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# Diagnosis of AIV by molecular diagnostic techniques

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**Abstract**
Avian Influenza Viruses (AIV) Type A is all mammalian virus and are most important economically. These viruses exhibits antigenic variation and therefore needs highly accurate and specific diagnostic tests. As they spread widely and quickly so a rapid and timely diagnosis is required. To fulfill these requirements, molecular diagnostics techniques are most suitable for AIV. As they are RNA viruses so they can be detected by RT-PCR which has additional step of reverse transcription and formation of cDNA from RNA and subsequently denaturation, annealing and extension multiplex PCR is a step ahead PCR by amplification by multiple targets at a time and hence save time. Apart from this DNA microarray is a chip based technique which has multiple spots of oligonucleoprotein or capture on a small – chip and can bind the multiple targets and are detected by reporter dye.

**Keywords:** AIV, PCR, RT-PCR, reverse transcriptase, DNA microarray, Multiplex-PCR, capture probes

## Introduction
Among the all exotic viruses Influenza Viruses have grabbed attention from last few decades because of their ability to spread as far as to pandemics and their variant nature. Mostly it involves Type A of influenza viruses which includes mammalian viruses like swine flu, avian flu etc. It has global impact due to heavy economic losses to commercial poultry sector and hence lots of advancements for early diagnosis of the disease. The conventional methods used for identification of AIV and detection requires special laboratory facilities and technically trained staff which makes the process very time consuming and cost intensive. But with the advancement in molecular technology, the use of molecular techniques based on nucleic acid amplification for genetic identification have improved the sensitivity and speed for diagnosis of AIV [1]. Also they have reduced human effort and time. The molecular diagnostic techniques used for AIV detection and identification are discussed as below.

## RT-PCR
Polymerase chain reaction (PCR) is a relatively simple and widely used molecular biology technique to amplify and detect DNA and RNA sequences, less time consuming also as it requires only a few hours. PCR is highly sensitive and requires minimal template for detection and amplification of specific sequences. Basic PCR methods have further advanced from simple DNA and RNA detection. For standard PCR, necessities are a DNA polymerase, magnesium, nucleotides, primers, the DNA template to be amplified and a thermocycler. The PCR mechanism has basic three steps:

1) The double-stranded DNA (dsDNA) is heat denatured,
2) Primers align to the single DNA strands and
3) The primers are extended by DNA polymerase, resulting in two copies of the original DNA strand.

The denaturation, annealing, and elongation process over a series of temperatures and times is known as one cycle of amplification. This cycle is repeated approximately 20-40 times and the amplified product can then be analyzed. PCR is widely used to amplify DNA for subsequent experimental use. As PCR is a highly sensitive method. The master mix must be well mixed and then split by the number of reactions, ensuring that each reaction will contain the same amount of enzyme, dNTPs and primers. Reverse transcription PCR, or RT-PCR, allows the use of RNA as a template. An additional step allows the detection and amplification of RNA. The RNA is reverse transcribed into complementary DNA (cDNA), using reverse transcriptase.

The RT step can be performed either as a separate reaction (two-step PCR) [2] or along with PCR reaction in one tube (one-step PCR) using a temperature between 45 °C and 60 °C depending on the properties of the reverse transcriptase. The quality and purity of the RNA template is essential for the success of RT-PCR. The first step of RT-PCR is the synthesis of a DNA/RNA hybrid. Reverse transcriptase also has an RNase H function, which degrades the RNA portion of the hybrid. The single stranded DNA molecule is then completed by the DNA-dependent DNA polymerase activity of the reverse transcriptase into cDNA and the first-strand reaction can affect the amplification process. This is followed by the standard PCR procedure is used to amplify the cDNA. The possibility to revert RNA into cDNA by RT-PCR has many advantages. To utilize the technique for AIV detection, a copy of DNA, complimentary (cDNA) to viral RNA is synthesised using a reverse transcriptase (RT) enzyme and random hexanucleotides or a sequence specific primer. The sequence of interest located within the cDNA is amplified using a heat stable polymerase enzyme from the bacterium Thermus aquaticus (TAQ- DNA Polymerase) along with primers.

The amplification of a DNA sequence typically involves 30-40 PCR cycles and each cycle requires three steps: denaturing, annealing and extension. In the first denaturing step, the double stranded DNA (dsDNA) is melted at approximately 94ºC to form single strands DNA (ssDNA). In the annealing step the specific primers binds (anneal) to the single strands of the target DNA. The annealing temperature is normally between 50 ºC to 65 ºC depending on the length and on the guanine/cytosine (GC) content of the selected primers. Finally, the extension step where the TAQ polymerase adds deoxynucleotide triphosphate bases (dNTP's) to the primer on the 3' side takes place. The extension occur around 72ºC at 50-100 nucleotides per sec. Depending on the aim of the study to detect, different genes of AIV are selected as the target for RT-PCR to detect AIV. The presence of AIV can be confirmed by targeting highly conversed genes in all influenza a viruses such as matrix (M) [3] or nucleoprotein (NP) [4, 3] First designed a set of primers, M52C and M253R, of matrix gene and used for diagnosis purpose.

The PCR is followed by gel electrophoresis to analyze the result. This procedure finds its application in clinical diagnostics and is about 100-folds more sensitive than the classical virus isolation method. It has been suggested that the increased sensitivity is likely due to the detection of RNA from incompletely packaged virus particles or viral RNA from infected cells **[5]**. Though Conventional end-point RT-PCR is disadvantageous on being time intensive non automated and risk of cross contamination. to overcome these disadvantages, a real-time RT-PCR (rRT-PCR) approach was introduced to detect AIV. The rRT-PCR eliminates the post-amplification steps increasing the reliability and reproducibility of the assay. In rRT-PCR the amplified DNA is detected by monitoring the fluorescence emitted as the reaction progresses in real time. The two common methods used for detecting PCR product are a) nonspecific intercalating dye (e.g. SYBR green) which becomes fluorescent when binding to double stranded DNA and b) sequence specific approach using TaqMan probes. The TaqMan probes are oligonucleotides longer than the primers and are designed to anneal to an internal region of a PCR product to increase the specificity of real-time PCR assays. TaqMan probes contain a report dye on the 5; base and a quenching dye on the 3' base. Real time RT-PCR with TaqMan probe. While the probe is intact, the close proximity of the reporter and quencher prevents the emission of fluorophore via fluorescence resonance energy transfer (FRET). During the primer extension 25 step, the 5' exonuclease activity of Taq polymerase cleaves the 5' end of probe which contains the reporter dye. This separates the reporter from the quencher and the reporter starts to emit fluorescence. The fluorescence intensity increases in each cycle and is proportional to the amount of amplified DNA. The rRT-PCR assay is characterized by a wide dynamic range of quantification of 7 to 8 logarithmic decades, a high technical sensitivity (< 5 copies) and a high precision (< 2% standard deviation) [6]. A single-step RT-PCR method also greatly reduces the risk of cross contamination since it is a closed system once the template is added. The application of real-time RT-PCR detection for identifying the AIV based on TaqMan probe chemistry was first described by [7]. The limit of detection in the assay is 1000 copies of target RNA, which is equivalent to 0.1 EID50 of virus. The matrix gene rRT-PCR was evaluated as highly sensitive, specific for AIV and selected as a method for surveillance by avian influenza reference laboratories [8].

**Subtyping of AIV by RT-PCR**

The antigenic differences of HA and NA forms the molecular basis for subtyping of AIV. The amino acid between H1 and H12 subtypes varies from 20% to 74% compared to 0-9% variation among the same subtypes [9]. As the amino acid sequences are determined by the nucleotide sequences, the presence of wobble in the codon usage (for e.g., Arginine can be CGU, CGC, CGA, CGG, AGA or AGG) will increase the difference in nucleotide sequences between HA\subtypes to more than 20–74% [10] Using conventional RT-PCR, Lee *et al*. (2001) designed the first subtype specific primers that were able to differentiate fifteen subtypes (H1–H15) of the AIV viruses. The major advantage of this method is the reduction of testing time from one week (HI typing) to one day. Among the sixteen HA subtypes of the AIV (H1-H16), H5 and H7 are the two known subtypes which are responsible for high pathogenicity. So the identification of these subtypes in the sample has been of prime importance in AIV diagnosis. The detection and differentiation of H5 and H7 have been developed using both conventional RT-PCR and real-time RT-PCR **[7]**. The developed rRT-PCR has been used for the quantification and competitive replication studies of AIV H5 and H7 subtypes [Lee & Suarez]. The quantitative rRT-PCR demonstrated high correlation between the amount of viral RNA determined by quantitative rRT-PCR and the virus titer determined by VI method using SPF eggs. The use of rRT-PCR for AIV quantification greatly reduces the risky handling of infectious materials. However, the rRT-PCR can only quantify the presence or absence but not the viability of the virus.

The phylogenetic analysis of HPAI viruses of H5 and H7 subtype showed two geographically distinct lineages of North American and Eurasian viruses [11]. The primers and probe sequences designed by [7] were optimally designed to detect North American H5 and H7 and failed to detect some of Eurasian H5 and H7 subtypes [8]. So modifications have been made to originally design, validate and shown to sensitively detect all H5 Eurasian lineages [8]. Adaption of primers and probe are also needed if some new AIV's emerge with genetic variation that could affect the primer and/or probe binding sequences. It is important to employ validated and updated

methods from the OIE (world organization for animal health) reference laboratories in the diagnosis of HPAI outbreaks [12].

**Multiplex RT-PCR**

Multiplex RT-PCR consists of simultaneous amplifications of more than one target using multiple primer sets in a single reaction. The produced RT-PCR amplicons vary in sizes that are specific to different DNA sequences. The advantages of the multiplex RT-PCR are rapid, sensitive, specific, and cost effective detection. A two step reaction of a multiplex conventional RT-PCR for identification of influenza AIV and simultaneous subtyping of H1N1 and H3N2 subtypes were developed 27 [13] and further utilized for a one step detection of H1N1, H3N2 and H5N1 subtypes [14]. Other multiplex conventional RT-PCR for subtyping of H5, H7 and H9 subtypes have been reported [15]. The multiplex rRT-PCR assay using TaqMan probes labelled with the two or three different reporter dyes for the simultaneous detection of combination of H5-H7 [7] and Matrix-H5-[16] genes have been developed. Recently, Hoffmann *et al*. (2008) developed a new rRT-PCR for rapid detection of H5 specific subtype and also for pathotyping of HPAI H5N1 of the Qinghai lineage [17]. In that study, a set of primers and two probes were designed for the amplification and detection of a fragment spanning the cleavage site HA0 sequence of the HA gene of H5 subtype.

Overall the methods based on rRT-PCR multiplexing offer rapidity and accuracy and are therefore attractive for large-scale screening of suspected cases of influenza A virus subtype ofH5, H7 or H9 in order to control potential outbreaks. However, the use of two or three fluorophore dyes increases the complexity in the detection system and it become more expensive.

**DNA Microarray**

Another approach for identification of PCR amplicon in multiplex assays is the use of DNA microarray. It consists of a solid support onto which arrays of spots from hundred to many thousands of oligonucleotides or capture-probes are fixed in an area of 1–2-$cm^2$ [18]. These spots contain multiple copies of capture-probes (CP) and the spots can range in size from 10 -500 microns. The CP can bind specifically to the target and the binding is detected using a 28 reporter molecule (e.g. fluorescent dye) of the target DNA sequence. The successful implementation of microarray technologies has required the development of many methods and techniques for fabricating the microarrays and spotting the CP, target preparation, hybridization reactions, and data analysis [18]. The DNA microarray enhances the assay capabilities by detecting multiple targets in parallel, and being more sensitive and specific. The first study on microarray for AIV detection was reported by [19]. That method the detection of H5 and H7 of AIV and New Castle Disease virus (NDV) was made using PCR products of a multiplex RT-PCR. Accuracy and sensitivity of the oligonucleotide microarray was 10- 100 times higher than the PCR end-point analysis by agarose gel electrophoresis. The hybridization signals on the microarrays were determined by colorimetry. Using DNA microarrays, Han *et al*. 2008 reported simultaneous subtyping of all influenza A viruses subtypes (sixteen serotypes of HA (H1–16 )and nine (N1–9) of NA) that have been identified in mammalian and avian species. In that study, an asymmetric multiplex PCR with 25 sets of primers was carried out in four separate reactions and the amplified target cDNA's (fluorophore labelled) were hybridized over the immobilized capture-probes. By this approach, all subtypes were identified with high sensitivity and the results were consistent with that of the virus isolation method. However, the hybridization procedure involved several steps and it was time consuming [19] Moreover, considering the variability between and within different HA and NA gene subtypes among the lineages, the designed capture-probe and oligonucleotide needs further optimization. Recently, an electronic DNA microarray system (NanoChip 400 system) has been used for fast detection of AIV as well as HA subtyping, and pathotyping [20]. In the assay, one-step amplification of HA0 cleavage site of all the 16 HA subtypes and matrix gene-specific of AIV were performed. The developed microarray comprised of 97 CP designed for HA subtyping. These CP targeting the HA0 cleavage site are conserved within a given subtype and have variation between subtypes. These CP can also distinguish between HPAI and LPAI viruses. One CP was designed to target the M-gene to identify AIV. The method was extensively validated with a high specificity and a high sensitivity (of 101 to 102 copies).This low-density microarray can be an approach to the problem of AIV classification and could be used as a diagnostic tool for the detection and typing of AIV. However, the method uses expensive equipment and it involves long processing steps. Sample preparation is an important step which is always required for removing inhibitors and to reduce the sample volume, and to homogenize sample for a reproducible and repeatable test.

Nucleic acid amplification methods based on RT-PCR need a preparation of AI viral RNA from different types of samples which includes swab, either pharyngeal or cloacal, and faecal droppings which is for routine surveillance work. The Sample preparation is therefore an important step which is always required for removing inhibitors and to obtain reliable diagnosing results. Other important objectives of sample preparation are to reduce the sample volume, to concentrate the total RNA into a workable volume and to homogenize sample for a reproducible and repeatable test.Two methods are used for extraction of RNA from AIV;

(a) Silica column extraction
(b) Magnetic bead based.

The most widely used method for extraction of RNA from AIV is based on a silica column extraction kits that are commercially available. The advantages of column based extraction are removal of PCR inhibitors and a homogenous RNA with high quality. The drawback of the method however, but this method is very laborious and has a limited throughput capacity and batch to-batch variation may exist in quality and quantity of the template A magnetic bead based RNA extraction has been introduced with automated platforms for pathogen detection. Magnetic carriers prepared from a biopolymer exhibiting affinity to the target nucleic acid are used for the extraction process. The magnetic bead based extraction method is a simple and efficient way to separate the RNA from PCR inhibitors. It also takes less time compared to column based extraction method due to the fewer manipulation steps and no centrifugation. Magnetic beads can be used for larger volume of sample up to 10 ml and the purified RNA can be down scale to 5 to 50µl. Magnetic bead based extraction method used silica-based magnetic beads with robotics for extraction of RNA from AIV and obtained a sensitivity and specificity comparable to virus isolation and manual silica column extractions. The main advantages of the automated robotic viral nucleic acid extraction are high throughput processing; hands-free operation; and reduction in human and technical errors.

**Conclusion**

Molecular diagnostics techniques are automated techniques which reduce human effort & saves time. They work with high on accuracy, sensitivity and specificity. These techniques facilitates the easy and rapid confirmatory diagnosis required for identification & detection of AIV which is required to prevent spread of the disease causing heavy economic losses. Though all these techniques are lab based and requires expensive equipment & an advanced set up to perform but still they are most popular choices because of their high sensitivity & specificity.

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