African Journal of Agricultural Research Vol. 4 (10), pp. 1093-1096, October 2009
Available online at http://www.academicjournals.org/AJAR
ISSN 1991-637X © 2009 Academic Journals

Full Length Research Paper

Detection the 4/91 strain of infectious bronchitis virus in testicular tissue from experimentally infected rooster by reverse transcription-polymerase chain reaction

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Accepted 7 September, 2009

Avian infectious bronchitis (IB) was initially considered to be a disease primarily of the respiratory and female reproductive tract of chicken. Avian infectious bronchitis virus has wide and variable tissue tropisms. The important variant 4/91 strain has been isolated from the broiler breeder flocks. The Massachusetts serotype has been isolated in the epididymal region of the rooster reproductive tract. No detailed information is apparently available about the tropism of 4/91 strain of AIBV to the rooster reproductive tract. Therefore, we hypothesized that the rooster reproductive tract is a target for 4/91 strain. This study confirms that IBV 4/91 strain has a potential to infect the testicular tissue in rooster.

Key words: Infectious bronchitis virus, testicular tissue, rooster.

INTRODUCTION

Avian infectious bronchitis (AIB) was initially considered to be a disease of the respiratory and female reproductive tracts of chicken. The virus predominantly replicates in the upper respiratory tract followed by viraemic spread to various organs, e.g. kidneys, reproductive and lymphoid tissues (Dhinakar and Jones, 1997, Crinion et al. 1972a). Thus, it is clear that strains of avian infectious bronchitis virus (AIBV) have wide and variable tissue tropisms. The major economic loss is from ovarian damage and the precipitous and prolonged decrease in egg production in laying flocks (Cunningham, 1970). AIBV infects the oviduct and causes lesions, degeneration of the epithelial lining, and the formation of cysts. The epithelial lining of the female reproductive tract contains numerous ciliated cells, which are target for AIBV. The important variant strain of AIBV, 793/B (4/91), has been isolated from the broiler breeder flocks (Gough et al, 1972, Cuiping et al. 2007). The appearance and spread of the economically important variant 793/B (4/91) has given us a reminder of the need for more detailed understanding of the immunopathogenesis of variants with unusual tissue tropisms and disease manifestation. Villarreal et al. (2007) reported the detection of IBV and avian metapneumovirus (aMPV) in the testes of roosters from a Brazilian poultry broiler breeder's flock with epididymal stones and low fertility. Samples of testis, trachea, and lungs from breeder males aged 57 weeks were positive for IBV by reverse transcriptase-polymerase chain reaction (RT-PCR). But It seems that no detailed information is available about the tropism of 4/91 strain of AIBV to the rooster reproductive tract. Therefore, we hypothesized that the rooster reproductive tract is a target for 4/91 strain of AIBV.

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MATERIALS AND METHODS

Animals

In total, ninety one day old male broiler breeder chicks (Ross 308 strain) were used in this experiment. Chicks were divided into two groups (A and B) and housed in separated rooms by group. The roosters were raised until 16 weeks of age and were fed a commercial laying hen diet. Feed and water were provided ad libitum. Group A (vaccinated, n = 45) was inoculated intranasally with the attenuated live AIBV. Vaccination began at 2 weeks of age. The control group B (unvaccinated, n = 45) was inoculated with 0.1 ml of normal saline. All equipments, sheds, and clothing were fumigated or washed with antiseptic or ethanol before use.

Vaccine virus

The vaccine was used in this study contained the live attenuated AIBV. The commercial AIBV 4/91 type was used for this study and is available from Intervet (Millsboro, DE). Serial 0100002 IBVac-H® titer is $10^{4.5}$ EID_{50} viral particles per dose. The roosters were administrated one dose in a volume of 0.1 ml intranasally at ages 2, 6, 10 and 14 wk.

Sample collection

Blood (n = 3) was drawn from each group (A, B) beginning at 1 week of age at 2 weeks intervals continuing until 15 weeks of age. Blood samples were obtained by cardiac puncture on days 0, 7 and 21. Blood samples (n = 3) were taken from the wing vein on days 35, 49, 63, 77, 91 and 105. Serum was removed from each sample and stored at -70°C for determination of antibody titers to AIBV. roosters from each group were killed on 3, 7, 11 and 15 wk. The whole blood was collected in the presence of EDTA. The whole Blood samples were processed within a few hours of collection for RNA extraction. One testis was cut and placed in 10% neutral buffered formalin for histologic evaluation, whereas the other testis was used for RNA extraction. The trachea, cecal tonsil, kidney, testis whithout epididym and epididymal region samples from each chicken were taken and stored at -70°C for RNA extraction.

RNA extraction

Tissue samples were freezed in liquid nitrogen and broken into suitably sized pieces. Tissue samples were homogenized in 1 mL of a lysis buffer (Tissue RNA Prep Mate- Bioneer) transferred the homogenates to 1.5 ml tubes. Appropriate chloroform was added and stored samples for 5 min at 4°C. Tubes were centrifuged at 12000 rpm for 10 min, the aqueous phase was collected and precipitated RNA with 0.45 ml of isopropanol. The RNA precipitates were centrifuged for 5 min and washed once with 0.45 ml of 80% ethanol. The obtained RNA pellet was dried for 5 min and dissolved in 30 µl of sterile water. The RNA was stored at -70°C until further use.

Primer

Based on the DNA sequences of genomic segments S_1, of IBV 4/91 from GenBank, a pair of primers XCE_2/BCE_1 was used to amplify the S_1 glycoprotein gen (235 bp). The primer sequences were as followed $XCE2^-$, 5' CCTCTATAAACACCCTTGCA3' and BCE_1^+, 5'AGTAGTTTTGTGTATAAACCA3' (Adzhar, 1997).

Reverse transcription – polymerase chain reaction (RT-PCR)

Reverse transcription was carried out at 37, 38, 39, 40, 41 and 42^0C for 30 min for each temperature , using 8 µl of RNA sample , 3 µl primer ($XCE2^-$, Bioneer–Korea) and 1 µl Revert Aid ™ M-MULV reverse transeriptase (Fermentas). PCR was performed in MJ Mini ™ Gradient Thermal Cycler (Blo RAD – USA) with 3 µl as template in a total of 25 µl reaction volume containing 10 pmol of each primer and 1 U Tag DNA polymerase (Cinna Gen). Reactions were carried out at 94°C for 3 min followed by 30 cycles of 94 ^{0}C for 45s, 50 ^{0}C for 45s and 72 ^{0}C for 1 min. Finally 72^0C for 5 min.

Analysis and gel extraction of PCR products

To fractionate DNA fragments, 10 µl of PCR product was loaded onto 2.5% agarose gel and electrophoresed for 50 min in TAE buffer, containing 0.5 Mg/Ml ethidium bromide. DNA marker of 100 bp was as size marker for determination of the length of the amplified fragments. In order to develop a general method for PCR reaction, nine different preparations were run each time: (1) Blank sample that had all reagents without cDNA (2) Positive control that was prepared from purified vaccine virus. (3) Negative control that was prepared from epididymis region of unuaccinated rooster (Group B). 4, 5, 6, 7, 8 and 9 samples were trachea, cecal tonsil, testis without epididym, epididymal region, whole blood and kidney that were prepared from vaccinated roosters.

Histopathology

Tesits with epididymis was fixed in 10% neutral buffered formalin for 3 days and then processed for paraffin embedding. Sections were cut at 5 µm and stained with hematoxylin and eosin for histopathological examination.

RESULTS

AIBV anibody titer

All birds that were killed bled for measurement of IBV antibodies level in the serum using ELISA (Flock check IBV. ELISA test kit, IDEXX laboratories Inc., Westbrook, ME 049092, USA). All roosters of vaccinated group (Group, A) that had been immunized, developed anti AIBV antibodies at 16 weeks of age, whereas the roosters from the unvaccinated group (Group, B) remained negative for IBV antibodies throughout of the experiment.

Histopathology

No histopathological differences of tesits with epididymis were observed between the unvaccinated and vaccinated groups at any age. In both groups spermatogenesis was first seen in a few roosters at 12 weeks more than roosters at 15 weeks (Figure 1 and 2).

Detection of AIBV by PCR

To verify the presence of AIBV, total RNA was extracted from the tissue explants of rooster 3 - 15 weeks of age and reverse transcribed into cDNA. The cDNA from the explants was amplified with primers specific to the S_1 gene. Amplified products from cDNA from AIBV exposed trachea, cecal tonsil, kidney, testis without epididym and epididymal region were detected at the predicted size of 235 base pair. None of the samples from control birds were positive. The whole blood and testis without the epidiymal region samples of infected chickens did not have a visible 235 bp product. IBV could be detected in the trachea, cecal tonsils, kidneys ,testis without the epidiymal region and epididymal region samples from experimentally infected chickens by RT-PCR using the S1- gene oligonucleotide pair (Figure 3). The detection

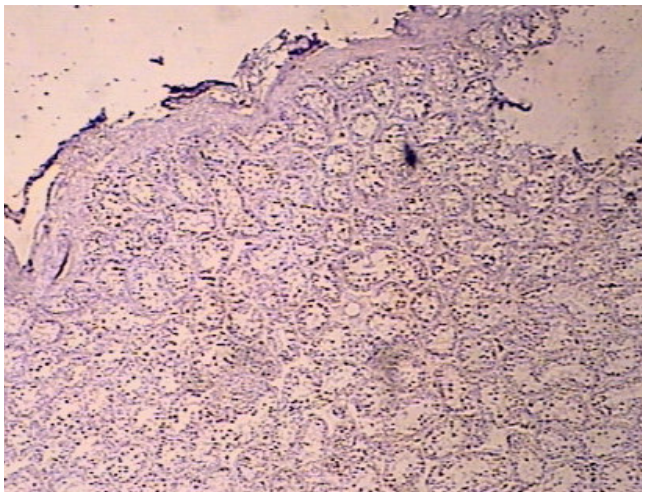

Figure 1. No histopathological differences of tesits were observed between the infected and uninfected groups at any age (H&E×200).

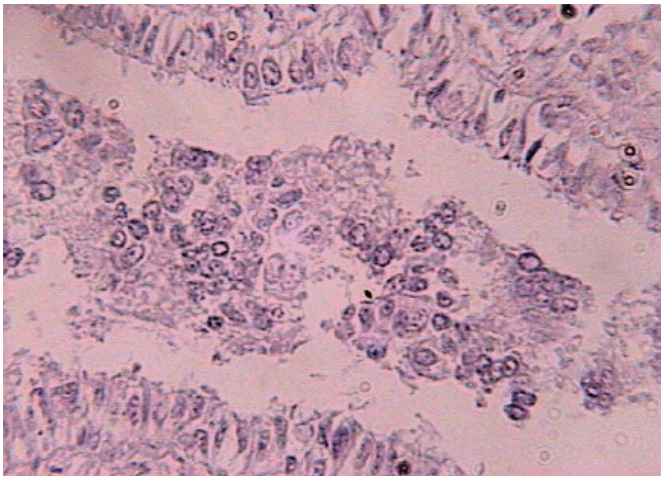

Figure 2. No histopathological differences of epididym region were observed between the infected and uninfected groups.(H&E×400)

rate varied between 42 % for cecal tonsils and 6% for testis without the epidiymal region samples (Table 1).

DISCUSSION

Infectious bronchitis is currently one of the major diseases in poultry production. The disease has occurred frequently in vaccinated and nonvaccinated flocks and caused severe economic losses in recent years in Iran uninfected roosters (Group B) were negative RT-PCR. The RT-PCR was performed on whole blood samples of infected roosters and not all of them were positive. The results of this study show that there has not been viremia at least during the time interval of sample collection. The (Haqshenas et al., 2005, Nouri et al., 2003; Seify abad

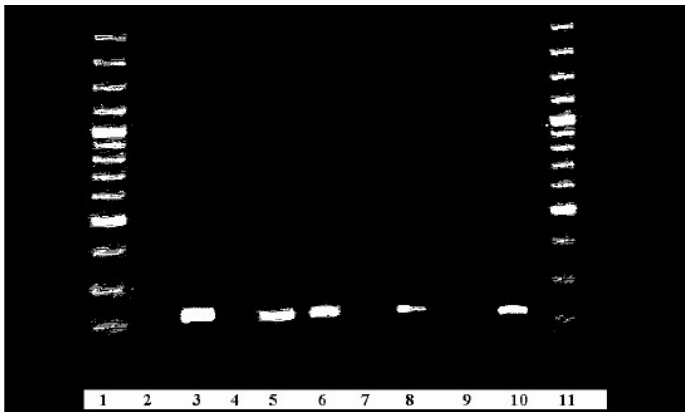

Figure 3. Detection of IBV by RT-PCR in different tissue samples from chicken that as infected by an attenuated IBV 4/91 strain viruse. Lane 1: Marker 100 pb, Lane 2: Blank (without cDNA), Lane 3: Positive control (IBV vaccine viruse), Lane 4: Negative control (group B; uninfected), Lane 5:Trachea, Lane 6: Cecal tonsil, Lane 7: Whole blood, Lane 8: Epididymal region, Lane 9: Testis without epididym, Lane 10: Kidney, Lane 11:Marker

Shapouri et al., 2002; Vasfi Marandi, 2000). The important variant strain of IBV, 4/91 was recently isolated from broiler and broiler breeder flocks in Iran (Seify abad Shapouri, 2002, Vasfi Marandi and M. H. Bozorgmehri Fard, 2000). The 4/91 strain has wide and variable tissue tropisms, include respiratory tract, gut tissues and pectoral muscles (Gough et al. 1992). All samples from detection rates were 42% , 39%, 25% , 19% and 6 % for cecal tonsils, trachea, Kidney , epididymal region and testis without the epidiymal respectively. RT-PCR examinations confirmed the presence of IBV in the epididymal region and testis. These observations contradict those previously reported, however, the roosters used in this experiment were young, compared to those used in other studies (Handberg et al. 1999, Boltz et al. 2004, Uletta et al. 2006, Boltz et al. 2007, Villarreal et al. 2007). The RT-PCR results, suggest that the 4/91 IBV strain is more enterotropic by virtue of its prolonged persistence in the cecal tonsil compared to the trachea. This fact was suggested earlier by Dhinakar and Jones, (1996) who reported 793/B (4/91) IBV strain was more enterotropic than pneumotropic and was associated with diarrhea Although, Darbyshire et al. (1976) reported that the IBV had a tropism for ciliated epithelium in epidiymal region but they had studied that avian infectious bronchitis virus was capable of infecting the epididymal region in vitro, whereas, we detected IBV directly from the male reproductive tract and other tissues. Also Villarreal et al. (2007) detected the IBV in the testis of rooster from a Brazilian poultry broiler breeder's flock by RT-PCR. They amplified a gen fragment that revealed a close relationship with European IBV genotype D274. But in the present study, 4/91 IBV strain was detected from male reproductive tract. The significant affinity of the live attenuated 4/91 IBV strain for the male reproductive tract

Table 1. IBV detected by S1-gene RT-PCR in whole blood, trachea, kidney, testis without epididymal region (Testis) and epididymal region (Epididym) tissues from experimentally infected chickens related to the day post-inoculation.

	Week 3		Week 7		Week 11		Week 15		Total	
Tissue	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Blood	0[a]/9[b]	0[a]/9[b]	0/9	0/9	0/9	0/9	0/9	0/9	0/36 (0%)	0/36 (0%)
Trachea	4/9	0/9	3/9	0/9	4/9	0/9	3/9	0/9	14/36(39%)	0/36 (0%)
Kidney	0/9	0/9	2/9	0/9	3/9	0/9	4/9	0/9	9/36 (25%)	0/36 (0%)
Cecal tonsil	1/9	0/9	5/9	0/9	6/9	0/9	3/9	0/9	15/36(42%)	0/36 (0%)
Testis	0/9	0/9	0/9	0/9	1/9	0/9	1/9	0/9	2/36 (6%)	0/36 (0%)
Epididym	0/9	0/9	2/9	0/9	2/9	0/9	3/9	0/9	7/36 (19%)	0/36 (0%)

A: Vaccinated group
B: Unvaccinated group
a: Number of positive preparations (chickens) by RT-PCR
b: The total number of preparations (chickens) examined.

suggests that it would be interesting to study effects of pathogen 4/91 IBV strain (nonattenuated virus) on the reproductive tract in rooster. It would still be difficult to confirm that the IBV can replicate in tissue cells in male reproductive tract, but IBV has a potential to infect the reproductive tract in rooster.

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