloped, single stranded, positive sense RNA virus. Since its first appearance in the late 1980s, PRRS has become endemic in most pig-producing countries worldwide. In 2006 in China, a highly pathogenic form of PRRS appeared, and pandemic disease has now rapidly spread
among Southeast Asian countries with severe economical losses reported. However, while this variant of PRRSV has been isolated in diseased animals, its pathogenicity has not yet been fully determined. Studies on pathological findings and cell tropism of the virus in experimental model may help elucidate the pathogenicity of this highly pathogenic PRRSV. Here, we review the difference in lesions between regular type and highly pathogenic PRRS and introduce our experimental model which uses an isolate of this variant in Vietnam.

1 Pathogenesis and lesions in PRRSV infection

PRRSV, a member of the Arteriviridae family in the order Nidovirales, is a positive, single-stranded RNA virus approximately 15.5 kb in length (Cavanagh, 1997). Two distinct genotypes of PRRSV sharing approximately 60% identity at the nucleotide level have been described-European type (genotype 1) and North American type (genotype 2) (Murtaugh et al., 1995)-and isolates of these genotypes demonstrate substantial genetic and antigenic diversity (Zimmerman et al., 2006). PRRSV has extremely restricted tropism for cells of monocyte lineage, and the primary cell target in the host is alveolar macrophages in the lung and macrophages in lymphoid tissues (Halbur et al., 1996; Duan et al., 1997). Replication of PRRSV in residential macrophages induces apoptosis with direct effects from viral gene products (Suárez et al., 1996) and indirect effects likely due to cytokines, reactive oxygen species, or nitric oxide released from infected cells. Secretion of proinflammatory cytokines from infected macrophages results in inflammatory responses such as recruitment of leukocytes, initiation of immune responses, and vascular permeability (Zimmerman et al., 2006). These pathological events due to the infection of PRRSV probably induce a wide variety of lesions, including interstitial pneumonia with alveolar thickening and alveolar proteinaceous and karyorrhectic debris, lymphadenopathy with necrotic macrophages and germinal center hyperplasia, and perivascular lymphohistiocytic inflammation in visceral organs, including the heart and brain (Rossow et al., 1995; Halbur et al., 1995).

2 Clinical and pathological features of highly pathogenic PRRS

Reports in China (Tian et al., 2007; Zhou et al., 2008; Zhou and Yang, 2010) and Vietnam (Feng et al., 2008) reviewed clinical signs and lesions in highly pathogenic PRRS. Characteristically, prolonged high fever exceeding 41 °C and rubefaction on the skin and ears were the common clinical signs in the diseased pigs, with other clinical features including depression, anorexia, respiratory distress, lameness, shivering and diarrhea. Neural signs were also noted in some diseased pigs. Gross findings in the diseased pigs were manifested as hemorrhagic and edematous lesions in multiple viscera, including severe pulmonary edema and consolidation with hemorrhagic spots, spleen infract, blood spots in the kidneys, hemorrhage and edema in lymph nodes, ulcers in the gastro-intestinal tract, edema and congestion in the brain, and arthritis with swollen joints. Few reports have described detailed microscopic findings in field cases. An immunohistochemical approach with a specific anti-PRRSV monoclonal antibody showed presence of PRRSV antigens in multiple organs including lung, brain, spleen, lymph node, liver, heart, tonsil, kidney, and hypoderm (Tian et al., 2006).

3 Experimental infection with highly pathogenic PRRSV

Results of experimental infection with isolates from the diseased pigs with highly pathogenic PRRS ranged from exhibition of mild to moderate clinical signs (similar to experiments with regular type PRRSV) to successful reproduction of the disease with fatal consequences. In a previous study, experimental infection with Chinese isolates induced high fever, blood spots, shivering, and limping
within 3 to 6 days after intranasal inoculation of the virus, and the inoculated pigs were all dead by 10 days post infection (Tian et al., 2006). Another study using a Chinese isolate with 60-day-old pigs (Zhou et al., 2008) reported a high fever (40-41 °C), lack of an appetite, red discoloration on the body, and cyanosis in the ear, with all pigs subsequently dying within 21 days after infection. Post-mortem examination showed pulmonary consolidation, hemorrhage in stomach, and lymph nodes, and histological findings were interstitial pneumonia with mild thickening of the alveolar septa accompanied with infiltration of mononuclear cells, mild perivascular cuffing and intumescence of vascular walls in the brain, and depletion of lymphoid follicle in the lymph nodes and spleen. The inoculated virus was also able to be isolated from the blood and tissues of the infected pigs.

In contrast to the relatively extreme findings above, experimental infection with a Vietnamese isolate (Metwally et al., 2010) induced no fatalities, although the infected pigs still developed clinical signs, including a fever of 40.6-41.1 °C lasting 1 week in duration, depression, low appetite, and a rough coat. Gross necropsy findings in pigs at 28 days after infection showed fibrinous peritonitis and pleuritis, pericarditis, and polyarthritis. Histologically, the animals had multifocal interstitial pneumonia, lymphoid hyperplasia, pericarditis, peritonitis, and meningoencephalitis. PRRSV was detected in the lungs and spleen of the inoculated pigs.

Amounts of virus in experimental model were evaluated in recent reports. Viral loads in blood and tissues in pigs infected with the highly pathogenic isolate were dramatically greater than in animals inoculated with an attenuated derivative strain (Liu et al., 2010). Further, interstitial pneumonia and brain edema related to high amounts of inflammatory cytokines in the blood at the early phase of infection were described in inoculated pigs. Other reports have also described thymus atrophy and lymphoid necrosis with enormous levels of virus present in infected animals (Wang et al., 2011). These findings suggest that the lesions in highly pathogenic PRRS may be related to the marked replication of the virus in its host.

4 Our experimental model

Here, we focused on examining lesion development after infection of our experimental model with a highly pathogenic variant of PRRSV. The variant PRRSV was isolated from a diseased pig in Vietnam and propagated using porcine alveolar macrophages. Thirteen 4-week-old specific-pathogen-free pigs were intranasally inoculated with 10^{5.5} TCID_{50} PRRSV, while 4 separate animals were kept as uninfected controls. All pigs were monitored daily for clinical signs. Three or four infected pigs each were necropsied on 3, 7, 14, and 21 days post-inoculation (dpi), and any pathological alterations and amounts of PRRSV RNA were assessed in the collected tissues. All infected pigs exhibited high fever above 41 °C and depression with anorexia, edema, and dyspnea. In addition, necropsy examination revealed consolidated pneumonia and lymphadenopathy in all infected animals. Renal petechiae and swelling of knee joints were observed in some pigs. One pig died at 10 dpi with severe hemorrhagic pneumonia and ileocecal ulcer. Microscopically, severe interstitial pneumonia characterized by marked alveolar exudates due to apoptotic or necrotic cells was observed at 7 and 14 dpi. In lymphoid tissues, focal necrosis or abundant single cell necrosis were prominent at 3 and 7 dpi. Immunohistochemical analysis revealed that PRRSV antigen was detected in necrotic cell debris and monocyte/macrophage lineage cells in multiple organs, while a large amount of PRRSV RNA was detected in serum and multiple organs as well. No clinical signs or lesions were observed in control animals.
Discussion

Previous reports regarding field cases and experimental infection with highly pathogenic PRRS mentioned that the characteristic lesions in the diseased pigs were hemorrhage and edema in multiple viscera, interstitial pneumonia, and lymphoid atrophy. In our model, striking necrotic lesions (including apoptosis in lung) related to elevated viral RNA amount were detected in infected pigs. These lesions were somewhat similar to regular type PRRS, but their increased severity was likely correlated to increased viral replication in monocyte/macrophage lineage cells. Further studies on pathological and pathophysiological findings are needed to elucidate the pathogenicity of highly pathogenic PRRS.

References


Tibial dyschondroplasia (TD) in ducks: Case reports

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Abstract

Tibial dyschondroplasia, TD, is a non-infectious disease causing lameness in poultry. It is commonly found in young fast-growing broilers, turkeys, and ducks. During the past 3 years, various strains of 20 ducks from farms in central Thailand were investigated for TD. All of ducks exhibited the similar clinical manifestations such as lameness and reluctance to walk. Proximal tibia deformities were seen as well. Additionally necropsy was performed in all ducks which revealed lesions especially at proximal tibia. Macroscopically a lesion was characterized by opaque tissue extending from epiphyseal growth plate to metaphysis in 12 ducks, and such lesion was found together with focus of tissue necrosis in 5 ducks. Besides, opaque tissue was also found in metaphysis of 3 ducks. Histologically, a number of eosinophilic immature chondrocytes accumulated in lacuna surrounded by uncalcified extracellular matrix. Furthermore, there were no bacteria, virus and fungi isolated from all ducks. Thus, in the definitive diagnosis tibial dyschondroplasia was made. Moreover, in Thailand we lack the knowledge in this field so that the objective of this study is to demonstrate histopathology of tibial dyschondroplasia in ducks, which will lay a foundation for pathological diagnosis in the future.

Key words: tibial dyschondroplasia, ducks

Background

Tibial dyschondroplasia (TD) is neither an infectious nor contagious animal disease. The disease is usually found in days-old fast growing broilers, turkeys and ducks worldwide. The disease results from abnormality of cartilage growth to become bone at epiphyseal growth plate of proximal tibia. TD is characterized by accumulation of cartilage extending from epiphyseal growth plate to transitional zone of metaphysis (Orth and Cook, 1994) together with avascularization, attributing to absence of bone ossification. Moreover, the disappearance of vascularization causes bone necrosis. This abnormal bone formation contributes to tibial deformity, leg weakness and finally easy to crack. There are no other clinical signs of tibial dyschondroplasia except lameness, reluctance to walk and occasionally sitting on the hock joints, making the hock joints swelling and arthritis can be observed. The affected animals had difficulty to find food, causing them to be cachexia followed by death. However, the mechanism of the disease is unclear (Michael et al., 1992; Orth and Cook, 1994; Riddell, 1991), but the putative factors resulting in the disease such as mycotoxin, gene and imbalance of pH as well as calcium and phosphorus in food have been reported (Hunter et al., 2008; Leach and Liburn, 1992; Praul et al., 2000; Riddell, 1987). Because of no treatment, prevention from the risk factors might be important. Thus, this report aims at presenting histological lesions of TD in ducks so that the information will be used to pave way for diagnosis in the future.
Materials and methods

History and clinical signs

Various strains of 20 ducks with 10-30 days of age in farms within central part of Thailand were examined. All of them exhibited lameness, reluctance to walk, inability to stand, and some were sitting on the hock joints (Figure 1). However, they still had normal appetite. Most of them were good in body condition. Besides, they did not respond to treatment by antibiotics and mineral supplement.

Physical examination

General physical examination was performed in 20 ducks. There was no abnormality being observed except the deformity, bowing, softening and easily to crack of tibial bones. Longitudinal sagittal plan sectioning was made by sharp knife at the bones in order to divide a bone into 2 pieces so that gross examination can be observed.

Necropsy

After performing necropsy by cervical disarticulation, specimens such as heart, lungs, liver, spleen, kidneys, intestine, pancreas, trachea, esophagus and brain were sent to isolate for viral infection, and a piece of tibial bone as well as such specimens were sent to isolate for pathogenic bacterial infection, and did fungal culture.

Virological, Bacterial and fungal examination

For viral isolation, samples such as heart, lungs, liver, spleen, kidneys, intestine, pancreas, trachea, esophagus and brain were diagnosed for avian influenza (OIE, 2009) and duck viral enteritis (Namba, 2003). For bacterial and fungal isolation heart, lungs, liver, spleen, kidneys, intestine, pancreas, trachea, esophagus and brain were culture aerobically on blood agar and MacConkey media at 37°C, and the proximal tibia was dipped in alcohol, flamed and put inside sterile plastic bags containing 0.01 M phosphate buffer saline (PBS), pH 7.2. The bone was crushed, and the suspension was swabbed on to blood agar and MacConkey media and culture in aerobic and anaerobic condition at 37°C for 24 hours. Meanwhile, fungal isolation was performed on Sabouraud’s dextrose agar at room temperature and at 37°C for 3 days, and they were observed for fungal growth.

Histological examination

The same type of specimens as mentioned above including the other piece of proximal tibial bone were fixed 24-48 hours in 10% neutral buffer formalin. Then, the proximal tibial bone was decalcified in formic acid/formalin method, and histopathological slides were made according to Luna, 1968. In brief after fixing, specimens were trim, dehydration by different concentration of alcohol series, clearing with xylene, infiltration with liquid paraffin and embedding in order to make paraffin blocks. Later paraffin blocks were sectioned 3 µm thick and stained with hematoxylin & eosin (H&E) and sealed by coverslips.

Results

Necropsy findings

Macroscopically lesions were observed in 20 ducks. There was opaque tissue extending from epiphyseal growth plate to metaphysis of proximal tibia (Figure 2B) in one leg of 7 ducks and in both legs of 5 ducks. Additionally such a lesion was found together with necrotic tissue in one leg of 5 ducks (Figure 2C). In addition to those lesions, in 3 ducks opaque tissue was found in metaphysis in one leg. Furthermore, no other lesions were seen from all specimens.

Histological lesions

There was accumulation of small eosinophilic immature chondrocytes in lacuna surrounded by basophilic uncalcified matrix, extending from epiphyseal growth plate to metaphysis (Figure
3) in 12 ducks. Such a lesion was also founded together with focal necrosis in 5 ducks (Figure 4). Additionally, in 3 duck immature chondrocytes were found in metaphysis with avascularization (Figure 5).

**Bacterial and viral isolation results**

No bacteria, virus and fungus were isolated from all specimens.

![Figure 1](image1.png)

**Figure 1.** A&B: Affected ducks sit on the hock joints, inability to stand and reluctant to walk.

![Figure 2](image2.png)

**Figure 2.** A: Longitudinal sectioning of proximal tibia. Normal characteristic of proximal tibia. B: Whitish tissue extending from epiphysis to metaphysis (arrow). C: Focus of necrotic tissue in metaphysis (arrow).

![Figure 3](image3.png)

**Figure 3.** Small eosinophilic immature chondrocytes located in lacuna surrounded by basophilic uncalcified matrix (arrow) (H&E, 40x).

![Figure 4](image4.png)

**Figure 4.** A large number of Immature chondrocytes (open arrow) and focal necrosis (close arrow) (H&E, 10x).

![Figure 5](image5.png)

**Figure 5.** Immature chondrocytes (arrow) in metaphysis (H&E 20x).
Discussion

There are 2 types of bone development namely, the intramembranous and endochondral ossification. The intramembranous ossification is the formation of flat bone from connective tissue such as, skull and mandible. In contrast to the intramembranous ossification, endochondral ossification is the formation of long bone from chondrocytes in epiphyseal growth plate region. This type of bone includes femur, tibia and humerus. Especially in poultry, longitudinal sectioning of tibia consists of a region of epiphysis at proximal and distal tibia, and a region at the middle between epiphysis is diaphysis. Furthermore, in epiphysis there is epiphyseal growth plate which has metaphysis area. Importantly, new bone formation will occur in this metaphysis in which there are differentiations of cartilage to become bone concurrence with deposition of calcium in extracellular matrix which finally becomes calcified matrix. During the deposition of calcium, there are developmental processes of tiny immature chondrocyte within lacuna to become mature chondrocyte. The process begins with enlargement of immature chondrocytes, and space of cytoplasm within lacuna also increases together with deposition of calcium in extracellular matrix until the new bones occur. This perfectly finishes the process of new bone development (Weather et al., 1987). However, if there are some factors including gene susceptible to the disease, contamination of mycotoxin, imbalance of pH or calcium and phosphorus in diet disturbed the process of bone development, immature chondrocytes will not develop to become bone, and calcium also will not deposit in extracellular matrix, and finally the extracellular matrix turns to uncalcified matrix. This affects to bone growth, causing softening and weakness of bone. Moreover, this type of bone has to bear the animal weight, contributing to bone deformity (Hunter et al., 2008; Leach and Liburn, 1992; Praul et al., 2000; Riddle, 1987).

Macroscopic lesions from the necropsies in this study found whitish tissue extending from epiphyseal growth plate to metaphysis corresponding to the report of Hunter et al. (2008) in that the study of broilers showing lameness, bone deformity, reluctant to walk, caused by tibia dyschondroplasia as well as the study of Riddle (1991) describing those lesions on the disease caused by metabolic disturbance. Furthermore, Praul et al. (2000) reported the histopathological lesion of disease correlating with gene that is susceptible to tibial dyschondroplasia which corresponds to microscopic lesion in this report in that there is large amount of immature chondrocyte and uncalcified matrix accumulating in metaphysis. This report also microscopically corresponds with the study of Throp et al. (1991) in broilers that feed with difference formula of gradient in diets as well as the study of Wise and Nott (1975) on incidence of tibial dyschondroplasia in strains of duck.

Summary

Hence, from history and clinical sign, macroscopic and microscopic findings tibial dyschondroplasia is the definitive diagnosis so that this information will pave the way for making a surveillance and control strategy in the future. Because tibial dyschondroplasia is usually overlooked in a lame duck, Most of infectious diseases gain more interest nowadays. We would like to suggest that in all cases of lame ducks tibial dyschondroplasia should be brought to the lists of differential diagnosis.
References


Adjuvant effect of CpG oligodeoxynucleotides on antibody responses to avian influenza vaccine in chickens

Effect of CpG ODNs on avian influenza vaccination

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Abstract

Most of the inactivated avian influenza (AI) vaccines currently used in a number of countries are supplemented with oil adjuvant. However, because oil adjuvant persists in the body long after vaccination, it has the potential to threaten the safety and quality of poultry products. Poultry that have received oil-adjuvanted inactivated AI vaccines must be held for long periods after vaccination before they can be shipped for slaughter (e.g., 20 weeks in Japan). In practice, this precludes the use of inactivated AI vaccines in meat poultry such as broiler chickens. To solve this problem, an alternative safe and degradable adjuvant is needed. Here, we examined the adjuvant effects of CpG oligodeoxynucleotides (ODNs) on antibody responses to inactivated AI vaccines. Chickens were inoculated with inactivated AI virus mixed with incomplete Freund adjuvant in combination with, or without, conventional mammalian-specific CpG ODNs. Addition of human-specific CpG ODN 2135 containing a GTCGTT motif or of mouse-specific CpG ODN 1826 containing a GACGTT motif enhanced serum antibody titers as measured by ELISA. However, these CpG ODNs did not enhance serum antibody titers as measured by hemagglutination inhibition testing. The ability of the CpG ODNs to stimulate chicken leukocytes in vitro was much lower than their ability to stimulate mouse leukocytes. To identify the optimum sequence of CpG ODNs for chickens, a panel of different CpG ODNs was screened for their ability to induce in vitro proliferation of chicken leukocytes. CpG ODNs containing a G(G/C/T)CGTT or a T(G/C/T)CGTT motif were modest stimulators of chicken leukocytes in vitro. Taken together, our results show that the development of a chicken-specific ODN motif that efficiently stimulates chicken leukocytes should be further explored so as to obtain a substantial adjuvant effect when the ODN is combined with inactivated AI vaccines.

Keywords: avian influenza, chicken, CpG oligodeoxynucleotide, vaccine

Background

Highly pathogenic avian influenza (HPAI) has spread worldwide and has caused huge economic losses in the poultry industry through direct mortality and preemptive culling (Suarez, 2010). Application of a “stamping out” policy is the major method for the control of HPAI. However, this traditional method requires sufficient veterinary infrastructure for diagnosis, euthanasia, and disposal of infected or euthanized poultry. As a result, incomplete “stamping out” has failed to control HPAI in several countries.
Recently, mass vaccination has been introduced as an additional control method in countries where HPAI has become endemic (Swayne et al., 2011). Most AI vaccines used in the field are inactivated vaccines consisting of whole inactivated AI virus and an oil adjuvant. However, because oil adjuvant persists in the body long after vaccination, it has the potential to threaten the safety and quality of poultry products (Droual et al., 1990). Poultry that have received oil-adjuvanted inactivated AI vaccines must be held for long periods after vaccination before they can be shipped for slaughter (e.g., 20 weeks in Japan). In practice, this precludes the use of inactivated AI vaccines in meat poultry such as broiler chickens. To solve this problem, an alternative safe and degradable adjuvant is needed.

Synthetic oligodeoxynucleotides that contain multiple unmethylated CpG motifs (CpG ODNs) demonstrate strong immunostimulatory effects in mammals (Kreig, 2000). CpG ODNs activate macrophages to secrete cytokines such as interferon-α. CpG ODNs promote B cells to proliferate, differentiate, and secrete antibodies. Injecting CpG ODNs in combination with antigens enhances antigen-specific antibody responses. Such immunostimulatory effects are best induced by CpG ODNs with a GTCGTT motif (in humans) or a GACGTT motif (in mice) (Rankin et al., 2001).

CpG ODNs are recognized by Toll-like receptor (TLR) 9 in mammals. However, the chicken genome contains no ortholog of mammalian TLR9. This suggests that, for CpG ODNs in chickens, recognition, signal transduction, and immunological outcomes are different from those in mammals (Brownlie et al., 2009; Keestra et al., 2010). Nevertheless, several studies have shown that CpG ODNs have adjuvant effects in poultry (Wang et al., 2009; Hung et al., 2011), although the optimum sequence of CpG ODNs for chickens remains unclear. Here, we tested the adjuvant effects of conventional mammalian-specific CpG ODNs on antibody responses to an inactivated AI vaccine in chickens. In addition, we screened a panel of different CpG ODNs to identify the optimum sequence of CpG ODNs for chickens.

Materials and methods

1 CpG ODN

A conventional human-specific CpG ODN 2135 consists of 3 copies of a GTCGTT motif. A conventional mouse-specific CpG ODN 1826 consists of 2 copies of a GACGTT motif. A negative control ODN 1911 contains no CpG motif. We screened a panel of 31 different CpG ODNs consisting of 3 copies of an (A/G/C/T)(A/G/C/T)CGTT motif or a GTCG(A/G/C/T)(A/G/C/T) motif.

2 Vaccination

Four-week-old white leghorn chickens were inoculated twice at a 4-week interval via a subcutaneous route with 1280 hemagglutination units (HAU) of inactivated H5N1 HPAI virus (A/chicken/Yamaguchi/7/2004) mixed with incomplete Freund’s adjuvant (50% volume/dose) in combination with or without a CpG ODN (50 mg/dose) (Wang et al., 2009).

3 ELISA

ELISA plates were coated with inactivated H5N1 HPAI virus (A/chicken/Yamaguchi/7/2004). Aliquots of serially diluted serum samples were incubated in wells for 1 h at room temperature. To detect antibodies bound to the virus, horseradish peroxidase–conjugated anti-chicken IgG (Bethyl Laboratory, USA) and TMB enzyme substrate (KPL, USA) were added. Optical density values were read at a wavelength of 450 nm after TMB stop buffer (KPL) was added.
4. Hemagglutination inhibition tests

Serum samples were 2-fold serially diluted with phosphate-buffered saline. Aliquots (25 µL each) of the diluted serum samples were incubated with 25 µL of 4 HAU of an inactivated H5N1 HPAI virus (A/chicken/Yamaguchi/7/2004) for 30 min at room temperature. Fifty microliters of 0.55% chicken red blood cells was added to each mixture, which was then further incubated for 45 min at room temperature. Antibody titers were expressed as the highest serum dilution causing complete hemagglutination inhibition (HI).

5. In vitro cell proliferation assay

Leukocytes (10^6) were cultured in 200 µL of RPMI medium containing 10% fetal calf serum and a CpG ODN (12.5 mg/mL) (Rankin et al., 2001). The cells were labeled with 1 µCi of[^3H] thymidine during the last 12 h of the 72-h culture period. The labeled cells were harvested on glass-fiber filters and radioactivity was measured with a liquid scintillation counter. The stimulation index (SI) was calculated by dividing the cpm values in the test wells by those in unstimulated control wells. A stimulation index of 2 or higher was considered positive.

Results

First, we tested whether conventional mammalian-specific CpG ODNs would enhance antibody responses to an inactivated AI vaccine. Chickens were inoculated twice subcutaneously with inactivated AI virus mixed with incomplete Freund’s adjuvant in combination with human-specific CpG ODN 2135, mouse-specific CpG ODN 1826, or a negative control (no-CpG ODN, 1911). Four weeks after the second vaccination, serum antibody titers were determined by using ELISA and HI tests. The ELISA antibody titers induced by vaccination with mammalian-specific CpG ODNs were higher than those induced by vaccination with no-CpG ODN (Fig. 1A). However, the HI antibody titers induced by vaccination with the mammalian-specific CpG ODNs did not differ from those induced by vaccination with no-CpG ODN (Fig. 1B).

Next, we tested the direct immunostimulatory effect of conventional mammalian-specific CpG ODNs on chicken leukocytes. Chicken leukocytes, and mouse leukocytes for comparison, were cultured in the presence of human-specific CpG ODN 2135, mouse-specific CpG ODN 1826, or the negative control no-CpG ODN 1911. The stimulation efficacy of the CpG ODNs was assessed on the basis of in vitro cell proliferation. Conventional mammalian-specific CpG ODNs had a stimulatory effect on chicken leukocytes (Fig. 2A), but the stimulatory effects of the conventional mammalian-specific CpG ODNs on chicken leukocytes were much lower than those on mouse leukocytes (Fig. 2B). These results suggested that the sequences of the conventional mammalian-specific CpG ODNs were not optimum for chickens.

Last, we screened a panel of 31 different CpG ODNs to identify the optimum sequence of CpG ODNs for chickens. Chicken leukocytes were cultured in the presence of different CpG ODNs. Stimulation efficacy was assessed on the basis of vitro cell proliferation. Several CpG ODNs had stimulatory effects on chicken leukocytes (Fig. 3). These relatively potent stimulators contained a G(G/C/T)CGTT or a T(G/C/T)CGTT motif. However, the overall stimulatory effects of these CpG ODNs on chicken leukocytes were still much lower than those of the conventional mammalian-specific CpG ODNs on mouse leukocytes (Fig. 2B).
Discussion

Conventional mammalian-specific CpG ODNs had an adjuvant effect on antibody responses to an inactivated AI vaccine in chickens. However, their efficacy was quite modest. This was likely because the direct immunostimulatory effects of these CpG ODNs on chicken leukocytes were much lower than those on mammalian leukocytes. The development of a chicken-specific ODN motif that efficiently stimulates chicken leukocytes should be further explored so as to obtain a substantial adjuvant effect when the ODN is combined with inactivated AI vaccines.

Fig. 1. Antibody titers induced by vaccination with conventional mammalian-specific oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs). Chickens were vaccinated twice with an inactivated H5N1 avian influenza virus (A/chicken/Yamaguchi/7/2004) mixed with incomplete Freund’s adjuvant in combination with or without a conventional mammalian-specific CpG ODN. (A) Antibody titers determined by using ELISA at 4 weeks after the second vaccination. Data are given as OD_{450} values of serum samples diluted at 1:25,600. (B) Antibody titers determined by using hemagglutination inhibition (HI) tests at 4 weeks after the second vaccination. Data are given as HI titers. Symbols indicate data from individual chickens. Horizontal bars indicate means. 2135, human-specific CpG ODN 2135; 1826, mouse-specific CpG ODN 1854; 1911, negative control (no-CpG ODN 1911).

Fig. 2. In vitro leukocyte proliferation in response to conventional mammalian-specific oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs). Chicken peripheral blood leukocytes or mouse spleen leukocytes were cultured in the presence or absence of a CpG ODN and labeled with [3H]thymidine during the last 12 h of the 72-h culture period. (A) Stimulation indexes of chicken peripheral blood leukocytes. (B) Stimulation indexes of mouse spleen leukocytes. Symbols indicate data from individual animals. Horizontal bars indicate mean values. 2135, human-specific CpG ODN 2135; 1826, mouse-specific CpG ODN 1854; 1911, negative control (no-CpG ODN 1911).
In vitro proliferation of chicken leukocytes in response to different oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs). Chicken peripheral blood leukocytes were cultured in the presence of a CpG ODN and were labeled with [3H]thymidine during the last 12 h of the 72-h culture period. White and black bars indicate stimulation indexes of cells derived from two representative chickens. Horizontal dotted bar indicates a stimulation index of 2.

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References


Identification of helminths in dolphins stranded along the Andaman coast of Thailand during 1992 - 2006

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Abstract

During 1992 – 2006, seven dolphins were stranded on the shores of the Andaman coast of Thailand. The seven cetaceans consisted of three striped dolphins (Stenella coeruleoalba), two spinner dolphins (Stenella longirostris), one spotted dolphin (Stenella attenuata), and one rough – toothed dolphin (Steno bredanensis). Autopsies of the dolphins were done for diagnose the cause of death. During autopsies, several helminths were found in all dolphins especially from gastro-intestinal tracts and respiratory tracts. Species of the helminths were identified. The veterinarian collected, fixed and preserved the parasites in 70% ethyl alcohol. The identification of the helminth's species was done according on morphology of the helminths at Parasitology Section, National institute of Animal Health. Most of the worms were identified under stereomicroscope and compound microscope while some worms were prepared for permanent and semi – permanent slides using Harris’ haematoxylin or borax carmine staining. The species of helminths were classified as follows: 57.14% (4/7) Anisakis spp., 14.29% (1/7) helminthes in the family Pseudaliidae, 14.29% (1/7) Anisakis spp. with Bolbosoma spp. and 14.29% (1/7) Anisakis spp. with helminths in the family Pseudaliidae. The detail of the results was as follows, the nematodes were in stomach and small intestine and the acanthocephalan were found. All of the nematodes were identified as Anisakis spp. The acanthocephalan was identified as Bolbosoma spp. In respiratory tracts, the nematodes helminths were found in the lungs. This investigation found only adult helminths because we examined only the worms in the internal organs of the dolphins. Furthermore, necropsy results showed three lesions including 14.29% (1/7) gastritis, 14.29% (1/7) gastritis with gastric ulcer, 14.29% (1/7) gastritis with verminous bronchopneumonia, and 42.86% (3/7) no lesion. It was possible that helminth infection might affect the dolphins’ health. This is the first report of helminths recovered from dolphins in the Andaman region of Thailand.

Keywords: helminths, dolphins, Andaman, Thailand

Background

Parasitic infection is one of the problems of marine mammals in many parts of the world such as USA (Stroud and Roffe, 1979), UK (Baker, 1992). The major causes of death in these 2 reports were hunting by gunshot or fishing gear, bacterial infection such as pneumonia and parasitic infection. The systems that found the helminths were gastro-intestinal tract, respiratory tract, uro-genital tract and connective tissue. Parasitic infection in marine mammals can be classified due to the systems found and type of helminths. Helminths in gastro-intestinal tracts were classified to nematode, cestodes,
Helminths in respiratory tracts include nematodes and trematodes. Helminths in reproductive tracts include nematode. Helminths in connective tissue include only cestodes.

In Thailand, on the shores of the Andaman coast of Thailand in Phuket and Phangnga provinces during 1992 – 2006, 176 dolphins and whales were stranded. In this study, we chose only 7 dolphins due to the completeness of the carcass. The dolphins used in this study consisted of three striped dolphins (Stenella coeruleoalba), two spinner dolphins (Stenella longirostris), one spotted dolphin (Stenella attenuata), and one rough – toothed dolphin (Steno bredanensis). The objective of this study was to identify the dolphin’s parasites in this area.

**Materials and methods**

Autopsies of the 7 dolphins were done to diagnose the cause of death. Inspecting the dolphin was done before autopsy. The dolphins were identified following the guideline of Adulyanukosol and Kittiwattanawong (2004). During autopsies several helminths were found in all dolphins especially from gastro - intestinal tracts and respiratory tracts. The stomachs, intestines (small and large) and lung were opened to search for the parasites. The veterinarian collected, fixed and preserved the parasites in 70% ethyl alcohol for transport the specimen for identification of the species.

The identification of the helminth’s species was done at Parasitology Section, National institute of Animal Health. Most of the worms were identified under stereomicroscope and compound microscope according to morphology with taxonomy key of Yamaguti (1958), while some worms were prepared for permanent and semi – permanent slides using Harris’ haematoxylin or Borax - carmine staining (Meyer and Penner, 1962). Glycerine was used to clarify the thick helminths.

Data analysis was done using descriptive statistics including length of the worm, quantity of the parasites, age of the dolphin, lesion found.

**Results**

The helminthes found were classified by type of dolphins and were shown in table 1.

**Table 1 : Taxonomic information of helminths found in 4 types of dolphins**

<table>
<thead>
<tr>
<th>Type of dolphins</th>
<th>Helminths type</th>
<th>Site</th>
<th>No. Infected</th>
<th>Length (cm.) ±</th>
<th>No. of parasites</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anisakidae</td>
<td>stomach, small intestine</td>
<td>2/3</td>
<td>2.8 ± 1.16</td>
<td>1</td>
<td>Non-mature</td>
</tr>
<tr>
<td>2</td>
<td>Anisakidae</td>
<td>Stomach, small intestine</td>
<td>2/2</td>
<td>4.7 ± 0.78</td>
<td>28</td>
<td>Non-mature</td>
</tr>
<tr>
<td>3</td>
<td>Anisakidae</td>
<td>stomach, small intestine</td>
<td>1/1</td>
<td>3.6 ± 3.54</td>
<td>2</td>
<td>Mature</td>
</tr>
<tr>
<td>4</td>
<td>Anisakidae</td>
<td>stomach, small intestine</td>
<td>1/1</td>
<td>4.6 ± 1.3</td>
<td>71</td>
<td>Mature</td>
</tr>
<tr>
<td></td>
<td>Bolbosoma sp.</td>
<td>small intestine</td>
<td>1/1</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Remarks : 1 = striped dolphins (Stenella coeruleoalba)  
2 = spinner dolphins (Stenella longirostris)  
3 = spotted dolphin (Stenella attenuata)  
4 = rough – toothed dolphin (Steno bredanensis)

The species of helminths found in 4 types of dolphins were classified as follows: 57.14% (4/7) Anisakis spp., 14.29% (1/7) helminths in the family Pseudaliidae, 14.29% (1/7) Anisakis spp. with Bolbosoma spp., and 14.29% (1/7) Anisakis spp. with helminths in the family Pseudaliidae.
Nematodes in gastro-intestinal tract were in the Phylum Nematheminthes, Class Nematoda, Family Anisakidae. The dominant feature of this family was cylindrical and slender shape, mount parts presents of 3 - lips, cuticular annulations along the body, long anterior muscular - part esophagus.

The acanthocephalan was identified as *Bolbosoma* spp. The morphology of the worms had a bulb that could divide into three parts, anterior, posterior and intermediate ring-bulb, in the proboscis containing 14 longitudinal rows of hooks and each contains 8-9 hooks according to Yamaguti (1958). This parasite had beeb reported to infect humans. The symptom was acute abdominal pain (Tada, 1983).

In respiratory tracts, the nematodes found in the lungs were classified into the family Pseudalilidae, superfamily Metastrongyloidea. The worms in this family have cuticular surface through the body surface, thread-like nematode, no-lips at proximal part, club-shaped esophagus and symmetry in male spicules. The necropsies finding and the parasites species found in individual dolphin were shown in Table 2.

### Table 2 Lesions found in each dolphin

<table>
<thead>
<tr>
<th>No.</th>
<th>Type of dolphins</th>
<th>Sex</th>
<th>Age of dolphin</th>
<th>Helminths</th>
<th>Lesion found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>striped dolphins</td>
<td>M</td>
<td>Non-mature</td>
<td>Anisakidae</td>
<td>NF</td>
</tr>
<tr>
<td>2</td>
<td>striped dolphins</td>
<td>F</td>
<td>Non-mature</td>
<td>Anisakidae</td>
<td>Gastritis and Verminous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pseudalidiae</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>3</td>
<td>striped dolphins</td>
<td>F</td>
<td>Non-mature</td>
<td>Pseudalidiae</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>4</td>
<td>spinner-dolphins</td>
<td>M</td>
<td>Non-mature</td>
<td>Anisakidae</td>
<td>NF</td>
</tr>
<tr>
<td>5</td>
<td>spinner dolphins</td>
<td>M</td>
<td>Non-mature</td>
<td>Anisakidae</td>
<td>NF</td>
</tr>
<tr>
<td>6</td>
<td>spotted dolphin</td>
<td>F</td>
<td>Mature</td>
<td>Anisakidae</td>
<td>Gastritis and ecchymotic hemorrhage in stomach wall</td>
</tr>
<tr>
<td>7</td>
<td>rough – toothed dolphin</td>
<td>F</td>
<td>Mature</td>
<td><em>Bolbosoma</em> sp.</td>
<td>Gastritis and gastric ulcer</td>
</tr>
</tbody>
</table>

Remark : NF = Not found

### Summary

In this study, the dolphins that stranded on the Andaman in 1992 – 2006 were 176 dolphins. But the complete dolphin carcasses that the helminths could be kept were only 7 dolphins because Thailand is in Tropical zone. The dolphin carcass can undergo rapidly autolysis. From Table 1, we found helminths in the family Anisakidae in all types of dolphins like Berón-Vera et al, 2007 and Abollo et al., 1998b. Berón-Vera et al. (2007) found in all 18 common dolphin species in Patagonia while Abollo et al, 1998b reported that *Anisakis* spp. is only one nematode type found in the stomach in dolphins in Spain. This may be because of feeding type in dolphin. Dolphins usually swallow the larvae of the parasites in infected tiny fish and squid. The importance of these parasites is that this parasite can infect humans. Symptoms of infection can lead to more severe cases with acute abdominal pain (Nuchjangreed et al, 2006). The *Anisakis* spp. nematodes were found in the stomach and small intestine while acanthocephalan were found in small intestine. *Bolbosoma* spp., the acanthocephalan had been reported to infect humans also. This helminths were in the family Polymorphidae, class Palaeacanthocephala. The symptom of human infection was acute abdominal pain (Tada, 1983). The helminths in Family Pseudalidiae (Metastrongyloidea) was found in the lung. The data showed that non-mature dolphin was infected with this worm as Dailey et al, 1991. He said that the young animals had a higher prevalence than adults.

Table 2 showed that the lesion in mature dolphins which Anisakidae was found was related to the helminths found in the sample, these can be described that the mature dolphin may have eaten the larvae of *Anisakis* spp. in fish or squid. Then, the larvae grew into mature parasites and caused...
lesions as they had grown up. But in non-mature dolphins, there was no-lesion; this may be explained that the non-mature dolphins were recently infected. In 1990–1996, Abollo et al, 1998a reported gastric ulcer caused by Anisakis simplex nematode in 17.2%. They concluded that gastric ulcers are non-fatal lesions in cetacean stranded in Northwestern Spain. The 2 in 7 dolphins which harbored helminths in Family Psudaliidae showed lesion of pneumonia like the report of Dailey et al, 1991. They reported that heavy infection may cause early postnatal death due to verminous pneumonia. In our experiment, the dolphin which was infected with Bolbosoma spp. did not show any specific symptom; this occurrence can be described like Perrin et al., 2002. He explained that Bolbosoma spp. infection has little relevance on host health, such as only local reactions produced by the proboscis of Bolbosoma spp.

The species of helminths were classified as follows: 57.14% (4/7) Anisakis spp., 14.29% (1/7) helminths in the family Pseudaliidae, 14.29% (1/7) Anisakis spp. with Bolbosoma spp. and 14.29% (1/7) Anisakis spp. with helminths in the family Pseudaliidae.

Furthermore, necropsy results showed four lesions including 14.29% (1/7) gastritis, 14.29% (1/7) gastritis with gastric ulcer, 14.29% (1/7) pneumonia, 14.29% (1/7) gastritis with verminous bronchopneumonia and 42.86% (3/7) no lesion.

From this study, the species of the helminths in dolphin stranded along the Andaman coast of Thailand, during 1992 – 2006 were identified and we also concluded that parasites can play an important role not only in dolphin’s health but also in dolphin’s population. This showed that parasitological information is valuable for conservation of cetaceans in the Andaman coast of Thailand. This is the first report of helminths recovered from dolphins in Andaman region of Thailand.

Acknowledgement

Some part of this paper was done by funding from Faculty of Veterinary Medicine, Chiang Mai University. Special staining of the helminths was done at Faculty of Science, Chiang Mai University. Special thanks to an autopsy team in Marine Endangered Species Unit, Phuket Marine Biological Center, Phuket province.

References

Figure 1  Spotted dolphin (*Stenella attenuate*), Gray, 1846

Figure 2  Spinner dolphin (*Stenella longirostris*), Gray, 1828

Figure 3  Striped dolphins (*Stenella coeruleoalba*), Mayen, 1833

Figure 4  Rough-toothed dolphin (*Steno bredanensis*), Lessen, 1828

Figure 5  Head of *Anisakis* spp., Fresh preparation (× 40)

Figure 6  Head of *Bolbosoma* spp., Harris’ hematoxylin staining (× 20)

Figure 7  Head of nematode in Family, Fresh preparation (× 40)
RNA-Seq Analysis of Embryo of the tick *Haemaphysalis longicornis*

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Abstract

**Background:** *Haemaphysalis longicornis* is a dominant, and a primary vector of bovine piroplasmosis. Because of the social awareness about the massive and frequent use of traditional acaricides, the development of an alternative strategy, such as vaccines or the tick-specific chemical compounds, becomes essentially necessary. To increase our knowledge about key molecules which play modulatory roles in tick’s physiology, outfitting of the gene catalogue is the fundamental instrument. In this study, *de novo* transcriptome sequencing of the tick embryo was conducted by using short read sequencing technology of Illumina platform to investigate the novel genes of *H. longicornis*.

**Method:** Embryonated eggs were incubated for 11 days prior to extract the total RNA. The mRNA selection, library preparation and sequencing were performed on an Illumina GAIIx sequencer according to the manufacturer’s instructions. High quality short read sequences were assembled *de novo* by using Velvet v1.1.02. Resulting contigs were searched in stages against the larger set of EST database (db) of this tick (HlESTdb), NCBI non-redundant (nr) protein db or *Ixodes scapularis* peptide db (I.scaPep.db) to identify the novel gene fragments.

**Results:** A first pass Velvet assembly yielded over 115 k contigs. Contigs filtered by blastn (HlESTdb) and blastx (I.scaPep.db) search were further analyzed by blastx search against nr db, and matched to an embryogenesis related genes controlling cell polarity or neurogenesis which was not listed in HlESTdb.

**Summary:** RNA-seq analysis was conducted by using *H. longicornis* embryo to investigate the novel gene products which were not listed in the HlESTdb. Given that, this technique is a powerful tool to collect gene data set from the organisms with insufficient genome information.

**Keywords:** *Haemaphysalis longicornis*, tick, embryo, RNA-seq, next generation sequencing

Background

Because of the social awareness about the extensive and frequent use of traditional acaricides, the development of an alternative strategy, such as vaccination or use of tick-specific chemical compounds, becomes essentially necessary. To get the candidate molecules, a comprehensive gene catalogue is the fundamental instrument for developing a better understanding of the key molecules that modulate tick physiology. Therefore, a systemic analysis of the genetic and genomic basis of any organism definitely relies on the analysis of a cDNA library of expressed sequence tags (ESTs) or full-genome sequencing. An annotated database of tick ESTs has been generated using several tick species, including *Amblyomma variegatum*, *Rhipicephalus (Boophilus) microplus*, *Ixodes scapularis*, and *Rhipicephalus appendiculatus* (http://compbio.dfci.harvard.edu/tgi/tgipage.html). A genome project focusing on only one species, *I. scapularis*, is now ongoing (Pagel Van Zee et al., 2007; Hill and
Haemaphysalis longicornis is a dominant and a primary vector of bovine piroplasmosis and is distributed around the north side of the pan-pacific area and part of Australia (Hoogstraal et al., 1985). Recently, we constructed organ-specific cDNA libraries from this species and randomly sequenced several clones to generate an EST database. Although extensive effort was devoted to the generation of the HIEST database, it does not contain rare transcripts, such as transcription factors.

The development of ESTs has historically relied upon Sanger sequencing technology, and some recent efforts depended upon longer-read next-generation sequencing technologies, such as Roche 454. Next-generation sequencing (NGS) technologies are revolutionizing molecular biology by lowering cost-per-sequenced-nucleotide and increasing throughput (Paszkiewicz and Studholme, 2010). Short-read platforms such as Illumina and SOLiD produce higher coverage and a lower cost-per-sequenced-nucleotide; however, due to the short reads associated with those platforms, their use has usually been restricted to resequencing applications, which rely on a reference sequence for sequence assembly. In absence of a reference sequence, a computational de novo assembly approach is required. With increased read lengths from technologies such as Illumina and the development of new computational tools, short reads can be assembled and used for transcriptome analysis (Paszkiewicz and Studholme, 2010).

The objectives of this study were 1) to sequence mRNA from of H. longicornis embryos using the Illumina platform, 2) to identify transcripts that are not listed in the current HIEST database, and 3) to confirm the technological efficiency of this approach with the goal of developing a molecular resource for marker development and gene identification.

Materials and methods

Tick colony

The parthenogenetic Okayama strain of the ixodid tick H. longicornis maintained at the Laboratory of Parasitic Diseases, National Institute of Animal Health (NIAH, Tsukuba, Ibaraki, Japan) was bred by feeding on rabbits. The rabbits employed for tick maintenance were adapted to the experimental conditions for 2 weeks prior to the experiment and were treated in accordance with the protocols approved by the Animal Care and Use Committee of the NIAH.

RNA extraction and sequencing

Eggs were laid by engorged female ticks and were then removed from individual ticks on the day of oviposition; eggs were then incubated for 11 days under controlled condition (25°C and 90% relative humidity). Embryonated eggs were rapidly frozen in liquid nitrogen (LN) and stored at -80°C until RNA extraction. The frozen eggs were pooled and ground in LN; total RNA was then extracted from these pooled samples using standard protocols (RNeasy mini kit, QIAGEN). Messenger RNA selection, library preparation, and single-end sequencing (one lane) were performed by Hokkaido System Sciences Co., Ltd., on an Illumina GAIIx sequencer, according to the manufacturer’s specifications.

De novo transcriptome assembly

Prior to assembly, we implemented a ‘quality filter’ by removing reads for which more than 33% of bases were ‘N’ and reads for which more than 34% of the nucleotides had Phred quality scores less than 20; here, a Phred score of 20 corresponded to a 1% expected error rate. The de novo sequence assembly was carried out using Velvet v1.1.02.
BLAST analysis

To identify novel coding RNA fragments, we first used BLASTN from the stand-alone bundle of BLAST algorithms v2.2.23+ (Altschul et al., 1997) to identify all contigs that showed significant similarity (e-value < 1 x 10^{-7}, minimal alignment length of 25 bp) with sequences from the H. longicornis EST database (HIESTdb), which was generated by our laboratory and contains approximately 4600 contigs and 11000 singletons. Contig-EST matches were discarded and remaining contigs were further analyzed using BLASTX to identify all contigs that showed significant similarity (e-value < 1 x 10^{-7}, minimal alignment length of 33 residues) with sequences in the I. scapularis peptide database (IscaPep.db), which was downloaded from Vectorbase (http://www.vectorbase.org/). Iscapep IDs of hit contigs were checked against the HIEST-IscaPepID list; matching contigs were discarded, but non-matches were analyzed further. Contigs that survived all of these filtering steps were then finally analyzed using BLASTX to identify all contigs that showed significant similarity (e-value < 1 x 10^{-6}, minimal alignment length of 33 residues) with sequences in the non-redundant (nr) protein database, NCBI (http://www.ncbi.nlm.nih.gov/guide/).

Results

Sequencing, assembly and filtering results

In this study, we sequenced single-end mRNA from the embryo of H. longicornis on a single lane of a flow cell using the Illumina Genome Analyzer Ix. Approximately 240000 reads, totaling roughly 2.2 GB of sequence, passed Illumina quality filtering. We then removed 27% of these 240000 reads because they were flagged as either low quality or low complexity. A first-pass Velvet assembly conducted using the parameters indicated in the Methods and a hash length of 47 yielded over 115447 contigs that were each over 100 bp and yield an N50 of 254 bp comprised of approximately 28 MB of sequence and was subjected to further downstream filtering and analysis as detailed in the Methods and Figure 1.

Novel expressed sequences relating to embryogenesis

The RNA reads were derived from developing embryos, which undergo dramatic organogenesis to form a tick body and express complex patterns of gene activity; therefore, the RNA reads should represent genes involved in a wide range of developmental processes. Indeed, the assembled contigs included over 150 genes with close sequence similarity to genes with important functions in the development of other arthropods. Briefly, these included the following major groups: 1) genes involved in axis specification, patterning, and morphogenesis, including many transcription factors (homothorax, extradenticle, spalt-like, bicaudal, teashirt-like, Sex comb on midleg, Ultrabithorax, cut, Abdominal B, hairy); 2) genes involved in several signaling pathways, including MAPK pathways (Epidermal growth factor-like protein, MAP kinase, discs large 1), the Wnt receptor signaling pathways (armadillo, Wnt oncogene analog 2), the Notch signaling pathways (Notch, delta, fringe, dishevelled, Enhancer of split, Strawberry notch), the Hedgehog signaling pathway (hedgehog); 3) genes involved in endocrine regulation of development, including ecdysone signaling (Ecdysone receptor, Ecdysone-induced protein 78C); and 4) autophagy-related genes (atg2, 7, 9 and 16l1).

Discussion

In the study of parasites, the expression profiling conducted in recent years has mainly focused on organisms with well-characterized genomes. However, for many organisms that play important roles with respect to biotechnological and pharmaceutical applications, only minimal and low-quality information on the genome and transcriptome is available; therefore, relevant new insights can be expected from expression
profiling in these organisms. EST databases are powerful tools for expression profiling, but developing EST databases with enough sequence information to cover the entire genome of a non-model organism is very costly. Here, we applied NGS to identify embryonic transcripts that are not housed in the HIESTdb, which contains approximately 4600 contigs and 11000 singletons (Tsuji, unpublished data).

In order to recover the genomic origin of as many mRNA reads as possible, we conducted a combined BLAST search that made use of the HIESTdb and data from a closely related ixodid tick species for which ample genomic information has been compiled. In total, about 15.5% of the reads could be assigned to tick genes or to the HIESTdb. Of the remaining contigs, 15.6% could be successfully annotated because they contained significant homology with well-characterized sequences from other organisms. The other 68.8% are likely to correspond to as-yet-unknown transcripts or splice variants, novel exons, UTRs, or non-coding RNAs.

As shown, novel NGS contigs that showed significant similarity to sequences from the nr protein database included rare transcripts, such as those for embryo-specific genes and transcription factors. We recovered many genes that participate in the Notch signaling pathway (e.g., Notch, Delta, Split, and fringe). The Notch pathway is conserved among all metazoans and plays a modulatory role in embryogenesis, especially neurogenesis (Fiuza and Arias, 2007). Embryonic samples used in this study were collected 11 days after oviposition. Therefore, the Notch pathway is likely involved in neurogenesis in tick embryos.

Furthermore, we recovered several autophagy-related genes (atg2, 7, 9, and 16l1). In Drosophila embryos, autophagy promotes caspase-dependent cell death in cells of an extra-embryonic tissue (Mohseni et al., 2009). Previously, five atg genes (atg3, 4, 6, 8, and 12) were reported to be included in the HIESTdb; these genes were expressed in several organs and in the embryo (Umemiya-Shirafuji et al., 2010; Kawano et al., 2011). Therefore, in tick embryos, these genes might function to eliminate cells that have served their function and are no longer needed.

Taken together, our results showed that RNA-seq analysis is a powerful tool for acquiring information on the transcriptomes and genomes of organisms that have not been the focus of extensive genome projects and, therefore, for which standard gene expression profiling and EST strategies are impossible or extremely time consuming and expensive.

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**Figure 1.**

De novo transcriptome assembly and analysis workflow. Illumina reads were assembled using Velvet assembly. Following assembly with Velvet, sequential BLAST programs were used to analyze the contig sequence similarity with sequences in the HIEST, *Ixodes scapularis* protein, and non-redundant protein databases.
Acknowledgements

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References


Pathogenicity analysis of highly pathogenic avian influenza virus by profiling host gene expression

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Abstract

Cyanosis in infected chicken is one of the typical clinical signs of highly pathogenic avian influenza virus (HPAIV) infection. Recombinant influenza viruses possessed surface antigens from HPAIV (HP) and internal genes from two low pathogenic avian influenza viruses (LPAIVs) were generated by reverse genetic technique to elucidate the mechanisms of cyanosis expression. Correlation between expression of cyanosis and host genes were analyzed in chickens infected with three recombinant viruses [rHP, WB (L/PB1) and LP (W/MNS)] and a field strain (HP). Infection with 10^6EID50/100ul of HP and rHP caused 100% mortality without cyanosis whereas that with WB (L/PB1) and LP (W/MNS) caused 100% mortality and cyanosis. Each of three chickens was inoculated intranasally with each virus and blood vessels from them were collected at 24 hours post inoculation. cDNA synthesized from blood vessels were applied to the microarray chip by Affymetrix where 38,535 probes considered from chicken genes were loaded. Chicken genes were annotated with human orthologs. Probes that were up-regulated or down-regulated significantly (P<0.05) and the expression value was log2 ratio<-0.585 or 0.585<log2 ratio versus PBS control group were extracted in each group. Subsequently among them, eighteen genes which expression was significantly affected by cyanosis were identified. Pathway analysis among those genes indicated that up-regulated genes, RGS5, MTMR8, SOX17 and down-regulated gene, BLVRA, were related each other. Three genes up-regulated by cyanosis possessed functions such as vascularization, blood vessel development, vasculature development and endothelial cell development, and RGS5 and BLVRA were known to be regulated by hypoxia. Cyanosis caused by HPAIV infection is considered to involve disseminated intravascular coagulation (DIC). DIC is thought be caused by consumption coagulopathy and involves disorder of endothelial cells. It is suggested, therefore, that genes identified in this study would play key roles in cyanosis expression.

Keywords: highly pathogenic avian influenza, chicken, cyanosis, reverse genetic technique, microarray analysis

Background

H5N1 subtype highly pathogenic avian influenza virus (HPAIV) has been spreading through Asian, European and African continents since the outbreak of goose flu in Guangdong province in 1996 (WHO, Xu et. al., 1999). Pathogenicity of those viruses in poultry has been analyzed to establish strategies of preventing the viral infection and the means of treatment. Multiple basic amino acids at the cleavage site of hemagglutinin proteins of H5 and H7 subtype are well known as a molecular basis of pathogenicity of HPAIV, causing highly mortality in chickens (Horimoto and Kawaoka, 1994, Senne et. al., 1996, Wood et. al., 1993). In our previous study, 17 recombinant viruses which possessed hemagglutinin (HA) and neuraminidase (NA) genes from an HPAIV and other internal gene segments from an HPAIV and two low pathogenic avian influenza viruses (LPAIVs) derived from chicken (LP) and wild bird (WB) were generated by a reverse genetic technique to elucidate the pathogenicity (Uchida
et. al., 2012). Chicken experiments using those recombinant viruses showed that exchange of an internal gene segment affected survivability of the chickens with statistical significance, indicating that internal genes are involved in survivability of infected host. Microarray analysis of the infected chickens revealed that several host gene expression also played an important role of survivability. Besides survivability, cyanosis is one of the typical clinical manifestations caused by HPAIV. Our study demonstrated that expression of cyanosis in HPAIV infected chicken was not correlated with survivability of infected hosts. Chickens infected with rHP and wild type HP did not express cyanosis at all although the infected with the other two viruses, WB (L/PB1) and LP (W/MNS), did, although survival period of chickens infected with either of them was within 2.25 days post inoculation (DPI). Host gene expression in lung of the infected was subjected for microarray analysis. As a result, genes that were possibly correlated with cyanosis were not able to identify in chicken lung. In this study, gene expressions in blood vessel among chickens infected with those strains were compared to identify host factors involved with cyanosis.

**Materials and methods**

Four-week-old specific pathogen free (SPF), white leghorn, L-M-6 strain, chickens were obtained from Nisseiken Co., Ltd. Viruses used for this study were a field strain, HP, and recombinant viruses, rHP, WB (L/PB1) and LP (W/MNS) (Table 1). Regarding cyanosis expression, viruses were categorized into two groups; HP and rHP were no-cyanosis and WB (L/PB1) and LP (W/MNS) were cyanosis-inducing group (Table 1 and Fig. 1). For the microarray analysis, three chickens were infected with $10^6$ EID50 / 100 ul of four respective viruses intranasally. At 24 hours post infection, the chickens were euthanized to collect blood vessel samples and they were individually homogenized by Precellys 24 (Bertin). The blood vessel homogenates were treated with TRIzol® Reagent (Invitrogen) to extract total RNA that was then purified using the RNeasy MinElute Cleanup Kit (Qiagen). Total RNA was reverse transcripted to cDNA with T7 oligo d(T) primer (Affymetrix, Inc.). The cDNA synthesis product was used for in vitro transcription reaction with T7 RNA polymerase and biotinylated nucleotide analog (pseudouridine base). Then, the labelled cRNA products were fragmented, loaded on to GeneChip(R) Chicken Genome array (Affymetrix, Inc) and hybridized according to the manufacturer’s protocol. Streptavidin-Phycoerythrin (Molecular Probe) was used as the fluorescent conjugate to detect hybridized target sequences. Raw intensity data from the GeneChip array were analyzed by GeneChip Operating Software (Affymetrix, Inc). Thirty eight thousand five hundred and thirty five probes were loaded on the microarray chip. The hybridized gene chip was scanned and the array data was collected. Filtering steps of probes were described as below. Step1: The probes of which values were suppressed or induced versus PBS significantly ($P<0.05$) and log2 ratio<-0.585 or 0.585<log2 ratio versus PBS control group were extracted from each group. Step2: Among them, probes which expression was similarly affected by the infection within each group were extracted. Step3: Then, probes affected similarly with all four viruses were excluded to restrict probes affected by only cyanosis. Step4: Furthermore, expression values from each chicken in cyanosis group were compared with those of no-cyanosis group to extract probes correlated with cyanosis. The probes of which the values of cyanosis group were suppressed or induced versus no-cyanosis group significantly ($P<0.05$) and log2 ratio<-0.585 or 0.585<log2 ratio versus no-cyanosis group were extracted. The chicken gene data was annotated with human orthologs using BLAST based on the chicken reference sequence mRNAs in NCBI, assuming that chicken genes function similarly to human orthologs. Annotated genes were applied for pathway analysis by software Pathway studio 8.
Table 1. Characterization of recombinant viruses used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Segment</th>
<th>Mean survival time (days)</th>
<th>Score of cyanosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>HA</td>
<td>2.00</td>
<td>0.00</td>
</tr>
<tr>
<td>HP</td>
<td>NA</td>
<td>2.00</td>
<td>0.00</td>
</tr>
<tr>
<td>WB (L/PB1)</td>
<td>PB1</td>
<td>2.00</td>
<td>0.00</td>
</tr>
<tr>
<td>LP (W/MNS)</td>
<td>NS</td>
<td>2.25</td>
<td>4.25</td>
</tr>
</tbody>
</table>

Figure 1. Representative pictures of cyanosis (A) HP and (B) LP (W/MNS) infection

Results

Chicken genes considered to be correlated to cyanosis were extracted through 3 steps as described in methods from all of 38,535 probes. Number of extracted probes at step1-3 was 1949, 329 and 142, respectively. As a result, 26 probes representing 18 chicken genes were revealed and they were annotated to 17 human genes by BLAST (Table 2). Two of chicken genes, WFDC2 and LY86, were annotated to none or different human gene, AQP12B. Therefore they were re-annotated to human genes by NCBI homologene. Human orthologs of WFDC2 and LY86 were considered as WFDC3 and LY86. Among the extracted 18 genes, 10 genes were up- and 8 genes were down-regulated significantly. All of eighteen genes were applied to pathway analysis to elucidate relation of those genes each other. Thirteen genes were connected each other via properties of cell process, disease and treatment although five genes had no relation among them at all. Pathway initially established was so complicated that it was difficult to see correlation regarding cyanosis expression because genes involved possess many functions besides cyanosis. Pathway was reconstructed by focusing on functions related to cyanosis expression. The properties of cell process, disease and treatment drawn in the pathway were restricted based on the keywords correlated with cyanosis. Keywords, then, were “blood flow, blood pressure, blood vessel, capillary, cell adhesion, cell proliferation, clotting, edema, erythrocyte, endothelial cell, heme metabolism, hemolysis, hemorrhage, hemodynamics, hypotension, hypoxia, infiltration, ischemia, leukocyte, permeability, platelet, thrombosis, vascularization, vasculature, vasodilation”. The reconstructed pathway illustrated that seven genes were correlated to cyanosis function, and they were connected among them via cyanosis function except MTMR8 (Fig. 2). Among those seven genes, ALB, HPX, NPTX2, LY86, RGS5, SOX17 and MTMR8 were up-regulated and CCDC88A and BLVRA were down-regulated. In this study, nine genes correlated with cyanosis expression were identified by microarray analysis of chicken blood vessel and following these filtering steps. Those extracted genes were reasonable to explain mechanisms of expression of cyanosis. Cyanosis caused by HPAIV infection is considered to be triggered by destruction of endothelial cells and adhesion of blood cells and platelets at blood vessel wall following to cytokine induction. After excessive initiation of tissue factor by these stimulations, redundant blood clotting invites disseminated intravascular coagulation (DIC). DIC causes disorder of microvascular circulation and hemorrhagic diathesis by consumption coagulopathy. Nine genes identified in this study would play key roles in cyanosis expression.
Table 2. Extracted genes correlated with cyanosis

<table>
<thead>
<tr>
<th>Chicken genes</th>
<th>Human genes</th>
<th>Pathway analysis for connection among genes</th>
<th>Pathway analysis on cyanosis expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL11A1</td>
<td>COL11A1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ALB</td>
<td>ALB</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HPX</td>
<td>HPX</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NPTX2</td>
<td>NPTX2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC44A3</td>
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Up-regulated genes

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</table>

Down-regulated genes

Discussion

Chicken genes considered to be involved in cyanosis were identified by microarray analysis. Eighteen genes were identified by comparing expression values between blood vessels of chickens infected with virus which induced cyanosis and virus without cyanosis. Furthermore, nine genes closely correlated with cyanosis expression were identified by pathway analysis. This strategy was useful to analyze pathogenicity on HPAIV infection in infected host side.
References


FMD antigenic profiling ELISA

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Abstract

An antigenic profiling of FMD field isolate virus was carried out by double antibody sandwich ELISA using polyclonal antibodies. Totally 50 samples of FMD type A field virus isolates causing outbreak in Thailand and Lao PDR during 2005-2007 and 2011 were studied. The aim of this study was to analyze the antigenic binding reactivity of field virus isolates and compare with several reference virus vaccine strains in order to select an appropriate ELISA reagent system that would be used in further investigation or other research such as vaccine matching test. The result of antigenic profiling test demonstrated that most of FMD type A field isolates from Thailand and Lao PDR during 2005-2007 gave a high antigenic binding reactivity to the reference virus vaccine strain A118/87 rather than A/Sakolnakorn/97, A22 Iraq 24/64 and A132/87 respectively. Meanwhile, the result of antigenic profiling test of FMD type A field virus isolates from Thailand in 2011 demonstrated that all viruses gave very high antigenic binding reactivity to the reference virus vaccine strain A/Sakolnakorn/97 rather than A118/87 and A132/87 respectively. In conclusion, the recent FMD type A virus outbreak in Thailand in 2011 was categorized as the most A/Sakolnakorn/97 viral related group rather than A118/87 or A132/87. This investigation was useful for preliminary study of viral grouping by antigenic binding reactivity principle in selecting a high specific reagent that would be used in the assay system. Hence, an ELISA reagent of A/Sakolnakorn/97 system was recommended to use in further investigating of vaccine matching test for FMD type A field virus isolate causing outbreak in 2011.

Keywords: foot and mouth disease virus, antigenic profiling, LP ELISA

Background

Foot and mouth disease (FMD) is a highly infectious viral disease of cloven-hooved livestock and is important in Thailand. Susceptible animals which include cattle, water buffalo, sheep, goats, pigs, and wildlife. The epidemiology of FMD have resulted in the slaughter of millions of animals, despite this being a frequently nonfatal disease for adult animals, though young animals can have a high mortality. FMD viruses isolated from different outbreaks showed much antigenic difference, which generally results in vaccine lacking effective protection against those FMD epidemic strains and even causing FMD outbreak. Therefore, the epidemiological program to assess regularly the antigenic characteristic of field isolates. The monitoring of the antigenic relationship of field isolates in relation to the reference vaccine strain can be show the efficacy of the vaccine virus in use and also has access to select the most suitable vaccine strains in case of vaccine selection. (Ma et. al.(2011). Linchongsubongkoch et.al. (2008), reported that FMD type A field isolated viruses indicated an antigenically changed from virus vaccine strain from time to time and associated with the re-circulation of serotype A in the field causing problem for the control of field virus through the
appropriate vaccine. Knowledge on the antigenicity of circulating viruses was important to select most suitable vaccine strains and ensure prompt identification of new variants. Hence the selection of suitable and specific diagnostic reagents corresponding to the current virus outbreak strain become an very important in assay system such as antigen titration, vaccine matching or other research purpose. An antigenic profiling ELISA was carried out in antigen titration process by using polyclonal antisera which was suitable approach for selecting an appropriate ELISA reagent system for further investigation of vaccine matching or other research studies. This study was to analyze the antigenic binding reactivity of field isolate viruses serotype A causing outbreaks in Thailand and Lao PDR during 2005-2007 and Thailand in 2011 comparing with several reference virus vaccine strains. The result would be calculated in percent binding reaction between test sample and reference virus.

Materials and methods

Reference viruses and field viruses
Reference viruses type A118/87, A132/87, A22-Iraq 24/64 and A/Sakolnakorn/97 were obtained from seed vaccine strains, except for A22-Iraq 24/64 was provided from World Reference laboratory for FMD (WRL), Pirbright Laboratory, UK. Field samples from FMD infected animals submitted for laboratory diagnosis which were from Lao PDR, Thailand during 2005-2007 and Thailand in 2011 which subjected for serotype identification using standard ELISA typing and the virus isolation test by inoculating to primary lamb kidney cell for 2-3 passages and further 4 or 5 passages in BHK-21 cell line. Then viral culture supernatant fluid was again confirmed by antigen typing test as described by Roeder and Le Blanc Smith (1987). The reference vaccine strain and field isolate viruses were used in this study.

Antigen titration by ELISA technique
Antigen titration was carried out by double antibody sandwich ELISA method as this following. 96-well ELISA plate was coated by rabbit trapping antiserum raised against FMD type A118/87, A132/87, A22-Iraq 24/64 and A/Sakolnakorn/97 in separated plate for each serotype at dilution 1:5000 in Carbonate/bicarbonate buffer (Sigma). Incubate at +4°C overnight. Prepare two fold dilution series of virus isolation fluid and reference virus (1:2, 1:4, 1:8, 1:16, ....., 1:128) in PBST buffer , 50 ul/well was added from row A to G, row H for blank, sample no. 1-10 was added in column 1-10, and column 11-12 for control reference virus. Plate were incubated at 37 °C incubator on rotary shaker for 1 hour, wash plate 4 times. Guinea pig detecting antisera against FMD type A118/87, A132/87, A22-Iraq 24/64 and A/Sakolnakorn/97 in blocking buffer were prepared and added to each corresponding plate, incubated at 37°C 1 hour, then wash as before. Horsedadish peroxidase conjugate was added across the plate, incubated at 37°C1 hour, then wash as before. TMB substrate was added, leave at room temperature for 20 minutes, then stop reaction with 1 M H2SO4. The color reaction was measure by reading optical density (OD) of ELISA reader at wave length 450 nm.

Antigenic binding reactivity
Antigenic binding reactivity was expressed as percentage of binding reactivity (% binding) between the OD of test sample and OD reference virus at the antigen dilution where as an OD in range 1.0-1.5, in this assay system, the appropriate dilution used in calculating percentage of binding reactivity was at 1:16 after subtract of background (Bg) in each plate.

\[
\text{% binding reactivity} = \frac{\text{OD sample-OD Bg}}{\text{OD reference virus-OD Bg}} \times 100
\]
Results

All field isolate virus and reference vaccine virus used in this study were listed in table 1. The result of antigenic profiling test of FMD type A field isolates from Thailand and Lao PDR during 2005-2007 compared with reference vaccine strain of A132/87, A Iraq 24/64, A/Sakolnakorn/97 and A118/87 were shown in figure 1, 2, 3 and 4 respectively.

Table 1. List of FMD field isolate viruses and reference virus vaccine strain used in this study in Thailand and Lao PDR during 2005-2007 and 2011

<table>
<thead>
<tr>
<th>Sample number</th>
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Figure 1. Result of % binding reactivity of FMD type A field isolate viruses from Thailand during 2005-2007 to the reference virus vaccine strain A132/87, A Iraq 24/64, A/Sakolnakorn/97 and A118/87. The result showed a high antigenic binding reactivity to the reference virus vaccine strain A118/87 rather than A/Sakolnakorn/97, A Iraq 24/64 and A132/87 respectively.
Figure 2. Show the percentage of binding reactivity field isolate virus in Thailand in 2011 react with Reference vaccine strain A118/87 system, all samples gave <40% binding reactivity.

![Figure 2](image)

Figure 3. Show the percentage of binding reactivity field isolate virus in Thailand in 2011 react with Reference vaccine strain A/Sakolnakorn/97 system, majority gave 50-80% binding reactivity.

![Figure 3](image)

Figure 4. Show the percentage of binding reactivity field isolate virus in Thailand in 2011 react with Reference vaccine strain A132/87 system, all samples gave <40% binding reactivity.

![Figure 4](image)

Conclusion

The antigenic profiling ELISA of FMD type A field isolate viruses from Thailand and Lao PDR during 2005-2007, majority was given a high antigenic binding reactivity to the reference virus vaccine strain A118/87 rather than A/Sakolnakorn/97, A22 Irag 24/64 and A132/87 respectively. In contrast, most field isolate viruses type A causing outbreak in Thailand in 2011 indicted the very high antigenic binding reactivity to the reference virus vaccine strain A/Sakolnakorn/97 (>50-80% binding reactivity)) rather than A118/87 (<40% binding reactivity) and A132/87 (<40% binding reactivity) respectively, it mean that filed isolate virus showed remarkable close antigenic reactivity to the homologous virus (Samuel et. al, 1991). However, Aggarwal et al. (2011) reported that antigenicity of FMDV could be changed because of frequent mutations in it's genome, thus...
evade the protective immunity provided by vaccine. Therefore, other research study was needed to support that occurrence. An antigenic profiling could provide a rapid indication of vaccine matching investigation and this results motivated by the necessity of large-scale immunological thoroughly. Figure 1 showed that propensity of antigenic binding reactivity of FMD type A field isolate viruses from infected samples of Thailand and Lao PDR during 2005-2007 were remarkable as high antigenic binding reactivity to the reference virus vaccine strain A118/87 rather than A/Sakolnakorn/97, A22 Irag 24/64 and A132/87 respectively. Furthermore, the field outbreak of type A during 2006-2007 were closely related to type A 118/87 vaccine strain (Linchongsubongkoch et al., 2008). However, in 2011 the antigenic profiling result was demonstrated high % binding reactivity to A/Sakolnakorn/97 system than other as shown in figure 2, 3 and 4. Interestingly, most of type A field outbreak in Thailand in 2011 were demonstrated the high antigenic binding reactivity with A/Sakolnakorn/97 system (> 50-80% binding reactivity as showed in figure 3 ) and less binding reactivity to A118/87 and A132/87 (<40% binding reactivity as showed in figure 2 and 4) . This result could be provided the preliminary basic information of viral grouping in selecting an appropriate ELISA reagent to use further vaccine matching.

In conclusion, antigenic profiling study of FMD type A from Thailand and Lao PDR during 2005-2007 was indicated that most of field viruses gave a high binding reactivity to the vaccine strain A118/87 while the FMD type A in 2011 was indicated that most of field viruses gave high binding reactivity to A/Sakolnakorn/97. This investigation was useful for preliminary study of antigenic grouping in basic principle for selecting a high specific reagent that would be used in the diagnostic assay system. Hence, an ELISA reagent of A/Sakolnakorn/97 system was recommended to use in further investigating of vaccine matching test for FMD type A filed isolate virus causing outbreak in 2011.

Acknowledgement

The author wish to acknowledge the RRL staffs for their help and participation in the successful implementation of this study. Special thanks to expert of National Institute of Animal Health (NIAH) for English proofreading of the manuscript.

References


Repeated and Intermittent Shedding of Rotavirus A during the Lives of Farm-raised Pigs

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Abstract

Rotavirus A (RVA) is a common cause of diarrhea in young pigs. Although implementing and maintaining appropriate health management practices are obviously crucial for controlling RVA-induced diarrhea in piglets, there is a lack of understanding of the viral ecology within these animals from birth to slaughter. Here, to understand RVA infection dynamics within a farm, we conducted a longitudinal observational study on RVA shedding in a group of ten pigs from birth to slaughter. A total of 400 fecal samples were collected from ten individual pigs from three sows at the intervals of twice a week or once in every two weeks. Seventy-one samples (17.5%) were positive for RVA by reverse transcription PCR designed to detect the VP7 and VP4 genes. At least 13 combinations of 5 G (G2, G4, G5, G9, and G11) and 6 P (P[6], P[7], P[13], P[23], P[27] and P[34]) genotypes were identified by direct sequencing of the PCR products. We were able to detect RVA VP7 sequences from each pig 4 to 6 times with intervals of 7 to 52 days (from 7 to 119 days of age). Each pig harbored RVAs with at least 3 to 6 different combinations of G and P genotypes, while repeated excretions of RVAs carrying the same combinations of G and P genotypes were also observed. Virus shedding and changes in G and P genotypes appeared to be associated with movement of the pigs into weaning, growing and finishing barns. These results indicated that, over their lifetimes, pigs raised for meat frequently and intermittently excrete genetically diverse RVAs.

Keywords: pig, rotavirus, genotype, infection dynamics, epidemiology

Background

Rotavirus A (RVA) is a major cause of severe diarrhea in children worldwide and is estimated to cause more than 453,000 deaths each year among children aged 5 years or younger (Tate et. al., 2012). Similar to findings in humans, RVA is also one of the most frequently detected enteropathogens associated with acute enteritis among young farm animals such as piglets and calves. RVA infections pose an economic threat to the livestock industry due to increased morbidity and mortality rates, and poor growth performance (Dhama et. al., 2009). Although implementing and maintaining appropriate health management practices are obviously crucial for controlling RVA-induced diarrhea in piglets and calves (Dhama et. al., 2009), there is a lack of understanding of the viral ecology within these domestic animals from birth to slaughter.

RVA is a member of the genus Rotavirus, within the family Reoviridae. Its genome consists of 11 segments of double-stranded RNAs. Since the two outer capsid proteins VP7 and VP4 independently induce neutralizing antibodies, they form the basis for the dual classifications system for G and P types, respectively, and play an important role in protective immunity (Estes and Kapikian, 2007). Recently, a new rotavirus classification system, which defines different genotypes for all the 11 genome segments based on the percent nucleotide sequence identity in each segment, was
proposed (Matthijnssens et. al., 2008). According to this scheme, 27 G and 35 P genotypes have been described for RVAs of humans and animals (Matthijnssens et. al., 2011). To date, a variety of genotypes including 12 G genotypes (G1-G6, G8-G12, and G26) and 14 P genotypes (P[1], P[5]-P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32], and P[34]) have been detected in pigs.

Although five G (G1 to G4, and G9) and three P genotypes (P[4], P[6], and P[8]) represent the majority of clinically important RVA strains in humans (Santos and Hoshino, 2005), the intensive RVA surveillance associated with introduction of the RVA vaccine into human populations has resulted in the detection of RVAs with unusual genotypes, including those commonly detected in pigs and cattle (Gentsch et. al., 2005; Iturriza-Gómara et. al., 2012). Animal RVAs are therefore regarded as a potential reservoir for the genetic diversity of human RVAs, and consequently their ecology has been of great concern (Martella et. al., 2005). Further, an increase has been noted in the incidence of porcine RVA diarrhea on large-scale pig farms (Dewey et. al., 2003), and emergence of different RVA genotype or new genetic variant of a specific genotype have been observed in association with diarrheal outbreaks in suckling pigs (Lorenzetti et. al., 2012; Miyazaki et. al., 2011). Understanding the viral ecology within a pig population is therefore crucial for preventing or reducing the incidence of porcine RVA diarrhea as well as transmission of porcine RVAs to humans. However, most studies of porcine RVAs have focused on the genotypes present in suckling and weaned pigs, which is not informative about viral ecology and genotypic characteristics when animals are raised on farms over an extended period.

In the present study, to understand the ecology of RVA infection within a farm over an extended period, we investigated the excretion dynamics and genotypic characteristics of RVAs isolated from animals over their lifetimes on a farrow-to-finish swine farm in Japan.

Materials and methods

Study design. The study was performed in a 900-sow farrow-to-finish farm managed by a continuous flow production system, from November 2002 to June 2003. Pigs were nursed with their sows in each farrowing pen until weaning at 28 days of age. They were comingleed with those from other litters at the time of weaning, transferred into a weaning barn, and distributed into pens in a weaning barn (10 to 12 pigs per pen). Pigs were next transferred into growing and finishing barns at 59 and 101 days of age without rearranging the group or commingling with other pigs. Fences that allowed for nose-to-nose contact separated the pens in each barn. Ten piglets from three litters (three or four piglets per litter) born on the same day were randomly selected and ear-tagged for identification. They housed together as one group after weaning without commingling of other pigs. Fecal samples were directly collected from the rectum of each pig twice weekly (from 7 to 119 days of age) or once every two weeks (after 119 days of age).

RNA extraction and reverse-transcription PCR (RT-PCR). Total RNA was extracted from fecal suspensions using TRizol-LS (Invitrogen Corp., Carlsbad, CA, USA) and subjected to RT-PCR. The VP7 gene and the VP8* fragments of the VP4 gene were amplified using a Qiagen OneStep RT-PCR kit (QIAGEN, Valencia, CA, USA) with primer pairs Beg9/End9 and Con2/Con3, respectively (Gentsch et. al., 1992; Gouvea et. al., 1990). Although the VP4 genes were further reamplified using the Takara ExTaq kit (TaKaRa Bio Inc, Shiga, Japan), the VP4 sequences were able to be determined in only 24 of the 71 samples in which VP7 sequences were detected. Therefore, the samples were further analyzed for the VP4 gene using a different primer pair VP4-13F/VP4- 821R, which was designed to detect porcine RVAs (Miyazaki et. al., 2012).
G and P genotyping. G and P genotypes were determined by direct sequencing of the VP7 and VP4 PCR products, respectively. The sequences were assembled, edited, and analyzed using MEGA 5 software. The nucleotide sequences of the VP7 or VP4 genes from the fecal samples were compared with those of reference strains available from GenBank (Matthijnssens et. al., 2011). Alignments of multiple sequences were performed using the CLUSTAL W algorithm. Genetic distances were calculated using the Kimura-2 correction parameter, and phylogenetic dendograms were constructed via the neighbor-joining method with 1,000 bootstrap replications (Matthijnssens et. al., 2008).

Results

Detection rate of RVAs. Of the 400 fecal samples collected from 10 individual pigs from 7 to 217 days of age, 71 (17.8%) were positive for the RVA VP7 gene (Table 1). The detection rate was highest during the weaning period (37.8%), followed by the growing (20.0%), finishing (6.7%), and suckling (3.3%) periods. The detection rate of RVA nucleotide sequences was significantly higher in soft to watery (20.0%, 4 of 20) than in solid (4.0%, 4 of 100) feces in the finishing period \((P < 0.01)\). RVAs were also detected at higher rates in soft to watery than in solid feces in the weaning (56.3%, 9 of 16 vs. 33.8%, 25 of 74) and growing (22.7%, 17 of 75 vs. 18.2%, 10 of 55) periods, although the differences were not judged to be statistically significant. The relationship between RVA detection and fecal consistency could not be evaluated during the suckling period because the records of fecal consistency were not available for feces collected from 7 to 17 days of age, and only two samples were positive for RVA sequences.

Genetic analysis of RVAs. The nucleotide sequences of the VP7 genes were successfully determined for 60 samples. Phylogenetic analysis allowed us to classify the 60 RVA sequences as one of five G genotypes (G2, G4, G5, G9, and G11) of 27 established G genotypes. The VP7 genes of the isolates belonging to the same G genotype were genetically closely related to each other with more than 99.7% nucleotide (nt) identities to each other, except those of the G5 isolates which shared 98.7% to 100% nt identities to each other.

The VP4 nucleotide sequences were determined in 65 of the 71 samples. Phylogenetic analysis enabled us to classify the 65 RVA VP4 sequences as one of six P genotypes (P[6], P[7], P[13], P[23], P[27], and P[34]) of 35 established P genotypes. The nucleotide sequences corresponding to the P[6], P[7], P[23], P[27], and P[34] genotypes shared greater than 99.0% nt identities, and each genotype was able to be assigned to a single branch. In contrast, the P[13] sequences were able to be easily separated into three clusters, with nt sequence identities within and between clusters ranging from 99.9% to 100% and 77.7% to 78.9%, respectively (data not shown).

Shedding pattern of RVAs. Each of the pigs started to excrete RVAs at an average age of 28 days old (ranging from 7- to 42-days-old) and ended at an average age of 109 days (range: 98 to 119 days). RVA shedding could be detected repeatedly at least 4 to 6 times within 7 to 52 days. The G and P genotypes detected in each pig are summarized in Table 1.

In the suckling period, virus shedding was detected in only one pig at 7 (pig #6, G4 P[6]) and 24 (pig #2, P[6] genotype) days of age, respectively.

Within one week after weaning, RVA shedding was detected in the feces of seven pigs (#1, #3, #5, and #7-#10) for the first time and in two pigs (#2 and #6) for the second time. This peak in RVA shedding included combinations of two G (G5 and G9) and three P (P[6], P[13] and P[23]) genotypes. Mixed infection with more than one RVA strain was observed in two pigs (#2 and #9 at 31 days). We detected a second peak of RVA shedding (from 42 to 45 days of age) soon after the first peak in the
same weaning barn. In nine pigs, RVAs had been detected on previous occasions, and in one pig (#4), this was the first instance. We detected RVAs sequences of G5 together with P[7] or P[13] genotypes in the second peak. Notably, the same G5 genotype RVAs were detected at the first and second peaks in five pigs (#3, #6, #7, #9, and #10). Among them, the same combinations of G and P genotypes were detected in two pigs (G5P[13] in #7 and #10). The mixed infection was also observed at this time in four pigs (#1, #3 and #5 at 45 days, and #8 at 49 days).

The third peak was characterized by predominant detection of G11 in combination with P[13], P[27], or P[34] genotypes starting from 56 days and peaked at 59 days, which were just before and after their movement into the growing barn. G2P[27] genotype RVAs were detected sporadically in the first half of the growing period (59 to 73 days). The mixed infection was observed again at this peak in pig #7 at 59 days.

The fourth peak was characterized by predominant detection of G5P[7] genotypes starting from 88 days and peaking at 98 days one week before their transfer into the finishing barn. G5P[7] genotypes were detected consistently in the feces of pigs #4 and #6 before they entered the finishing barn, and in pig #4 even after its entry. P[7] sequences sharing a close genetic relationship with those identified at the second peak were detected in pigs #2, #4, #6, and #7 again at this peak. Re-excretion of the same G5P[7] genotype was observed in pigs #4, #6, and #7. Other genotypes were identified in pigs #2, #5, and #9 and included G5P[13], G5P[27], G11P[7], and G11P[13]. Mixed infection was also observed at this peak in pigs #7 and #10 at 98 days of age. During the finishing period, G2 and G11 combined with P[7], P[13], and P[34] genotypes were detected sporadically from 112 to 119 days.

A total of 13 different combinations of G and P genotypes (G2P[27], G2P[34], G4P[6], G5P[6], G5P[7], G5P[13], G5P[27], G9P[6], G9P[23], G11P[7], G11P[13], G11P[27], and G11P[34]) were identified among pigs aged 7 to 217 days. We identified RVAs with at least three to six different combinations of two or three G and two to five P genotypes in individual pigs. Repeated excretion of RVAs with the same G or P genotypes occurred in eight pigs (#2-#4, and #6-#10). Among them, six pigs (#3, #4, #6, 7, #9, and #10) excreted RVAs twice with the same combinations of G and P genotypes with intervals ranging from 10 to 56 days.

Discussion

Here, we demonstrate the repeated and intermittent lifetime shedding of genetically diverse RVAs into feces by individual pigs raised for meat production in a longitudinal observational study. Virus excretion and changes in the G and P genotypes tended to occur in association with movement of the pigs into different barns. These facts suggest that such repeated virus shedding and changes in G and P genotypes may have resulted from exposure to different RVA strains that persist in each barn, because the farm described in the present study was managed by a continuous-flow system, and consequently not every pen in the barns could be washed and disinfected to eliminate excreted viruses. Further, viruses could also be transmitted from neighboring pigs, because pens were separated by fences that allowed the pigs to come into direct contact. The frequency of exposure and excretion of RVAs within a pig herd might therefore be reduced by changing the management system and structure of the pens. In addition, our study also demonstrated that mixed infection occurs frequently throughout the lifetime of the pigs. The genetic diversity of porcine RVAs within a herd might be maintained through the mixed infection and subsequent gene reassortment events.

The zoonotic potential of porcine RVAs has been a great concern, and an increase has been noted in the incidence of porcine RVA diarrhea on large-scale pig farms. Together with continuous monitoring of genotypic characteristics of porcine RVAs, understanding the viral ecology within a pig population is crucial for preventing or reducing the incidence of porcine RVA diarrhea as well as transmission of porcine RVAs to humans. We believe that our data illuminate one set of mechanisms influencing the distribution of RVA in a pig population.
Acknowledgments

We thank Drs. Hidetoshi Ikeda and Koshi Yamamoto for providing the fecal samples and information about the farm's history. We would like to also thank Dr. Itsuro Yamane for advice on statistical analysis and Hiroko Kunifuda for technical support. This research was supported in part by a research grant from the National Institute of Animal Health, Japan.

Table 1. G and P genotypes of RVAs detected in feces collected from 10 pigs from 7 to 217 days of age.

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The * indicates the samples were negative for RT-PCR targeting the VP7 and VP4 genes. Gx and P[x] indicate that VP7 and VP4 sequences could not be determined because of mixed infection with more than one strain of RVAs, respectively. Gnd and P(nd) indicate that the VP7 and VP4 nucleotide sequences could not be determined because of unsatisfactory quality of the sequence trace data.
References


Molecular characterization and phylogenetic study of caprine arthritis encephalitis viral sequences from goats in Northern Thailand

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Abstract

Caprine arthritis-encephalitis virus (CAEV) is Lentivirus infection that causes chronic disease in goat. This study focused on the partial of gag gene of 3 Thai CAEVs isolated from normal healthy, more than six-month-old goats. The infected goats were diagnosed by the presence of CAEV-specific antibody by ELISA assay. The gag gene was sequenced and created the phylogenetic tree. The results showed that all Thai CAEV strains sharing nucleotide sequence identities 92.5-98.0 % within the strain, 90.1-91.4 % with wild type strain CAEV-Co (USA) sharing and 61.8-62.6 % with prototype SA-OMVV (South Africa). Deletion or insertion within the gag gene of all Thai isolates was not found. The prototypic strains CAEV-Co, CAEV-Gansu (China), and CAEV1GA (Norway) closely related to 3 Thai isolates were in 1st cluster of CAEV, whereas the wild type strains SA-OMVV, Swiss Goat (Switzerland), P10LV (Portugal), EV1 (Scotland), 85/34 (USA), and K1514 (Iceland) were classified into the 2nd cluster of MVV. The results suggested that all Thai CAEV isolates might have originated from the same the prototypic CAEV-Co strain ancestor.

Keywords: caprine arthritis encephalitis (CAEV), phylogenetic analysis, gag gene, Thailand

Background

Caprine arthritis-encephalitis virus (CAEV) is lifelong persistent viral infection that causes chronic polyarthritis in adult animals, interstitial pneumonia, progressive weight loss and mastitis with decreased milk production in adult goats. CAEV also causes leukoencephalomyelitis in young goats (Cork, 1974; Crawford, 1980a; Crawford and Adams, 1981; Rowe and East, 1997; Smith and Sherman, 1994; Matthews, 1999; Pugh, 2002). Most goats infected with CAEV become asymptomatic carriers. The major route of viral transmission usually occurs vertical from infected dam to kid through milk or colostrums and horizontal through contact between infected goat and healthy goat via secretions (uterus, vagina, saliva and respiratory) including infected blood, and breeding (Rowe, 1991, 1992; Rowe and East, 1997). CAEV is a member of group genus Lentivirus, family Retroviridae that also includes the human immunodeficiency virus (HIV), simian-immunodeficiency virus (SIV), bovine immunodeficiency virus (BIV), maedivirus virus (MVV), equine infectious anemia virus (EIAV), and feline immunodeficiency virus (FIV). It is an enveloped, single stranded RNA virus. The genome of this is approximately 6200 bp in length The genome the CAEV genome contains between two long terminal repeats (LTR), gag, pol and env genes encoding for structural and enzymatic proteins. The vif and rev genes encoding for regulatory proteins. and a vpr-like encoding an accessory protein (Villet et al., 2003). CAEV was first isolated in 1980 from a goat with arthritis (Crawford et al., 1980 a). The CAE has been reported in most goat countries in France, Italy, Spain, Norway, Iceland, Wales, Switzerland, Poland, Mexico, Brazil, Jordan, Saudi Arabia, Syria, Turkey, Australia, India, Hungary, USA, Japan and China. (Kusza, 2004; Torres-Acosta, 2003; Angelopoulou, 2005; Konishi, 2004, Kuzmak, 2007, Nord,
In Thailand, Tantaswasdi et al. (1985) reported CAE-Like virus in Saanen goats during 1984-1985. In this study, the \textit{gag} gene of CAEV, the highly conserved across strains, was investigated. This \textit{gag} gene encodes information for one major Capsid or p28 and two minor Matrix and nucleocapsid core proteins. The capsid protein is highly immunogenic (Clements and Zink, 1996). These informations are useful for a further study of molecular biology of CAEV that is prevalent in northern provinces of Thailand and using modern techniques in biotechnology will provide the means for the effective control and eradication of this important disease of goats.

\**Methods**

\textit{Animals and blood collection}: Four hundred cross breed goats of a variety's age (> 6 month) were convenient selected from four provinces (Chiang Mai, Lampang, Pha Yao, and Nan). Heparined blood collection from Jugular vein were collected between 1 to 4 February 2011. Sampling was based on a minimum sample size of 385 animals, assuming an expected prevalence of 50 \%, a confidence interval of 95\%, and an error of 5\% (Win Episcope 2.0).

\textit{Viruses}: A total eight animals sero-positive as determined by competitive ELISA kit. The peripheral blood leukocytes were isolate from 10 mL of blood that drawn into EDTA-coated tubes by centrifugation at 1500 × g for 25 min. Erythrocytes were haemolyzed by osmotic shock with H$_2$O and 2× phosphate-buffered saline (PBS). Following two washes with PBS, the cell pellets were extracted by using Qiagen DNA viral kit (Qiagen, Germany). The genomic DNA isolation was resuspended in Diethylpyrocarbonate (DEPC) treated water.

\textit{Reverse transcription-polymerase chain reaction}: The nested PCR were applied to detected the 512 bp of fragment proviral DNA, focused on the \textit{gag} gene of CAEV genome. The outer primers were GEX5 5-GAAGTGGTGCTGGAGAGGTCTTG-3 and GEX3 5-TGCCTGATTGGCTTCGGA-3 and inner primers were GIN5 5-GATAGAGCATGGCGAGGCAAGT-3 and GIN3 5-GAGGCCCATGCTGGCTACTGTG-3 (Guiguen, 2000). First-round amplifications were performed by using 5 uL of genomic DNA were added in a 50 µL PCR reaction containing 25 µL of 2× reaction buffer (Invitrogen), 1 µM of each primers, 1 µL SuperScript III/Platinum Taq High Fidelity enzyme mix (Invitrogen) and 17 µL of DEPC-treated water. PCR cycling conditions were as follows: 5 min at 95°C, followed by 35 cycles of 15 sec at 94°C, 30 sec at 50°C, 60 sec at 68°C, with a final extension of 15 min at 68°C. Second-round PCRs were performed with 2 uL of the first-round reaction used as template under the following same conditions. All PCR reac-tions were carried out in MJ research PCR machine (Bio-Rad, USA.). The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide (EtBr), and visu-alized under ultraviolet transillumination.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Isolates & Accession Number & Province/Country \\
\hline
CAEV-Co & M33677 & U.S.A. \\
CAEV-Gansu & AY900630 & China \\
CAEV1GA & AF32109 & Norway \\
NAN-6/2011 & JF714255 & Nan/Thailand \\
LAMPANG-56/2011 & JF714253 & Lampang/Thailand \\
LAMPANG-1/2011 & JF714254 & Lampang/Thailand \\
Swiss Goat & AY445885 & Switzerland \\
P10LV & AF479638 & Portugal \\
SA-OMVV & M31646 & South Africa \\
EV1 & S51392 & Scotland \\
85/34 USA & AY101611 & USA \\
KV1514 & M10608 & Iceland \\
\hline
\end{tabular}
\caption{GenBank accession numbers of Thai CAE viruses isolates and reference strains}
\end{table}
Sequencing and phylogenetic analysis: The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany), calculated the concentration of them by using an ND-1000 NanoDrop spectrophotometer (NanoDrop Tech., USA) at 260 nm, ligated with a pGEM®-T vector system (Promega, USA), and transferred into *E. coli* DH5α by using Gene Pulser (Bio-RAD, USA). The clones were determined by using both directions of M13 primers (pGEM®-T vector) and Bigdye® Terminator version 3.1 chemistry (Applied Biosystems, USA), according to the manufacturer’s instructions. Reactions were run on a 3130 DNA analyser (Applied Biosystems, USA). After sequencing, assembly of sequences, removal of primers and low quality sequence data by using SeqScape® software version 2.5 (Applied-Biosystems, USA), multiple nucleotide sequence alignments with the Clustal W algorithm, translation nucleotide into protein sequence, and processing were performed with the Bioedit software version 7.0.4.1 (Thompson, 1994). The phylogenetic analysis, based on partial genome nucleotide and amino acid sequences were constructed with Molecular Evolutionary Genetics Analysis (MEGA, version 4) (Tamura et al., 2007) using neighbor joining tree inference analysis with the Tamura-Nei model. The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985) and 1000 bootstrap replications were used.

Results

Analysis and comparison of inferred amino acid sequences: The three in eight sero-positive goats were also PCR positive which were described in Table 1. The three Thai CAEV strains sequenced consisted of 496 nucleotides of *gag* gene and 164 amino acid residues. All sequences were deposited in the GenBank database and given the accession numbers (Table 1). Nucleotide and amino acid sequence homology results are described in Table 2. We found that the all Thai CAEV strains sharing 92.5-98.0% nucleotide sequence and 91.0-97.4% amino acid sequence identities within strain, with the prototypic CAEV-Co sharing 90.1-91.4 % nucleotide sequence and 89.7-91.6% amino acid sequence identities and with SA-OMVV sharing 61.8-62.6 % nucleotide sequence and 53.3-53.9 % amino acid sequence identities. Moreover, we did not found deletion or insertion within the *gag* gene of these 3 isolates. The amino acid sequence alignments of all Thai isolates comparing with those of reference strains revealed the presence of amino acid sequence conservation as shown in Fig 1. These regions were amino acid sequence YPEL (Motif I) at position between 8-11, LTEGNCLW  (Motif II) at position between 31-38, EPWT (Motif III) at position between 53-56 and FQQLQ (Motif V) at position between 160-164 that showed a perfect similarity in CAEV-Co, CAEV-Gansu, CAEV1GA, SO-MVV, EV1, 38/54 USA, P10LV and K1514 strain. In contrast, we found a high heterogeneity that the three Thai isolates related other CAEV isolates, like the prototype CAEV-Co strain but unlike MVV-related strains have a deletion of a sequence of 7 amino acids QLYPNLE (Motif IV) at positions 128-134 of the MA protein.

<table>
<thead>
<tr>
<th>Name of sequences</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
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<td>58.8</td>
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<td>57.0</td>
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<td>89.1</td>
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<td>7.Swiss Goat</td>
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<td>85.0</td>
<td>86.9</td>
<td>86.7</td>
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</tr>
</tbody>
</table>

Table 2. Comparison of specific nucleotide and deduced amino acid sequences of *gag* gene. Nucleotide identity (%) in lower triangle; Deduced amino acid identity (%) in upper triangle.
Phylogenetic analysis: The phylogenetic analysis of 12 lentivirus isolates was performed from Thailand, while 9 of which were known prototypic strains of ovine/caprine lentiviruses obtained from GenBank. Based on the partial gag genes, performed using the neighbor-joining method helped to identify, well supported by the bootstrap values, and derived phylogenetic tree as shown in Fig. 2. It was found that lentivirus clustered into 2 major clusters. The 1st cluster belong to CAEV group consisted of 3 Thai strains, prototypic strain CAEV-Co, CAEV1GA and CAEV-Gansu strain and the 2nd cluster belong to MVV group comprised wild type strain SA-OMVV, K1514, P10LV, 85/34 USA and EV1.

Fig. 1. Multiple alignment of the deduced amino acid sequence of the gag gene encoding matrix and the N-terminal part of capsid protein. Dots indicate identity with the consensus sequence, whereas dashes indicate deletions. Motifs I, II, III, IV, V are described in the text.

Fig. 2. Phylogenetic relationships of the gag gene of CAEVs isolated from Thailand and compared with other lentivirus sequences in GenBank. Tree was generated by using MEGA 4 program (neighbor-joining analysis method and with Tamura nei model). Numbers below branches indicate bootstrap values from 1000 replicates. Analysis was based on nucleotides 496 bp.
Conclusions

In this study, the nucleotide sequences of a 496 bp from 3 Thai CAEV isolates from naturally infected goats were determined. The present study focused on the sequences of \textit{gag} gene for our comparison and phylogenetic analysis because this region is well known to be specific of the group of lentiviruses and have less divergences intra-group compared to those of the envelope gene (Rolland, 2002). This region has also been well studied by PCR that is used for diagnosis of the infection with high sensitivity and specificity (Kuzmak, 2003). CAEV provirus in 65 % of sero-positive goats were not detected by PCR. This could be because of the a very low copy number of proviruses present in the samples tested with corresponding to a very low rate of infected monocytes in peripheral blood, that correspond also to a greater number infected cells in organs rather than blood as already observed in HIV-1 infection in humans (Edgington, 1993; Kuzmak, 2007). Indeed, in contrast to primate lentiviruses that replicates both in CD4+ T cells and monocyte/macrophage cell lineage, goat and sheep lentiviruses are restricted in vivo to infection of monocyte/macrophage cell lineage, and the replication occurs only in tissue when infiltrating monocytes differentiate into macrophages (Gendelman, 1986; Narayan, 1988). Finally, this region was of interest because it encodes almost the entire matrix and N-terminal part of capsid protein of the \textit{gag} gene (Kwang, 1996). Our result indicated that most of these goat CAEV isolates from northern part of Thailand are closely related to CAEV-Co sharing 90.1-91.4% and 89.7-91.6% similarity in nucleotide and amino acid level, respectively. These isolates share greater similarity and cluster mainly with CAEV-Co strain as was evidenced by the phylogenetic tree, probably having unique ancestor that is closely related to CAEV-Co strain. We found long peptide (Motif I, II, IV and V) was a significant value for detection of lentiviruses present in sheep and goats living in mixed flocks and those in close contact. In addition, these peptides may elucidate the events of cross-species transmission of these viruses following the jump of the species barrier (Kuzmak, 2007). This study is the first to report the sequence of goat lentiviruses from Thailand. Whether the high proportion of CAEV sero-positive goats in Thailand that we found in this study result from the spread of isolates that cause rapid infection and diffusion is unknown. The high prevalence probably also results from the lack of any eradication program.

Acknowledgments

We gratefully acknowledge Dr. Boonprom Enkvetchakul for critical reading of manuscript. The technical assistance of National Institute of Animal Health, Bangkok and Reginal Veterinary Development Center, Lampang for their assistance and also the livestock officer of Chiang Mai, Pha Yao, Nan and Lampang province for their kind assistances.

References


Reactivity of monoclonal antibodies raised against an A(H1N1)pdm2009 virus with swine influenza viruses of different lineages

Antigenicity of swine influenza viruses

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Abstract

A new influenza virus strain, which was designated as ‘Pandemic A(H1N1)2009 (A(H1N1)pdm09) virus’ afterwards, caused the first pandemic in humans in the 21st century. The virus was arisen from a genetic reassortment between two different swine influenza viruses (SIVs) circulating in Eurasian and North American pigs, respectively. In consequence, the virus acquired an HA gene descended from a classical H1 SIV. The A(H1N1)pdm09 virus can be transmitted from human to domestic animals; several cases of the human-to-pig transmission have been reported worldwide since the widespread in human population. The antigenic relationship among human and swine A(H1N1)pdm09 viruses and their ancestral classical H1 SIVs is not well studied. In this study, to understand the antigenicity of A(H1N1)pdm09 viruses, we produced the monoclonal antibodies (mAbs) against A/Narita/1/2009 (H1N1) which was the first A(H1N1)pdm09 virus isolated from human in Japan. Nineteen monoclonal antibodies with hemagglutination inhibition (HI) activities against A/Narita/1/2009 (H1N1) were established with standard procedure for mice hybridoma production. HI test using a panel of the mAbs against two human and four swine A(H1N1)pdm09 viruses, four classical swine H1 viruses, an avian-like swine H1 virus and a human seasonal H1 virus was performed. Sixteen mAbs reacted with all of the A(H1N1)pdm09 viruses used. Ten out of them recognized one or more classical SIVs with titers of 1:32 to 1:256. It was suggested that the A(H1N1)pdm09 and classical SI viruses shared conserved epitopes. In addition, 3 mAbs reacted with much lower HI titers against the human A(H1N1)pdm09 isolate, A/California/04/2009 (H1N1). Only 2 mAbs recognized the avian-like SIV with titers of 1:32. No mAbs showed HI activities against the human seasonal virus. Taken together, the mAbs produced in this study would be useful for antigenic characterization of A(H1N1)pdm09 viruses.

Keywords: Influenza virus, Pig, Pandemic A(H1N1)2009, Antigenicity

Background

Swine influenza is an acute respiratory disease caused by influenza A virus in pig. Clinical signs including fever, coughing and nasal discharge are observed in pigs affected (Olsen et.al., 2006). Due to a high morbidity rate (approximately 100%) and a loss of appetite causing weight loss of pigs, swine influenza has been estimated to be one of the most costly diseases in pig industry (Bennett and Ijpelaar, 2005). Therefore, control of swine influenza in pig populations is a major concern for pig producers.

Swine influenza viruses (SIVs) of three subtypes, H1N1, H1N2 and H3N2, are co-circulating in pig population in the world. H1 SIVs possessing ‘classical’ swine H1 genes derived from 1918
Spanish influenza viruses have been predominant in North America and Asia (Olsen et. al., 2006). 'Avian-like' H1 SIVs derived from the introduction of avian viruses into pig population have been predominant in Europe since 1979 (Olsen et. al., 2006). In 2009, 'Pandemic A(H1N1)2009' (A(H1N1)pdm09) virus caused the first influenza pandemic in human in the 21st century. This novel influenza virus was resulted from a reassortment of two parent SIVs (Novel Swine-origin Influenza A(H1N1) Virus Investigation Team, 2009). HA, PB2, PB1, PA, NP and NS genes were from the North American 'triple reassortant' H1 SIV, remaining genes (NA and M) were from the Eurasian avian-like SIV. HA genes of the 'triple reassortant' SIVs were originally derived from those of the classical H1N1 SIVs (Vincent et. al., 2008). Human-to-pig transmission of the A(H1N1)pdm09 viruses have been frequently reported worldwide since after the emergence of it (Howden, et. al., 2009; Pereda et. al., 2010).

Vaccination has been considered to be the primary means for the control of swine influenza in pigs (Olsen et. al., 2006). HA protein expressing on the viral surface is a major determinant for the antigenicity (Kyriakis et. al., 2010b). Thus, emergence of an antigenic variant from vaccine strains is a crucial issue for pig industry (Kyriakis et. al., 2010a). In this study, we produced 19 monoclonal antibodies raised against A/Narita/1/2009 (H1N1) which was the first A(H1N1)pdm09 virus isolated in Japan. Hemagglutination inhibition (HI) assay using a panel of the mAbs was performed to analyze the antigenic relationship among human and swine A(H1N1)pdm09 viruses, classical H1 SIVs and the other different lineage viruses.

**Materials and methods**

**Virus**

A/Narita/1/2009 (H1N1) and A/California/04/2009 (H1N1) represent the A(H1N1)pdm09 viruses isolated from human in Japan and USA, respectively. A/swine/Osaka/1/2009 (H1N1), A/swine/Yamagata/12/2010 (H1N1) and A/swine/Yamagata/19/2010 (H1N1) represent the A(H1N1)pdm09 viruses isolated from domestic pigs in Japan. A/swine/Iowa/15/1930 (H1N1), A/swine/Kyoto/3/1979 (H1N1) and A/swine/Tochigi/1/2008 (H1N2) represent the classical swine H1 viruses. A/swine/Korea/CAN01/2004 (H1N1), A/swine/Belgium/1/1998 (H1N1) and A/Niigata/08F219/2009 (H1N1) represent the triple reassortant H1 SIV, avian-like H1N1 and human seasonal H1N1 virus, respectively. California, Yamagata and Niigata strains were propagated in MDCK cells, the rest strains were propagated in embryonated chicken eggs. A/Narita/1/2009 (H1N1) and A/California/04/2009 (H1N1) were kindly provided from National Institute of Infectious Diseases, Japan and CDC, USA, respectively. A/swine/Korea/CAN01/2004 (H1N1), A/swine/Belgium/1/1998 (H1N1) and A/Niigata/08F219/2009 (H1N1) were kindly provided from Chungbuk National University, Korea, Ghent University, Belgium and Niigata University, Japan, respectively.

**Serological analysis**

Monoclonal antibodies (mAbs) raised against A/Narita/1/2009 (H1N1) were prepared with standard procedure for mice hybridoma production. HI activities against the homologous virus were used for the selection of hybridomas. Hybridomas were cultured in RPMI1640 with 10% FBS at 37°C with 5% CO₂. Supernatant containing mAbs were collected, subsequently subjected to HI assay. HI assay using a panel of the mAbs against the viruses stated above was performed according to the techniques previously described (Takemae et. al., 2012). The erythrocytes of guinea pig (0.5%) were used in HI assay.
Results

Results: Nineteen mAbs (a0042, a0341, a0392, a0420, a0584, a0598, e1578, j0098, e1103, a0964, a0568, a1065, e1027, e1126, a0579, a0937, j0549, j0781, e1149) showing the HI titers from 1:64 to 1:256 against Narita/1/2009 (H1N1) were analyzed (Table 1).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titters with monoclonal antibodies of</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Narita/1/2009</td>
<td>256 256 256 256 64 256 128 128 128 256 256 128 128 64 128 256 256 256 256</td>
</tr>
<tr>
<td>A/swine/Yamagata/12/2010</td>
<td>256 256 256 256 64 256 128 128 128 256 128 128 128 128 128 128 128 128</td>
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<tr>
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<td>256 256 256 32 256 256 64 64 256 256 256 256 256 256 256 256 256 256</td>
</tr>
<tr>
<td>A/swine/Yamagata/19/2010</td>
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<tr>
<td>Classical swine virus</td>
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</tr>
<tr>
<td>A/swine/Kyoto/3/1979</td>
<td>2 16 4 16 2 &lt;2 256 32 128 128 8 256 128 64 128 256 128 32</td>
</tr>
<tr>
<td>A/swine/Tochigi/1/2008</td>
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</tbody>
</table>

It was demonstrated that the A(H1N1)pdm09 viruses isolated from human and pigs were antigenically similar. All of the mAbs used except for three j0549, j0781 and e1149 recognized all of the A(H1N1)09 viruses with similar titers to those of homologous virus (Table 1). This result was supported by the fact that only one or two substitutions of the deduced amino acids within the predicted antigenic sites (Sb, Ca, Cb) in the HA1 protein were observed between Narita/1/2009 (H1N1) and the other A(H1N1)pdm09 viruses used in this study (Fig. 1).

Figure 1. Deduced amino acid substitutions within the predicted antigenic sites (Sa, Sb, Ca, Cb) in HA protein with A/Narita/1/2009 (H1N1).
Antigenicity of the A(H1N1)pdm09 viruses was distinguishable from those of the classical SIVs, although the presence of the common epitopes conserved between the A(H1N1)pdm09 and classical SIVs was suggested. Thirteen out of 19 mAbs recognized one or more classical SIVs including the triple reassortant virus with titers of 1:32 to 1:256 (Table 1). On the other hand, six mAbs (a0042, a0341, a0392, a0420, a0584, a0598) showed titers less than 1:16 with any viruses possessing classical H1 genes (Table 1). Such antigenic differences between A(H1N1)pdm09 virus and classical SIVs were supported by the evidence that 9 to 10 amino acid substitutions were found within the four antigenic sites (Sa, Sb, Ca, Cb) between Narita/1/2009 (H1N1) and the classical SIVs used (Fig. 1). In addition, the possibility that the antigenic drift have occurred among the classical SIVs was suggested. Different reactivity of the classical SIVs against the mAbs was shown. The mAbs (j0098, e1103, a0964, e1027 and e1126) did not react with A/swine/Tochigi/1/2008 (H1N2), a recent classical SIV in Japan; on the other hand, those mAbs reacted with A/swine/Kyoto/3/1979 (H1N1), a vaccine strain in Japan, with titers higher than 1:32 (Table 1). It was true that the 8 amino acid substitutions within the antigenic sites (Sa, Ca, Cb) between A/swine/Tochigi/1/2008 (H1N2) and A/swine/Kyoto/3/1979 (H1N1) were found.

Major antigenic differences of the A(H1N1)pdm09 virus from avian-like SIV and human seasonal virus were demonstrated. A/swine/Belgium/1/1998 (H1N1) reacted with only two mAbs with the titers of 1:32, on the other hand, A/Niigata/08F219/2009 (H1N1) did not react with any mAbs (Table 1).

Discussion

We produced 19 monoclonal antibodies raised against the human A(H1N1)pdm09 virus in this study. HI assay using a panel of the mAbs against the viruses including human and swine A(H1N1)pdm09 viruses, the classical SIVs, the avian-like SIV, and the human seasonal virus was performed. The similar antigenicity between human and swine A(H1N1)pdm09 viruses was demonstrated. On the other hand, the A(H1N1)pdm09 viruses were antigenically distinguishable from the classical SIV which was the ancestor to A(H1N1)pdm09 virus, avian-like SIV and human seasonal virus. Thus, the mAbs produced in this study would be useful for antigenic characterization of SIVs of H1 subtype including A(H1N1)pdm09 viruses.

Acknowledgement

We would like to thank Drs. M. Tashiro, T. Odagiri, T. Kageyama at National Institute of Infectious Diseases, Japan, Y-K Choi at Chungbuk National University, Korea, K. V. Reeth at Ghent University, Belgium and R. Saito at Niigata University, Japan, for providing viruses used in this study.
References


Genetic analysis of Japanese porcine reproductive and respiratory syndrome virus strains from 1992 to 2010

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Abstract

Background: Porcine reproductive and respiratory syndrome (PRRS) causes reproductive failure of sows and respiratory disorder of piglets and growing pigs, and is one of the most economically significant swine diseases in the pig production countries. As for PRRS virus (PRRSV) causing this disease, European (EU, type 1) and North American (NA, type 2) genotypes are classified by approximately 60% nucleotide identity at genome level. Both genotypes are antigenically and genetically diversity. The analysis of genetic diversity for PRRSV field isolates is necessary information for control of PRRS. We analyzed the sequence variation of NA-type PRRSV strains in Japan and compared them with those of PRRSVs from other countries. In addition, the genomes of EU-type PRRSVs isolated for the first time in Japan in 2009 were analyzed.

Methods: The ORF5 genes of 49 NA-type PRRSVs isolated from lung samples of pigs with respiratory signs in the various areas of Japan from 2007 to 2010, were sequenced. The published ORF5 sequences of 32 Japanese strains from 1992 to 2001 and those of viruses from other countries were also used for phylogenetic analysis. EU-type PRRSVs detected by RT-PCR were isolated using porcine alveolus macrophages, and their genomes were sequenced.

Results: In phylogenetic analysis, ORF5 sequences of NA-type PRRSVs were classified into five clusters. Cluster III consisted of 46 Japanese PRRSVs and 9 viruses in Japan belonged to Cluster II constructed with viruses from other countries and RespPRRS/Repvo vaccine strain. In addition, viruses in these clusters showed genetic diversity. On the other hand, ORFs5, 6 and 7 genes of EU-type Japanese PRRSV isolates were similar with EU-type PRRSVs isolated from the US.

Conclusions: PRRSVs classified in Cluster III have been prevalent since the virus was first isolated in Japan in 1992. EU-type Japanese PRRSVs might be introduced from the US recently.

Keywords: PRRS, ORF5, NA-type, EU-type

Background

Porcine reproductive and respiratory syndrome (PRRS) causes reproductive failure of sows and respiratory disorder of piglets and growing pigs, and is one of the most economically significant swine diseases in the pig production countries. This syndrome is caused by PRRS virus (PRRSV), a member of the family Arteriviridae in the order of Nidovirales. PRRSV is an enveloped single-stranded positive-sense RNA virus and has the genome of about 15 kb in length, encoding at least nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b and ORFs 3 to 7) (Snijder and Meulenberg, 1998). Among them, ORF5 encoding the major envelope protein is used for the phylogenetic analysis because it is most variable. PRRSV can be divided into two genotypes,
European (EU, type 1) and North American (NA, type 2). Both genotypes are antigenically and genetically diverse (Murtaugh et. al. 1995) and each shows wide genetic variation (Shi et. al. 2010a, b). The analysis of genetic diversity for PRRSV field isolates is necessary information for control of PRRS.

The Japanese first strain, EDRD1, was isolated in 1993 and closely related to NA-type antigenically (Murakami et. al. 1994). Only NA-type PRRSVs had been detected and isolated until 2001 since virus was isolated for the first time in Japan (Yoshii et. al. 2005). Most of Japanese PRRSV strains belonged to cluster III which was one of five clusters (Yoshii et. al. 2005). In this paper, we analyzed the sequence variation of NA-type PRRSV strains in Japan after 2001 and compared them with those of PRRSVs from other countries. In addition, the genomes of EU-type PRRSVs isolated for the first time in Japan in 2009 were analyzed.

**Materials and methods**

Lung samples were collected from pigs with respiratory signs in 11 prefectures of Japan from 2007 to 2010. The samples were provided from Livestock Hygiene Service Centers in 11 prefectures. Viral RNA was extracted from 10% lung homogenate using TRI reagent LS, according to manufacturer’s instruction. To detect and differentiate PRRSV, conventional RT-PCR and nested PCR method (Kono et. al. 1996) was carried out. The ORF5 genes of NA-type PRRSVs were amplified by one-step RT-PCR using one step RT-PCR kit (Qiagen) with the published primer pair (P420: 5’-CCATTCTGTTGCAATTTGA-3’, P620: P620: 5’-GGCATATATCATCAGCCG-3’) (Andreyev et. al. 1997). For the DNA sequencing, the PCR products were purified using MinElute PCR Purification Kit (Qiagen), and directly sequenced using the same primers with a BigDye Terminator v3.1 Cycler Sequencing Kit (Applied Biosystems) on an ABI 3130x genetic analyzer (Applied Biosystems).

Viral RNA extracted from two lung samples from 70 and 156 days old pigs in a farm in a prefecture at 2008, were detected as EU-type PRRSVs by RT-PCR (Kono et. al. 1996). These homogenates were inoculated on porcine alveolus macrophages (PAM) obtained from 4 week-old specific pathogen free pig. PAM was cultured in RPMI1640 supplemented with 10% fetal bovine serum and antibiotics (25 U/ml penicillin, 25 µg/ml streptomycin, 25 µg/ml neomycin and 300 U/ml polymyxin). The cultures were observed daily for cytopathic effect (CPE) and were frozen at -80°C when 70% CPE was reached. Two isolates in Japan were designated as Jpn EU 4-37 and -38.

Viral RNA was extracted from cell culture supernatant of PAM inoculated Japanese EU-type PRRSVs using TRI reagent LS. The total RNA was reversetranscribed using ReverTra Ace-alpha (Toyobo) and PCR amplification was performed using high-fidelity DNA polymerase KOD-plus (Toyobo, Japan) and oligonucleotide primers were prepared as a base of the published sequence of the European prototype PRRSV strain Lelystad (LV; Genbank accession No. M96262) and further synthesized based on the Jpn EU 4-37 sequence for 5’ RACE and sequencing. The 3’-ends of the PRRSV genome were determined using 3’ RACE kit (Takara), respectively. The PCR products were purified using a High Pure PCR Product Purification Kit (Roche) and sequenced with a BigDye Terminator v3.1 Cycler Sequencing Kit on an ABI 3100 genetic analyzer.

Sequence identities and alignments were calculated by GENETYX software. Phylogenetic trees were constructed by the neighbor-joining method implemented in MEGA 4 software (Tamura et. al. 2007). Bootstrap analysis was performed with 1000 replications.
Results

To analyze the genetic variation of Japanese PRRSVs, we sequenced the ORF5 genes of 49 NA-type PRRSVs from 11 prefectures of Japan (17 viruses from 2007 to 2008, 15 in 2009, 17 in 2010). In phylogenetic analysis with these sequences and 32 sequences of the reported Japanese PRRSV strains (Yoshii et al. 2005, Iseki et al. 2011), the ORF5 sequences of Japanese NA-type PRRSVs were classified into five clusters. Only one sample, Kochi08-1 detected from 2007 to 2008, was classified in nothing of these five clusters. Cluster I consisted of four viruses from 1992 to 1993, four from 2000 to 2001 and one in 2009. Two PRRSVs from 2000-2001, three from 2007-2008, two in 2009 and three in 2010 belonged to Cluster II constructed with RespPRRS/Repro vaccine strain (MLV). Cluster III consisted of 57 Japanese PRRSVs (1992-1993: 7, 2000-2001: 14, 2007-2008: 12, 2009: 10, 2010: 14), and clusters V was composed of one virus from 2000 to 2001 and one in 2009. In our previous study (Yoshii et al. 2005), cluster IV virus was not found in Japan. However, Jpn5-37, which classified in cluster IV, was first detected from a sample collected during 2007 to 2008 (Iseki et. al. 2010). The Japanese NA-type PRRSVs in cluster III show genetic diversity.

Recently, the ORF5 gene sequences of NA-type PRRSVs, comprised of 8,624 field samples, 3 attenuated live vaccine and one laboratory attenuated strain, were analyzed by phylogeny construction (Shi et. al. 2010b). These sequences were divided into 9 monophyletic lineages. Over 85% of all sequences were classified into four large lineages, lineages 1, 5, 8 and 9, while the remainder was classified into 5 small lineages. Among these small lineages, lineages 3 and 4 are Asian specific lineage; lineage 3 consisted of Chinese and Taiwanese strains and lineage 4 had only Japanese strains. In phylogenetic analysis of Japanese PRRSVs based on the reported historical samples (Shi et. al. 2010b), all strains in cluster III classified by phylogenetic analysis of Japanese PRRSVs were included in lineage 4. The cluster II viruses were located in sublineage 5.1, which belonged to MLV along with a large number of sequences in all parts of world. A large number of vaccine-related sequences were present in sublineage 5.1, so that the genetic diversity of sublineage 5.1 was less than other non-vaccine-associated sublineages (Shi et. al. 2010b). It might be difficult to distinguish vaccine descendants from field viruses since vaccine strain, MLV, is used at many farms in Japan. Two cluster IV and two cluster V viruses were included in lineage 1 and sublineage 5.2, respectively. Four strains in cluster I were belonged to lineage 9 and two cluster I viruses were closely related with lineage 9. In addition, Kochi08-1, not classified by our analysis, was similar to lineage 9. Three cluster I viruses were not able to be classified clearly. These results show that NA-type PRRSVs in cluster III has been presence only in Japan and few Japanese PRRSVs was included in the large lineages 1, 5, 8 and 9, classified by most of viruses from the US and many countries. Since the cluster III viruses show genetic diversity and are genetically different from MLV (cluster II), this finding suggests that PRRSVs could be antigenically diverse and it could complicate control of PRRS.

Two EU-type PRRSVs, Jpn EU 4-37 and -38, were first isolated in Japan. Partial genome sequence was determined for these isolated viruses (The sequence of Jpn EU4-37 was almost same as Jpn EU 4-38,), and the protein-coding region containing 9 ORFs of Jpn EU 4-37 was 14,692 nucleotides (nt). Compared with all ORFs of EU-type PRRSV prototype strain LV, those of Jpn EU4-37 genome was of the same length except that non-structural protein (nsp) 2 region in ORF1a and nsp12 region in ORF1b had deletions. Furthermore, this deletion (20-nt) of nsp12 region in ORF1b of Jpn EU 4-37 was found in comparison with EuroPRRSV and SD01-08 isolated in the US and KNU-07 isolated in Korea. NSP12, which is 152 amino acids (aa) in length by EU-type, of both genotype of PRRSV have unknown its properties and functions. This deletion could cause a frame shift of C-terminal part.
of NSP12, probably generating of NSP12 consisting of 149 aa. However, this short form NSP12 of Jpn EU 4-37 could have no influence its properties and functions in comparison with NSP12 of EU-type PRRSVs. On the other hand, there was 5-nt deletion in nsp2 region of ORF1a in a comparison between Jpn EU 4-37 and LV. This deletion was a genetic feature of EU-type PRRSVs from the US (Shi et al. 2010a). In phylogenetic analysis based on ORF5, Jpn EU 4-37 was included in the group of the US viruses associated with 51-nt deletion in nsp2 region. In addition, sequence comparison with regard to ORF5 showed that, compared to LV, EuroPRRSV and SD01-08, Jpn EU 4-37 exhibited 91.4, 91.1 and 96% (91.5, 90.5 and 97.5%) identity in nt (aa), respectively. Further, the ORF6 and ORF7 sequences of Jpn EU 4-37 showed 97.1 and 97.5% identity in nucleotide with SD08-01. These results showed that Japanese first EU-type PRRSV strain, Jpn EU 4-37, was similar with EU-type PRRSVs isolated in the US.

Discussion

Japanese PRRSVs classified in cluster III (lineage 4) have been prevalent since the virus was first isolated in Japan in 1992. Since cluster III viruses showed genetic diversity and were mainly circulating in Japan, these viruses could be antigenically diverse and make control of PRRS with vaccine difficult. On the other hand, EU-type PRRSVs were first isolated in Japan and closely related with EU-type PRRSVs isolated in the US. Japanese EU-type PRRSVs might be introduced from the US recently.

References

Epidemiological study of *Burkholderia pseudomallei* isolates from animals and soil in an endemic area of Ratchaburi province by pulsed-field gel electrophoresis

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Abstract

**Background:** *Burkholderia pseudomallei* is an environmental saprophytic bacterium found in water and soil in tropical and subtropical regions. It is the causative organism of melioidosis in humans and animals.

**Method:** This study analyzed genetic diversity of *B. pseudomallei* isolates from animals and soil in an endemic area in Ratchaburi province. A total of 36 isolates originated from 22 clinical animal cases occurred during 2006-2010 and 14 soil samples that were collected during January-March 2011 in the goat farms where melioidosis cases have been identified. The chromosomal DNA of all isolates were cut with enzyme *Spe*I and run by Pulsed-Field Gel Electrophoresis (PFGE) to analyze for DNA fingerprint patterns.

**Results:** PFGE patterns of the 36 isolates appeared in 7 different genotypes. The dominant PFGE pattern belonged to 28 isolates which consisted of 14 isolates from animals and 14 isolates from soil.

**Summary/Conclusions:** This study indicated the possible role of *B. pseudomallei* contaminated soil as a source of *B. pseudomallei* infection in animals. Melioidosis outbreaks in animals in Ratchaburi province confirmed the presence of *B. pseudomallei* in soil.

**Keywords:** *Burkholderia pseudomallei*, animals, soil, pulsed-field gel electrophoresis, PFGE

**Background**

Melioidosis is a potentially severe tropical disease occurring in humans and animals and is caused by *Burkholderia pseudomallei* (Koonpaew et al., 2000; Sprague and Neubauer, 2004; Kaestli et al., 2007). The bacterial infection occurred as a result of exposure to contaminated soil or water (Dance, 2000; Inglis et al., 2004; Koonpaew et al., 2000). It is isolated frequently from soil in regions where the disease occurs. The most likely transmission is direct percutaneous abrasion, inhalation and ingestion (Currie et al., 2000). The epidemiology of melioidosis is complicated due to the environmental persistence of the organism and is subject to distinct differences in the organism’s distribution in soil, disease presentation and incidence rates among different areas of endemicity (Roberta et al., 2006). In Thailand, endemic areas melioidosis have been labeled as the main area (Dance, 1991). The greatest number has reported with an estimate of 2,000-3,000 cases each year (Leerarasamee, 2000). The risk of the disease varies according to the region of the country because of uneven distribution of organism, with most cases being found in the northeastern part of Thailand (Chaoawagul et al., 1989). The epidemiological study about the distribution of virulent strains and the source of melioidosis infection are available, environmental sampling has been widely used to determine the presence of *B. pseudomallei* in an effort to identify geographical distribution of the organism and related risk of infection (Smith et al., 1995; Wuthiekanun et al., 1995; Brook et al., 1997; Vuddhakul et al., 1999). The genotyping of isolates from the environment and cases of disease is an
essential component of outbreak investigations to link isolates to a common contaminated source (Currie et al., 2001). Understanding the epidemiology of melioidosis can be undertaken by molecular typing which offers a very discriminatory tool for differentiating strains, several studies have used Pulsed-Field Gel Electrophoresis (PFGE) to study clonal relatedness because of its high discriminative power. (Currie et al., 2001; Azura et al., 2011; Chua et al., 2011).

In this study, we attempt to examine distribution of *B. pseudomallei* which contaminated in soil surrounding the goat farms from the disease outbreak in Ratchaburi province and compare the genetic relatedness between isolates from infectious animal strains and soil isolates by using PFGE.

**Materials and methods**

**Soil and Infectious Animals isolates**

A total of 459 soil sampling were collected from 51 soil sites, 153 holes in Ratchaburi province, within 1,500 m² surrounding goat farms where the melioidosis occurs during the dry season (January to March 2011). Approximately 500 g of each soil sample were removed from each hole at a depth of 15, 30 and 45 cm and transported immediately to the laboratory. Soil was taken to homogeneity and cultured for the presence of *B. pseudomallei*, using 5 g of each soil sampling put into a sterile tube, mixing well with 40 ml of sterile distilled water, and shaken vigorously for 15 min, allowed to settle for sedimentation 15 min, 2 ml of the soil supernatant were transferred into 10 ml of Ashdown’s selective enrichment broth and incubated aerobically at 37°C for 48 h. Further, a 1 ml of supernatant was diluted 10-fold to concentrations of 10⁻² and 10⁻³ and 100 µl of each dilution were spread onto modified MacConkey agar which 5 mg/liter of gentamicin, incubated aerobically at 37°C for 48-72 hr. The culture plates were observed daily for the morphological appearance of *B. pseudomallei* colonies.

A total of 22 isolates of *B. pseudomallei* were available from melioidosis cases of 13 goats, 4 deer, 4 pigs, and 1 porcupine in Ratchaburi province during 2006-2011. Identity was confirmed by conventional method and preserved in cryobank (Mask Diagnostics, Germany) at -80°C. Working culture were maintained in 5% sheep blood agar, incubated aerobically at 37°C for 48 h.

**Identification of isolates**

All isolates were identified by biochemical tests, which were positive to oxidase, motility, nitrate reduction, oxidative fermentative to glucose, maltose and lactose. The positive colonies were confirmed as *B. pseudomallei* using polymerase chain reaction for determining the presence of the specific amplicons of the 16S rRNA gene (411 bp), used primer BS4R (5’-CAC TCC GGG TAT TAG TAG G-3’) and U33 (5’-AAG TCG AAC GGC AGC ACG G-3’), followed by Dharakul et al. (1996). PCR amplification was performed in a total volume of 50 µl containing PCR buffer (contain 1.5 mM MgCl₂), 1.25 unit HotStarTaq DNA Polymerase(QIAGEN, Germany), 200 µM of each dNTP mix, 2 mM MgCl₂, distilled water and 0.5 µM each of primer BS4R/U33, using the reference strain DMTC 27191 for positive control and distilled water for negative control. The reaction mix was activated initially 95°C for 15 min, followed by 35 cycles of denaturation 95°C for 30 sec, annealing 62°C for 30 sec and extension 72°C for 30 sec, and a final extension 72°C for 10 min. PCR products were separated on 1.5% agarose gel.

**Pulsed-field gel electrophoresis and agarose block preparation**

DNA and block were prepared by modification of the method of Thong et al. (1994). Pure colonies were grown in 5% sheep blood agar at 37°C for 24 h. Cells were suspended and adjusted to optical density at 600 nm of 1.2 in 10 mM Tris-HCl (pH 7.5) and 1 M NaCl, washed once and mixed
with an equal volume of 2% low melting agarose, then loaded into block moulds, allowed to solidify in 4°C for 10 min and incubated overnight at 37°C in lysozyme solution (6mM Tris-HCl, 1mM NaCl, 100 mM EDTA, 0.5% Brij, 0.22% deoxycholate, 0.5% Sarkosyl and 2 mg/ml lysozyme). Agarose block was then transferred to proteinase K buffer containing 1 mg/ml proteinase K, 0.5 M EDTA, 0.5% Sarkosyl and incubated at 50°C for 48 h. The cell debris and proteinase K were then removed by five wash in TE buffer for 20 min at room temperature and stored at 4°C until use.

**Digestion and Electrophoresis**

DNA plugs were equilibrated in RE buffer at 37°C for 30 min, then digested with 10 units of SpeI in 100 µl of RE buffer at 37°C overnight. The digests were washed once in TE buffer and run on 1% agarose gel on a Bio-Rad CHEF DRIII (Biorad, USA), with a 3-50 sec pules ramp at 6v/cm for 30 h. Gels were stained with ethidium bromide, visualized with ultraviolet light in GelDoc.It (UVP, USA), and analyzed with the molecular analyst program on Bio1D ++ (Vilber Lourmat, Germany).

**Results**

A total of 459 soil samples were collected from 153 holes in 51 sites surrounding goat farms in Ratchaburi province. The isolation of *B. pseudomallei* at a depth of 15 to 45 cm was observed at 3.05% (14/459). The isolates of sample collected at a depth of 15 cm were 4.58% (7/153) more frequent than the isolates at a depth of 30 and 45 cm which were found at 2.61% (4/153) and 1.96% (3/153). (Table 1)

The PFGE of macrorestricted chromosomal DNA from a total of 14 soil samples and 22 infectious animal isolates revealed 7 different pattern types. For major isolates, 28 out of 36 [11 goats (within soil sampling sites), 2 deer (2 km nearby), 1 porcupine (35 km distant), and 14 soil isolates] exhibited a predominant type A. Other distinct patterns [1 goat (within soil sampling sites) and another one from a 75 km distance] were type B and type C. Two out of 4 deer-isolate showed type D and type E; these deer were raised within the same farm in which type A was found. Interestingly, 4 swine isolates from a site located 3 km nearby showed 2 different pattern types; 3 isolates were found to be type F and the other one was type G. (Table 2)

A dendrogram depicting the estimated phylogenetic relationship was constructed based on pairwise comparison of PFGE banding. Overall, 28 predominant isolates namely type A revealed 100% genetically identical. The isolates from infectious animals were found to be genetically diverse; more than 75% displayed genetic similarity between type A and type B. These goats were raised within the same farm. Isolates among type C from goat from a site farther located 75 km in distance and type F from 3 out of 4 swine isolates showed genetic relatedness for more than 85%. Moreover, approximately 75% were observed to play genetic similarity between 1 out of 4 swine isolates and 1 out of 4 deer isolates as type E and type G, while there was one deer namely type D showed 55% genetic similarity amongst all isolates. (Figure 1)

**Table 1** Detection of *B. pseudomallei* in soil samples collected from an endemic area in 1,500 m² surrounding goat farms in Ratchaburi province

<table>
<thead>
<tr>
<th>Depth of soil sampled (cm)</th>
<th>Number of detected samples / number of collected samples (Percentage of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>7/153(4.58%)</td>
</tr>
<tr>
<td>30</td>
<td>4/153(2.61%)</td>
</tr>
<tr>
<td>45</td>
<td>3/153(1.96%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>14/459(3.05%)</td>
</tr>
</tbody>
</table>
Table 2  Number of isolates and distribution of *B. pseudomallei* SpeI-pulsotypes

<table>
<thead>
<tr>
<th>Sources</th>
<th>Distance far from soil sampling site (km.)</th>
<th>Number of isolates</th>
<th>Total of pattern types</th>
<th>Number of isolates and PFGE pattern types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>within sampling sites</td>
<td>12</td>
<td>2</td>
<td>11 1 1 - - - - - -</td>
</tr>
<tr>
<td>Goat</td>
<td>75</td>
<td>1</td>
<td>1</td>
<td>- - 1 - - - - - -</td>
</tr>
<tr>
<td>Deer</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>2 - - 1 1 - - - -</td>
</tr>
<tr>
<td>Porcupine</td>
<td>35</td>
<td>1</td>
<td>1</td>
<td>1 - - - - - - - -</td>
</tr>
<tr>
<td>Swine</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>- - - - - 3 1</td>
</tr>
<tr>
<td>Soil sampling sites*</td>
<td></td>
<td>14</td>
<td>1</td>
<td>14 - - - - - - - -</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>36</td>
<td>7</td>
<td>28 1 1 1 1 1 3 1</td>
</tr>
</tbody>
</table>

* sampling sites: 1,500 m² surrounding the goat houses

Discussion

This study investigated the distribution of *B. pseudomallei* in soil during the early dry season over a period of 3 months (January to March, 2011). Soil sampling site was selected in a 1,500 m² area surrounding goat houses in Ratchaburi province in which the melioidosis in animals occurred. The isolation rate of *B. pseudomallei* from the soil samples collected at a depth of 15 down to 45 cm was different; 4.58% isolates appeared at a depth of 15 cm, whereas 2.61% and 1.96% were observed at a depth of 30 and 45 cm. A total of 3.05% positive isolates from 14 out of 459 samples collected were less than that found in previous studies. Specifically, in the northeastern Thailand at different time periods during the rainy season, Vuddhakul et al. (1999), Chantratita et al. (2008) and Wuthiekanun et al. (2009), reported a higher presence of *B. pseudomallei* at 20.4%, 80% and 28%, respectively at a depth of approximately 30 cm in soil. This is not surprised because the northeastern Thailand was marked as a hyperendemic area; in fact, the soil in this region harbors more *B. pseudomallei* than the soil in the other regions (Vuddhakul et al., 1999). In addition, the prevalence of isolates in the wet season was higher than that of the dry season because the surviving bacteria in the ground were brought by the rising water to an upper soil layer and multiply under a more favorable condition. However, a seasonal variation is not a major determinant of *B. pseudomallei*. Rolim et al. (2009) reported that during the dry season in the northeastern Brazil the positive culture was present at 58.8% of soil samples collected at a depth between 20-40 cm, whereas Wuthiekanun et al. (1995) and Brook et al. (1997) reported that in the northeastern Thailand and the northern territory of Australia the positive isolates were recovered from soil during the dry season. However, the increasing numbers and survival of the organisms in the soil layers were correlated with the soil type and high rainfall.
(Strauss et al., 1969), while heavy clay soils are much better to support bacterial persistence than well-drained, light and sandy soils (Inglis and Sagripanti, 2006). Soil with a water content of less than 10% led to the death of *B. pseudomallei* within 70 days, while soil with a water content of more than 40% maintained bacterial life for 726 days (Tong et al., 1996). The difference of physical, biological, and chemical soil features appear to influence the survival of isolates (Dance, 2000; Inglis and Sagripanti, 2006). Additionally, this discrepancy could be due to the differences of specimen collection, isolation and identification techniques, or due to ecological factors influencing the environmental presence of *B. pseudomallei* (Tong et al., 1996).

The genotyping analysis of melioidosis was performed on SpeI macro-restriction pattern which was found to be more discriminatory in PFGE typing for *B. pseudomallei* (Chua et al, 2011). For a dendrogram depicting the estimated phylogenetic relationship based on the comparison of genetic banding patterns, a total of 36 isolates were identified at 7 PFGE-different pattern types; 28 dominant isolates demonstrated 100% genetically identical. Most 14 soil isolates were observed to have a single fingerprint pattern. In contrast with previous studies of Chantratita et al. (2008) and Wuthiekanun et al. (2009), the genetic variability isolates to 12 PFGE pattern types in soil samples from disused land in the northeastern Thailand and 10 PFGE pattern types isolates from rice paddy soil were revealed. These difference might have explained that there were soil isolates from a distinct geographical location and our isolates were investigated in the soil taken from a small geographic area where was not genetically diverse.

The isolates obtained from infectious animals were mainly from 12 goats which were raised within the soil sampling sites and 4 deer were from a site located 2 km in the field nearby in which 2 and 3 fingerprint pattern types were observed. Eleven out of 12 goat isolates and 2 out of 4 deer isolates were found to have 100% identical genetic pattern with the soil isolates. This is not evident whether the transmission mode of organism is. We speculated that the soil which is contaminated with *B. pseudomallei* might be the sources of animal infection in this outbreak. However, some isolates isolated from animals nearby such as deer showed genetic similarity. Likely, these were the descendant of clones of the same progeny from soil isolates, which were disseminated from different areas especially during the wet season (Strauss et al., 1969; Haase et al., 1995; Struelens, 1996). Interestingly, it was found that from 4 swine isolates from a site located 3 km nearby (soil sampling sites) showed 2 genetically distinct pattern types from the others. It is difficult to explain about the source of infection because the piggery were situated onto a concrete platform and did not expose to soil environment. It is possible that there was introduction of infected animals from the other place or the transmission modes of infection may have come from various routes such as contaminating water supply, percutaneous abrasion, inhalation of aerosolized bacteria, and ingestion (Currie et al., 2001).

The infectious animal isolates recovered from several locations were found to be genetically diverse and heterogeneous. For instance, as a case of porcupine from a site located 35 km distant from soil sampling sites, it was 100% genetically identical with dominant pattern isolates. Meanwhile, the isolates of a goat from a site situated at 75 km distant from soil sampling sites and deer from a site located nearby appeared genetically different. This suggested that several strains of *B. pseudomallei* were distributed in the environment. But it is difficult to speculate the transmission between animals and the contaminated environmental. Except that there is an environmental study determining the possibility of soil or water being a source as compared to the isolate from an emerging disease area. The presence of the identical genetic patterns among the clinical and environmental isolates evaluated suggested a link between the pathogens present in contaminated soil and the emergence of melioidosis (Chen et al., 2010).
In conclusion, severe strain of *B. pseudomallei* is distributed in soil as an endemic organism in this goat farm. The occurrence of the disease in animals was associated with isolates in soil. This was supported by the molecular typing of soil isolates which revealed the same clonality of outbreak strains. Interestingly, it is likely that endemicity may extend to expose to other animals in the nearby boundaries.

**Acknowledgements**

We are grateful to Dr. Pacharee Thongkamkoon and staff of Bacteriology & Mycology section of National Institute of Animal Health (NIAH) for the support of PFGE data analysis. Thanks all of our colleagues of Veterinary Research and Development Center (Western Region) for field collection of soil samples and laboratory assistance.

**References**


**Pasteurella multocida infection in lion (Panthera leo)**

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**Abstract**

**Background:** *Pasteurella multocida* causes several diseases in the various kinds of animal as the primary infection such as hemorrhagic septicemia and fowl cholera. Mostly, this organism is an opportunistic pathogen that the effects of predisposing factors such as stress are integrated. However, *P. multocida* infection in wild animals has rarely been reported. In this study, a 1-year-old male lion (*Panthera leo*) from a zoo in the Northern part of Thailand presented the posterior limb paresis for 3 months. It was submitted to the Veterinary Research and Development Center (Upper Northern Region). The causes of this sign were investigated.

**Method:** At post mortem examination, gross lesion observation, histopathology and laboratory testing were performed. From bacterial culture, colonies of *P. multocida* grown on blood agar were harvested and differentiated for capsular typing based on molecular technique. In addition, somatic types were identified using serology method.

**Results:** There was no external lesion presented except the carcass was cachexia. Internal lesions including congestion of spleen and intermediate lobe of lung, hemorrhage of gall bladder and reddening of caecal mucosa were found. *P. multocida* was found in the bacterial culture from lung, heart, intestine and brain. Capsular and somatic types of this organism were A: 3, 14, 16, respectively.

**Conclusions:** This study aimed to report the rare case of *P. multocida* in a lion. Although the posterior limb paresis in this lion did not relate to the disease mechanism of *P. multocida* and the reason for its poor condition was unknown, this report can be used as a database for monitoring wildlife’s health, and the transmission of organisms via environment, food, and animals to animas should be considered.

**Keywords:** *Pasteurella multocida*, lion, *Panthera leo*

**Background**

*Pasteurella multocida* is a Gram-negative, nonmotile rod or coccobacillus. Its size is approximately 0.2 µm by up to 2.0 µm (Hirsh and Biberstein, 2004). This bacterium could be classified into 5 different capsular polysaccharide types (A, B, D, E and F) and 16 somatic lipopolysaccharide antigenic types (as numbers 1 to 16) (Rimler and Rhoades, 1987, Boyce et al., 2000). Although this organism is mostly an opportunistic pathogen, despite that the effects of predisposing factors such as stress, environmental changes are integrated, it can be a primary causative agent of several diseases (Chen et al., 2002, Miller, 2001). As the primary infection, each type can be associated with various diseases according to the principle hosts. *P. multocida* type A causes fowl cholera in avian, while type F affects turkeys. Type D relates to atrophic rhinitis in swine. In cattle, haemorrhagic septicemia results from type B infection as same as type E which found in Africa (Quinn et al., 1994). Other animals including wildlife have been reported (De-Alwis, 1999). For human, at much less frequency, *P. multocida* causes osteomyelitis, septic arthritis, sepsis and meningitis via bites or scratches especially from wild animals (Burdge et al., 1985, Kizer, 1989).
The pathogenesis of *P. multocida* infection in animals includes three conditions of disease that are respiratory tract involvement, septicaemia and trauma-associated type (Hirsh and Biberstein, 2004). Haemorrhagic septicaemia in cattle and atrophic rhinitis in swine shows the clinical signs involving pneumonia and upper respiratory tract collapse. Similarly, septicaemia type also appears in haemorrhagic septicaemia and avian species resulting in severe losses of economic worldwide (Ewers et al., 2006).

The present study aimed to report of *P. multocida* infection in lion in a zoo of Thailand. Moreover, type of the organisms was identified.

## Materials and Methods

### Case history

A 1-year-old male lion (*Panthera leo*) that was housed in a captivity with ten lions in a zoo settled in the northern region of Thailand presented the posterior limb paresis for 3 months. Antibiotics and vitamin B complex were administered for treatment to retrieve this sign but no response was observed. Due to the poor condition, it was humanely euthanized with an intravenous sodium pentobarbital solution. Then, the carcass was submitted to Veterinary Research and Development Center (Upper Northern Region) for a post mortem examination.

### Laboratory examination

Gross lesions were observed. The internal organs including parts of brain, heart, lung, liver, spleen, kidney, lymph nodes and all parts of gastrointestinal tract were submitted for bacterial culture. Additionally, they were fixed in 10% neutral buffered formalin for histopathological examination with Haematoxylin and Eosin staining (H&E).

Brain was diagnosed for the presence of rabies virus using fluorescence antibody technique (FAT) while parts of respiratory tract were submitted to isolate influenza virus by egg inoculation. Blood parasites were examined using Giemsa’s staining of thin blood smear, whereas fecal sample was performed by flotation and simple sedimentation techniques.

### Typing of *Pasteurella multocida*

Polymerase chain reaction (PCR) was used to differentiate *P. multocida* capsular type from a pure colony. After an overnight bacterial culture at 37°C, the suspected colonies grown on blood agar were harvested and suspended in 50 µl of deionized water. For DNA extraction, the bacterial suspension was boiled at 95°C for 10 minutes, followed by an immediate chill. Then, the cell lysate was centrifuged at 12,000 rpm for 10 minutes and the supernatant was used as the DNA template.

A multiplex PCR was performed to confirm *P. multocida*-specific and type A, using previously described primers (Townsend et al., 1998, Townsend et al., 2001). Each reaction mixture contained 1xPCR buffer, 2.0 mM MgCl$_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 3.2 pmol of each primer and 1.0 U Taq DNA polymerase (HotStarTaq, Qiagen, Germany). DNase/RNase free water was added to adjust as 25 µl of total volume and also used as negative control. PCR conditions consisted of initial denaturation at 95°C for 5 minutes. Then, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds were performed with a final extension at 72°C for 5 minutes. The amplified product was electrophoresed on 2.0% agarose gel in 1xTAE running buffer and stained with ethidium bromide. A specific band was observed under UV transillumination.

Furthermore, the *P. multocida* colonies were submitted to the National Institution of Animal Health (NIAH) for capsular type insistency and somatic type differentiation based on serology technique.
Results

Pathological findings
There was no external lesion presented except the carcass was cachexia. Several internal organs showed lesions including congestion of the intermediate lobe of lung (fig.1) and spleen (fig.2), hemorrhage of gall bladder, reddening of caecal mucosa. Moreover, round worms were found at the junction between stomach and duodenum. For histopathological findings, the moderate interstitial pneumonia, severe edema of gastric submucosa and severe hemorrhage of spleen were found.

Virology findings
Influenza and rabies causative agents were not found from any diagnosed organs.

Parasitological findings
Ascaris egg was found in fecal sample, whereas blood parasite was not found.

Bacteriological findings
P. multocida from lung, heart, intestines, and brain was grown on blood agar on an overnight culture. The molecular typing of this organism revealed A: 3, 14, 16 for capsular and somatic type (Fig. 3).

![Figure 1 Congestion in the intermediate lobe of lung (arrow)](image1)

![Figure 2 Congestion of spleen](image2)

![Figure 3 Agarose gel electrophoresis of PCR product amplification based on multiplex PCR for capsular typing of P. multocida. Lane M, 100 bp DNA marker; lane S, sample; lane A, capsular type A reference sample; lane B, D, E and F, capsular type B, D, E and F reference samples, respectively; lane N, reagent as negative control. The numbers on the right of panel indicated product size of each primer as 1,044, 760, 657, 511, 851 and 460 bp for capsular type A, B, D, E, F and P. multocida-specific, respectively.](image3)
Discussion

*P. multocida* infection in captive wild animals in Thailand has rarely been reported. This organism is found as a commensal in the nasopharynx and gastrointestinal tract of many wild and domesticated animals (Chen et al., 2002). A previous study, however, reported that *P. multocida* was cultured from the upper respiratory tract of healthy domestic cats at 52% to 70% and this rate increased up to 100% in sick animals (Kizer, 1989). Similarly, Burdge et al. (1985) found that two patients developed serious *P. multocida* infection after being attacked by large cats such as lion indicating that the organisms usually reside in the oral cavity of wild animals.

Although *P. multocida* does not often cause of disease in its animal hosts, several diseases may occur according to the capsule polysaccharide types. A previous study reported that the capsule plays an important role in host and disease specificity (Davies, 2004). In wild animals, there was no report on which the capsular type is specific to and causes the diseases in any hosts.

In this study, the capsular and somatic types of *P. multocida* are A: 3, 14, 16 based on the molecular characterization. Similarly, there was a report that wild mammals commonly yielded untypable or cross-reacting isolates such as A: 3, 4 (Williams and Barker, 2001). Hoshuyama et al. (1995) revealed that the predominant capsular and somatic types causing purulent skin lesions in household cats which are the same family (*Felidae*) of lion were A: 3, 4. The study of *P. multocida* isolated from a lion in India presented types A: 3, 4 which were founded to be pathogenic to mice at the mortality rate of 70 to 90%. Unlikely, type B: 2 caused 30% mortality to mice in the same study (Saxena et al., 2006).

As described previously, several diseases can be developed according to the capsular types and host specific. More frequently, *P. multocida* is a secondary invader when resistance of the animal reduced by various factors such as trauma, stress, intercurrent diseases, overcrowding and environmental stressors (Carter and Wise, 2004). In this case, the distinct clinical sign of lion, posterior limb paresis, was not probably related to the disease mechanism of *P. multocida* and the reason for its poor condition was unknown. Nevertheless, the presence of *P. multocida* in several internal organs including lung, heart, intestine and especially brain suggested the role of organism as a possible infective agent. Furthermore, the congestion of lung and spleen may be the initial state of septicemia due to the vascular damage. In order to conclude the causes of this disease in this lion, more samples or more parameters such as complete blood count and blood chemistry profiles should be examined.

Since this organism is relatively vulnerable in the environment, contaminated excreta, exudates, water, feed and other fomites, they can be sources of infection for hours or weeks. Then, the route of infection should be monitored. Especially, ingestion or food-borne transmission has caused of pasteurellosis (Williams and Barker, 2001). Moreover, Saxena et al (2006) reported that *P. multocida* types in wild animals had the similar pattern to domestic animals, suggesting that the infection in wild animals may pass through domestic animals.

This study reported the *P. multocida* infection in a lion. Although the disease mechanisms of this case are still unknown, this report could be used as a database for monitoring wildlife’s health. Furthermore, the transmission of organism via environment, food, and animals to animals should be considered.

Acknowledgement

We thank Dr.Wattanasak Chamlakorn and the staffs of Pathology and Bacteriology sections of Veterinary Research and Development center for their support, and the Bacteriology section of the National Institutional of Animal Health (NIAH) for *P. multocida* typing. We are also grateful to Dr. Pacharee Thongkamkoon for her helpful comments concerning the manuscript.
References


Serological survey of antibody to anthrax in livestock from Mae Hong Son

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Abstract

Background: Anthrax is a zoonotic disease caused by Bacillus antracis. It is the most common in domestic and wild herbivores such as cattle, sheep, goats, camels and antelopes. It can also be seen in humans who exposed to the tissues of infected animals. Anthrax is nearly worldwide in distribution; the spore form of this organism can survive in the environment for many decades. Certain environmental conditions are likely to produce ‘anthrax zones’ where in the soil is heavily contaminated with anthrax spores. In addition, because of its persistence in soil, anthrax is a rather important veterinary disease especially in domestic herbivores (Cieslak and Etzen, 1999).

Method: An ELISA assay was performed to detect antibodies against anthrax protective antigen recombinant of B. antracis in serum samples. Briefly, 50 µl diluted serums (1:100) were bound to anthrax anti-toxin antigen coated plate by protein G-HRP as conjugate. These complexes were detected using 3,3,5,5'-tetramethylbenzidine (TMB) substrate. Absorbance at 450 nm was measured by an ELISA reader, represented in Optical Density (OD) value. The cutoff value was defined as 2 times of the negative control's OD value.

Results: The results of ELISA test for 3,315 serum samples revealed that the OD values were between 0.06-0.20, whereas the negative control serum and the anthrax anit-serum positive control revealed the OD value of 0.12 and 0.6, respectively. Serum samples present the OD value less than targeted cutoff at the OD value of 0.24.

Conclusion: No antibody to B.antracis was found in all tested samples; hence, the overall prevalence of anthrax in Mae Hong Son livestock was zero.

Key words: antibody, anthrax, livestock, Mae Hong Son

Background

Anthrax is a zoonotic disease caused by the spore-forming bacterium Bacillus antracis. It is the most common in domestic and wild herbivores such as cattle, sheep, goats, camels and antelopes. It can also be seen in humans who exposed to the tissues of infected animals, contaminated animal products or contacted directly to B. antracis spores under certain conditions (Kahn, 2010). B. antracis is a large, non-motile, gram-positive rod that forms environmentally resistant endospores when exposed to air (Groves and Harrington, 2006). This causative agent enters the body by ingestion, inhalation, or through skin. However the exact route of infection is often in doubt; it is generally considered that most animals are infected by the ingestion of contaminated food or water (Radosits et al., 2000). Anthrax is nearly worldwide in distribution; the spore form of this organism can survive in the environment for many decades. Certain environmental conditions are likely to produce ‘anthrax zones’ where in the soil is heavily contaminated with anthrax spores. In addition, because of its persistence in soil, anthrax is a rather important veterinary disease especially in domestic herbivores (Cieslak and Etzen, 1999).

Anthrax is enzootic in most countries of Asia called ‘anthrax belt’. Outbreaks of anthrax in animal population were reported from Bangladesh, Bhutan, Myanmar, Nepal and Sri Lanka (Krishnan and Gangel, 2008). For many decades, Thailand has imported animals from neighboring countries, as it’s a part of traditional border trade. However, importation of these animals without history or record of
vaccination causes risk of transboundary animal diseases to livestock in Thailand, such as foot-and-mouth disease, brucellosis, tuberculosis and anthrax.

Mae Hong Son province, a border province between Thailand and Myanmar, has several entry points which allow importation of cattle and buffalo into Thailand, mainly for domestic consumption. Many efforts were carried out to minimize risk caused by importing animals from neighboring countries, including application of animal identification and record, vaccination, immunological tests, treatment of external and internal parasites, as well as quarantine. Nevertheless, the routine serological examinations are mainly on foot-and-mouth disease, brucellosis and paratuberculosis. Therefore, there could be a chance to introduce anthrax, if it exists in imported animals, to local livestock. This is a study which carried on to provide the prevalence of anthrax in order to indicate the risk of the disease in local livestock.

Materials and methods

Study area and Serum samples

The study area was in Mae Hong Son province in the northern Thailand, the mountainous province which every district shares a common border with Myanmar. This province is one of the main routes to import livestock across 4 entry points along its border.

The serum samples were collected from Mae Hong Son livestock in 2010. The total of 3,315 serum samples comprised 2,142 cattle, 565 buffaloes, 393 goats and 215 sheep. The samples were tested at Veterinary Research and Development Center (Upper Northern Region).

Serological Assays

The ELISA assay according to Vitoorakool et al. (1999) was used to detect antibodies against anthrax protective antigen recombinant of *B. antracis* in serum samples. Briefly, 50 µl diluted serums (1:100) were bound to anthrax anti-toxin antigen coated plate by using protein G-HRP as conjugate. These complexes were detected by using 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The yellow color was developed, the level of darkness refer to quantity of antibody in serum. Absorbance at 450 nm was measured by ELISA reader, represented in Optical Density value (OD value). Interpretation was based on comparison of absorbance for the sample with defined cutoff value as 2 times of negative control OD value.

Results

Table 1 shows OD values of 3,315 serum samples which were between 0.06-0.20. The negative control serum and anthrax anti-serum as positive control presented the OD values of 0.12 and 0.6, respectively. From the results, the cutoff value should be 0.24 as two times of the negative control’s OD value. Therefore, no antibodies to *B. antracis* were found in all kinds of tested animals (Table 2).

<table>
<thead>
<tr>
<th>Samples</th>
<th>OD value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Livestock serums</td>
<td>0.06-0.20</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.12</td>
</tr>
<tr>
<td>Anthrax anti-serum</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 2 Test results to *B. antracis* protective antigen by animal species

<table>
<thead>
<tr>
<th>Samples</th>
<th>Serum samples (n)</th>
<th>OD value results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>2,142</td>
<td>negative</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>565</td>
<td>negative</td>
</tr>
<tr>
<td>Goats</td>
<td>393</td>
<td>negative</td>
</tr>
<tr>
<td>Sheep</td>
<td>215</td>
<td>negative</td>
</tr>
</tbody>
</table>

* negative means OD value less than cutoff value
Discussion

The serological surveillance of antibodies to anthrax in cattle, buffaloes, goats and sheep in Mae Hong Son revealed the OD values of the tested samples less than the cutoff value as well as the positive control’s; therefore, no antibodies to *B. antracis* were found (Hardjoutomo et al., 1990). As a result, there was suspected that there was no infection in these animals. This is similar to the study of Vitoorakool et al. (2004) which presented the result of no antibodies to anthrax were found in imported cattle and buffaloes from Chiang Rai and Mae Hong Son during 2002 to 2003. The animals that had passed through animal quarantine stations were held for a quarantine period of at least 21 days, which is enough for incubation period of the disease at 3-7 days (ranging 1-14 days), whereas the OIE terrestrial code suggests the incubation period shall be 20 days (Kahn, 2010 and World organization for animal health, 2010). In the quarantine period, the animals will present clinical signs if infected. The last anthrax outbreaks in animals in the northern Thailand were reported in 1996 (Office of disease prevention and control, 1997). Furthermore, Bureau of Emerging disease (2010) reported that there was no anthrax human case in Thailand for 8 years from 2001 to 2010. According the results of this study, it could assume that imported animals were not infected or transmitted anthrax disease in the country. Because of the closed border policy, however, no imported animal moved through the entry points at the study period. Therefore, it is recommended that a future study should be carried out on the post-imported animals.

Acknowledgement

We would like to thank Mae Hong Son provincial veterinary staff for their kind support on sample collection and the Immuno-serology Section’s staff of Veterinary Research and Development Center (Upper Northern Region) for the serological tests.

References


Histology and Immunohistochemical Detection of Porcine Circovirus Type2 for the Diagnosis of Postweaning Multisystemic Wasting Syndrome

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Abstract

Nowadays it is recognized that porcine circovirus type2 (PCV2) infection can be detected in the absence of postweaning multisystemic wasting syndrome (PMWS). This report describes histological lesions and immunohistochemical detection in 3 weaning pigs affected with PCV2 infection. Histologically, the unique lesions including severe lymphoid depletion, interstitial pneumonia, myocarditis, hepatitis, nephritis and enteritis were found in all pigs (3/3). Importantly, typical basophilic intracytoplasmic inclusion bodies were revealed in lymphoid organs of 1 pig (1/3). The PCV2 antigen was immunohistochemically detected in lymphoid organs and other organs associated with the lesions in 2 pigs (2/3). The predominant viral antigen-positive cells are mostly macrophages and less frequently bronchial epitheliums, kidney tubular epitheliums as well as cardiac myocytes. In molecular method, PCV2 nucleic acid was also detected from all pigs using PCR (3/3). This study suggests that histology is useful for PCV2 diagnosis in the case that typical inclusion bodies were found. PCR is the most sensitive technique but IHC correlates better with the presence of characteristic lesions. Thus, together with typical histopathology, IHC should be performed to confirm the diagnosis of PMWS.

Keywords: porcine circovirus type2, postweaning multisystemic syndrome, histology, immunohistochemistry

Background

Porcine circovirus type 2 (PCV2) is considered to be a key agent in the development of several diseases, which lead to the term PCV2-associated Disease (PCVAD) including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), proliferative necrotizing pneumonia (PNP) and other diseases (Opriessnig et al., 2007). Among these conditions, PMWS has caused the greatest economic losses to the pig industry (Allan and Ellis, 2000). The predominant clinical signs are weight loss, dyspnea, lymphadenomegaly, skin pallor, diarrhea, and occasionally jaundice (Kennedy et al., 2000). Histological lesions include lymphoid depletion, granulomatous inflammation with multinucleated giant cells formation and occasionally with intracytoplasmic inclusion bodies in lymphoid tissues (Chianini et al., 2003). To confirm PMWS, the criteria are set: 1) clinical signs of wasting and/or weight loss; 2) histological lesions of lymphoid organs and inflammation in other organs such as lung, liver, kidneys and intestine; and 3) PCV2 antigen or nucleic acid demonstration within characteristic lesions (Sorden, 2000).

This study reports histological and immunohistochemical characterizations in 3 weaning pigs affected with PCV2 infection.
Materials and Methods

Tissue samples and histopathology

The study was conducted from 3 pigs in the 5-12 week of age range with clinical signs of weight loss, dyspnea and skin pallor. Hemorrhage, necrosis and plaque were generally found over the skin of pig number 1. At necropsy, tissues samples of internal organs were collected and fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin. Five micron thick sections were cut and stained with hematoxylin and eosin (H&E) for histological examination.

Immunohistochemistry

Tissue sections were deparaffinized in xylene, rehydrated through graded alcohols. Endogenous peroxidase was removed by 10 min reaction of 3% hydrogen peroxide in phosphate-buffered saline (PBS) and digested with proteinase K (Dako, USA). Polyclonal rabbit antiserum against PCV2 (provided by Virology Section, NIAH, Thailand) at 1:1,200 dilution was used as a primary antibody. Following the reaction, tissues were reacted with the appropriate secondary antibody and detection system from EnVision+System-HRP Labelled Polymer kit (Dako, USA) and using AEC (3-amino-9-ethylcarbazole) as a chromogen before counterstaining with hematoxylin.

PCR

For each pigsample, tissues were extracted using DNA extraction kit (Quigen, Germany). The forward and reverse primers 5’-CGGATATTGTAGTCCTGGTCG-3’ and 5’ACTGTCAAGGCTACCACAGTCA-3’ were used for the PCR amplification (481bp products). The amplified products were run in a 1.5% agarose gel, and visualized by staining with 0.5 µg/mL ethidium bromide.

Results

The results of histopathology and immunohistochemistry in lymphoid tissues and non-lymphoid tissues were shown in Table 1.

Subacute hemorrhagic epidermitis and necrotic dermatitis with vasculitis were found in the skin of pig number 1. Numerous multiple grape-like intracytoplasmic inclusion bodies were found in lymph nodes of pig number 2 (Fig. 1). The types of predominant viral antigen-positive cells are mostly macrophages in lymphoid organs more than in non-lymphoid tissues. However, other viral antigen-positive cells are bronchiole epithelium, renal tubule epithelium (Fig. 2).

According to the PCR analysis, PCV2 nucleic acids were detected in all pigs.

Discussion

In this study, all pigs had prominent microscopic lesions in both lymphoid and non-lymphoid tissues (3/3). Numerous multiple grape-like intracytoplasmic inclusion bodies were found in the lymph nodes of pig (1/3). The inclusions are reliable indicator of PCV2 infection but they are not present in all PCV2 infected cases (Sorden, 2000). Thus, together with characteristic histological lesions, IHC should be performed to confirm the diagnosis.

PCV2 antigen was detected in pig No. 2 and 3 (2/3) by IHC and PCV2 nucleic acid was detected in all pigs (3/3) by PCR. To compare IHC and PCR, the detection rate of PCV2 antigen in this study was lower than PCR similar to the study by Kim and Chae (2004) which revealed that PCR was more sensitive than IHC. In addition, the previous report showed that in case of mild microscopic lesions, PCV2 was detected by PCR and not by IHC (Calsamiglia et al., 2002). Apart from that, IHC is more specific with the presence of PCV2 in associated lesions and should be considered as a
better technique to diagnose clinical PMWS. Results from this study indicated that 2 out of 3 pigs were diagnosed PMWS based on the requirement of Sorden’s criteria. However, it was possible that the pig number 1 could be diagnosed as PDNS because the presence of glomerulonephritis couple with the skin lesion of epidermitis and dermatitis are specific for the disease. Importantly, diagnosis of PDNS is not reliant on demonstration of PCV2 in the tissue (Done et al., 2000).

The types of predominant viral antigen-positive cells are mostly macrophages. This is closely resembled the findings reported by Kennedy et al (2000) and Choi et al (2000). It is noticeable that the antigen-containing macrophages in lymphoid tissues were abundant compare to the positive macrophages in non-lymphoid organs. For this reason, lymphoid tissues must be considered as organs of choice to performed IHC. However, antigen-containing epithelium of other organs may be found as well. Therefore, the demonstration of PCV2 antigen in lymphoid and non-lymphoid tissues associated with characteristic histological lesions is useful for the diagnosis of PMWS.

Table 1 Results of histopathology and immunohistochemistry (IHC) in tissue samples of 3 pigs infected with porcine circovirus type2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lesion</th>
<th>Pig No.</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H&amp;E</td>
<td>IHC</td>
<td>H&amp;E</td>
<td>IHC</td>
<td>H&amp;E</td>
</tr>
<tr>
<td>Lymphoid tissue</td>
<td>Lymphoid depletion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- With inclusion body</td>
<td>+</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Without inclusion body</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Non-lymphoid tissue</td>
<td>Bronchopneumonia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Heart</td>
<td>Myocarditis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatitis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>- Interstitial nephritis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>- Fibrinous glomerulonephritis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>Enteritis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Brain</td>
<td>Vasculitis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

H&E+: lesion; H&E-: no lesion

IHC-: no staining, IHC+: focal staining, IHC++: multifocal staining , IHC+++: diffuse staining

Fig. 1 (a): Lymph node; numerous multiple grape-like intracytoplasmic inclusion bodies in macrophages of a pig number 2. H&E 400X. (b) Lymph node; abundant PCV2 antigen stained red in macrophages of pig number 2, IHC 100X.

Fig. 2 (a) Lung; PCV2 antigen stained red in macrophages, bronchiole epithelium and cellular debris. IHC 200X. (b) Kidney; PCV2 antigen stained red in renal tubule epithelium. IHC 200X.
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References


Rapid and visual detection of white spot syndrome virus by loop-mediated isothermal amplification

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Abstract

Background: White spot syndrome virus (WSSV) is the most threatening virus that causes mass mortality in shrimp aquaculture worldwide. In this present study, we developed a rapid and visual detection of WSSV based on the principle of loop-mediated isothermal amplification (LAMP) using a set of specifically designed six primers that recognize eight distinct target sequences.

Methods: The amplification of all reagents simultaneously added with fluorescence reagent (calcein and MnCl₂) is performed within a single closed tube on a regular water bath under a constant temperature of 65°C for 60 min. Upon the completion of reaction, the emission of green fluorescence allows immediate visualization for positive reaction by naked eyes without requirement of additional devices or reagents.

Results & Conclusion: This established LAMP protocol is highly specific to WSSV, whereas it is not specific to all other tested shrimp pathogens, including IHHNV, HPV, MBV, BP, TSV, YHV, and IMNV as well as shrimp genomic DNA. In addition, the detection sensitivity of this LAMP assay (10 viral copies) is as comparable as that of nested polymerase chain reaction (PCR), yet it possesses greater advantages than the nested PCR due to its inherent specificity, cost effectiveness, and obviation of handling with the mutagen ethidium bromide for electrophoresis.

Keywords: White spot syndrome virus; Loop-mediated isothermal amplification; WSSV; LAMP; Shrimp

Background

White spot syndrome virus (WSSV) is the most devastating pathogen causing mass mortality in penaeid shrimp population worldwide. Since the emergence in 1992, this virus has continued over the last decade to affect crustacean industries with significant economic losses. With regard to detection of WSSV infection at both the lifelong carrier stage and severe acute stage, many methods have been developed and validated in order to help prevent and control the disease transmission or outbreak.

Loop-mediated isothermal amplification (LAMP) is a novel alternative nucleic acid amplification method based on the principle of strand displacement DNA synthesis and production of stem-loop DNA structures under a constant temperature. This method has drawn a lot of attentions as it offers a rapid, accurate, and cost-effective genetic diagnosis for infectious microorganisms.

In this present paper, we report the development and validation of LAMP-based assay for WSSV in penaeid shrimp coupled with the simple visual detection of products; meanwhile, its applicability was assessed with field samples. 🌟
**Materials and methods**

**Design of primers for LAMP assay.** The highly conserved genomic region of WSSV (GenBank accession number AF332093; genome positions 224,261 to 225,707) which has been used in the conventional nested PCR was selected as the target for the primer design. Primers for LAMP assay were designed using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) and synthesized as high performance liquid chromatography (HPLC)-grade by BioDesign Co. Ltd.

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<td>LB</td>
<td>CTT CAG CCA TGC CAG CCG T</td>
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</table>

**DNA extraction.** Shrimp specimens used in this study were sent to our laboratory from different areas of Thailand without clinical manifestation of WSSV infection, and their appropriate target organs, i.e. pleopods for WSSV, were sampled and submitted for the examination of diseases. DNA extraction was performed using both a commercial kit and a tissue boiling method.

**LAMP assay.** The LAMP assay was performed in a 25 µL of reaction mixture containing 1.6 µM each FIP and BIP, 0.08 µM each F3 and B3, 0.64 µM each LF and LB, 800 µM each deoxyribonucleotide triphosphate (dNTP), 1 M betaine (Sigma), 20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 10 mM KCl, 4 mM MgSO₄ (Sigma), 0.1% Triton X-100, 8 U Bst DNA polymerase (New England Biolabs) and 2 µL template DNA except for the negative control for which the template DNA was omitted from the reaction. To visualize the reaction, 25 µL calcein (Sigma) and 0.5 mM MnCl₂ (Sigma) had been previously added to the reaction mixture, as previously described (Tomita et al., 2008; Peng et al., 2011). The amplification was carried out in a conventional water bath. The reaction was terminated by heating at 80°C for 2 min.

**Results**

**Optimization of the LAMP assay.** The optimal temperature for the LAMP reaction was determined to be between 60 and 65°C, which could yield the color change to green as observed by naked eyes. The optimal time and concentrations of magnesium, betaine, dNTPs, and MnCl₂ were identified as 60 min, 4 mM, 1 M, 0.8 mM, and 0.5 mM, respectively. With a simultaneous addition of diluted calcein (1.25 µL) plus diluted MnCl₂ (0.625 µL) to the reaction tube, positive reactions (generated pyrophosphate) turned green, while negative reactions remained faint orange.

**Specificity & Sensitivity.** The specificity of LAMP was confirmed by the digestion using HincII and SspI restriction enzymes. The amplified products with WSSV resulted in a series of bands by agarose gel electrophoresis. The resultant digested products of 167, 171, and 253 bp with HincII, and 121, 153, and 267 bp with SspI were in accordance with the predicted sizes. The LAMP reaction was tested using 10-fold serial dilutions of WSSV DNA from purified recombinant plasmid and compared against results from the nested PCR assay. The detection limit of both the LAMP and nested PCR assay was 10 copies.
Applicability of the LAMP assay. A total of 102 field samples were tested for WSSV by visualized LAMP and nested PCR. The results revealed that 73 out of 102 were tested positive with both the LAMP and nested PCR, while the other 29 samples were negative. None of these samples that were negative with the LAMP was tested positive with the nested PCR, and vice versa.

Fig.1. The optimal condition and concentration of LAMP for the detection of WSSV. The optimal condition and concentration with six primers is monitored by visual detection of color change and agarose gel electrophoresis. A. Optimal temperatures. M: 100-bp DNA molecular weight marker; 1–6: 60, 61, 62, 63, 64, and 65ºC, respectively. B. Reaction times. 1–7: 30, 40, 50, 60, 70, 80, and 90 min, respectively. C. Concentrations of magnesium. 1–5: 2, 3, 4, 6, and 8 mM, respectively. D. Concentrations of betaine. 1–4: 0, 0.5, 1.0, and 1.5 M, respectively. E. Concentrations of dNTPs. 1–5: 0.2, 0.4, 0.8, 1.4, and 1.6 mM, respectively. F. Concentrations of MnCl\(_2\). 1–4: 0, 0.5, 1.0, and 1.5 mM, respectively. (+): the color change to green, i.e. positive reaction of the visualized LAMP; (–): the starting orange color remains unchanged.

Conclusion

In this study, the LAMP assay with high sensitivity was executed in a water bath for 60 min, and the amplification was visualized by adding fluorescent reagent before amplification in order to observe the change of color by naked eyes. In conclusion, the newly established LAMP assay represents a reliable tool to detect WSSV in field conditions without requirement of specialized equipment.
Equine nasal granuloma caused by *Halicephalobus deletrix*: a case report

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Abstract

Abnormal mass in the nasal cavity from a 5 years-old horse was collected in 10% neutral buffer formalin. It was submitted to histologically investigate without clinical history except for sudden death. Routine histopathological slides were performed. Microscopic examination of the abnormal mass revealed that severe granulomatous inflammation was observed. Many small nematodes characterized by rhabditiform esophagus with anterior corpus, an isthmus, and posterior bulb, dorsoflexed ovary with uninucleated ovum were found in the center of lesion. These nematodes are morphologically consistent with *Halicephalobus deletrix*. To our knowledge, equine nasal granuloma caused by *H. deletrix* has not been reported in Thailand. The aim of this report is to present the microscopic findings and the identification of *H. deletrix* in a horse with clinical manifestation of nasal granuloma.

Key words: Equine nasal granuloma, *Halicephalobus* (Syn. *Micronema*) *deletrix*, horses.

Background

*Halicephalobus deletrix* previously known as *Micronema deletrix* is a small nematode belonging to the order Rhabtida. Normally it is a free-living parasite found in soil and humus (Gardiner et al., 1981). In this order, three genera consisting of *Rhabditis* (*Peodera*), *Halicephalobus* (*Micronema*) and *Strongyloides* are reported to parasitize domestic animals (Bowman, 1999). *Halicephalobus deletrix* was first described as *Micronema deletrix* as a causative agent of bilateral naso-maxillary granuloma in a horse by Anderson and Bemrick (1965). Then, the parasite was first isolated from the mandibular lesions of a horse. Morphological diagnosis of that parasite, based on rhabditiform esophagus with anterior corpus, isthmus, and posterior bulb, dorsoflexed ovary with uninucleated ovum, was *Halicephalobus gingivalis*, which is a synonym of *H. deletrix* (Anderson et al., 1998). At present, several cases of *H. deletrix* infection have been reported in both horses and humans. Organs associated with the infection in horses include brain, kidneys, nasal cavity, spinal cord, lymph nodes and adrenal glands (Vasconcelos et al., 2007). Most of the cases were diagnosed by tissue slide sections, and in the cases of humans, there were at least four cases of halicephalobiosis reported (Ondrejka et al., 2010). All of them are involved in the brain infection that contributes to meningoencephalitis. The disease in horse has been reported from many places such as U.S.A, Egypt and Canada (Jordan et al., 1975). The parasite infestation at the nasal mucosa results in a formation of granuloma called equine nasal granuloma. To our knowledge, equine nasal granuloma caused by *H. deletrix* has not been reported in Thailand. The aim of this study is to present the microscopic findings and the identification of *H. deletrix* in a horse with clinical manifestation of nasal granuloma.
Methods

A formalin fixed 3x3x3 cm mass was collected from the nasal cavity of a 5-years-old horse in the central Thailand without clinical history. The tissue sample was submitted to Pathology Section, National Institute of Animal Health for diagnosis. Histopathologic slides for microscopic examination were performed by a routine process according to Luna, 1968. The slides were sectioned at 3 µm thick, stained with H&E, PAS and Masson’s trichrome, and observed under a light microscope.

Results

Microscopic lesions of the mass revealed severe granulomatous inflammation characterized by the presence of epithelioid macrophages, multinucleated giant cells with fibrous tissue formation (Figure 1A). Many small nematodes characterized by rhabditiform esophagus with anterior corpus, an isthmus, and posterior bulb (Figure 1B), dorsoflexed ovary (Figure 2A) with uninucleated ovum (Figure 2B) were found in the center of the lesion. These nematodes are morphologically consistent with *Halicephalobus deletrix*.

**Figure 1.** A: Nasal granuloma characterized by fibrous formation, presence of epithelioid macrophages and multinucleated giant cells (close arrow); the nematodes located in the lesion (open arrow) H&E, 4X. B: *H. deletrix* morphologically comprises of rhabditiform esophagus characterized by corpus (C), isthmus (I), and bulb (B) H&E, 40X.

**Figure 2.** A: The lesion containing *H. deletrix* depicts dorsoflexed ovary (arrow head); the parasite’s keratin shows purplish red, PAS stain, 40X. B: Granulomatous lesion shows uninucleated ovum of *H. deletrix* (open arrow), and intestine (close arrow), the strong purplish red, is composed of glycogen and mucin depositions, PAS stain, 20X.

**Figure 3.** Granulomatous lesion contains a large number of nematodes (arrow); fibrous tissue stains blue; parasites composed of keratin stains red, Masson’s trichrome, 20X.
Discussion

The diagnosis is based on morphology found in histological slides which was described by Chitwood and Lichtenfels (1972). Furthermore, the morphology of the nematodes found in our report is consistent with that of nematodes reported by many previous studies (Rubin and Woodard, 1974; Simpson et al., 1988; Chalmers et al., 1990; Trostle et al., 1993; Rames et al., 1995 and Akagami et al., 2007). Moreover, there were many nematodes located in the lesion (Figure 3); however, the only female nematodes were observed in the slides. This suggested that the parasites have ability to reproduce by parthenogenesis. However, we could not obtain the complete history. Therefore, the death of this horse was possibly due to the multiplication and distribution of this parasite to vital organs.

Summary

Only *H. deletrix* has been reported to infest the nasal mucosa in horses. Additionally we wish to raise the public concern about the halicephalobiosis because the nematodes may pose threat to human health. Hence, the mechanism and the route of infestation should be studied in the future. To our knowledge, this is the first report of equine nasal granuloma caused by *H. deletrix* in Thailand.

References


Serological survey of neosporosis in dairy cattle in the central region of Thailand in 2011

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Abstract

Neosporosis is one of the most important diseases causing abortion in dairy cattle worldwide. The economic impact of neosporosis includes reproductive losses, reduction in milk production, premature culling and reduced weight gain, increasing the cost to the farmer. A cross sectional serological survey was conducted on a total of 834 blood samples collected from dairy cattle from 79 farms in Saraburi, Lopburi, Chainat and Supanburi which are the first four provinces in term of the highest population of dairy cattle in the central region of Thailand in August 2011 in order to estimate the seroprevalence of neosporosis in dairy cattle in the central Thailand and determined the age associated with antibodies against Neospora caninum (N. caninum) infection in dairy cattle. Sera were collected and tested for antibodies against N. caninum infection by competitive enzyme linked immunosorbent assay (cELISA). The result showed that the seroprevalence of neosporosis in dairy cattle was 20.4% (170/834) and herd prevalence of neosporosis was 64.6% (51/79) by a commercial cELISA. The result showed that dairy cattle which were raised in Supanburi had the highest seroprevalence of N.caninum infection at 24.0% (12/50) followed by Saraburi at 23.8% (106/445), Lopburi at 17.0% (48/283) and Chainat at 7.1% (4/56), while the herd prevalences in Saraburi, Lopburi, Chainat and Supanburi were 75.9% (22/29), 65.5% (19/29), 36.4% (4/11) and 60.0% (6/10), respectively. The age associated with antibodies against N. caninum infection in dairy cattle showed the seroprevalence of cows aged 1-2 year, 3-4 year, 5-6 year and >6 year were 21.2% (39/184), 20.9% (53/254), 17.6% (36/204) and 21.9% (42/192) respectively. The seroprevalence of dairy cattle in each age group in Saraburi presented nearly the same value range from 22.0% (29/132) to 25.9% (22/85), while Lopburi and Chainat showed the highest seroprevalence of neosporosis in dairy cows in age group > 6 year as 20.0% (17/85) and 12.5% (1/8) respectively. Conversely, the highest seroprevalence of bovine neosporosis in Supanburi was in age group 1-2 year at 46.7% (7/15). This information is useful for local livestock official to set preventive and control measures for the disease.

Keywords: Serological survey, Neosporosis, dairy cattle

Background

Neosporosis is an important disease causing abortion and stillbirth in cattle worldwide (Dubey and Lindsay, 1996). The major cause of neosporosis is Neospora caninum (N. caninum) which is a protozoan parasite of animals. The definitive hosts of N. caninum are the domestic dog and the Australian dingo (both Canis domesticus) and the coyote (Canis latrans) (McAllister et al., 1998; Gondim et al., 2004; King et al., 2010). Thus, dogs and related canids play an important role in disease spreading. Neospora oocysts are environmentally resistant and are the key in the epidemiology of neosporosis (Neto et al., 2011). The dairy cattle can be infected by accidental ingestion of sporulated oocysts from
the environment and by vertical transmission from the dam to the fetus (McCann et al., 2007). However, ingestion of infected tissues is the most likely source of infection for carnivores (Dubey and Schares, 2011). In cattle, a few days after primary infection specific IgM and IgG antibodies appear. The specific IgM levels peak after 2 weeks of infection, while IgG levels increase during the first weeks up to 3-6 months after infection. Levels of specific antibodies may persist for life but fluctuate and sometimes below the detection limits of serological tests (Dubey and Schares, 2006). The effects of bovine neosporosis include reproductive losses, reduction in milk production, premature culling and reduced weight gain (Joao et al., 2005). Cows of any age may abort at three months of gestation to full term with mostly abortion occurring at five to six month gestation. Infected cows with *N. caninum* are two to seven times more likely to abort than uninfected cows (Dannatt, 1997). In addition, the risk of abortion is two to three times in seropositive cows than in seronegative cows (Joao et al., 2005). Cattle remain infected for life and might be repeated abortion due to neosporosis (Dubey and Schares, 2011). The main serological examination methods detecting antibody against *N. caninum* are indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA). However, the IFAT is time-consuming and more expensive than ELISA. While, a competitive ELISA (VMRD, USA) showed that no cross reaction to two closely related apicomplexan protozoa, *Toxoplasma gondii* and *Sarcocystis cruzi* (Baszler et al., 1996).

The objectives of this study are to estimate the seroprevalence of neosporosis in dairy cattle in central Thailand and to determine the age associated with antibodies against *N. caninum* infection in dairy cattle. This information will be useful for local livestock official to set preventive and control measures for the disease.

**Materials and methods**

**Study design**

A cross-sectional study was conducted on dairy cattle in four provinces: Saraburi, Lopburi, Chainat and Supanburi where are the first four rank of dairy cattle population in central region of Thailand in August 2011. Blood samples were collected from jugular vein or caudal vein of 834 dairy cattle from 79 farms. Sera were kept in -20 °C prior to test. Dairy cattle and the farm distribution in those 4 provinces are summarized in table 1.

**Laboratory procedures**

A commercial competitive enzyme–linked immunosorbent assay (cELISA-VMRD, Pullman, USA) was used to detect antibodies against *Neospora caninum* in bovine sera. Fifty microliters (µl) of serum sample, positive control and negative control were added to antigen-coated plate and incubated the plate at room temperature for one hour. After that washed the plate three times with washing buffer solution, followed by added 50 µl of antibody–peroxidase conjugate then incubated at room temperature for 20 minutes. After incubation, washed the plate three times with washing buffer solution. Fifty microliters of substrate was transferred to the plate and kept at room temperature for 20 minutes then added 50 µl of stop solution to the plate. Finally, read the absorbance at 620 nm optical density by ELISA reader. The result interpretation was determined by the percentage of inhibition. The negative sera showed < 30% inhibition, while the positive sera showed ≥ 30% inhibition according to the manufacturer’s instructions.

**Statistical Analysis**

The sample size was calculated using the Win Episcope 2.0. The estimated seroprevalence of neosporosis in dairy cattle raising in central region of Thailand was 50% at 95% confidence interval and a 3.5% accepted error. The proportional selected dairy cattle in each provinces was performed depending on dairy cows population. Tha data were analyzed using STATA version 16. Chi-square ($\chi^2$) was applied to determine the difference in seroprevalence among provinces, and age group as well as herd prevalence among provinces.
Table 1 The number of dairy cattle and farms in the selected provinces in central region of Thailand during 2011

<table>
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<tr>
<th>Province</th>
<th>Number of dairy cattle</th>
<th>Number of farm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saraburi</td>
<td>445</td>
<td>29</td>
</tr>
<tr>
<td>Lopburi</td>
<td>283</td>
<td>29</td>
</tr>
<tr>
<td>Chainat</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>Supanburi</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>834</strong></td>
<td><strong>79</strong></td>
</tr>
</tbody>
</table>

Results

The overall seroprevalence of *N. caninum* infection in dairy cattle was 20.4% (170/834) by cELISA. While the seroprevalence of bovine neosporosis in each districts was 23.8% (106/445) in Saraburi, 17.0% (48/283) in Lopburi, 7.1% (4/56) in Chainat and 24.0% (12/50) in Supanburi. Additionally, the overall herd prevalence of neosporosis was 64.6% (51/79). Herd prevalence in Saraburi, Lopburi, Chainat and Supanburi were 75.9% (22/29), 65.5% (19/29), 36.4% (4/11) and 60.0% (6/10) respectively. (table 2)

Table 3 shows the age associated with antibodies against *N. caninum* infection in dairy cattle. The seroprevalence of neosporosis in dairy cattle aged 1-2 year, 3-4 year, 5-6 year and >6 year were 21.2% (39/184), 20.9%(53/254), 17.6%(36/204) and 21.9%(42/192) respectively.

Table 4 shows the distribution of age associated with antibodies against *N. caninum* infection in dairy cattle in each provinces in central Thailand. The neosporosis seroprevalence of dairy cows in each age groups in Saraburi presented nearly the same value as 22.0% (29/132) in 1-2 year, 24.6% (30/122) in 3-4 year, 23.6% (25/106) in 5-6 year and 25.9%(22/85) in >6 year. The highest seroprevalence of bovine neosporosis in Lopburi was 20.0% (17/85) in >6 year followed by 19.0%(19/100) in 3-4 year, 12.9% (9/70) in 5-6 year and 10.7% (3/28) in 1-2 year. Similarly, the highest seroprevalence of *N.caninum* infection in dairy cows in Chainat was 12.5%(1/8) in >6 year followed by 8.7% (2/23) in 3-4 year and 6.3% (1/16) in 5-6 year. Additionally, the seroprevalence of neosporosis in dairy cattle in age group 1-2 year, 3-4 year, 5-6 year and >6 year were 46.7% (7/15), 22.2% (2/9), 8.3% (1/12) and 14.3% (2/14) respectively.

From data analysis, the result showed statistically significant difference of seropositive cattle among the provinces ($\chi^2=11.734$, $p=0.008$); there was statistically significant difference between Saraburi and Chainat ($\chi^2=8.074$; $p=0.004$), Saraburi and Lopburi ($\chi^2=4.779$; $p=0.029$) and Chainat and Supanburi ($\chi^2=5.857$; $p=0.016$). While, there was no statistically significant difference of herd prevalence among the provinces ($\chi^2=5.544$; $p=0.136$). Moreover, there was no statistically significant difference of seropositive cattle among age groups ($\chi^2=1.316$; $p=0.725$).

Table 2 Seroprevalence and herd prevalence of neosporosis in dairy cattle in Central region of Thailand during 2011

<table>
<thead>
<tr>
<th>Province</th>
<th>Number of examined</th>
<th>Number of positive</th>
<th>Prevalence (%)</th>
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<tr>
<td>Saraburi</td>
<td>445</td>
<td>106</td>
<td>23.8*</td>
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<tr>
<td>Lopburi</td>
<td>283</td>
<td>48</td>
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<td>Supanburi</td>
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<tr>
<td></td>
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<td><strong>79</strong></td>
<td><strong>51</strong></td>
<td><strong>64.6</strong></td>
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*Same letters in the same column means significance difference ($p<0.05$)
Table 3  Age associated with antibodies against *N. caninum* infection in dairy cattle

<table>
<thead>
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<th>Ages (year)</th>
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<th>Prevalence (%)</th>
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<tr>
<td>&gt;6</td>
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<td>21.9</td>
</tr>
<tr>
<td>Total</td>
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<td>170</td>
<td>20.4</td>
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Table 4  Distribution of age associated with antibodies against *N. caninum* infection in dairy cattle in each province in central region Thailand

<table>
<thead>
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<th>Provinces</th>
<th>Ages (year)</th>
<th>Number of examined</th>
<th>Seropositive</th>
<th>Prevalence (%)</th>
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<td>0</td>
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<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>56</td>
<td>4</td>
<td>7.1</td>
</tr>
<tr>
<td>Supanburi</td>
<td>1-2</td>
<td>15</td>
<td>7</td>
<td>46.7</td>
</tr>
<tr>
<td></td>
<td>3-4</td>
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<td>5-6</td>
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<td>8.3</td>
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<tr>
<td></td>
<td>&gt;6</td>
<td>14</td>
<td>2</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>12</td>
<td>24.0</td>
</tr>
</tbody>
</table>

Discussion

From this study, the seroprevalence of neosporosis in dairy cattle in central region of Thailand was 20.4% and herd prevalence of neosporosis was 64.6 % by cELISA. This finding was higher than the previous study of Suteeraparp et al. (1999) and Jittapalapong et al. (2008). Suteeraparp et al. (1999) found 6% seropositive to neosporosis in dairy cattle in 11 provinces of central Thailand by IFAT. Besides, Jittapalapong et al. (2008) investigated the seroprevalence of *N.caninum* infection in dairy cows in 3 provinces of the northeast of Thailand using cELISA found 11.7% seroprevalence and 58.2% herd prevalence of neosporosis. In Thailand, there was a case report studied by Kyaw et al. (2003). They reported 41.7% (5/12) of aborted cows from two farms in Chonburi and Saraburi provinces were positive to *N.caninum* infection by using cELISA. Moreover, *Neospora* tachyzoites were detected in placenta of a seropositive aborting cows which confirmed that neosporosis was the possible cause of abortion. The present study revealed that dairy cows raised in Supanburi had the highest seroprevalence of *N.caninum* infection at 24.0% followed by Saraburi at 23.8%, Lopburi at 17.0% and Chainat at 7.1%. In addition, the highest herd prevalence of neosporosis was 75.9% from Saraburi, followed by 65.5% from Lopburi, 60.0% from Supanburi and 36.4% from Chainat. It was indicated that dairy cattle which raised in central region of Thailand had a high chance to expose to *N.caninum* oocyst by the definitive host since oocyst are environmentally resistant and play a vital role in spreading of bovine neosporosis (Neto et al., 2011). Consequently, the pathogen may circulate...
and infect to dairy cows in the region. Besides, transmission via milk and semen should also be concerned (Dubey and Schares, 2011).

The age associated with antibodies against *N. caninum* infection in dairy cattle in this study revealed nearly the same percentage of seroprevalence in each age group. The seroprevalence of cows aged 1-2 year, 3-4 year, 5-6 year and >6 year were 21.2%, 20.9%, 17.6% 21.9%, respectively, and there was no statistically significant difference in each age group ($\chi^2=1.316; p=0.725$). This finding was contrast with the previous study of Jittapalapong et al. (2008). They found that the highest proportion of seropositive of neosporosis was in the oldest age of dairy cattle. Additionally, Eras et al. (2011) stated that seroprevalence of neosporosis increased with age. The seroprevalence of dairy cows in all age groups in Saraburi presented nearly the same value range from 22.0% to 25.9%. While, Lopburi and Chainat revealed the highest seroprevalence of neosporosis in dairy cattle in age group > 6 year as 20.0% and 12.5% respectively. It was surprisingly that the highest seroprevalence of bovine neosporosis in Supanburi was in age group 1-2 year at 46.7%. This result was inconsistent with the study of Jittapalapong et al. (2008) and Eras et al. (2011). The reason why the seroprevalence of bovine neosporosis in all age groups were found nearly the same percentage (17.6% - 21.9%) from all four provinces is that most of the dairy cattle in central Thailand are raised in the farm where are open to the environment and dogs can easily enter and defecate at the raising area so the dairy cattle in all age groups could be easily infected with the pathogen. Therefore, dog is considered as risk factor for neosporosis especially young dogs because they produce more oocysts significantly higher than adult dogs (Cavalcante et al., 2011). In contrast, the study of Thurmond and Hietala (1997) revealed that the risk of abortion is age –dependent and they also found that seropositive cows were 1.7 times more likely to abort in their 2nd pregnancy and Dubey et al. (2006) found that the transmission rate increases with the age of gestation period.

Conclusions

The overall seroprevalence and herd prevalence of *N. caninum* infection in dairy cattle in central Thailand in this study was 20.4% and 64.6% respectively by cELISA, which was higher than the previous study. In addition, the age associated with antibodies against *N. caninum* infection in each age group of dairy cattle revealed nearly the same percentage of seroprevalence. The strategies to reduce the disease should be applied in order to preventive and control measure for example maintain the close herd, use serological screening test (Dubey and Schares, 2011) and restrict the number of the dog (Joao et al., 2005).

Acknowledgement

I wish to express my acknowledgement to Dr. Ladda Trongwongsa and Dr. Watcharachai Narongsak, National Institute of Animal Health for their guidance of the manuscript, the local staff from Saraburi, Lopburi, Chinat and Supanburi provincial livestock office which communicated to the farmer for collecting the samples and the staff from Parasitology section, National Institute of Animal Health for collecting the sample and laboratory work. Finally, I would like to thanks Mr.Kijsart Aonngemthayakorn, Center for Agricultural Information, The Office of Agricultural Economics for suggesting the statistical analysis.


Genetic diversity and antimicrobial susceptibility of *Listeria monocytogenes* isolated from chicken meat from fresh markets in Bangkok

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Abstract

**Background:** Twenty isolates of *Listeria monocytogenes* (*L. monocytogenes*) obtained from a survey of *L. monocytogenes* contamination in duck and chicken meat collected from fresh market in Bangkok during 2006-2007 were evaluated for genetic diversity by pulsed-field gel electrophoresis (PFGE).

**Methods & Results:** Combined result from PFGE patterns of *Apa*I, *Sma*I and *Asc*I revealed total 12 genetic patterns of which pattern 1 predominated and covering 8 isolates, suggesting the existence of high similarity clones around the Bangkok areas. Antimicrobial susceptibility testing of these 20 isolates resulted as minimum inhibitory concentration (MIC) ranges were as follows: ampicillin 0.5-1 µg/mL, penicillin 0.5-2 µg/mL, ciprofloxacin 1-16 µg/mL, gentamicin 0.25-0.5 µg/mL, erythromycin 0.25-0.5 µg/mL, tetracycline 0.5-64 µg/mL and trimethoprim/sulfamethoxazole <0.125/2.375 µg/mL.

**Summary:** In general, most of the isolates were susceptible to the antimicrobials tested. However, resistances to penicillin and tetracycline were found in one isolate each and resistance to ciprofloxacin was found in 7 isolates. Thus, surveillance and monitoring antimicrobial resistant *L. monocytogenes* should be carried out at regular intervals.

**Keywords:** genetic diversity, antimicrobial susceptibility, *Listeria monocytogenes*, pulsed-field gel electrophoresis
RT-PCR Survey of emerging paramyxoviruses in cave-dwelling bats

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2 Eastern Veterinary Research and Diagnosis Centre, Baan Boung, Chonburi 20220 Thailand
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Abstract

Background: Bats are the reservoir hosts for several paramyxoviruses including two serious zoonotic viruses, Hendra virus and Nipah virus which are responsible for fatal infections in animals and humans. These two viruses are sufficiently different from previously described paramyxoviruses and are included in a new genus, henipavirus. We report here a survey of cave-dwelling, insectivorous bats in Thailand for the presence of henipaviruses.

Method: Pooled urine samples were collected in nine caves inhabited by six different bat species in the northern (Chiangmai and Nakornsawan) and southern (Songkla and Satoon) provinces of Thailand. A reverse transcription PCR (RT-PCR) assay using henipavirus-specific primers derived from the conserved region of the RNA polymerase (L) gene was used to detect known and unknown viruses in this genus. Samples from seven out of nine caves surveyed tested positive by RT-PCR.

Results: Nucleotide sequences of the PCR bands revealed the presence of diverse strains (three clusters and seven divergent genotypes) of previously uncharacterised paramyxovirus(es). Phylogenetic analysis based on the deduced L protein sequence revealed close correlations between the positive samples and the recently described but unclassified paramyxoviruses: Beilong virus and J-virus.

Summary: This is the first report on the prevalence of paramyxovirus variants in cave-dwelling bats and highlights the importance of further epidemiological surveillance in bats.

Keywords: bats, natural reservoir, paramyxovirus, RT-PCR, Thailand, zoonoses
Potential risk factors of porcine reproductive and respiratory syndrome outbreak in Lower North of Thailand

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Abstract

Background: Since an outbreak of porcine reproductive and respiratory syndrome (PRRS) has occurred in Lower Northern Thailand, high morbidity and high mortality rate, especially in small scale pig farms were observed. Veterinary Research and Development Center (Lower Northern Region) conducted an epidemiology study in the outbreak area.

Objective: The objective of the study is to identify potential risk factors of the PRRS outbreak in pig farms in Lower North of Thailand during September 2010 - June 2011.

Method: Case - control study was conducted by reviewing PRRS cases from the laboratory records. Pig farm owners whose farms are located in the outbreak area were interviewed. Potential risk factors of PRRS outbreak were identified by odd ratio at the 95% confidence interval.

Results: From the 55 laboratory cases of reported PRRS, 78.2 % were from Phitsanulok province and 12.8% were from Uttaradith. Bacterial co-infection such as Escherichia coli, hemolytic E. coli and Streptococcus suis was found. Antimicrobial drugs that bacteria resisted consisted of streptomycin, tetracycline, kanamycin, and ampicillin. The potential risk factors of the case-control (28:92) were that pig farm owners lacked knowledge and personnel sanitation and that seller’s trucks drove into farm and shared boar for breeding.

Conclusion: Because pig farm owners lack knowledge about the use of medical equipments, many bacteria may have resisted antimicrobial drugs. Some of bacteria co-infecting in the PRRS cases can harm humans as well. Health education, farm management and farm biosecurity should be provided to farm owners to prevent the possibility of a PRRS outbreak.

Keywords: outbreak, risk factors, PRRS, antimicrobial drug, pig
Carrier Rate of Streptococcus suis in Tonsils of Slaughtered Pigs in Lampang and Phayao Provinces of Thailand

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Abstract

Background: Streptococcus suis (S. suis) is an important swine and human pathogen. In Thailand at least 300 cases of S. suis infection in humans have been reported. From the 35 capsular serotypes currently known, serotype 2 is the major cause of human infection however serotype 14, the relatively less prevalent serotype was reported in human cases in this region. Method: Since there was high prevalence of human cases occurred sporadically in the Northern part where eating of raw pork products is common especially in Lampang and Phayao provinces, a survey of S.suis from tonsils of slaughtered pigs was done during September 2009 – July 2010. Totally 236 and 559 tonsil samples of apparently healthy pigs from slaughterhouses of the respective provinces were cultured. S.suis was isolated and identified by conventional method and polymerase chain reaction (PCR) using primers specific to 16S rRNA, and capsular gene types 1(14) and 2(1/2). Additional confirm was done by slide agglutination and capillary tube precipitation with S. suis type 1, 2 and 14 antisera. Result: The results revealed that of 236 tonsil samples from Lampang, 64.8%, 3.8% and 2.1% of them were positive with S. suis, S. suis type 2 and S. suis type 14, respectively. Whereas 61.4%, 5.9% and 1.2% of 559 pig tonsil samples from Phayao were positive with S. suis, S. suis type 2 and S. suis type 14, respectively. There was no significantly different among the prevalence of S.suis in pig tonsils and the ambient temperature and humidity. Additional results of virulence-associated genes investigation including suilysin (sly), muramidase released protein (mrp) and extracellular protein factor (epf) revealed that of 151 S. suis isolates from Payao the genotypes with sly+, mrp+ and epf+ were detected at 64.9%, 58.9% and 3.9% respectively. Summary: Even though the route of transmission from pigs to humans is unclear, the high prevalence of S.suis carrier in pigs especially serotypes 2 and 14 and the presence of virulence-associated genes might be risk factors for S. suis infection in humans.

Keywords: Streptococcus suis, tonsils, pigs, Lampang, Phayao
Brucellosis OIE Laboratory Twinning Programme France/Thailand.

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\(^2\)EU/OIE/FAO Brucellosis Reference Laboratory, French Agency for Food, Environmental and Occupational Health Safety (ANSES), Maisons-Alfort, France.

**Background:** The OIE Laboratory Twinning Project aims at improving global capacity for disease prevention, detection, and control through better veterinary governance. The discussion and preparation of the twinning project on brucellosis started during the 1\(^{st}\) and 2\(^{nd}\) FAO-APHCA / OIE Regional Workshops on Brucellosis Diagnosis and Control in 2008 and 2009 (directed by Dr. B. Garin-Bastuji and collaboration with DLD-NIAH). A series of objectives were developed for the twinning process.

**Methods:** The objectives of the laboratory project have been endorsed by the directors of both laboratories and respective national CVOs. Main objectives are to strengthen the level of expertise in brucellosis (serological diagnosis/screening and Bacteriological isolation of *Brucella* by culture) and implementing reference activities (i) control and standardisation of diagnostic reagents and vaccines, (ii) organization of Regional serology proficiency ring-trials and (iii) *Brucella* typing and molecular detection and identification. The work programme includes 3 annual training sessions of NIAH staff at Anses and 3 visits of Anses experts to NIAH.

**Results:** The project was approved in June 2010 for a 3 year-duration (2010-2013). The first year programme was fully achieved with:

- Anses standard operating procedures (SOP) (serological and bacteriological diagnosis, *Brucella* biotyping and control of antigens and ELISA kits) were translated in English and sent to NIAH
- A 1\(^{st}\) training session at Anses for 3 NIAH scientific and technical staff on serological diagnosis and control of diagnostic antigens and ELISA kits and first discussions regarding the organisation of the bacteriological diagnosis in adequate quality and biosafety conditions.
- The transfer of secondary reference standard sera (iELISA) as well as of a freeze-dried control serum panel from Anses to NIAH;
- NIAH has also started the quality control of RBT antigen pilot batches, as well as of National positive control standard sera, some of same giving adequate resultants according to Anses checks;
- NIAH started the collection of sera from infected cattle, buffaloes, sheep and goats in order to prepare a future common serum collection for further standardisation and attest evaluation studies.
- The first visit of Anses to NIAH (1 week – 2 experts) took place on April 9-15, 2011 for a first audit of the organisation and work activities in place with exchanges (i) on the RBT antigen local production, (ii) laboratory management for serological testing, bacterial culture and molecular biology, (iii) the preparation and standardisation of national standard sera and positive control sera and (iv) on Investigation of brucellosis infected herds in the field.

Finally, a common poster regarding brucellosis field surveys performed in Thailand was presented at the last Brucellosis international Conference in Buenos Aires, Argentina in September 2011.

**Summary/conclusions:** Achievement of all project objectives is expected to strengthen the Asia Pacific regional impact of the NIAH brucellosis laboratory and to ensure the capability for the already ongoing implementation of those regional activities that are expected from an OIE Reference Laboratory, by stimulating an active network of regional diagnostic expertise for the surveillance and control of brucellosis.

Keywords: OIE, laboratory twinning, brucellosis
Brucellosis seroprevalence of livestock in Lower Northeastern region, Thailand from 2009 to 2011

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Abstract

Brucellosis is a zoonotic disease of global importance infecting humans, livestock and other domestic animals. Between 2009 and 2011, a total of 59,554 samples from 2,129 herds were submitted to Veterinary Research and Development Center (Lower Northeastern Region) for serological analysis by RBT cooperated with CFT to determine the seroprevalence of brucellosis. The 26,624, 21,135, 5,511, 4,914 and 1,370 samples were collected from dairy cattle, beef cattle, goat, buffalos and sheep, respectively. The overall individual-level seroprevalence of brucellosis was 0.66 % while from individual species was 2.79, 0.76, 0.57, 0.36, and 0.16 % for goat, beef cattle, buffalos, sheep and dairy cattle, respectively. The herd-level seroprevalence was 5.45 % while from individual species was 17.26, 13.04, 6.78, 4.35 and 2.96 % for goat, sheep, beef cattle, buffalos and dairy cattle, respectively. Both herd-level and individual-level seropositive were increased from 2009 to 2011. The herd-seropositive in goat and the individual-seropositive in beef cattle were highly increased statistically significantly. Ubonratchathani province was the highest seroprevalence area in individual-level (2.05%) and Chaiyaphum province was the highest seroprevalence area in herd-level (12.26%). This study showed that brucellosis was still prevalent and the trend of the disease has increased from 2009 to 2011. Brucellosis remains a problem disease for the livestock in Lower Northeastern Regional of Thailand. The implementation of brucellosis, tuberculosis, paratuberculosis (BTP) disease-free farms project is necessary continue to prevent and eliminate it completely.

Keywords: brucellosis, seroprevalence, RBT, CFT

Background

Brucellosis caused by bacteria of the genus Brucella is considered one of the most widespread zoonosis in the world. Brucella spp. is the rod-shaped bacteria, quite round (coccobacilli), staining gram negative (gram negative) and not sporulate (Ekatat, 2009). Dormant reside within the cell and sometime be found outside the infected cells. In cattle and buffaloes mainly caused by Brucella abortus, in goats caused by B. melitensis, in sheep caused by B. melitensis and B. ovis. The pathogen of cattle and goats can be transmitted from animals to humans (zoonosis) by consuming milk from infected cows and goats because of the bacteria are excreted with the milk and living up to 2-3 days. People can be infected by consuming raw milk or unhygienic processing milk or contact with the vaginal discharge (Hungerford, 1975). Direct contact with the calves that are aborted in the womb or eviscerated carcasses of infected cattle. The pathogens is usually not found in muscle. The bacteria can be destroyed by normal cooking temperatures. The disease can be contacted by people who killed and dissected the carcasses and internal organs (Chobjit and Taecho, 2008). Symptoms in patients infected with the liver and spleen, pneumonia, arthritis, muscle weakness, fatigue, testicular
inflammation and undulate fever (Jorge et al., 1998). The symptoms in cattle, goats, sheep that infected the testes can cause inflammation or tumor growth. Female often miscarriage in pregnant women and inflammation. The low fertility rate problems occur in the herd. In dairy cattle, feeding rate is relatively low. Both male and female with the disease can spread disease to other animals easily by contact with the vaginal discharge, feeding, or water mixed with bacteria (Jensen and Mackey, 1979).

There are many serological diagnostic methods such as Rose bengal test (RBT), Rapid plate test (RPT), EDTA-Tube agglutination test (EDTA-TAT), Complement fixation test (CFT), Enzyme-linked immunosorbent assay (ELISA) etc., which are highly effective in sensitivity, specificity, and fast for the detection of antibodies in animals. In addition, the determining disease needs to use animal age and vaccination history. All of the test, CFT is standard method to confirm the disease (OIE, 2009). However the disadvantage of this method is much difficulties technique and take a long time while RBT as a screening method that was simple, easy and faster. According to Nittaya and colleagues were found RBT tested in the cattles sera showed sensitivity, specificity and accuracy up to 99.11%, 99.88% and 99.84%, respectively, consistent with the study of the Muktaderul and colleagues (2011) found RBT in the cattle and goats sera showed sensitivity 100.00%, specificity 93.40%. Concluded that the method of RBT was highly effective compared to CFT which was the standard method. The disease can be tested in laboratory and in the area as well.

Veterinary Research and Development Center (Lower Northeastern region) where is the agency responsible for the diagnosis in Brucellosis in the Northeastern region such as Chaiyaphum, Nakhon Ratchasima, Buriram, Yasothon, RoiEt, Surin, Ubon Ratchathani and Amnatcharoen province. The implementation of brucellosis, tuberculosis, paratuberculosis (BTP) disease-free farms project that the policy of National Economic and Social Development Plan the 8th edition, 1997. The purpose of this study to explored the prevalence of Brucellosis in Lower Northeastern region in Thailand. The data have been used to control Prevention and eradication from the area.

**Materials and methods**

**Collection of samples:** A total of 59,554 serum samples from 26,624, 21,135, 5,511, 4,914 and 1,370 were collected from dairy cattle, beef cattle, goat, buffalos and sheep, respectively. The serum samples were submitted to immunology laboratory of Veterinary Research and Development Center (Lower Northeastern Region) for Rose Bengal Test (RBT) and the positive reaction by RBT were sent for Complement Fixation Test (CFT).

**Serological Tests:**

**Rose bengal Test:** RBT was performed with a 8% cell suspension of B. abortus 1119-3 in rose bengal (Bureau of Veterinary Biologics, Pakchong Nakhon Ratchasima). The sample and antigen were brought to room temperature for 30 min before use. One Brucella positive and one negative reference samples were used on each plate. In The serum sample from goats and sheep, 75 and 25 µl of serum and antigen was taken on the plate glass. Equal volumes (30 µl) of serum (dairy cattle, beef cattle and buffalos serum samples) and antigen were mixed and rotated on a glass plate for 4 min. Agglutination values were recorded as negative (–) and positive (+, + +, + + +, and + + + +) representing different degrees of agglutination.

**Complement Fixation Test:** CFT was performed in microplates according to OIE’s manual (6th Edition 2009). Briefly, in 96-well microtiter plates, a 25 µl aliquot of each serum and controls (negative and positive) were serially diluted in veronal saline buffer (commercial reagent). A 25 µl volume of previously titrated antigen (B. abortus biotype 1, strain 99; Antifix, Synbiotics Europe, France) was then added to each well, followed by 25 µl of complement (commercial reagent). After incubation at 4°C, overnight, 25 µl of sensitized sheep erythrocytes were added to each well and plates were again
incubated at 37°C for 30 min. After incubation, the plates were spun (500 g for 3 min) or keep at 4°C, overnight and the results evaluated as follows: 100% hemolysis was considered a negative reaction, while reactions showing 75, 50 or 25% of hemolysis were considered positive. Sera with positive fixations at a titer equivalent to or higher than 20 international complement fixation units (ICFTU), as prescribed by the European Union, were considered to be positive.

Results

Between 2009 and 2011, Both herd-level and individual-level seropositive were increased statistic significantly (Table 1)

### Table 1. Herd and individual seroprevalence of brucellosis in Lower Northeastern region, Thailand during 2009 – 2011.

<table>
<thead>
<tr>
<th>Year</th>
<th>Herd-level</th>
<th></th>
<th></th>
<th>Individual-level</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number of sample</td>
<td>positive</td>
<td>%</td>
<td>number of sample</td>
<td>positive</td>
<td>%</td>
</tr>
<tr>
<td>2552</td>
<td>851</td>
<td>46</td>
<td>5.41</td>
<td>18,106</td>
<td>136</td>
<td>0.75</td>
</tr>
<tr>
<td>2553</td>
<td>799</td>
<td>32</td>
<td>4.01</td>
<td>23,469</td>
<td>144</td>
<td>0.61</td>
</tr>
<tr>
<td>2554</td>
<td>479</td>
<td>38</td>
<td>7.93</td>
<td>17,979</td>
<td>111</td>
<td>0.62</td>
</tr>
<tr>
<td>Total</td>
<td>2,129</td>
<td>116</td>
<td>5.45</td>
<td>59,554</td>
<td>391</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Between 2009 and 2011, Chaiyaphum province was the highest seroprevalence area in herd-level at 12.26% (32/261) (Table 1) and Ubonratchathani province was the highest seroprevalence area in individual-level at 2.05% (36/1,754) (Table 2).

### Table 2. Herd seroprevalence of Brucellosis in Lower Northeastern – region, Thailand during 2009–2011.

<table>
<thead>
<tr>
<th>Province</th>
<th>Buffalos</th>
<th>Sheep</th>
<th>Dairy cattle</th>
<th>Beef cattle</th>
<th>Goat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>%</td>
<td>Positive</td>
<td>%</td>
<td>Positive</td>
</tr>
<tr>
<td>Chaiyaphum</td>
<td>3/16</td>
<td>18.75</td>
<td>1/7</td>
<td>14.29</td>
<td>3/103</td>
</tr>
<tr>
<td>Nakhon atchasima</td>
<td>0/11</td>
<td>0</td>
<td>1/3</td>
<td>33.33</td>
<td>25/664</td>
</tr>
<tr>
<td>Buriram</td>
<td>0/7</td>
<td>0</td>
<td>1/1</td>
<td>100.00</td>
<td>1/247</td>
</tr>
<tr>
<td>Roi et</td>
<td>0/15</td>
<td>0</td>
<td>0/1</td>
<td>0</td>
<td>0/2</td>
</tr>
<tr>
<td>Yasothon</td>
<td>3/81</td>
<td>3.70</td>
<td>0/3</td>
<td>0</td>
<td>0/2</td>
</tr>
<tr>
<td>Srisaket</td>
<td>2/79</td>
<td>2.53</td>
<td>0/0</td>
<td>0</td>
<td>1/36</td>
</tr>
<tr>
<td>Surin</td>
<td>2/32</td>
<td>6.25</td>
<td>0/6</td>
<td>0</td>
<td>2/26</td>
</tr>
<tr>
<td>Amnatcharoen</td>
<td>0/3</td>
<td>0</td>
<td>0/0</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td>Ubonratchathani</td>
<td>1/9</td>
<td>11.11</td>
<td>0/2</td>
<td>0</td>
<td>0/0</td>
</tr>
<tr>
<td>Total</td>
<td>11/253</td>
<td>4.35</td>
<td>3/23</td>
<td>13.04</td>
<td>32/1,080</td>
</tr>
</tbody>
</table>

### Table 3. Individual seroprevalence of Brucellosis in Lower Northeastern region, Thailand during 2009-2011

<table>
<thead>
<tr>
<th>Province</th>
<th>Buffalo</th>
<th>Sheep</th>
<th>Dairy cattle</th>
<th>Beef cattle</th>
<th>Goat</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Positive</td>
<td>%</td>
<td>Positive</td>
<td>%</td>
<td>Positive</td>
</tr>
<tr>
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<td>1/642</td>
<td>0.16</td>
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<td>1.21</td>
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<td>Buriram</td>
<td>0/164</td>
<td>0</td>
<td>2/18</td>
<td>11.11</td>
<td>1/5339</td>
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<td>0</td>
<td>0/11</td>
<td>0</td>
<td>0/192</td>
</tr>
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<td>0</td>
<td>1/566</td>
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<td>0/285</td>
<td>0</td>
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<td>0/13</td>
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<td>5/1,370</td>
<td>0.36</td>
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The trend of seroprevalence Between 2009 and 2011, the herd-seropositive in goats was increased statistic significantly (Fig. 1) and individual-seropositive in beef cattle was highly increased statistic significantly (Fig. 2).

Figure 1 Herd seroprevalence during 2009–2011
Figure 2 Individual seroprevalence during 2009–2011

Discussion

Between 2009 and 2011, a total of 59,554 samples from 2,129 herds of dairy cattle, beef cattle, goat, buffalos and sheep were test by using the RBT and confirmed with CFT. The seropositive in herd-level and individual-level were increased statistic significantly especially in herd-level. The herd-seropositive in goats were increased statistic significantly (Fig. 1) and individual-seropositive in beef cattle were increased statistic significantly (Fig. 2). Ubonratchathani province was the highest seroprevalence area in individual-level 2.05% (36/1,754) and Chaiyaphum province was the highest seroprevalence area in herd-level 12.26% (32/261) (Table 1). In the beef cattle were found the most of seropositive in herd-level and individual-level in Chaiyaphum province 14.85% (15/101) and 2.38% (64/2, 693), respectively. Dairy cattle were found the most of seropositive in herd-level and individual-level in Surin 7.69% (2/26) and 0.55% (2/366), respectively. To compared with the report of Juthamas, Panya, Amarin they study were found 0.01% seropositive 0.1% of brucellosis in cattle in Yasothon province in 1997-1999 and Chobjit and Taecho (2008) studied in cattle in Roi Et province in 2007–2008 they found 0.34% seropositive. This study showed that the present study is higher than that Yasothon and Roi Et province. In the buffaloes were found seropositive in herd-level and individual-level in Chaiyaphum province 18.75% (3/16) and 5.53% (12/217), respectively which higher than the report of Chobjit and Taecho in 2007 – 2008 they found 0.73% seropositive. Goats and sheep were found seropositive in herd-level and individual-level in Buriram province 35.29% (6/17), and 5.84% (49/839), 100.00% (1/1) and 11.11% (2/18), respectively (table 3). In Nakhon Ratchasima province where the intensively area of the livestocks found that the seropositive in herd-level was 5.21% (40/768) that less than of result in herd-level in Chaiyaphum province 12.26% (32/261) and individual-level was 0.37% (95/25,769) that less than the result of herd-level in Ubonratchathani province 2.05% (36/1,754) (Table 2).

This study showed that brucellosis remains a livestock problem in the northeastern region and seem to be increased in some species. The implementation of brucellosis, tuberculosis, paratuberculosis (BTP) disease-free farms project is necessary continue to prevent and eliminate it completely.
Acknowledgement

Thanks Dr. Monaya Ekatat; consultant of National Institute of Animal Health, Dr. Aroonpan Doonsoongnern and laboratory staffs in immunology and serology section of Veterinary Research and development Center (Lower Northeastern region) to help the task successfully.

References


Standard Operating Procedure Brucellosis Complement Fixation Test (French technique), Rev.001 05/06/2009, OIE/FAO Brucellosis Reference Laboratory, 1-12.


The Laboratory Surveillance of Brucellosis in cattle, buffalo, goats and sheep during 2009 to 2011 in Region 1

Sarayutth Kaewkalong1, Reka Kanitpun1, Surapong Wongkasemjit1, Suree Thammasart1, Monaya Ekgatat1, Bruno Garin-Bastuji2 and Vimol Jirathanawat1

1National Institute of Animal Health (NIAH) Department of Livestock Development (DLD), Bangkok, Thailand.
2EU/OIE/FAO Brucellosis Reference Laboratory, French Agency for Food, Environmental and Occupational Health Safety (Anses), Maisons-Alfort, France.
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Background: Brucellosis is regarded as one of the major zoonotic infections worldwide and one of the notifiable diseases according to the Animal Epidemic Act B.E.2499. This report aims at reviewing and updating the epidemiological situation of animal brucellosis in region 1 of Thailand.

Methods: The serological surveillance is one activity among various strategies of NBCEP. 125,801 serum samples from 4,171 herds were collected from cattle (58,034 samples/2,639 herds), buffaloes (3,141 samples/107 herds), goats and sheep (64,626 samples/1425 herds) in region 1 from 2009 to 2011 and were tested at NIAH using Rose Bengal Test, ELISA and Complement Fixation Test.

Results: The brucellosis prevalence rates in 2009-2011 in region 1, were 4.03%, 29.91%, 7.48%, 19.72 and 32.63% at herd level and 0.73%, 3.14%, 0.32%, 2.86% and 12.0%, at individual level, in dairy cattle, beef cattle, buffaloes goats and sheep, respectively. In 2011 the herd prevalence rates in dairy cattle, beef cattle, buffaloes, sheep and goats decreased to 1.12 %, 5.67%, 0%, 16.74% and 16.22% respectively. The individual prevalence rates were 0.07%, 0.84%, 0%, 4.22% and 1.77%, respectively. The overall herd and individual rates were 11.82% and 2.36%.

Discussion and conclusions: The herd prevalence decreased gradually from 17.8% to 7.0 % in all species from 2009 to 2011. In Chainat, Lopburi, Nonthaburi, Pathum Thani, Phra Nakhon Si Ayutthaya and Sing Buri both of herd and individual prevalence decreased. However, the serum samples were collected from only 8.69% dairy cattle, 1.64% beef cattle and 3.46% buffaloes, whereas, 18.2% goats and 10.8 % sheep, which are not enough to be representatives of the whole population in the region. Therefore, the policy of brucellosis control programme should be strengthened and implemented continuously to reach a better coverage of the surveillance.

Keywords: brucellosis, laboratory surveillance, region 1
Brucellosis: seroprevalence in upper northern region of Thailand during 2007 to 2011

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Abstract

Background: For a decade that Brucellosis in Thailand has been in process of being eradicated since it is one of the most important diseases in livestock and the fact that it is transmissible to human makes it more serious. The disease is caused by a group of bacteria known as the genus Brucella, three of which cause the most concern: B. abortus primarily affecting cattle and buffaloes, B. melitensis affecting goats and sheep and B. suis affecting swine. This retrospective study reviewed the past five-year serological situation of Brucellosis in upper northern region of Thailand from 2007 to 2011.

Methods: Serum samples were collected from adult beef cattle, dairy cattle and buffaloes (>1 year-old), goats, sheep and swine (>6 month-old). The average samples were approximately 0.38, 33.07, 0.59, 26.86, 87.60 and 0.03 percentages of the population from beef cattle, dairy cattle, buffaloes, goats, sheep and swine, respectively. Serological tests were performed based on the methods of RBT, EDTA-TAT, I-ELISA and CFT.

Results: The average seroprevalence percentages of brucellosis in beef cattle, dairy cattle, buffaloes, goats, sheep and swine were 5.02, 0.06, 1.02, 2.30, 0.06 and 0.22, respectively. Beef cattle presented the highest prevalences every year with marked rise while in dairy cattle remained stable at 0.03 for the last 4 years. The prevalences of buffalo were fluctuated between 0.66-1.55. The prevalences of goat were increasing rapidly from 0.2 to 6.39. In sheep, the prevalences were found every other year at 0.21 and 0.09 in 2008 and 2010, respectively. The prevalence in swine was found only in 2008.

Conclusion: These results revealed the prevalence of brucellosis in economic domestic livestock of upper northern Thailand. It could be useful information in order to improve control and eradication measures.

Key words: brucellosis, serological, upper northern region, Thailand
Seroprevalence of *Brucella abortus* in the eastern region of Thailand, 2009 – 2011

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1Veterinary Research and Development Center (Eastern Region), Department of Livestock Development, Chonburi, Thailand. E-mail: vrd_ep@dld.go.th

**Background**

Brucellosis is a worldwide zoonotic disease caused by the *Brucella* bacteria. *Brucella abortus* is the most common cause of brucellosis in livestock which mainly affects cattle. However, buffaloes, goats, sheep and dogs may also contact *B. abortus* infection. Infection usually leads to abortion in the host, which may result in massive direct economic losses and may also become a potential barrier to international trade (1). The infection is transmitted to humans by animals through direct contact with infected materials like afterbirth tissues or indirectly by ingestion of animal products and by inhalation of airborne agents (2). Cattle and small ruminant are the major source of human infection known as undulant fever. Cases of human brucellosis in the eastern region of Thailand through contact with the discharges of goats (3) have also been reported in 2010. The objective of this study was to determine the seroprevalence of *B. abortus* antibody in livestock sera in the eastern region of Thailand during 2009-2011 and also to monitor the trend of brucellosis in this region.

**Method**

Specimens from beef cattle, dairy cattle, buffaloes, sheep and goats sera were collected from 9 provinces in the eastern region of Thailand. (Figure 1). A total of 7,432 animals from 224 herds in 2009, 7,412 animals from 302 herds in 2010, and 9,506 animals from 722 herds in 2011 were tested for *B. abortus* antibody. Serological tests were performed according to OIE (4). All sera were screened by rose bengal test (RBT) and complement fixation test (CFT) was used as a confirmatory test for RBT positive sera, using in-house RBT antigen and commercial CF antigen.

**Results**

The overall seroprevalence was found to be 2.4% of 7,432 animals from 16.5% of 224 herds in 2009, 1.7% of 7,412 animals from 11.9% of 302 herds in 2010 and 1.4% of 9,506 animals from 7.6% of 722 herds in 2011. The highest prevalence rate was from buffalo sera (4.7%) in 2009, sheep and goat sera (2.3%) in 2010 and beef cattle (2.1%) in 2011, whereas sheep and goat herds were found to have the highest rate each year at 21.5%, 17.9% and 10.0%, respectively (Table 1).

**Summary**

Seroprevalence rate of *B. abortus* in the eastern region of Thailand tended to decrease at both individual animal and herd levels. For the control of brucellosis, all seropositive livestock were removed that may lead to the eradication of brucellosis. However, brucellosis is a notifiable disease according to Thailand’s Animal Epidemics Act B.E.2499 (1956) and its revision B.E.2542 (1999) for animal disease control and eradication.
References


Figure 1: 9 Provinces in eastern region of Thailand.

Table 1: Individual animal and herd prevalence based on CFT 2009-2011

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<tr>
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<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animals (%)</td>
<td>Herds (%)</td>
<td>Animals (%)</td>
</tr>
<tr>
<td><strong>Beef Cattle</strong></td>
<td>45/1,597 (2.8)</td>
<td>12/61 (19.7)</td>
<td>29/1,475 (2.0)</td>
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<tr>
<td><strong>Dairy Cattle</strong></td>
<td>42/2,511 (1.7)</td>
<td>8/69 (11.6)</td>
<td>19/2,276 (0.8)</td>
</tr>
<tr>
<td><strong>Buffaloes</strong></td>
<td>24/509 (4.7)</td>
<td>3/29 (10.3)</td>
<td>5/604 (0.8)</td>
</tr>
<tr>
<td><strong>Sheep &amp; Goat</strong></td>
<td>68/2,815 (2.4)</td>
<td>14/65 (21.5)</td>
<td>71/3,057 (2.3)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>179/7,432 (2.4)</td>
<td>37/224 (16.5)</td>
<td>124/7,412 (1.7)</td>
</tr>
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</table>
Seroprevalence of brucellosis in livestock in western Thailand during 2009-2011

Philaiphon Chetiyawan¹ Chongmas Antarasena¹

¹Veterinary Research and Development Center (Western Region), PO. BOX 18 Chombung, Ratchaburi 70150, Thailand

Background

Brucellosis is a worldwide zoonotic disease and is a systemic infection caused by facultative intracellular bacteria of the genus Brucella involving many organs and tissues. It causes losses to the livestock industry in the affected areas, which are mainly due to abortion and infertility. It is transmitted from animals to humans by ingestion of unsterilized milk or contact with secretions from infected animals. Eradication by testing and culling is the way to the elimination of brucellosis in regions with a low prevalence.

Methods

This report was to determine the seroprevalence of brucellosis in livestock (beef cattle, dairy cattle, buffalo, sheep, goats and swine) in 5 provinces in western Thailand, namely; Nakhonpathom, Ratchaburi, Kanchanaburi, Petchaburi and Prachuabkhirikhan between 2009 and 2011. Serum samples were submitted to Veterinary Research and Development Center (Western Region). A total of 128,308 livestock serum samples (59,192 sheep and goats, 54,982 dairy cattle, 11,090 beef cattle, 2,128 buffalo and 916 swine) from 4,834 herds were collected. Samples were tested for Brucella spp. antibodies using 3 serological methods. Rose bengal test (RBT) was used for screening. Indirect ELISA (iELISA) and complement fixation test (CFT) were used as a confirmatory test.

Results

The overall seroprevalences at the individual and herd levels of brucellosis were 1.81% (2,326/128,308) and 11.47% (555/4,838), respectively. On an individual level, the highest prevalence of brucellosis was found in beef cattle at 4.70% (521/11,090), followed by sheep and goats at 2.37% (1,404/59,192), dairy cattle at 0.71% (391/54,982), swine at 0.44% (4/916) and buffaloes at 0.28% (6/2,128). On the herd level, the highest prevalence of Brucella spp. antibodies in beef cattle was 26.82% (96/358), followed by sheep and goats at 23.10% (271/1,173), swine at 8.33% (2/24), dairy cattle 5.87% (182/3,099) and buffalo 2.17% (4/184). For the overall seroprevalences at the individual and herd levels, the highest prevalence of brucellosis was found in Kanchanaburi at 3.19% (632/19,840) and 23.37% (122/522), followed by Nakhon Pathom at 3.02% (551/18,219) and 20.27% (60/296) , Ratchaburi at 2.36% (401/16,986) and 13.9% (108/777), Prachuap Khiri Khan at 1.06% (521/48,952) and 9.33% (182/1,950) and Phetchaburi at 0.91% (221/24,311) and 6.42%(83/1,293).
Table 1: Estimated prevalence of brucellosis, at herd and animal levels for beef cattle, dairy cattle, buffaloes, sheep, goats and swine in western Thailand during 2009 – 2011.

<table>
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<tr>
<th>Provinces</th>
<th>Status</th>
<th>Beef cattle</th>
<th>Dairy cattle</th>
<th>Buffaloes</th>
<th>Sheep and Goats</th>
<th>Swine</th>
<th>Total</th>
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<tr>
<td></td>
<td>Herds</td>
<td>2.70%</td>
<td>5.26%</td>
<td>0%</td>
<td>30.77%</td>
<td>ND</td>
<td>20.27%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1/37)</td>
<td>(3/57)</td>
<td>(0/20)</td>
<td>(56/182)</td>
<td></td>
<td>(60/296)</td>
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<tr>
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<td>Individual</td>
<td>0.09%</td>
<td>1.54%</td>
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<td>3.32%</td>
<td>ND</td>
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<td>(12/779)</td>
<td>(0/194)</td>
<td>(538/16,184)</td>
<td></td>
<td>(551/18,219)</td>
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<td>0%</td>
<td>17.48%</td>
<td>5.56%</td>
<td>13.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(28/122)</td>
<td>(4/79)</td>
<td>(0/129)</td>
<td>(75/429)</td>
<td>(1/18)</td>
<td>(108/777)</td>
</tr>
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<td></td>
<td>Individual</td>
<td>5.29%</td>
<td>2.4%</td>
<td>0%</td>
<td>1.82%</td>
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<td>(158/2,986)</td>
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<td>(1/693)</td>
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<td>100%</td>
<td>23.07%</td>
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<td>(30/135)</td>
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<td>(0/4)</td>
<td>(62/205)</td>
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<td>6.67%</td>
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<td>(16/3,023)</td>
<td>(0/771)</td>
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<td>Kanchanaburi</td>
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<td>(5/13)</td>
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<td>(16/3,023)</td>
<td>(36/231)</td>
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<td>(83/1,293)</td>
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<td>3.95%</td>
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<td>Herds</td>
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<td>7.13%</td>
<td>12.9%</td>
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<td>0%</td>
<td>9.33%</td>
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<td>(12/51)</td>
<td>(12/14,138)</td>
<td>(4/31)</td>
<td>(42/226)</td>
<td>(0/4)</td>
<td>(182/1,950)</td>
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<td>(38/1,936)</td>
<td>(245/34,046)</td>
<td>(6/620)</td>
<td>(232/12,345)</td>
<td>(0/5)</td>
<td>(521/48,952)</td>
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<td>Herds</td>
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<td>5.87%</td>
<td>2.17%</td>
<td>23.1%</td>
<td>8.33%</td>
<td>11.47%</td>
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<tr>
<td></td>
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<td>(96/358)</td>
<td>(182/3,099)</td>
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<td>(271/1,173)</td>
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<td>(555/4,838)</td>
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<td></td>
<td>Individual</td>
<td>4.7%</td>
<td>0.71%</td>
<td>0.28%</td>
<td>2.37%</td>
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<td>1.81%</td>
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<td>(6/2,128)</td>
<td>(1,404/59,192)</td>
<td>(4/916)</td>
<td>(2,326/128,308)</td>
</tr>
</tbody>
</table>

Summary

This study indicated that the Brucella spp. infection exists with widespread distribution in livestock in western Thailand. It was recommended that regular disease surveillance, control and eradication measures should be taken urgently to reduce brucellosis in Thailand.

Acknowledgements

The author would like to thank staff of Immunology section Veterinary Research and Development Center (Western Region) for their cooperation and help to the completion of serological test.
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