

PCR-RFLP ANALYSIS OF HA AND NA GENES OF A H5N1 ISOLATE BEING A POSSIBLE ESCAPE MUTANT

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ABSTRACT

New AIV strain continue to emerge because of genetic instability of influenza virus and lack of proof reading and repair mechanism makes AIV genome easily prone to error that occur during replication. This study provide an update of H5N1 being an escape mutant which escaped from a immunized bird in aimmunization experiment conducted on birds with virus strain A/Duck/Tripura/103597/2008(H5N1). This article provide an update of H5N1 virus as it was evolving, creating the potential for new strain that is efficiently transmitted from birds to birds, birds to human or human to human remains highly lethal. The emergence of escape mutant may be either because of antigenic drift or genetic shift which results in H5N1 variants.

Key Words: *Avian influenza*(AIV), H5N1 , PCR -RFLP, HA, NA

INTRODUCTION

Avian influenza (AI) is one of the most important diseases of poultry that negatively impacts poultry health and international trade of poultry and poultry products. *Influenza* Avirus continues to spread from the wild bird reservoir to poultry and produce disease ranging from asymptomatic infection to severe, systemic disease with high mortality. The response to an AI outbreak varies depending upon the pathotype of the virus, the subtype of the virus and whether poultry exports are affected.

Avian influenza Avirus strains are further classified as Low Pathogenic (LPAI) or Highly Pathogenic (HPAI) on the basis of specific molecular genetics and pathogenesis criteria that require specific testing. Most avian influenza A viruses are LPAI viruses that are usually associated with mild disease in poultry. In contrast, HPAI viruses can cause severe illness and high mortality in poultry. More recently, some HPAI viruses (e.g., H5N1) have been found to cause no illness in some poultry, such as ducks.

New *Avian influenzavirus* strains continue to emerge very often because of the genetic instability of the influenza viruses¹ and the lack of proofreading and repair mechanism makes the AIV genome easily prone to errors that occur during replication. Antigenic drift in mammalian viruses arises from point mutation in HA and/or NA genes that result in minor antigenic changes in the coding proteins.²⁻⁴ The ability of various influenza strains to show species selectivity is largely due to variation in the haemagglutinin genes. The influenza genome encodes for 10 proteins. Among them, Haemagglutinin (HA-1,734 base pair) is responsible for both attachment and fusion of virus with cellular receptors and organelles. Neuraminidase (NA- 1,398 base pair) is responsible for virus transport through mucin layers and release of new virus particles. Both induce neutralizing antibodies. Genetic mutations in the haemagglutinin gene that cause single aminoacid substitutions can significantly alter the ability of viral haemagglutinin proteins to bind to receptors on the surface of host cells. Such mutations in avian H5N1 viruses can change virus strains

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from being inefficient at infecting human cells to being as efficient in causing human infections as more common human influenza virus types⁵.

Change/mutation in the HA gene at vicinity of HA Receptor Binding Site (RBS) effect the recognition of the third saccharide residue and change the affinity pattern of binding⁶. In a report Several H9 and H5 escape mutants with a new glycosylation site showed both a decrease in the affinity of the hemagglutinin to the high molecular weight sialic substrates and reduced lethality in mice. The phenotypic effects of mutations in HA gene of H5 virus may be of importance to appraise the extent and direction of H5 influenza viruses' antigenic evolution⁷.

AIMS AND OBJECTIVES

To explore the possibility of being an escape mutant a re-isolated virus (formalin inactivated and sterility checked) from an experimental immunization study, to check for any change in restriction enzyme profile pattern of HA and NA gene. Since point mutation in surface proteins (antigenic drift) may result in new and immunologically different strains of influenza viruses that are responsible for influenza outbreaks and epidemics⁸.

MATERIAL AND METHODS

Revival of virus, RNA isolation, PCR amplification, RE digestion and analysis

The virus strain A/Duck/Tripura/103597/2008(H5N1) was obtained from HSADL repository and revived by inoculating into 9-11 day old embryonated chicken eggs through amnio-allantoic route. The experimental eggs were incubated at 37°C for 72 hrs and dead were chilled and harvested. HA titre was assessed. Viral RNA was extracted from allantoic fluid using QIAamp Viral RNA Mini kit (Qiagen, Germany) following the manufacturer's instructions and stored at 80°C until further use. cDNA was synthesised using cDNA synthesis kit (MBI Fermentas) as per the manufacturer's instructions. HA and NA genes were amplified by PCR with the following cycling conditions: 5 min at 94°C followed by 35 cycles of amplification with denaturation at 95°C for 30

sec. annealing at 48°C (for NA) and 50°C (for HA) for 30 sec, extension at 72°C for 2 min. and a final extension of 10 min at 72°C. Primer pairs used were IFH5F-1-AGCAAAGCAGGGGTGTTTAACTAC AAT, and IVRH5R-1773-AGTAGAAACAAGGGTGT

AGCAAAGCAGGAGATTAAAATGAATCAA and NAI VAGTAGAAACAAGGAGTTTTTT.

PCR products were confirmed by 1% agarose gel electrophoresis. The PCR amplified products were purified by QIAquick gel extraction kit (Qiagen, Germany) as per the manufacturer's instructions. The amplified HA and NA gene products were cloned into T/A cloning vector (Fermentas, USA) as per protocol (vector insert ratio 1:3). The amplified HA and NA PCR products were digested with the following restriction enzymes. *EcoRI*, *PstI*, *SacI*, *KpnI*, *XbaI*, *BamHI* and *NcoI* respectively. Restriction Fragment length polymorphism genotyping was carried out using hexacutter restriction enzymes *EcoRI*, *PstI*, *SacI*, *KpnI*, *XbaI*, *BamHI* and *NcoI* for PCR amplified fragment size of 1734 bp of HA and 1398 bp of NA genes. The published sequences for HA and NA gene of A/Duck/Tripura/103597/2008(H5N1) was retrieved from NCBI and the Restriction enzymes digestion sites were predicted using Bioedit software.

RESULTS AND DISCUSSION

Influenza A viruses are immunologically significant from an evolutionary standpoint. Antigenic diversity of influenza A viruses occurs primarily at two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The AI hemagglutinin appears to be unique in its capacity to accept basic amino acids at its Proteolytic Cleavage Site (PCS).

India evidenced the presence of H5N1 (HPAI) strain in 2006 when it leads to a major outbreak in Navapur sub-district in the Western state of Maharashtra, India. Since then it is prevailing in India and regular outbreaks are being documented from the

country. The disease rapidly spread mainly in backyard poultry and ducks in 14 out of 19 districts⁹. An outbreak recorded was in West Bengal in January 2008 followed by another outbreak within a span of month, occurred in Tripura in April, 2008 in backyard ducks and geese.

A rapid PCR-RFLP genotyping method capable of distinguishing the internal genes of human H1N1, H3N2 and avian H5N1 influenza A viruses was developed and used to screen viruses from clinical samples isolated in Hong Kong during and immediately following the 1997 H5N1 avian influenza outbreak¹⁰. RT-PCR method, using RT-PCR followed by digestion of PCR products with restriction enzymes, was very beneficial for analyzing the genome of reassortant influenza viruses¹¹. Fereidouniet al., have developed a restriction fragment polymorphism-based assay that enables the pathotyping of influenza A of subtype H5 independent of sequencing or animal experiments after RT-PCR and restriction enzyme digest of the amplified product using *MboII* restriction enzyme.¹² Validation of the method using 28 H5 subtype reference isolates from different animal species revealed good performance characteristics regarding sensitivity and specificity, especially when targeting recent highly pathogenic AIV (HPAIV) of subtype H5N1 and Asian origin. The PCR-RFLP/ PCR-RE study was

carried out to study restriction enzyme pattern of HA gene and NA gene to infer broadly/indirectly any change in the HA and NA genes using randomly selected RE enzymes. For HA gene, *KpnI*, *SacI*, *PstI*, *EcoRI* and *XbaI*(**Fig. 1**) and for NA gene, *BamHI* and *NcoI*(**Fig. 2**) were used. The RE sites of the enzymes were predicted by Bioedit software. From the results of the study, there were no changes observed in the predicted cut sites of the enzymes from the original virus sequence. Using RFLP, any nucleotide mutation that occurs at the restriction site alters the genotype identification. Nucleotide mutations that occur outside the restriction site cannot be assessed. Moreover, success of the RT-PCR and RFLP genotyping depends on primer design and restriction enzyme selection. The other possible reason may be few of virions may be trapped deep inside the conc. protein which might have multiplied later to exhibit clinical symptoms in the bird. From this limited study, we could not be able to conclude completely that this may not be an escape mutant because the gravity of an escape mutant is more crucial and the future studies can be extended to use more advanced and elaborate studies like sequencing, predicting sequence changes in the antigenic sites and their appropriate implications etc.¹³⁻¹⁷

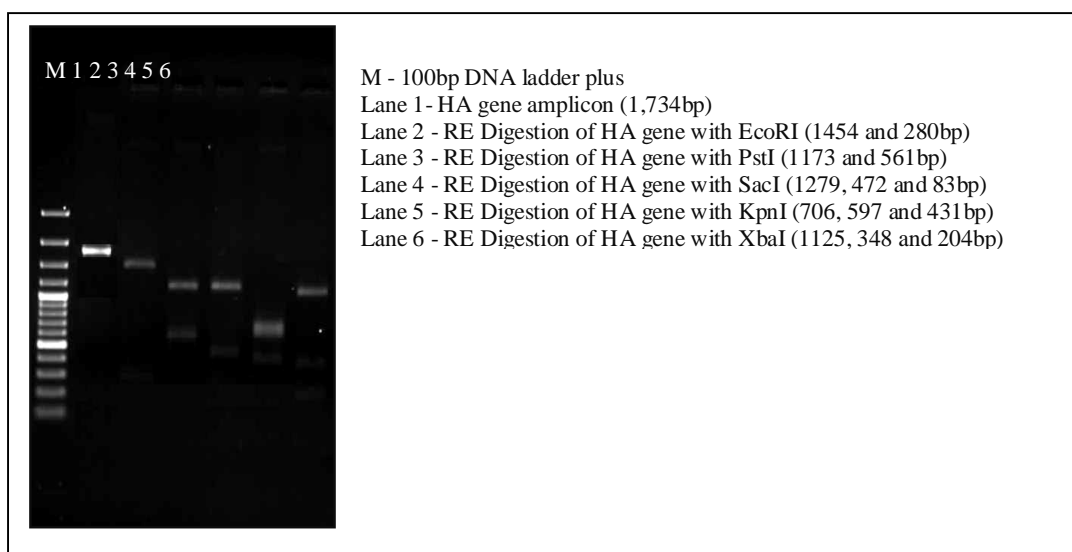


Fig. 1:Restriction enzyme profile of HA gene

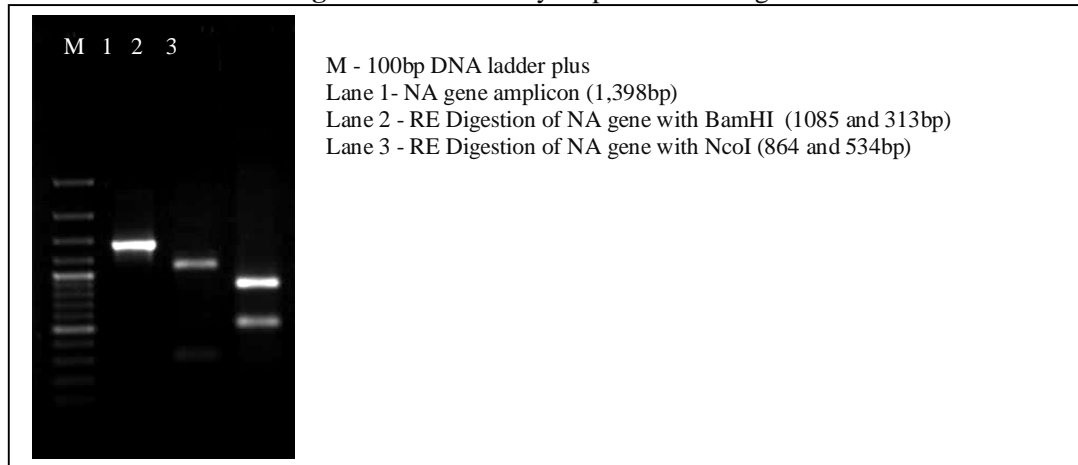


Fig. 2 : Restriction enzyme profile of NA gene

CONCLUSION

Avian influenza (AI) is a highly contagious acute viral disease of chicken caused by Type A Influenza virus. Avian H5N1 influenza A viruses are considered to be of high pandemic potential as they are able to cross the avian-human species barrier and cause disease in humans. The study was carried out with a vision to detect the possible reason i.e. antigenic drift or mutation in viral HA and NA gene of viral isolate of A/Duck/Tripura/103597/2008(H5N1) being an escape mutant since it was re-isolated from a bird which was immunized with inactivated virus. Both HA and NA genes were amplified with gene specific primers of H5N1. The amplified HA and NA gene products were then cloned into T/A vector. The recombinants were then selected on the basis of blue/white colony selection. The amplified PCR products were analyzed for any changes in HA and NA genes by restriction enzyme profiling pattern using randomly selected enzymes. The HA gene was analysed with a panel of restriction enzymes and NA gene was analyzed for Bam HI and NcoI. The RE profiling was thrice repeated to confirm any variation in the cut sites. From the results, no variation/changes were observed in both HA and NA gene from the originally predicted RE sites.

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