Application of Digital PCR for Sensitive Detection of Avian Influenza Viral RNA in Poultry Matrices with Low Viral Loads

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Abstract

Background: Avian influenza virus (AIV) is a highly contagious pathogen in poultry that causes substantial economic losses and poses significant risks to animal and public health worldwide. Rapid and accurate detection is essential for early outbreak recognition, effective control, and prevention of virus spread. Although real-time RT-PCR is the current standard for AIV diagnosis, its performance may be limited in specimens with low viral loads and complex poultry-derived matrices. Digital PCR (dPCR), which enables absolute quantification without reliance on standard curves, has emerged as a promising approach to improve sensitivity and precision in challenging diagnostic settings.

Objectives: To evaluate the performance of dPCR for sensitive and precise detection of the AIV genome in diverse poultry sample matrices, with particular emphasis on low viral load specimens.

Methods: A series of 10-fold and 2-fold serial dilutions of quantified synthetic AIV RNA corresponding to the influenza A matrix (*M*) gene were prepared in a no-effect matrix to evaluate analytical performance, including quantification range, limit of detection, linearity, and precision. Specificity was assessed using inclusivity, cross-reactivity, and negative pathogen panels. To assess matrix effects, RNA was spiked into poultry-derived sample matrices, including oropharyngeal swabs, cloacal swabs, and tissue homogenates (n = 20 each), and categorized into four viral load groups (10^4, 10^3, 10^2, and 50 copies/µL, representing high, intermediate, low, and very low viral loads, respectively). Quantification was performed using real-time RT-PCR and dPCR with primers and probes targeting the M1 gene. Analytical performance was evaluated across the dilution series, and agreement between methods was assessed using correlation and Bland–Altman analyses, including estimation of limits of agreement (LoA).

Results: Real-time RT-PCR demonstrated a wider quantification range (50 -10^6 copies/µL), whereas dPCR exhibited a narrower quantification range but a lower limit of detection (1 -10^4 copies/µL). Both methods showed strong linearity across 10-fold serial dilutions ($R^2 > 0.99$). dPCR demonstrated improved precision at low and very low viral loads, as indicated by a lower coefficient of variation. Specificity analysis showed almost perfect agreement, with 100% concordance (11/11) and Cohen's $\kappa = 1.0$. In matrix-derived samples, no significant difference was observed between dPCR and real-time RT-PCR at high, intermediate, and low viral loads in oropharyngeal and cloacal swabs, whereas a significant difference was observed at very low viral loads, with dPCR demonstrating improved detection performance. No significant differences were observed across all viral loads in tissue homogenates. Correlation analysis demonstrated strong agreement between the two methods in oropharyngeal swabs ($R^2 = 0.983$), cloacal swabs ($R^2 = 0.984$), and tissue homogenates ($R^2 = 0.982$). Bland–Altman analysis showed LoA from −0.256 to 0.179 $\log_{10}$ at high viral loads, −0.312 to 0.232 $\log_{10}$ at intermediate viral loads, −0.265 to 0.269 $\log_{10}$ at low viral loads, and −0.398 to 0.350 $\log_{10}$ at very low viral loads, indicating overall good agreement, with increased bias and wider limits of agreement observed at very low viral loads.

Conclusion: dPCR provides a sensitive and precise platform for the detection and absolute quantification of AIV genomes in diverse poultry sample matrices. Its improved performance in very low viral load specimens supports its use as a complementary or confirmatory method to real-time RT-PCR for early diagnosis, surveillance, and outbreak preparedness in avian influenza control programs.

Keywords: Avian influenza virus, Digital PCR, Real time RT-PCR

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