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Evaluation of RT-PCR for the Detection of Influenza Virus Serotype H9N2 among Broiler Chickens in Pakistan

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Abstract: A Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was standardized and compared with virus isolation techniques for the detection of avian influenza virus serotype H9N2 among Broilers suffering from respiratory tract infection. In this regard 50 tissues and cloacal swabs were tested for the isolation of avian influenza viruses. Out of this, 17 samples from tissues and 3 from cloacal swabs showed the presence of AIV H9N2 through virus isolation test while six tissue samples showed the presence of NDV. Out of the negative samples another 15 samples were positive for AIV H9N2 when tested by RT-PCR while seven samples were positive for NDV by RT PCR. Detection of AIV from the cloacal swabs by RT-PCR also confirmed the shedding of AIV among the infected flocks. Apart from detecting the presence of AIV H9N2 from clinical specimens, the RT-PCR was able to detect higher number of AIV, from healthy flocks, which would otherwise have been missed by routine lab methods or wrongly diagnosed and treated adversely.

Key words: AIV, H9N2, RT-PCR, differential diagnosis

INTRODUCTION

Avian influenza viruses (AIV) of serotype H5, H7 and H9 have attained great significance in recent years due to emergence of pathogenic form of these viruses in poultry and also due to their zoonotic potential (Claas *et al*., 1998; Naeem *et al*., 1999; Cameron *et al*., 2000). Although, avian influenza virus serotype H9N2 does not fall under the definition of Highly Pathogenic Avian Influenza (HPAI) viruses, it has caused severe infection in broilers, layers and broiler breeders in various countries (Naeem *et al*., 1999). Its subsequent isolation from chickens in Hong Kong and involvement in human infection in China and Hong Kong revealed its zoonotic significance (Uyeki *et al*., 2002).

Since its isolation from Pakistan in 1999, the virus has caused many outbreaks in the young broilers and laying birds in this country. A study reported later revealed 98% homology in the sequence of H9N2 isolates from Pakistan with that of Hong Kong isolates recovered from children (Cameron *et al*., 2000). This signifies the pathogenic potential of H9N2 isolates both from human and poultry.

In immunosuppressed chickens the virus is capable of inducing severe respiratory tract infections with high mortality in young chicks and severe decline in egg production in laying chickens (Bano *et al*., 2003). The H9N2 serotype of AIV appears to persist in chicks and spread to non-affected flocks through fecal-oral route without showing much of severe clinical signs. This makes it extremely difficult to diagnose H9N2 infection under field conditions. Due to this, it is not possible to assess the true prevalence rate of AIV H9N2 in a particular population, resulting in our inability to adopt effective control measures including vaccines.

Based on the field experience of AIV H9N2 infection, it appears that this infection has very close clinical signs and symptoms with those caused by mild strains of NDV and IBV. It, therefore, becomes very difficult to differentiate between these diseases under field conditions. Furthermore, due to difficulty in clinical diagnosis of AIV H9, there is very little tendency among the field veterinarians to incriminate AIV in various respiratory or reproductive tract infections, which does not convince them to recommend regular vaccination against AIV H9N2 in the field. In most of such situations the preferred approach is to opt for NDV or IBV re-vaccination, which usually results in severe reaction in case the flock is exposed to AIV.

With the knowledge of its involvement in human infection in some parts of the world, it becomes important to develop more sensitive and less time consuming methods to identify the H9N2 viruses both from chickens and their products. With the advent of PCR as an efficient tool for the diagnosis of viruses, it has become essential to employ it in the detection of AIV H9N2 infection or contamination. Although, the egg inoculation procedure has been successfully employed for identifying the circulating AIV in poultry populations, due to lack of access to SPF eggs, inherent risk of contamination of environment/workers while propagating the influenza viruses and difficulty in getting proper field specimens, for virus isolation, it has become necessary to introduce PCR for the diagnosis of AIV H9N2 in local situations. The present study was undertaken to evaluate the use of

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for the detection of influenza virus subtype H9N2 from the clinical cases of respiratory tract infections in broilers in addition to employing other routine techniques.

MATERIALS AND METHODS

Source and collection of specimens: Broiler flocks suffering from respiratory tract infection, without any involvement of other vital organs, were included in this study. The following types of samples were collected from the diseased flocks:

- Organs including spleen, trachea and lungs were collected from 50 broiler flocks. The organs collected from three chickens per flock were pooled together for this test.
- Cloacal swabs from five chickens per flock were collected from only ten flocks among the above sampled birds, by using sterile swabs and BHI (Oxide) transport medium. The cloacal swabs taken from five chickens of a single flock were pooled in a single vial.

The above samples were transported on ice in the dark to the laboratory and stored at 4°C for two weeks until used.

Virus isolation: Organ types were pooled and blended to prepare a 20% suspension in PBS (pH 7.2) solution containing Penicillin (2×10^8 IU/L) Gentamycin (250 mg/L), Streptomycin (200 mg/L) and Enrofloxacin (200 mg/L). It was centrifuged at 800xg for 10 min at 10°C. The supernatant was filtered through 0.22 um syringe filter (Gelman, USA) and inoculated into allantoic fluid of 9-days-old embryonated eggs. The eggs were incubated at 37°C for 3 days. A part of filtered material was stored at 4°C for PCR testing.

The Cloacal swabs were placed in the transport medium containing BHI broth (Oxide) with Penicillin (2×10^6 IU/L) Gentamycin (250 mg/L), Streptomycin (200 mg/L) Enrofloxacin (200 mg/litre). The tubes containing swabs were vortexed, centrifuged at 800xg and the supernatant was also processed as above for egg inoculation.

After inoculation, the eggs were chilled for 4 h and the allantoic fluid was harvested and tested for Haemagglutination (HA) activity. The positive samples were further tested using AIV H9N2 and NDV reference antisera, in a standard virus neutralization test (Beard, 1989).

RT-PCR application: The following procedure was used by employing selected primers (Table 1) and standardized protocol (Table 2).

RNA extraction: The above prepared tissue homogenate and cloacal swabs were spun at 800×g for 10 min and 800 µL of the supernatant was placed in a microfuge. To this 200 µL of DEPC-treated distilled water was added, followed by 600 µL of Trizol LS (Fermentas, USA) reagent. It was incubated for 5 min at RT followed by addition of 200 µL of chloroform and centrifuged at 15000 xg for 15 min. The supernatant was treated with 300 µL of isopropyl alcohol, followed by centrifugation and treatment of pellet with 500 µL of 70% alcohol. It was again centrifuged and the pellet was suspended in 20 µL of distilled water and stored at 4°C. The template RNA was later on quantified using a Bio-Photometer (Eppendorf Germany) and then used in the PCR procedure.

PCR protocol: A one step RT-PCR protocol was used to test the samples employing cMaster™ RT Kit (Eppendorf Germany) following the manufacturer's instructions.

The reaction mixture contained the AIV H9 specific primers along with other reagents from the Kit and it was run in a thermal cycler (MacMaster, Eppendorf, Germany), using the temperature parameters shown in Table 2. The amplified product was run on an agarose gel along with standard DNA markers at 100 V for 1.5 h and photographed as described by Sambrook *et al.* (1989).

To verify the specificity of RT-PCR for AIV subtype H9N2 detection, known NDV (Lasota) and IBV (Mass 41) and AIV H5 specific primers were used. For this purpose, the type specific primers for each virus serotype were employed in this study (Table 1).

RESULTS

Avian Influenza Virus (AIV) subtype H9N2 was successfully isolated from the clinical samples through *in ovo* inoculation. Total 17 isolates were recovered out of 50 tissue samples (34%) and 3 out of 10 from cloacal swabs (30%) upon inoculation into embrocated eggs (Table 3). Furthermore, 6 NDV isolates were also recovered from tissue samples. Out of the remaining 27 tissue samples, 15 AIV H9 and 7 NDV isolates were

Table 1: Specific primers used for PCR against AIV, NDV and IBV

Name/ID of virus	Sequence of forward primer	Sequence of reverse primer
AIV (H9 serotype)	AGCAAAAGCAGGGGAAYWWC	CCATACCATGGGGCAATTAG
IBV (M-41 serotype)	CATAACTAACATAAGGGCA	TGAAAACTGAACAAAAGACA
NDV (Lasota)	GGAGGATGTTGGCAGCATT	GTCAACATATACACCTCATC
AIV (H5 Serotype)	GGAATGATAGATGGNTGGTAYGG	GTGTTTTTAAYTAMAATCTGRACTMA

AIV: Avian Influenza Virus, IBV: Infectious Bronchitis Virus, NDV: Newcastle Disease Virus

detected by RT-PCR (Fig. 1). On the other hand, out of 7 remaining cloacal swabs, 2 samples had AIV serotype H9 (Fig. 2). So, as a whole 37 isolates (62%) of AIV H9 and 13 isolates (22%) of NDV were detected from the 60 clinical specimens. Table 4 reflects the effect of repeat passaging on the recovery rate of viruses from clinical samples. The data indicate that the virus could be recovered from 18% samples at the first passage where as from 31% samples after the second passage. 11, 15 and 10% of samples were negative for AIV or NDV by VI (P_1), VI (P_2) and the RT-PCR techniques, respectively (Table 4).

For conducting RT-PCR, the RNA component of a known AIV serotype H9N2 was extracted and used for standardization of RT-PCR, following the instructions provided by the kit manufacturer. The gel electrophoresis of the amplified PCR products detected from tissues showed characteristic bands of 808 base pairs of AIV subtype H9N2 and 320 base pairs of NDV (Fig. 1). The gel electrophoresis of amplified PCR products from clinical specimens, showed characteristic bands of 808 base pairs of AIV subtype H9N2 detected from tissues and swabs (Fig. 2). By adopting the standardized procedures, the specificity of the test was established by using RNA from IBV, H5 and NDV (Fig. 3). The sensitivity of RT-PCR for H9N2 was standardized to detect 2.99 ng of viral RNA (Fig. 4).

DISCUSSION

Avian influenza viruses are the major cause of acute respiratory and reproductive tract infection of chickens and every year bring about high morbidity and mortality worldwide. Clinically it is impossible to distinguish the avian influenza virus infection of upper respiratory tract from those caused by Infectious Bronchitis (IB) and Newcastle Disease (NDV). It is therefore, essential to diagnose the role of avian influenza viruses through certain specific laboratory assays. The conventional laboratory diagnosis of avian influenza such as viral isolation either in embryonated eggs or in cell culture is time consuming. Therefore, it is necessary to develop a diagnostic test for the rapid identification of influenza viruses directly from clinical specimens.

Transfer of influenza A viruses from animal host to human has presumably led to the emergence of new human pandemic strains. This includes subtypes H5, H7 or H9 of AIV. The early detection and identification of such viruses are, therefore, paramount in the surveillance of influenza viruses in a situation. To detect and partially characterize influenza A viruses from different animal species, RT-PCR has earlier shown to be sensitive and specific for the detection of human, avian and swine influenza A viruses (Joanna *et al*., 2001).

The conventional egg inoculation method employed for virus isolation in this study is basically considered as a gold standard for the isolation of influenza A viruses. Although, some recent studies have pointed out that the assay of Polymerase Chain Reaction (PCR) is the most sensitive and rapid test for the detection of influenza viruses, it also provides better detection of the virus from the clinical samples which might otherwise appear negative due to inappropriate sampling or loss of infectivity during shipment. This clearly reflects the role of good sampling for the recovery of the influenza viruses from clinical specimens (Vabret *et al*., 2000).

The virus isolation technique can only detect the live influenza viruses and the subsequent determination of its isolation is made by HA, which also requires higher concentration of virus particles in a specimen. On the other hand, RT-PCR has the ability to even detect a single virus particle, whether active or inactive. Avian Influenza viruses of low pathogenicity have high rate of in-apparent infection, short incubation and high infectivity. In routine practice, on the basis of clinical symptoms, avian influenza viruses of low pathogenicity cannot be differentiated from other respiratory tract viruses such as NDV and IBV. There is also no practice in Pakistan of conducting lab investigations on the clinical specimens to identify the causal agents. Some viruses such as Newcastle disease viruses also give Haemagglutination (HA) activity. It, therefore, becomes almost impossible to evaluate the impact of various influenza viral infections, alone or in coordination with other agents. Furthermore, due to the ability of AI virus shedding, it is important to screen a particular flock using a highly sensitive technique. As reflected from the results of this study, VI techniques have also shown less

Table 2: Program Parameter for one step RT-PCR

# of cycles	Step	Temperature	Time
1	1	42°C	55 min
1	2	94°C	2 min
30	3	94°C	15 sec
	4	50°C	10-20 sec
	5	68°C	2 min
1	6	72°C	2 min
1	7	4°C	10 min

Table 3: Rate of AIV H9N2 detection from clinical specimens examined

Type of samples	Total samples	Isolation through egg inoculation		Negative by VI	Virus detection be RT-PCR	
		AIV (H9)	NDV		AIV (H9)	NDV
Tissues	50	17	6	27	15	7
Cloacal Swabs	10	3	0	7	2	0
Total	60	20	6	34	17	7

VI: Viral Isolation, RT-PCR: Reverse Transcriptase Polymerase Chain Reaction, AIV: Avian Influenza Virus, NDV: Newcastle Disease Virus

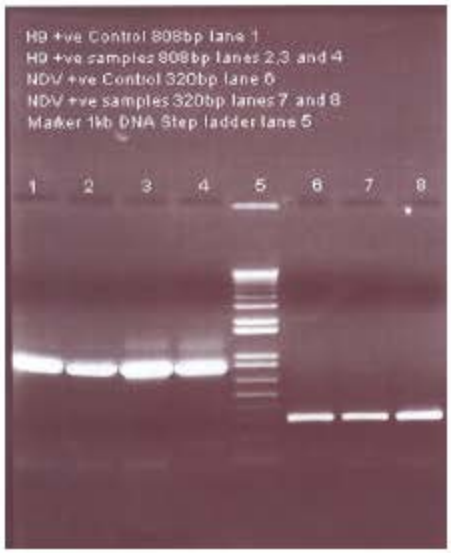

Fig.. 1: Agarose gel electrophoresis of AIV subtype H9N2 and NDV. Lane 1 represents H9 positive control 808bp. Lanes 2, 3, 4 represents samples positive for H9. Lane 6 represents NDV positive control 320 bp. Lanes 7 and 8 represents samples positive for NDV. Lane 5 represents marker 1 kb plus DNA step ladder

Fig. 2: Agarose Gel Electrophoresis of Subtype H9N2. Lane 1 represent H9 negative control. Lane 2 represents H9 positive control 808bp. Lanes 3, 5, 6, 8 represents H9 positve samples. Marker 1kb plus DNA ladder lane 4

sensitivity, as 7 out of 10 (70%) of the cloacal swabs were found negative by conventional VI techniques. It means that by using less sensitive techniques one can miss the AIV shedding flocks. There is a common practice in the field to carry out live vaccination against the virus suspected to be infecting the flocks. This usually includes ND or IB vaccines. So, in case of a misdiagnosis of AIV or due to the wrong diagnosis one may recommend IBV or NDV vaccine eventually resulting in severe vaccine reaction.

Table 4: Comparison of different diagnostic tests for the detection of AIV subtype H9 from clinical specimens

	Type of tests employed		
Description of results	V.I(P_1)	V.I(P_2)	RT PCR
Total samples tested at each attempt	60	49	34
Samples showing virus isolation	11	15	24
Samples showing no virus isolation	49	34	10
Percentage positive	18%	31%	70.5%

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction, VI: Virus isolation by egg inoculation, P Egg passage level

In current studies, the avian influenza virus H9N2 was primarily detected from the tissues of trachea, lungs and spleen, as well as in some cases from cloacal swabs. Its isolation indicates the systemic nature of H9N2 infection. It further suggests that under field conditions in chickens, AIV H9N2 is an invasive agent. Following natural exposure a systemic disease occurs, involving many vital organs of the birds. In many outbreaks of low pathogenic AIV, the mortality and lesions in respiratory and urogenital tracts have been extensively reported (Alexander and Gough1986; Halvorson, 1980; Meulemans, 1981).

The detection of AIV in cloacal swabs is an indication that birds shedding virus can be detected using PCR procedure. Therefore, the RT-PCR can be successfully used in surveillance of influenza to provide accurate and sensitive diagnosis on clinical specimens, as reported by others in the past (Ellis *et al*., 1997; Boivin *et al*., 2001, 2002, 2004).

In the present study, out of the 34 negative samples left after the completion of egg inoculation procedure, an additional 63% samples were found positive for the presence of AIV H9N2 through RT-PCR. This way the RT-PCR was able to detect more positive samples, which would otherwise have been missed by routine methods. This reflects higher level of sensitivity of this test as earlier reported by other workers (Liolios *et al*., 2001; Herrmann *et al*., 2000).

Some earlier studies about the genetics characterization of the gene segments have indicated that H5N1 viruses were generated by reassortment. In this regard avian influenza serotype H9N2 virus is a major donor of the internal genes including three polymerase genes (PB_2, PB_1 and PA) and Nucleoproteins (NP), Matrix (M) and Nonstructural (NS genes). This indicates that in the presence of different serotypes of influenza viruses in the field, there is always the likelihood of generating new viruses by gene reassortment between serotypes

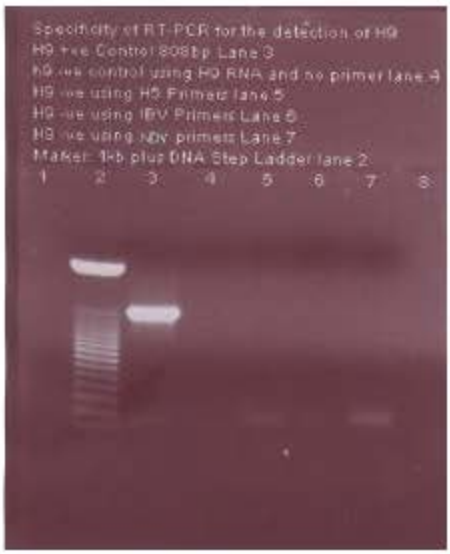

Fig. 3: Specificity of RT-PCR for the detection of H9. Lane 3 represents H9 positive control 808bp. Lane 4 represents H9 negative control using H9 RNA and no primer . Lane 5 represents H9 negative using H5 primers. Lane 6 represents H9 negative using IBV primer. Lane 7 represents H9 negative using NDV primers. Lane 2 represents marker 1kb plus DNA ladder

Fig. 4: Sensitivity of RT-PCR for the detection of subtype H9N2. Lane 3 represents the detection of 29.99ug of H9 RNA template. Lane 4 represents the detection of 2.99ug of H9 RNA template. Lane 5 represents the detection of 299.9ng of H9 RNA Template. Lane 6 represents detection of 29.9ng of RNA template. Lane 7 represents detection of 2.99ng of H9 RNA template .Lane 2 represents marker 1kb plus DNA step Ladder

pathogenic to birds and mammals (Guan *et al*., 1999). During recent years, H5N1 subtype of AIV has been found to transmit from poultry to humans causing many deaths in Far East countries (Claas *et al*., 1998). This signifies the potential of H9N2 as a reservoir of genes capable to cause infection in humans.

In this scenario, recently identified presence of H9N2 and H7N3 in Pakistan and H9N2 alone in various countries in the Middle East and South East Asia poses a continuous threat for the emergence of more pathogenic strains of influenza viruses. The isolation of H9N2 serotype from poultry, in this part of the world, signifies its pathogenic potential and therefore, suggests these viruses to be a possible candidate for future human pandemics originating in Asia. It, therefore, will be appropriate to launch comprehensive surveillance of live bird markets in the region using both VI and RT-PCR techniques, so as to assess the burden of various serotypes of AIV in a particular area, in order to devise appropriate control measures against the prevalent AIV serotypes in this region.

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