Research Article

Isolation, identification and molecular characterization of virulent avian infectious bronchitis virus in Khyber Pakhtunkhwa, Pakistan

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Abstract

Infectious bronchitis virus (IBV) of the *Corona viridae* family is a single stranded RNA virus; a causative agent of respiratory and urogenital tract diseases in chickens. This pathogen causes huge economic losses throughout the world where chickens are produced for commercial purposes. Embryonated egg inoculation was used for detection and amplification of IBV RNA, (specific pathogen free) SPF eggs (n=50, 9-11 days old) were inoculated with IBV suspected samples. After incubating the eggs for 72-144hours, the infectious allantoic fluids were harvested when signs of curling, stunting, dwarfing and death of the embryos appeared after inoculation. Viral RNA used in the RT-PCR was extracted from virus containing allantoic fluid and reverse transcribed to cDNA for detection of IBV and their serotypes. PCR products were electrophoresed to visualize the results. A high percentage (36/50eggs) of the eggs ($P<0.05$) were found infected with the virus in embryonic cells. The main necrotic lesions caused by the IBV in eggs were curling, stunting, dwarfing and mortality. First round PCR showed 75% samples were positive for IBV, whereas second round PCR confirmed 77.77% samples were positive for the Massachusetts serotype. In summary, despite of vaccination against IBV, this viral pathogen still circulates in Khyber Pukhtunkhwa. In addition, Massachusetts serotype is emerging with new variants leading to vaccine failure.

Keywords: Curling; Embryonated eggs; Infectious bronchitis disease; Massachusetts; Poultry; RT-PCR

Introduction

Avian Infectious Bronchitis disease (AIB) is an acute viral respiratory disorder of poultry with high contagious nature. The disease mainly attacks the respiratory tract and causes inflammation of the bronchi,

reproductive disorders and nephritis. Clinical signs vary based on the age of birds, pathogenicity of the virus strain and existing level of immunity [1, 2]. In broilers (2-6 weeks) the main clinical signs of IBV are difficulty in breathing, tracheal rales,

coughing and sneezing with or without nasal discharge, weakness accompanied by depression, reduced feed consumption and body weight loss [3]. The nephritic form of IBV is characterized by mild and transient respiratory signs followed by depression, ruffled feathers, hunched stance, reluctance to move, excessive water intake, rapid weight loss and diarrhea [3]. In reproductive form, many flocks show a decline in egg production associated with eggs of smaller size and inferior external and internal quality such as eggs softness, pale-shelled with thin albumen [4]. IBV incidence was first reported in 1931 in USA in a group of young chickens [5]. From that time, the malady has been recognized in layers, breeder and broiler chickens worldwide. IBV has a considerable economic impact with a high mortality rate (25-60%) in infected flocks. Poor weight gain, mortality, sub-optimal and down grading of eggs production leads to significant losses in the poultry industry [6]. The serological differences between the vaccine strains and the strain that causes the infection are responsible for the frequent epidemics of IBV [7]. Different IBV serotypes in Asia are Mass, Conn, Gray, Ark 99,793/B-India, NRZ-China, HV-China, and A1121-Taiwan [8]. The post translational changes within the IBV spike protein lead to the formation of S1 and S2 subunits [9]. The changes in the nucleotide sequence of S1 are said to be responsible for the appearance of variants which cannot

be effectually controlled by various vaccines. Among IBV proteins, the S protein and its subunit S1 are the most variable and key inducer of defensive immunity [10]. Poultry industry, with an investment of Rs 732 billion and with an annual growth rate of 10-12%, is one of the most vibrant segments of the agriculture sector of Pakistan (Eco, survey of Pak, 2013-2014 [11]. Studies conducted at the molecular level on IBV in KP, Pakistan are scant therefore, the present study aimed to characterize IBV from allantoic fluids of embryonated eggs and investigate its genotyping, serotyping and protectotyping.

Material and methods

Sample collection and virus isolation

This study was carried out on commercial chickens at Department of Poultry Science, Faculty of Animal Husbandry and Veterinary Sciences Peshawar, Khyber Pakhtoonkhwa Pakistan. Trachea, lungs and kidneys samples were collected aseptically from the post mortem section where birds died due to suspected IBV outbreaks, brought from the commercial chicken flocks across different parts of Khyber Pakhtoonkhwa Pakistan. Pooled 20% tissue suspensions were inoculated intra-allantoically into 9-11 day-old embryonated chicken eggs to observe the dwarfing, curling and death of embryos and the allantoic fluid was then harvested and used for RNA extraction as shown in the plate 1.

Plate 1. Collection of allantoic fluids as harvest for RNA extraction

RNA extraction and RT-PCR

The entire egg samples positive on inoculation were used for RNA extraction.

For each sample, two eppendorf tubes were used. Primers were diluted at 10X with RNAs free water. RNA was extracted

according to (Norgen) KIT procedure. In each tube 6 µl of RNA carrier was taken. RL Solution was added to all the tubes in amount of 600µl. After that 150 µl of each sample was added to all the tubes containing mixture respectively. Pulse vortexing of all tubes was carried out 20 times. All the tubes were incubated at 15-25°C for 10 min.

The tubes were then centrifuged 4000 rpm for 10 minutes and 600 µl of ethanol was added to all the tubes. Again pulse vortexing was done and the tubes were briefly centrifuged 5000 rpm for 15 minutes. An amount of 700 µl of the total mixture was moved to the columns inserted in the collection tubes and then centrifuged at 10,000xg for 60 sec. The flow through in the tubes was removed and the columns were reinserted into new collection tubes. Now 700 µl of washing solution W1 was added to all the tubes and centrifuged at 10,000 xg for 1 min. The tube containing flow through was discarded and the columns were inserted into new collection tubes. W2 solution (700 µl) was added to all the columns and again centrifuged at 10,000 rpm for 1 min. The tubes containing the remaining fluid were disposed off and the columns were placed into new collection tubes.

The tubes were then centrifuged at 14,000 rpm for 30 second. The spin columns were then shifted to 1.5 ml collection tubes. Sol E 50 µl was added to all the tubes and then placed at room temperature for incubation for 3 mins. The tubes were then centrifuged at 14,000 rpm for 1 min. At the end the spin columns were discarded and the elute in the micro centrifuge tubes containing viral RNA was collected. The collected RNA was directly used in RT-PCR application. Extracted RNA was screened for the presence of IBV by using nested RT-PCR. Briefly 10 µl of template RNA, 2 µl of dNTPs, with 1 µl of Random primer (ER) were mixed together and poured into a 0.2 ml of PCR tube. After cooling 4 µl of 10x RT-Buffer and 1µl reverse transcriptase enzyme (Thermoscientific, USA) was

added. Time set and incubation temperature was 10 min at 25°C, 60 min at 42°C and 5 min at 85°C as described earlier [12]. The first amplification reaction was carried out with one-step RT-PCR using the general pair of primer, BVEF

(5-CACTGGTAATTTTTCAGATGG-3) and IBVER

(5-CCTCTATAAACACCTTTACA-3).

The second amplification reaction was carried out using the specific sets of primers, IBVD

(5TTCCAATTATATCAAACCAGC-3), IBVM

(5-AATACTACTTTTACGTTACAC-3) and IBV4

(5-AGTAGTTTTGTGTATAAACCA-3).

Thermal conditions for PCR reaction were; initial denaturation at 94°C for 3min, 30 cycles of 94°C for 30sec, 55°C for 1min, 72°C for 1min and a final extension at 72°C for 7 min. The same thermal conditions were used for second round PCR.

Gel electrophoresis

Amplified PCR products were run over 2% Agarose gel. Various bands corresponding to different IBV serotypes were visualized using an ultraviolet gel documentation system (Uvitec Limited, Cambridge, UK).

Statistical analysis

The data was statistically analyzed through using SPSS software (SPSS, Inc., Chicago, IL, USA). (SPSS, Inc., Chicago, IL, USA). The variables were compared using a chi-square test. $P < 0.05$ was perceived as statistically significant.

Results

Replication of virus in embryonated eggs

After 72-144 hrs of incubation the (pathological signs) curling and death of embryos was observed in eggs. The main necrotic lesions caused by the IBV in these eggs were curling, stunting, dwarfing and mortality. These were the strongest signs sufficient to judge the multiplication of the virus in the embryonated eggs. Out of 50 eggs 28 were found with these signs which constitute 56% of the sample size, while in 8 eggs only death of the embryos was caused which constitute 16% of the sample

size with no specific signs and were considered as less positive as described in table 1. On the other hand the lesions were not observed in those eggs which were either rotten or with no embryo formation which might be affected by some incubation factors i. e incubation

temperature, humidity and turning frequency. A higher percentage ($P<0.05$) was found in 36 eggs as a result of virus replication in the embryonic cells as shown in the table 2. The pool harvested from positive eggs was used for virus isolation and RNA extraction.

Table 1. Egg Inoculation of tissue samples

9-11 days old embryonated eggs	No.	Candling time after inoculation	Positivity/negativity	%age
Dead embryos(Dwarfism, stunting, curling)	28	5-6 days	++	56%
Dead embryos	8	2-3 days	+	16%
Rotten eggs	3	2-3 days	—	6%
No embryo at all	4	1 day	— —	8%
Unidentified signs	7	5-6days	—	14%
Total	50			100%

Table 2. Presence of infectious bronchitis virus on RT-PCR

Samples	No.	Percentage%
Positive	27	75.00
Negative	9	25.00
Total	36	100
$P<0.05$		

1st Round PCR

To amplify the nucleotide sequence of IB virus, the process was carried out with the help of a set of general primers. Amplification of 27 samples of 466bp showed that RT-PCR was performed correctly. It also proved the presence of IBV in the samples as shown in (Figure 1).

2nd Round PCR

The 1st round PCR positive 27 samples on 2nd round PCR showed that among the three serotypes only Massachusetts serotype was identified in 21 samples attaining a band size of approximately 297bp while the remaining samples were unidentified as shown in the figure 2.

The table 3 shows the presence of mass serotype in 21 samples out of 27. This was confirmed by 2nd round PCR. It was also confirmed that IBV infection in poultry is caused by mass serotype which is prevalent in this region. Second round PCR specified the prevalent serotype.

Discussion

Infectious bronchitis is one of the most important and common infectious diseases of respiratory system of chickens [13]. In the concerned study 50 different samples originating from suspected cases at postmortem section Veterinary Research Institute, Peshawar were selected for isolation of virus and molecular characterization. The present study was aimed at investigating the isolation of infectious bronchitis virus from allantoic fluids of embryonated eggs and its genotyping followed by propagation of the virus as vaccine strain. Table 1 shows that on inoculation of samples in 9-11 days old embryonated eggs, 72% of total eggs were positive ($P<0.05$) while 28% of eggs give negative results. 56% of the positive findings were strongly positive while the remaining were said to be less positive based on macroscopic lesion. Similar results were found (79% positive) for IBV

by the real-time assay [14]. It was suggested in one of the research work that a range of strains of IBV could be detected in the allantoic fluid obtained from chick embryos after inoculation [15]. The results showed by Jahantigh [12] are supporting our current study as 4 out of 11 (36.36%) of the sampled flocks were positive to IBV by RT-PCR. The specific nested PCR performed on RT-PCR positive flocks proved that the circulating serotype of infectious bronchitis virus in Zabol was Massachusetts which is further assisting our results. On the other hand the results

described by Wang *et al.*, (1998) [16] prove that RT-PCR require a number of individual steps for the identification of different serotypes which make it time consuming and cumbersome. The lesions on embryo caused by IBV after sample inoculation are usually observed within 5-7 days but are not pathognomonic for IBV spotting [1]. RT-PCR was applied on RNA extracted from the suspected tissue samples and allantoic fluids. This technique has proved to be very efficient in identifying the IBV and different serotypes of IBV [15].

Table 3. Detection of Mass serotype on Nested-PCR 2nd round

Samples	No.	Percentage%
Positive	21	77.77
Negative	6	22.22
Total	27	100
<i>P</i><0.05		

In the present study Real time PCR on 36 positive samples showed the presence of IBV in the region. It was confirmed through molecular study that 27 samples (75%) were amplified to 466 bp which is a clear evidence of the presence of IBV in this region as shown in the figure 1. On the basis of statistical analysis ($P<0.05$), it was confirmed that infection has been circulating in the region which need further molecular study. Before 1956 it was observed that a single genotype is the causative agent of IBV acquisition however it was confirmed later that virus originates in different serotypes due to antigenic variations [17]. Previously, a total of 945 serum samples were analyzed in Pakistan and three IBV strains (Connecticut, M-41, Arkansas) were reported [18]. It was further analyzed from 2nd round PCR that present infection of IB is caused by the serotype that is prevalent in this area as shown in the figure 2. The product run on gel electrophoresis identified 21 samples of 297bp ($P<0.05$) which showed that the current infection is caused by the Massachusetts serotype. The same serotype

is responsible for heavy loss in poultry industry due to the havoc caused by this virus. The remaining 6 samples were unidentified which may be a sign of new variants having different genotype and change nucleotide sequence. Jahantigh *et al.* conducted a work to identify the infectious bronchitis virus with group-specific primers in Zabol, south-east of Iran. Their results showed that 36.36% of the sampled flocks were positive to IBV by RT-PCR [19]. It has been studied that any minor change in the nucleotide sequence of S protein interfere with the effect of vaccine which ultimately results in vaccine failure and leads to usual IBV outburst [20]. Vaccines that contain serotypes which differ in its antigenic and genotypic characters from the confront virus can cause better protection [21]. One possible clarification of the vaccination failure is the continuous emergence of new strains and serotypes of IBV [22]. Antigenic characterization of IBV isolates is important for selecting new and appropriate vaccines for the corresponding geographical regions [23]. In summary,

despite of available vaccine, IBV infection is still wide spread in KP, Pakistan. In addition, Massachusetts serotype is new

emerging IBV variant leading to vaccine failure.

Figure 1. Detection of IBV in different samples using RT-PCR

Figure 2. Presence of Massachusetts genotype showed on gel electrophoresis

Authors' contributions

Conceived and designed the experiments: Shabina, Sarzameen & SU Hayat, Performed the experiments: Shabina, Analyzed the data: Attaullah, Contributed materials/ analysis/ tools: S Gul, S Gul & Haseena, Wrote the paper: Attaullah.

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