Development, Optimization, and Validation of a Multiplex Real-time RT-PCR Assay for the Simultaneous Detection of BVDV, BTV, and BLV in Cattle

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Abstract

Background: The National Institute of Animal Health (NIAH) is responsible for the disease diagnostic screening of imported live cattle to prevent transboundary and emerging diseases into Thailand. Bovine Viral Diarrhea Virus (BVDV), Bluetongue Virus (BTV), and Bovine Leukemia Virus (BLV) are classified under the Animal Epidemics Act B.E. 2558 (2015). They are considered high-risk diseases that require mandatory health screening and certification prior to international movement into the country. Currently, the NIAH utilizes singleplex real-time PCR, which requires separate testing for each pathogen. This process is resource-intensive, extends operational timelines and increases costs which increases the turnaround time necessary for rapid biosecurity screening.

Objective: This study aimed to develop, optimization and validation a multiplex Real-time RT-PCR assay for the simultaneous detection of BVDV, BTV, and BLV in a single reaction to enhance the efficiency and speed of diagnostic services for imported cattle.

Materials and Methods: Specific primers and probes were designed targeting the 5'-UTR (BVDV), NS3 (BTV), and env (BLV) regions in accordance with WOAH Terrestrial Manual guidelines. Probes were labeled with reporter dye (FAM, VIC, and Cy5) to ensure non-overlapping signals. Synthetic plasmids containing these target sequences were used as positive controls. The one-step RT-qPCR master mix was used for the simultaneous detection of RNA and DNA targets. This assay optimization focused on primer/probe concentrations and thermal cycling conditions to maximize fluorescence signals. The Limit of Detection (LOD) was determined using serial dilutions of synthetic plasmids. Performance was evaluated using 60 reference samples (20 positive samples for each virus). Diagnostic sensitivity (D-Sn), diagnostic specificity (D-Sp), and Cohen's kappa statistics were calculated following WOAH validation standards.

Results: The optimized multiplex real-time RT-PCR demonstrated high performance in the simultaneous detection of BVDV, BLV, and BTV with the ability to distinctly identify each target through unique fluorescence signals and non-overlapping amplification plots. Assay optimization revealed that a primer and probe concentration of 400 nM provided the most optimal performance. The thermal cycling conditions were established with a reverse transcription step at 50°C for 10 minutes, followed by pre-amplification at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. The LOD was 3.21 x 10^5 copies/reaction for BVDV, BTV, and BLV. Consequently, the analytical sensitivity was consistently high, and both D-Sn and D-Sp reached 100% when validated against 60 reference clinical samples. Statistical analysis yielded a Cohen's kappa coefficient of 1.0, confirming perfect agreement with reference singleplex assay.

Conclusion: The preliminarily validated Multiplex Real-time RT-PCR provides a rapid, cost-effective, and highly reliable diagnostic tool. It significantly strengthens the biosecurity measures of NIAH by accelerating the health certification process for imported live cattle and ensuring the effective prevention of transboundary disease outbreaks into Thailand.

Keyword: Multiplex real-time RT-PCR, Optimization, Imported cattle, BVDV, BTV, BLV

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