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Article

Detection of avian reovirus antibodies in layer birds of small scale commercial farms in Dinajpur district of Bangladesh

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Abstract: The present study was conducted on layer birds of different age groups to determine specific antibody titer level against avian reovirus (ARV) by indirect enzyme linked immunosorbent assay (iELISA) at Dinajpur district of Bangladesh. This study showed that ARV specific antibody positive cases were 84 out of 90 blood serum samples and the highest antibody titer was 26120 and lowest antibody titer was 288. The total 93.33% sera samples were showed positive result. The study showed that 100% sera sample were positive against ARV at 6 weeks of aged group and the highest, lowest and mean antibody titer were 13917, 4895 and 10269 respectively. On the other hand 88.88% sera sample were positive against ARV at 10 weeks of aged group and the highest, lowest and mean antibody titer were 9779, 288 and 5689.89 respectively. The sera sample collected from 14 weeks of aged group showed 88.88% positive and the highest, lowest and mean antibody titer were 11727, 871 and 5250 respectively. The sera sample collected from 18 weeks of aged group showed 88.88% positive against ARV and the highest, lowest and mean antibody titer were 24440, 1234 and 12648.89 respectively. The sera sample collected from 22 weeks of aged group were 100% positive against ARV and the highest, lowest and mean antibody titer were 26120, 1752 and 11373.89 respectively. The sera sample collected from 26 weeks of aged group showed 100% positive against ARV and the highest, lowest and mean antibody titer were 8566, 1630 and 4327.44 respectively. The sera sample collected from 30 weeks of aged group showed 100% positive against ARV and the highest, lowest and mean antibody titer were 13431, 1989 and 5890.56 respectively. The sera sample collected from 40 weeks of aged group showed 77.77% positive against ARV and the highest, lowest and mean antibody titer were 14618, 433 and 5103.22 respectively. The sera sample collected from 48 weeks of aged group showed 88.88% positive against ARV and the highest, lowest and mean antibody titer were 14553, 957 and 7436.5 respectively. In conclusion it is evident that avian reovirus-specific antibody was successfully detected through commercially available avian reovirus antibody test kit (ELISA kit) and the virus induced a significant antibody titer indicating the affecting virus was absolutely ARV.

Keywords: avian reovirus (ARV); indirect enzyme linked immunosorbent assay; antibody titer; layer birds

1. Introduction

Poultry is considered as an important source of animal protein all over the world. The production and consumption of eggs and poultry meat has been increasing worldwide over the last three decades as the consumption of eggs has doubled and that of chicken meat has tripled (Jordan and Pattison, 2001). In Bangladesh, poultry contributes a major share of animal protein simply because of the limitations and religious taboos in case of pork and beef. In Bangladesh people consumes the lowest percentage of protein than the minimum requirements because of inadequate supply of protein-generating food products.

According to the Food and Agriculture Organization (FAO), each person should take 56 kg of meat and 365 eggs every year. But in Bangladesh, per head intake of meat is only 11.27 kg and egg 30 per year. As a result, people suffer from malnutrition. Poultry meat and eggs provide approximately 38% total animal protein in the country (FAO, 1999). Poultry sector has a tremendous employment generating opportunity in reducing unemployment problem in Bangladesh and other countries of the world. Poultry meat now accounts for more than 30% of all meat consumed in Bangladesh. The world's average annual per capita poultry meat consumption is currently 9.5 kg. The country's pervasive poverty may limit the number of people who can afford to consume chicken as suggested by the simple relationship between per capita GDP and chicken consumption. If population growth continues at this rate, protein deficiency will rise (Islam *et al.*, 2014). The magnitude of the contribution of the livestock sub-sector to the country's gross domestic product (GDP) is 3.1 percent and to agricultural GDP it is about 11 percent.

Commercial poultry industry is growing rapidly in Bangladesh. Estimates show that poultry population is increasing at the rate of 6.5% per year in the country. There are approximately 38000 commercial poultry farms housing 12410000 layers and 107845000 broilers in Bangladesh according to the census report 2006, completed by the Department of Livestock Services (DLS) and the Poultry Sector Development Project (PSDP).

The development of poultry industry has been seriously threatened by the outbreaks of acute, contagious and fatal diseases (Ali, 1994). Among the infectious diseases like Newcastle disease, Gumboro, Infectious Bronchitis, Collibacillosis, Salmonellosis, Fowl Cholera, Avian Influenza, Avian reo virus and Mycoplasmosis outbreak occurs. Avian reovirus is the most important disease of chickens that reduce the egg production. It is economically important to the poultry industry worldwide due to increased susceptibility to other diseases and negative interference with effective vaccination.

Avian reoviruses have a worldwide distribution in different species of birds and are associated with viral arthritis/ tenosynovitis, malabsorption syndrome, stunting/ runting syndromes, enteric disease, immunosuppression and respiratory disease in poultry (Jones and Kibenge, 1994). Reoviruses are non-enveloped double-stranded RNA viruses with a segmented genome and vary between 75 and 80 nm in diameter. They are not always pathogenic and have been found on routine examination in apparently healthy poultry (Robertson *et al.*, 1984). Avian reoviruses may cause serious disease in birds; especially in poultry they can cause important losses (Tang *et al*., 1987).

Avian reoviruses (ARV) are members of the Orthoreovirus genus in the family Reoviridae (Eric Guhyun Nham, 2013). The name reovirus derives from the acronym for Respiratory Enteric Orphan, because they were first isolated from these sites in humans with initially no apparent association with disease. In chickens, the most recognized form of ARV associated diseases is tenosynovitis also known as viral arthritis characterized by swelling in the hock joint. Depending on the degree of severity of the inflammation, an affected bird may be unable to move towards feed and water resulting in poor growth or death. Birds that survive to slaughter may be downgraded because of inflamed hock joints. Avian reoviruses are also associated with a variety of other diseases in chickens, such as respiratory and enteric disease, hydropericardium, pericarditis, myocarditis, and hepatitis. Many of these diseases affect chicken producers financially due to production losses, poor feed conversion, increased culling, and carcass condemnations. Thus, in modern agricultural industries where high-tech production and cutting-edge research go hand-in-hand to optimize productivity, control and prevention of these production-limiting diseases must be of utmost importance.

The initial avian reovirus was isolated by Fahey and Crawley in 1954 from the respiratory tract of chickens (Closas *et al.*, 1986). This isolate produces viral arthritis/tenosynovitis when inoculated into chickens (Clark, 2003). In field situation viral arthritis is seen primarily in meat type strains of chickens, but has been reported in egg type chickens and turkeys. While birds are usually affected with the disease at 4-8 weeks of age, older birds can also be affected naturally and younger birds experimentally. As would be expected, birds with the disease, varying degrees of lameness are a typical sign of the disease. Some birds may also be stunted in size. The lesions observed are swelling and inflammation of the hock joint and tendon sheath with a yellow colored fluid present in the hock. The fluid may be tinged with blood or occasionally it contains purulent (pus) exudates. As the inflammation progresses over time; scar tissue forms and may fuse tendons and sheaths together. Bones of the joint may also become eroded or pitted and rupture of the gastrocnemius tendon may be present.

In avian reovirus infection Sigma C protein was involved in induction of neutralization antibody. Monoclonal antibody may therefore be useful for the development of an antigen-capture Enzyme-Linked Immuno Sorbent assay for rapid detection of field isolates. Avian reovirus infections are typically subclinical in weaned mice,

and are best detected using serologic tests like Enzyme-Linked Immuno Sorbent Assay (Wright *et al*., 2004). The occurrence of avian reovirus in Bangladesh detected on June 1997 in Animal Health Research Division, Bangladesh Livestock Research Institute (Islam *et al.*, 2003). The present study was conducted with a view to determine the specific antibody titer level against ARV of chickens in small scale commercial layer farm by using indirect ELISA.

2. Materials and Methods

The research work was conducted in the Virology Laboratory of the Department of Microbiology, HSTU, Dinajpur, Bangladesh during the period of January to June, 2014.

2.1. Collection, transportation and preparation of samples

For detection of antibody titer, a total of 90 blood samples were collected from the selected layer bird of different small scale layer farm having average population 1000 that were situated at Sadar upazilla under Dinajpur district of Bangladesh. The birds were categorized into ten age groups. Group A1 included birds aged 6 weeks, group A2 included aged 10 weeks, group A3 included aged 14 weeks, group A4 included aged 18 weeks, group A5 included aged 22 weeks, group A6 included aged 26 weeks, group A7 included aged 30 weeks, group A8 included aged 40 weeks, group A9 included aged 48 weeks and finally group A10 included aged 60 weeks. The blood samples were collected aseptically from the wing vein using 3 ml disposable sterile syringe. Soon after collection of blood the syringes with blood were kept slantly at 4-8°C for overnight, so that blood can clot in one side of the syringe. Then the clotted blood was removed carefully with sterile needle and sera were poured into sterilized graduated centrifuge test tubes and shipped to the laboratory in ice box 3 hours after collection. For each syringe, individual needle was used. The sera were subjected to centrifugation at 1000 rpm for 10 minutes for purification. Then the clear sera were collected and kept in clean sterilized Eppendorf tubes and stored at -20°C for performing the indirect ELISA (iELISA).

2.2. Detection of the antibody titer level

ARV antibody test kit manufactured by Proprietario e Fabricante [BioCheck (UK) Ltd.] was used for the estimation of antibody titer. The indirect enzyme-linked immunosorbent assay (iELISA) was performed according to the manufacturer's instruction using ARV pre coated plates and pre-diluted, ready to use reagents and buffer. In case of iELISA, the titer was predicted from the absorbance value of 1:500 dilution of a serum using the formula supplied with the kit. To make substrate reagent, 1 tablet was added to 5.5 ml substrate buffer and was allowed to mix for 3 minutes or until fully dissolved. The prepared reagent was made on day of use. One wash buffer sachet was emptied and mixed into one liter of distilled water and allowed to dissolve fully by mixing. All other kit component were ready to use but were allowed to adjust the room temperature. The test samples were diluted to 1:500 by adding 1 µl to 0.5 ml of sample diluents. The mixture of the tube was mixed well by vortexing or shaking. The fresh Eppendorf tube was used for each separate sample.

ARV coated plate was removed from sealed bag and recorded location of samples on template. 100 µl of negative control was added into wells A1 and B1. 100 µl of positive control was added into wells C1 and D1. Then 100 µl of diluted samples were added into the appropriate wells and the plate was covered with lid and incubated at room temperature (22-27°C) for 30 minutes. The contents of wells was aspirated and washed 4 times with wash buffer (350µl per well). The plate was inverted and tapped firmly on absorbent paper. Then 100 µl of Conjugate reagent added into the appropriate wells. Then the plate was covered with lid and incubated at room temperature (22-27°C) for 30 minutes. The procedure was repeated as in previous. Then 100 µl of Substrate reagent was added into the appropriate wells and the plate was covered with lid and incubated at room temperature (22-27°C) for 15 minutes. Then 100 µl of Stop solution was added to appropriate wells to stop reaction. The ELISA plate was read by the microtiter plate reader in the Virology Laboratory, Department of Microbiology, HSTU, Dinajpur and recorded the absorbance of controls and samples by reading at 405 nm. For the test result to be valid the mean negative control absorbance should read below 0.30 and the difference between the mean negative control and the mean positive control should be greater than 0.15. The ARV positive control has been carefully standardized to represent significant amounts of antibody to ARV in chicken serum. The relative amounts of antibodies in chicken samples can then be calculated by reference to the positive control. This relationship is expressed as S/P ratio (Sample to Positive Ratio). Samples with an S/P of 0.2 or greater contain anti-ARV antibodies were considered positive.

Calculation of S/P ratio

$$\frac{\text{Mean of Test Sample - Mean of negative control}}{\text{Mean of Positive control - Mean of negative control}} = \text{S/P}$$

Calculation of antibody titer

The following equation relates the S/P of a sample at a 1:500 dilution to an end point titer.

Log_{10} Titer = 1.0 * Log (SP) + 3.156

Antilog = Titer

S/P value	Titer Range	Antibody status
0.184 or less	1234 or less	Negative
0.237 or greater	1630 or greater	Positive

3. Results and Discussion

3.1. iELISA antibody titer level determination from collected sera samples

Collectively, 93.33% of the sera samples were positive for antibody by iELISA (Table 1).

3.2. Detection of antibody titer level from Group A1

The results of the sera collected from 6 weeks of aged birds showed that highest titer was 13917 and the lowest titer 4895 and the mean titer was 10269. The detailed results are shown in Table 2.

3.3. Detection of antibody titer level from Group A2

The results of the sera collected from 10 weeks of aged birds showed that highest titer was 9779 and the lowest titer 288 and the mean titer was 5689.89. The detailed results are shown in Table 3.

3.4. Detection of antibody titer level from Group A3

The results of the sera collected from 14 weeks of aged birds showed that highest titer was 11727 and the lowest titer 871 and the mean titer was 5250. The detailed results are shown in Table 4.

3.5. Detection of antibody titer level from Group A4

The results of the sera collected from 18 weeks of aged birds showed that highest titer was 24440 and the lowest titer was 1234 and the mean titer was 12648.89. The detailed results are shown in Table 5.

3.6. Detection of antibody titer level from Group A5

The results of the sera collected from 22 weeks of aged birds showed that highest titer was 26120 and the lowest titer 1752 and the mean titer was 11373.89. The detailed results are shown in Table 6.

3.7. Detection of antibody titer level from Group A6

The results of the sera collected from 26 weeks of aged birds showed that highest titer was 8566 and the lowest titer 1630 and the mean titer was 4327.44. The detailed results are shown in Table 7.

3.8. Detection of antibody titer level from Group A7

The results of the sera collected from 30 weeks of aged birds showed that highest titer was 13431 and the lowest titer 1989 and the mean titer was 5890.56. The detailed results are shown in Table 8.

3.9. Detection of antibody titer level from Group A8

The results of the sera collected from 40 weeks of aged birds showed that highest titer was 14618 and the lowest titer 433 and the mean titer was 5103.22. The detailed results are shown in Table 9.

3.10. Detection of antibody titer level from Group A9

The results of the sera collected from 48 weeks of aged birds showed that highest titer was 14553 and the lowest titer 957 and the mean titer was 7436.5. The detailed results are shown in Table 10.

Table 1. Results of antibody titer of ARV suspected sera samples from the layer birds of different aged group.

SL. No.	Farms with area	Groups	Age of bird(weeks)	No. of samples	No. of Positive titer	% of positive cases (total)
01.	Jahid poultry Farm, pollibiddut, Dinajpur	A1	6	9	9	93.33%
02.	Sobus Layer Farm, Jamtoli, Dinajpur	A2	10	9	8	
03.	Lotifur Layer Farm, Ramdubi, Dinajpur	A3	14	9	8	
04.	Khalek poultry Farm, Ramdubi, Dinajpur	A4	18	9	8	
05.	Danesh Poultry Farm, Basherhat , Dinajpur	A5	22	9	9	
06	Manik Layer Farm, Ranigonj, Dinajpur	A6	26	9	9	
07	Shohid Layer Farm, Rajbari, Dinajpur	A7	30	9	9	
08	Safiqul Layer Farm Gopalgonj, Dinajpur	A8	40	9	7	
09	Mokbul Layer Farm, Chehalgazi , Dinajpur	A9	48	9	8	
10	Matasagar Poultry Farm, Rajbari, Dinajpur	A10	60	9	9	
	Total			90	84	

Table 2. Serum antibody titer of ARV suspected field sera samples from Group A1.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Jahid poultry Farm, pollibiddut, Dinajpur	6	01	7282	+	4895-13917	10269	100%
		02	13917	+			
		03	9467	+			
		04	11474	-			
		05	4895	+			
		06	13001	+			
		07	12891	+			
		08	8848	+			
		09	10646	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 3. Serum antibody titer of ARV suspected field sera samples from Group A2.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Sobus Layer Farm, Jamtoli, Dinajpur	10	01	288	-	288-9779	5689.89	88.88%
		02	6488	+			
		03	5323	+			
		04	4770	+			
		05	8998	+			
		06	2827	+			
		07	3844	+			
		08	9779	+			
		09	8892	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 4. Serum antibody titer of ARV suspected field sera samples from Group A3.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Lotifur Layer Farm, Ramdubi, Dinajpur	14	01	3689	+	871-11727	5250	88.88%
		02	6308	+			
		03	10457	+			
		04	871	-			
		05	2089	+			
		06	1836	+			
		07	8583	+			
		08	11727	+			
		09	1691	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 5. Serum antibody titer of ARV suspected field sera samples from Group A4.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Khalek poultry Farm, Ramdubi, Dinajpur	18	01	18031	+	1234-24440	12648.89	88.88%
		02	11510	+			
		03	14378	+			
		04	2740	+			
		05	17966	+			
		06	15851	+			
		07	7690	+			
		08	1234	-			
		09	24440	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 6. Serum antibody titer of ARV suspected field sera samples from Group A5.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Danesh Poultry Farm, Basherhat, Dinajpur	22	01	18333	+	1752-26120	11373.89	100%
		02	12791	+			
		03	26120	+			
		04	16802	+			
		05	2385	+			
		06	1752	+			
		07	13248	+			
		08	5856	+			
		09	4578	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 7. Serum antibody titer of ARV suspected field sera samples from Group A6.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Manik Layer Farm, Ranigonj, Dinajpur	26	01	8478	+	1630-8566	4327.44	100%
		02	8566	+			
		03	2875	+			
		04	6086	+			
		05	1630	+			
		06	3292	+			
		07	2307	+			
		08	3171	+			
		09	2542	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 8. Serum antibody titer of ARV suspected field sera samples from Group A7.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Shohid Layer Farm, Rajbari, Dinajpur	30	01	9352	+	1989-13431	5890.56	100%
		02	5096	+			
		03	3187	+			
		04	8372	+			
		05	2346	+			
		06	1989	+			
		07	3885	+			
		08	5357	+			
		09	13431	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 9. Serum antibody titer of ARV suspected field sera samples from Group A8.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Safiqul Layer Farm, Gopalgonj, Dinajpur	40	01	3567	+	433-14618	5103.22	77.77%
		02	5138	+			
		03	1912	+			
		04	433	-			
		05	3812	+			
		06	1153	-			
		07	1920	+			
		08	13376	+			
		09	14618	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 10. Serum antibody titer of ARV suspected field sera samples from Group A9.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Mokbul Layer Farm, Chehalgazi, Dinajpur	48	01	8910	+	957-14553	7436.5	88.88%
		02	2661	+			
		03	14553	+			
		04	5189	+			
		05	957	-			
		06	10010	+			
		07	7221	+			
		08	10682	+			
		09	6755	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

Table 11. Serum antibody titer of ARV suspected field sera samples from Group A10.

Name of farm and area	Age of the Bird (week)	Sample No.	Antibody titter	Interpretation	Titer range	Mean titer	Percent Positive (%)
Poultry Rajbari, Matasagar Farm, Dinajpur	60	01	7795	+	2448-22003	7926.33	100%
		02	3527	+			
		03	2740	+			
		04	2448	+			
		05	8180	+			
		06	10798	+			
		07	5763	+			
		08	22003	+			
		09	8083	+			

Legend

Titer range	**Antibody status**
1234 or less	Negative
1630 or greater	Positive

3.11. Detection of antibody titer level from Group A10

The results of the sera collected from 60 weeks of aged birds showed that highest titer was 22003 and the lowest titer 2448 and the mean titer was 7926.33. The detailed results are shown in Table 11.

The present study further revealed that a total 93.33% sere samples were positive for ARV antibodies related to the findings of Yuan *et al.*, 2011 (92.23%) and Juan *et al.*, 2008 (over 92%) which observed on layer chicken and higher than Roussan *et al.*, 2013 (21.4%) which observed on commercial broiler chicken and Owoade *et al*, 2006 (41%) which observed on breeder, broiler, pullet, layer and cockerel flock. Healthy birds can harbor the reovirus without exhibiting clinical signs and the virus is relatively resistant to certain disinfectants; for example, one strain survived 2% formaldehyde at 4^0C (Meulemanns *et al.*, 1982), another was only partially inactivated by 2% phenol after 24 hour at room temperature, but 100% ethylalcohol was effective (Petek *et al.*, 1967). Both vertical and horizontal transmissions of avian reoviruses are recognized. Egg transmission has been confirmed after experimental infection (Al-Mufarrej *et al.*, 1996) but the rate of transmission is probably very low in nature. Congenitally infected chicks are thought to act as a nucleus of infection for the rest of the hatch, since most are likely to become infected via the faecal-oral route (Jones and Onunkwo, 1978) although infection via the respiratory tract may also occur. In addition, reoviruses may enter broken skin of the feet of chicks from the litter and become established in the hock joints (Al-Afaleq and Jones, 1990). Therefore, avian reovirus infection associated with poor feed conversion and flock uniformity, reduced weight gain, dropped in egg production and somewhat lameness occurred. The highest antibody titer was found 26120 whereas the

lowest antibody titer was found 288 among the all sera sample. The highest mean titer was found in group A4 as 12648.89 of 18 weeks aged birds.

4. Conclusions

From the findings of the present research ARV antibodies were successfully detected from the field outbreak through commercially ARV antibody test kit (ELISA kit) and the virus induced a significant antibody titer indicated that the affecting virus was absolutely ARV. ARV infections resulting in decreased productivity and important economic loss need protective initiative for combating the resulted cases. Further studies are needed in order to assess the real impact of reovirus infection in birds in other parts of Bangladesh and also on vaccination against ARV infection.

Conflict of interest

None to declare

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