Development and Laboratory Validation of Bird-side Molecular Assays for Avian Influenza Virus

Avian influenza A viruses (AIV) significantly impact both animal and human health, necessitating reliable diagnostics for effective control, particularly for highly pathogenic strains such as H5Nx. This study details the development and laboratory validation of the Alveo Sense Poultry Avian Influenza Tests, designed for rapid, on-site detection of the AIV M-gene and subtypes H5, H7, and H9 using reverse-transcription loop-mediated isothermal amplification (RT-LAMP) and impedance-based measurements. These tests, using single-use microfluidic cartridges, deliver results within 45 minutes.

Laboratory validation demonstrated 100% specificity for AIV subtypes without cross-reactivity with non-AIV pathogens. The tests showed a limit of detection (LoD95) for H5, H7, and H9 subtypes ranging between RT-PCR Ct values of 29–33 in cloacal and oropharyngeal samples and could detect AIV in both spiked and field samples. Reproducibility and repeatability studies confirmed perfect agreement across operators and laboratories, maintaining accuracy under different pre-analytical conditions. These tests provide rapid, accurate, and reliable diagnostics for AIV subtypes, valuable for early detection and outbreak control, with further field validation needed to enhance understanding across various avian species.

Introduction

Avian influenza viruses (AIV), particularly subtypes H5Nx, pose significant threats to both animal and human health. The primary reservoir for AIV is aquatic birds, with the viruses classified into subtypes based on haemagglutinin (H) and neuraminidase (N) surface glycoproteins. In poultry, AIV strains are categorised into low pathogenicity (LP) and high pathogenicity (HP), with pathogenicity mainly determined by the number of basic amino acids in the HA0 cleavage site, influencing the proteases that can cleave HA and the tissues where AIV can replicate.

Historically, all AIV strains detected in aquatic birds were of low pathogenicity, causing minimal clinical signs. However, in poultry LP H5 and H7 strains can mutate into highly pathogenic forms, causing severe disease and death in poultry. The emergence of the goose Guangdong (Gs/GD) H5Nx lineage in China in 1996 marked a significant shift, with these viruses spreading globally via wild birds and causing mass deaths in various bird species, significant poultry outbreaks, and deaths in mammals, posing an increasing risk to humans.

Materials and Methods

Alveo Sense Technology

The Alveo Sense Poultry Avian Influenza Tests employ RT-LAMP technology combined with electrical impedance sensors, using single-use microfluidic cartridges. Each cartridge contains six assays for broad detection of avian influenza virus (AIV) through the M gene and specific detection of H5 and H7 or H9 subtypes. These tests yield qualitative results from oropharyngeal or cloacal samples and are validated for pooled samples of up to five cloacal or ten oropharyngeal swabs.

LAMP Assay Development

Influenza A virus HA sequences for H5, H7, and H9 assays and M sequences for the generic assay were sourced from the GISAID EpiFlu™ database. Conserved regions were identified through multiple sequence alignments, and RT-LAMP primers were designed accordingly. Specificity was confirmed via *in silico* mapping and primer BLAST, ensuring no cross-reactivity with non-target sequences.

Strains and Sample Collection

A diverse panel of archival AIV strains and other avian pathogens were used for validation. Swab samples were collected from specified-pathogen free (SPF) chickens and processed according to standard protocols. Limit of Blank (LoB), Limit of Detection at 95% (LoD95), and technical specificity studies were conducted using these samples.

Influenza A Real-Time PCR

RNA extraction from samples was done using the MagMax RNA/DNA isolation kit on a KingFisher™ Flex Purification system. Detection was carried out using a generic PCR targeting the Influenza A matrix gene. PCR assays were performed with the AgPath-ID™ One-Step RT-PCR kit on a QuantStudio™ 5 system, following established protocols for accurate detection and quantitation.

Laboratory Validation

LoB testing involved four unique pools of cloacal and oropharyngeal matrices, each tested in five replicates, showing 100% negativity across influenza-negative samples, indicating no cross-reactivity. Specificity testing across different Influenza A strains and non-influenza pathogens demonstrated 100% specificity, correctly identifying all influenza-positive and negative samples without cross-reactivity. The LoD95 was determined using H5N2, H5N8, H7N7, and H9N2 strains, with cloacal samples showing Ct values between 28.5 and 33.5 and oropharyngeal samples between 29.0 and 33.2.

Reproducibility and repeatability were assessed across different operators and laboratories, showing 100% agreement with expected outcomes, indicating robust and reliable assays. Post-rehydration reagent stability was evaluated by subjecting the reconstituted reaction mix to prolonged incubation at 30°C before testing, confirming the assays' robustness for field use.

Field samples from influenza-negative flocks and spiked positive samples were tested to validate the system. All negative field samples tested negative, while spiked samples showed robust detection across various matrices and pool sizes. Additionally, samples from an H5N1 LPAI positive flock were correctly identified, further validating the assays' performance.

Results

For the M assay development, 1474 full M sequences were analysed across serotypes like H3N8, H5N1, H5N2, H6N6, H7N3, H7N7, and H9N2. Twelve M designs were tested, with two selected for their broad detection and specificity. For the H5 assay, 9532 full HA sequences were analysed, resulting in three selected designs to cover all H5 clades. The H7 assay involved 384 sequences, leading to one selected design for broad H7 strain detection. For H9, 3856 sequences were

analysed, with one design chosen for its broad detection and specificity.

Limit of Blank (LoB) testing showed no cross-reactivity with non-target RNA or DNA, indicating a low risk of false positives. The Alveo Sense tests demonstrated 100% specificity, correctly identifying all influenza-positive and negative samples without cross-reactivity, confirmed by testing various avian influenza strains and pathogens.

The Limit of Detection (LoD95) was defined as the lowest concentration with ≥95% positive replicates. For H5N2, the LoD95 was Ct33 for both sample types; for H5N8, it ranged between Ct31-32; for H7N7, between Ct29-30; and for H9N2, between Ct31-32.

Reproducibility and repeatability assessments showed 100% agreement with expected outcomes, confirmed by a Cohen's kappa value of 1.0. Post-rehydration reagent stability testing confirmed assay robustness and suitability for field conditions. Field samples from influenza-negative flocks and spiked positive samples validated the system, with all negative samples testing negative and spiked samples demonstrating robust detection. Samples from LPAI H5N1 infected animals were correctly identified, validating the assays' real-world performance.

Discussion

Reliable diagnostics are crucial for controlling AIV outbreaks, especially for highly pathogenic strains like H5Nx. Traditional RT-PCR, while accurate, requires specialised equipment and trained personnel, making it less accessible in resource-limited settings. The Alveo Sense Poultry Avian Influenza Tests overcome these limitations with RT-LAMP technology, providing rapid, on-site detection.

The tests demonstrated high specificity and sensitivity in laboratory validation, with LoD95 values indicating effective detection of AIV in samples with moderate to high viral loads. The robust performance across various conditions and matrices suggests these tests offer rapid, accurate, and reliable on-site diagnostics for AIV subtypes H5, H7, and H9 on samples from fresh dead and sick birds, valuable for early flock-level detection and outbreak control.

Further field validation is necessary to fully understand the diagnostic performance across different avian species and in real-world conditions. This will help determine the best applications for these tests, whether for screening or as decisive diagnostics.

Conclusions

The Alveo Sense Poultry Avian Influenza Tests represent a significant advancement in the rapid, on-site detection of avian influenza viruses. These tests, employing cartridge-based RT-LAMP technology and electrical impedance detection, offer a portable, efficient, and reliable method for identifying AIV subtypes H5, H7, and H9 in unprocessed cloacal and oropharyngeal samples. The high specificity and sensitivity demonstrated during laboratory validation, along with robust reproducibility and stability of the assays, highlight their potential to enhance early detection in avian influenza outbreaks when using samples from fresh dead or sick birds. The ability to obtain results within 45 minutes supports faster decision-making and intervention, crucial for minimising the spread of infection and reducing economic impact and public health risks. As field validation continues, the Alveo Sense tests can complement existing diagnostic methods and significantly improve monitoring and surveillance programs, especially in resource-limited settings and areas lacking suitable laboratory facilities.

More information: www.gdanimalhealth.com/alveo-sense

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