

OCCURRENCE OF PASTEURELLOSIS AND NEWCASTLE DISEASE IN INDIGENOUS CHICKENS IN SIRAJGONJ DISTRICT

S. M. S. H. Belal

Veterinary Surgeon, District Veterinary Hospital, Sirajgonj

ABSTRACT

A study was carried out on indigenous layer chickens to know occurrence of Avian Pasteurellosis (AP) and Newcastle disease (ND) in Sirajgonj during the period from January/2012 to December/2013. The clinical signs showed before death was recorded by taking history and the birds were subjected to post mortem examination. In addition to the clinical and necropsy findings, ND was detected by Anigen® rapid antigen detection kit. The AP was confirmed by isolation and identification of *Pasteurella (P.) multocida* from liver, spleen and heart samples. The *P. multocida* was found to grow on nutrient broth, nutrient agar, blood agar and Eosin Methylene Blue agar where it produced whitish, opaque, round, flat, translucent colonies. It produced turbidity on nutrient broth. The organism was not found to grow on MacConkey agar media and did not cause hemolysis on blood agar media. The impression smear of liver and heart blood were stained by Gram's staining, Leishman staining and Methylene blue staining techniques to detect *P. multocida*. *P. multocida* organism was also found to ferment dextrose, lactose and mannitol with the production of only acid but did not ferment maltose and lactose. *P. multocida* was found non-motile, indole positive and urease negative. On triple sugar iron test it produced H₂S and fermented only glucose. It was found negative to both methyl red test and Voges Proskauer test. Out of 360 birds tested 59.72% (215) was AP positive and 40.28% was ND positive. Age, Sex, season and mortality due to other diseases were not considered.

Key words: Indigenous chicken, Avian pasteurellosis, Newcastle disease, mortality

INTRODUCTION

Indigenous chickens, otherwise known as traditional or backyard chickens, are local breeds of chickens (*Gallus gallus domesticus*) reared in rural areas of most parts of the world (Say, 1987). In these areas they are kept mostly for meat, a source of protein (Gueye, 1998). Usually there is very little nutritional supplementation and no proper housing is provided for the chickens. They range freely, scavenging for food and water (Sonaiya *et al.*, 1999). Bangladesh is an agro-based country having poultry density of 1460 birds/sq. kilometer of which 50% is backyard poultry (Dolberg, 2008 and Biswas *et al.*, 2008) as people rear traditionally. Commercial poultry favoring the term 'Poultry industry' plays an important role which started practically during 1980s in Bangladesh (Huque, 2001). There has been a tremendous development of this sector in the country (Rahman, 2004), but the contribution of backyard poultry has been started from the ancient period. In rural area of Bangladesh, about 80-90% household rear poultry and it is important not only as a source of income for majority of women among poor but also as the context of many social and cultural activities (Sultana, 2009). Although indigenous chickens are generally of low body weight, they can be a valuable source of tasty meat and eggs. There is scanty information on the health status and productivity of indigenous chickens. Newcastle disease (ND), also known as Ranikhet disease (RD), is a highly contagious viral disease that attacks many species of domestic and wild birds (Al-Garib *et al.*, 2003). The causal agent is the Newcastle disease virus (NDV) which is a negative sense single-stranded RNA virus belonging to the family Paramyxoviridae. The strains are classified into highly virulent (velogenic), intermediate (mesogenic) or avirulent (lentogenic) based on their pathogenicity in chickens (Beard and Hanson, 1984). The major clinical signs of ND are depression, weakness, loss of appetite, dehydration, inability to stand, cyanosis of comb and wattle, greenish watery diarrhoea, nasal and eye discharges, decreased egg production, loss of weight followed by death (Pazhanivel *et al.*, 2002). Gross lesions are petechial hemorrhages and ulcers with raised borders on the mucosa of proventriculus, pneumonic lungs, and hemorrhages in trachea, air sacs, brain and spleen (Pazhanivel *et al.*, 2002). The infection causes up to 40-60% mortality in commercial poultry population (Chowdhury *et al.*, 1985). Fowl cholera is a contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida* which hamper the profitable poultry production (Raji *et al.*, 2010). The disease is also known as avian cholera, avian pasteurellosis, and avian hemorrhagic septicemia (Richard and Pocard, 1998). It usually appears as a septicemic disease associated with high morbidity and mortality, but chronic or benign conditions often occur. The clinical signs of fowl cholera were anorexia, fever, ruffled feathers, mucus discharge from mouth, rapid respiration and diarrhea which was watery to yellowish initially and greenish with mucus finally (Rhoades and Rimler, 1990).

*Corresponding e-mail address: shariful.belal@yahoo.com

The gross lesions of fowl cholera are extensive congestion, enlarged and necrotic foci on spleen, necrotic parenchymatous hepatitis, congestion and hemorrhages in intestinal mucosa, petechial hemorrhages on the pericardium with serofibrinous pericarditis (Sharma *et al.*, 1974). The organism were Gram negative, non motile, non-spore forming rod occurring singly or pairs and occasionally as chains or filaments in Gram's-staining method. The organisms show bipolar character in Leishman's stain. The bacterium grows well on blood agar, nutrient agar and nutrient broth media and produce whitish, opaque, circular and translucent colonies with no hemolysis on blood agar media (Buxton and Fraser, 1977). The isolates consistently produced acid from dextrose, sucrose and mannitol but not fermented maltose or lactose. Biochemical characteristics of the organism are performed by fermentation reaction, Methylene Red (MR), Voges Proskauer (VP) and Indole test.

Newcastle disease (ND) and Fowl cholera cause approximately 40-60% and 25-35% mortality respectively in commercial poultry population (Chowdhury *et al.*, 1985). There was no clear and adequate data on the mortality caused by these two important diseases in backyard poultry. As a result it was very difficult both to estimate the economic loss and to take necessary action to combat it. So I planned to conduct a study on backyard poultry to detect this mortality.

MATERIALS AND METHODS

A comparative study between the clinical and laboratory diagnoses of Newcastle and Avian Pasteurellosis of poultry of Sirajgonj district was conducted during the period from January/2012 to December/2013 in the laboratory of District Veterinary Hospital, Sirajgonj

Sample collection

A total of 360 dead birds were submitted to the laboratory of the district veterinary hospital, Sirajgonj. The birds were subjected for postmortem examination and liver, spleen and heart samples were collected for isolation and identification of *Pasteurella multocida*. Tracheal swab was taken as sample to perform the Anigen® Rapid Antigen Detection Test (Chromatographic immunoassay) for the Newcastle disease to be detected. Clinical findings and post mortem lesions were recorded during collection of sample.

Clinical diagnosis of diseases

Clinical diagnosis was made on the basis of clinical history from the owners of the birds, recorded clinical signs of the remaining sick birds and post mortem lesions of dead birds.

Laboratory diagnosis of diseases

Rapid kit test (Chromatographic Immunoassay) for Newcastle disease

The samples were subjected to Anigen® Rapid Antigen Test Kit (Chromatographic immunoassay) for the detection of Newcastle disease antigen. Samples giving positive results (Figure 4) were then sent to the FDIL (Field Disease Investigation Laboratory) and CDIL (Central Disease Investigation Laboratory) for the confirmation of the results. As, for the interpretation of test results, positive reactions were indicated by the appearance of two distinct red lines (Figure 4), the chances of misinterpretation were little or absence. Among 20 samples sent for confirmation of diagnosis, all were found to be the same as found in District Veterinary Hospital, Sirajgonj. So the kit test was reliable and the kit had, as defined by the manufacturer, high degree of accuracy.

Isolation and identification of organism for Avian Pasteurellosis

Used media and biochemical reagents

Culture media

Nutrient broth (NB), Nutrient agar (NA), EMB agar, MacConkey agar and Blood agar (BA) media were used. Yeast extract and beef extract were also used with NB.

Chemicals and reagents

Gram's iodine, Leishman's stain, Methylene Blue stain, Hydrogen peroxide (3% H_2O_2), Sodium chloride (NaCl), Potassium chloride (KCl), Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 \cdot 12H_2O$), Potassium di-hydrogen phosphate (0.2M, $KH_2PO_4 \cdot 2H_2O$), Dehydrated sodium citrate, Phosphate buffered saline (PBS), Alsever's solution, Tannic acid solution, Acetone alcohol, Crystal violet, and Safranin were used.

Reagents for biochemical tests

Different types of sugar media such as Sucrose, Glucose, Dextrose, Maltose, Lactose, and Mannitol were used in this study. Methyl Red (MR), Voges-Proskauer (VP), Indole, Triple Sugar Iron (TSI) and Motility Indole Urease (MIU) were also used.

Isolation and identification of the organisms

P. multocida organisms were cultured according to the standard method described by Cowan (1985). The collected organisms were inoculated in BA, NA, MacConkey agar, EMB agar and NB enriched with yeast extract and beef extract for better growth. The inoculating media was incubated at 37°C in bacteriological incubator for characteristic colony formation. Subsequent subculture was performed for getting pure culture.

Morphological characterization

Gram's staining

Gram's staining was performed according to the method described by Merchant and Packer (1967) and observed for the staining characteristics of the organism.

Leishman's staining

Bacterial smear was prepared and fixed by gentle heating. The smear was flooded by Leishman's stain. Then an equal amount of distilled water was added. The mixture of stain and water was kept blowing for 5 minutes with the help of a pipette. The preparation was then washed with water, blotted, dried in air and examined under microscope.

Methylene blue staining

Bacterial smear was prepared and fixed by gentle heating. The smear was flooded by methylene blue stain. The slide was then allowed for 1 minute. The preparation was then washed with water, blotted, dried in air and examined under microscope.

Determination of motility of the bacteria

Motility test of the bacteria was performed by hanging drop slide preparation as described by Merchant and Packer (1967) and was observed for the motility of the organism.

Identification of isolated Pasteurella organism by using specific biochemical tests

Fermentation reaction with five basic sugars

The organism was subjected for fermentation reaction with five basic sugars such as dextrose, sucrose, lactose, maltose and mannitol and results were observed.

Methyl red (MR) test

The test was performed by inoculating a colony of the test organism and result was observed for the appearance of a bright red color which is an indication of acidity.

Voges-Proskauer (VP) test

Voges-Proskauer (VP) test was done with the organism and was observed closely for the slow development of a pink color for positive cases.

Indole test

The organism was subjected to Indole test and was observed after 1 minute for a red color in the reagent.

Motility Indole Urease (MIU) Test

Motility Indole Urease (MIU) test of the organism was done and observed for turbidity (motility), red ring (Indole) and urease production.

Triple Sugar Iron (TSI) agar slant

Triple Sugar Iron (TSI) agar slant reaction was carried out with suspected colonies of organism and the results were observed for the appearance of alkaline reaction (red) in slant, acidic reaction (Yellow) in the butt and H₂S production (black).

RESULTS

Clinical history and signs

The recorded clinical history and signs were drowsiness, whitish diarrhea and leg and neck paralysis in case of ND. Whereas anorexia, fever, ruffled feathers, mucus discharge from mouth, rapid respiration and diarrhea (watery to yellowish initially and greenish with mucus finally) were recorded in case of AP.

Post mortem examination of chickens

Gross lesions were petechial hemorrhages and ulcers with raised borders on the mucosa of intestine and proventriculus, pneumonic lungs, and hemorrhages in trachea, air sacs, brain and spleen in the samples suspected for Newcastle disease (Figure 1 and 2).

The samples suspected for Avian Pasteurellosis showed extensive congestion, enlarged and necrotic foci on liver, spleen, necrotic parenchymatous hepatitis, congestion and hemorrhages in intestinal mucosa, lungs, petechial hemorrhages on the pericardium with serofibrinous pericarditis (Figure 3 and 4).

Rapid kit test (Chromatographic Immunoassay) for Newcastle disease

The samples were subjected to Anigen® Rapid Antigen Test Kit (Chromatographic immunoassay) for the detection of Newcastle disease antigen. Among 360 samples, 145 were found positive (Figure 5) to Newcastle disease (40.27%). Samples sent to the FDIL (Field Disease Investigation Laboratory) and CDIL (Central Disease Investigation Laboratory) for confirmation of the results, revealed positive.

Cultural examinations for Avian Pasteurellosis

On nutrient broth

The growth of *P. multocida* in NB was characterized by diffused turbidity of the medium (Figure 6) and in few occasions pellicle was formed.

On nutrient agar

The presumptive *P. multocida* produced small colonies on NA media. The other characteristics of these colonies included whitish, discrete, opaque, circular and translucent in appearance (Figure 7).

On blood agar

The presumptive *P. multocida* produced small colonies on BA media. The other characteristics of these colonies included whitish, discrete, opaque, circular and translucent in appearance. No hemolysis was noticed on BA media (Figure 8).



Figure 1. Hemorrhages in the proventriculus



Figure 2. Hemorrhages in the intestinal mucosa



Figure 3. Necrotic foci in the liver



Figure 4. Hemorrhage and congestion of the lungs

On MacConkey agar

Culture on MacConkey agar plates yielded no colonies (Figure 9).

On EMB agar

Culture of *P. multocida* on EMB agar yielded small, circular, smooth, convex, translucent, glistening colonies which had a tendency to coagulase. Metallic sheen was absent (Figure 10).

Staining characteristics

Gram's staining technique

The organisms were Gram negative, cocco-bacillary or short rod shaped and bipolar generally arranged singly or in pairs (Figure 11).

Leishman's staining technique

Rod shaped bacteria with bipolar appearance were observed after Leishman's staining of fresh culture of the organisms, which was indicative of *P. multocida*. The rod shaped Gram negative organisms arranged singly, paired or in short chain indicated *E. coli*, *Staphylococci* were Gram positive and arranged in cluster.

Methylene blue staining technique

The organisms were appeared blue with bipolar character having coccobacillary shape.

Fermentation reaction with five basic sugars

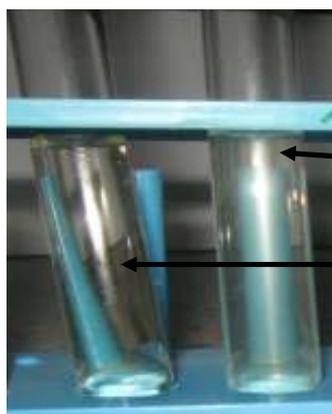
All the isolates fermented dextrose, sucrose and mannitol producing only acid. No fermentation reaction was seen with lactose and maltose. Acid production was indicated by the color change from reddish to yellow.



Figure 5a: Rapid kit test (Negative result)



Figure 5b: Rapid kit test (Positive result)



Turbidity due to growth of organism

Control

Figure 6. Growth of organism in Nutrient Broth

TSI slant reaction

In the stab culture of TSI agar, the isolated *Pasteurella* organisms produced an alkaline reaction (red) in slant and acid reaction (yellow) in the butt and slightly black color due to H_2S production (Figure 14).

Indole test

There is development of yellow colored ring with all the isolates indicating the positive test (Figure 15).



Figure 7. Growth of organisms in Nutrient agar media



Figure 8. Growth of Organism in Blood agar Media

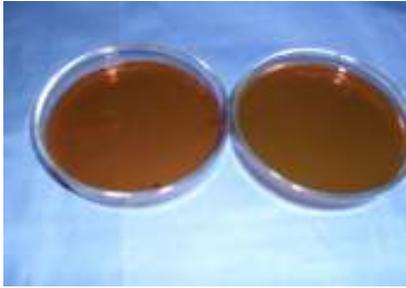


Figure 9. No growth of Organism in MacConkey agar media

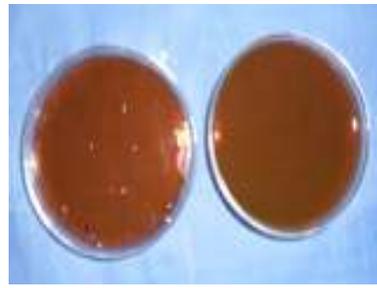


Figure 10. Growth of organisms in EMB agar media



Gram negative organism

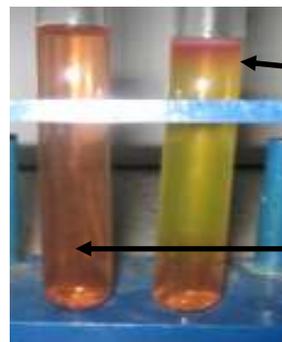
Figure 11. Gram stain showing organisms



No Color Change
VP (-ve)

Control

Figure 12. VP test



Red colour
ring
Indole (+ve)

Control

Figure 13. MIU test

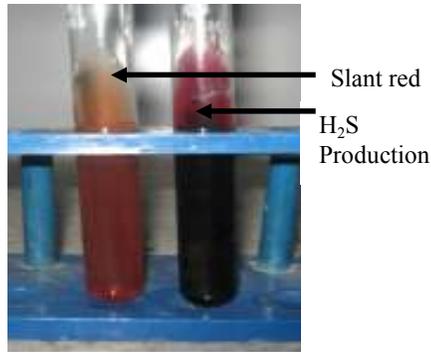


Figure 14. TSI test

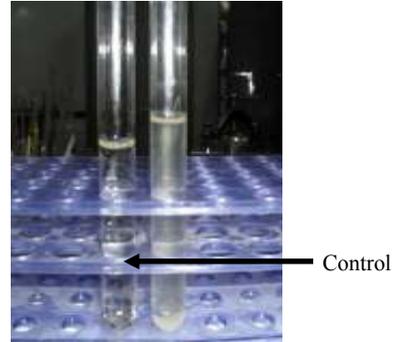


Figure 15. Indole test

Biochemical tests

Motility Indole Urease (MIU) tests

All the isolates in the MIU medium remained clear (non-motile). There is red color in the neck (Indole positive) and no urease production (Table 13).

Methyl Red (MR) tests

There is development of red color with all the isolates which was the indication of negative test (Figure 16).

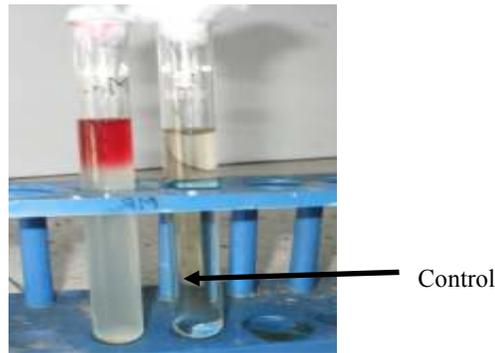


Figure 16. MR test

Table 1. Cultural and staining characteristics of *P. multocida* chicken isolates

Sources of isolates	Colony characteristics				Staining characteristics	
	Nutrient media	agar	Blood agar media	MacConkey agar media		EMB agar media
Dead chickens of Sirajgonj district	Whitish, opaque, circular and translucent appearance		Whitish, opaque, circular and translucent appearance without hemolysis	No colony appears	Small, circular, convex, glistening colonies, no metallic sheen..	Gram negative, coccobacillary and bipolar

Table 2. The results of different biochemical tests

Tests		Results
Fermentation reaction with five basic sugars	Dextrose/Glucose	+
	Maltose	-
	Lactose	-
	Sucrose	+
	Mannitol	+
	Control	-
Indole		+
MR		-
VP		-
MIU	Motility	-
	Indole	+
	Urease	-
TSI	H ₂ S	+
	Carbohydrate fermentation	Only glucose

+ = Fermentation with production of acid

- = No fermentation / negative

MIU = Motility Indole Urease

MR = Methyl red

VP = Voges Proskauer

TSI = Triple Sugar Iron

DISCUSSION

The aim of this study was to ascertain the mortality due to ND and AP in backyard poultry where death due to other diseases was neglected. The diagnostic procedures for Newcastle disease were clinical history, clinical signs, post mortem lesions, and rapid antigen detection kit test.

The major clinical signs of ND were depression, weakness, loss of appetite, dehydration, inability to stand, cyanosis of comb and wattle, greenish watery diarrhea, nasal and eye discharges, decreased egg production, loss of weight followed by death whereas gross lesions included petechial hemorrhages and ulcers with raised borders on the mucosa of intestine and proventriculus, pneumonic lungs, and hemorrhages in trachea, air sacs, brain and spleen as described by Pazhanivel *et al.* (2002). Although the kit had 100% specificity to ND, further confirmation was done by sending samples to the FDIL (Field Disease Investigation Laboratory) and CDIL (Central Disease Investigation Laboratory). There were 360 deaths due to these two diseases where Newcastle disease constituted 40.28% (145). Newcastle disease (ND) causes approximately 40-60% mortality in commercial poultry population (Chowdhury *et al.*, 1985) but, in this study, this rate was found 40.28%. This lower rate may be due to less density of backyard poultry, difficulty in the transmission of disease from one household to another household, small flock size and having some consciousness of traditional rearers regarding hygienic management, vaccination, treatment and providing nutrition to poultry. On the other hand, Lack of proper training among backyard poultry rearers has made them unable to maintain the birds properly which may be a cause of incidence of the disease.

The clinical signs of the samples recorded for Avian Pasteurellosis were the same as described by Rhoades and Rimler (1990) such as anorexia, fever, ruffled feathers, mucus discharge from mouth, rapid respiration and diarrhea which was watery to yellowish initially and greenish with mucus finally. The gross lesions of the same samples were the extensive congestion, enlarged and necrotic foci on spleen, necrotic parenchymatous hepatitis, congestion and hemorrhages in intestinal mucosa, petechial hemorrhages on the pericardium with serofibrinous pericarditis which resembled the lesions as described by Sharma *et al.* (1974). The organism was Gram negative, non motile, non-spore forming rod occurring singly or pairs and occasionally as chains or filaments in Gram's-staining method. The organisms show bipolar character in Leishman's stain.

The bacterium grows well on blood agar, nutrient agar and nutrient broth media and produce whitish, opaque, circular and translucent colonies with no hemolysis on blood agar media as described by Buxton and Fraser (1977). The isolates consistently produced acid from dextrose, sucrose and mannitol but not fermented maltose or lactose. Biochemical characteristics of the organism are performed by fermentation reaction, Methylene Red (MR), Voges Proskauer (VP) and Indole test. Although about 25- 35% mortality in chickens of Bangladesh is due to fowl cholera (Choudhury *et al.*, 1985) in commercial poultry population, but in backyard poultry this rate was found 59.72% in this study. This increased rate may be due to exclusion of mortality due to other diseases, absence of vaccination against FC and introduction of commercial poultry near households.

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