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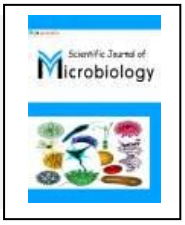

Original article

Isolation, identification and antibiogram profiles of *Staphylococcus aureus* from commercial broiler flocks in Dinajpur District of Bangladesh with special focus on the determination of lethal effect of extracted toxin

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ABSTRACT

The present study was designed with a view to isolate, identifies and characterizes *Staphylococcus aureus* from commercial broiler flocks having typical symptoms in Dinajpur District of Bangladesh with special focus on the determination of lethal effect of extracted toxin in day old chicks. The samples (pus/fluid) were subjected to bacterial isolation and identification by using cultural and biochemical techniques. Furthermore, the isolated *Salmonella* species were characterized by antimicrobial susceptibility testing. The study shown that the highest percentage (52.17%) of *Staphylococcus aureus* was obtained from group A followed by group B (41.67%) and group C (20.0%). Prevalence of toxicity in case of day old chicks those were given toxin orally was 30% and those were inoculated subcutaneously was 10%. Out of 30 day old chicks 04 died and average prevalence of toxicity is 20%. In postmortem examination it was found that tenosynovitis, most commonly in the

plantar area of the foot or just above the hock joint. This was progress to abscess formation in these areas. Infected joints had clear exudate with fibrin clots. The abdomen felt soft, mushy, flabby and enlarged. Lesions associated with heavy challenge of infection generally consist of congestion of internal organs, including the liver, spleen, kidneys, and lungs, accompanied by areas of tissue death. The isolates were resistant to cephradine, ampicillin, bacitracin, amoxacillin and sulphamethoxazole-trimethoprim. It was evident that ciprofloxacin, nitrofurantion, gentamycin and penicillin can be of better value in the treatment of *Staphylococcus aureus* infection.

1. Introduction

Poultry is essential to the national economy of Bangladesh and the welfare of human beings as well. Several constraints like the diseases, poor husbandry, low productivity and shortage of food affect the optimal performance of this industry in Bangladesh (Haque et al., 1991). Among the economic important diseases of poultry, Staphylococcosis (bumble foot) is one of the most important bacterial diseases in broiler industry causing heavy economic losses through mortality (0-15%) and reduced production performance/meat production. *Staphylococcus aureus* is a normal inhabitant of the skin and upper respiratory tract of diseased and healthy chickens (Shiozawa et al., 1980). *Staphylococcus aureus* is an important opportunist that can cause superficial to life-threatening illnesses in a variety of animal species. Infection is usually with an incubation period of 2-3 days seen after artificial infection. Toxins are involved in the development of staphylococcal infections and effective for lethal effect of bird through necrotizing and hemolytic activity. In poultry, this organism has been implicated in arthritis, osteomyelitis, synovitis, cellulites, dermatitis, endocarditis, septicaemia, wound infection, ophthalmitis and omphalitis (Bergmann et al., 1980; Shah et al., 2003; White 2003). Besides *Escherichia coli, Staphylococcus aureus* is next most important bacterium associated with yolk sac infection (Choudhury et al., 1993; Deeming 1995; Rehman et al., 1996; Kabir 2010).

Staphylococcus infections tend to occur more frequently during the following four periods of a breeder's life: 0 - 2 weeks femoral head necrosis (or bacterial chondronecrosis) are often related to egg or hatchery contamination and minor surgeries at 4 - 6 weeks infection hock and stifle joints secondarily related to other infection, at 10 - 20 weeks Infection hock and stifle joints is due to the stress of vaccination, feed restriction and sexual maturation. Overcrowding, poor feed distribution and insufficient feeder space exacerbate these problems. When the birds reaches 24 - 30 weeks of age than there might be hock and stifle joints infection associated with bumble foot. Male aggression and injuries associated with feed equipment, nest boxes and slats also contribute to the development of staphylococcal infections. The *Staphylococcus aureus* infection has become an increasingly great problem in industrialised poultry farming (Bergmann, 1980).

Most of the poultry farms in Bangladesh are not organized and scientifically planned. Many of the farmers here are not well aware of poultry farm management or ignorant about maintenance of bio-security measure and conditions attributed to farming system. Moreover, food poisoning by *Staphylococcus aureus* affects thousands of people each year. *Staphylococcus aureus* also causes invasive diseases such as arthritis (in poultry) and septicemia in poultry and humans (Hazariwala et al., 2002).The disease can be controlled by strictly maintaining hygienic management at the farm level, routine antibiogram study before treatment and by immunization. Therefore, the present study was undertaken with a view to investigate *Staphylococcus aureus* from commercial broiler flocks in Dinajpur District of Bangladesh with special focus on the determination of lethal effect of extracted toxin in day old chicks

2. Materials and methods

2.1. Study area

This study was conducted during the period from July to December 2012 at Parbatipur upazila of Dinajpur District. The samples were collected from the suspected birds of the broiler farms and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur for bacteriological examination.

2.2. Isolation and identification of *Staphylococcus spp.*

2.2.1. Collection of Sample

Samples were collected from the broiler birds of different age groups; group–A: 0 – 10 days, group–B: 11–20 days and group–C: > 20 days. The total numbers of samples were 131.

2.2.2. Culture of sample

Each sample inoculated separately in nutrient agar (NA) and blood agar (BA) to promote growth of bacteria. Each group of this media was incubated at 37^0C for overnight. The colonies on primary culture were subcultured by streak plate method until pure cultures with homogenous colonies were obtained. Then morphological characterizations of organism were performed by Gram's staining method.

2.2.3. Culture into different media

A loopful aliquot of culture was taken from the Nutrient broth culture and streaked on Staphylococcus agar no. 110 and Mannitol salt base agar media for getting the pure culture. All inoculated media were kept in an incubator at 37^0C for overnight. Then the representative isolates were stained by Gram's stain (Merchant and Packer, 1967).

2.2.4. Biochemical test

Several biochemical tests such as triple sugar iron (TSI) agar slant reaction, MR-VP reaction, motility indole urea (MIU) medium, indole test, catalase test and coagulase test were performed according to procedures described by Merchant and Packer (1967) and OIE (2000).

2.2.5. Maintenance of stock culture

The stock culture was maintained following the procedures of Choudhury et al. (1987). During the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of the isolated organisms were stored in sterilized 80% glycerin and used as stock culture. The equal volume of 80% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at -70°C in freezer for future use.

2.3. Antimicrobial susceptibility test

Antimicrobial susceptibility tests were performed using Kirby Bauer's disc diffusion method according to performance standards of CLSI (Clinical and Laboratory Standards Institute, 2006). The panel of antimicrobial agents tested included those that are recommended by CLSI or are commonly used for the treatment of *S. aureus* infections. The antimicrobial agents used are amoxicillin, cephradine, ciprofloxacin, gentamicin, kanamycin, nitrofurantion, penicillin, levofloxacin, tetracycline, doxycycline, azithromycin, sulphamethoxazole-trimethoprim, ampicillin and bacitracin.

2.4. Toxin extraction from Staphylococcal field isolate

Colonies of *Staphylococcus* species grown on Staphylococcus Agar No.110 or Mannitol Salt Agar at 37˚C for 24 hours were separated. Then the separated colonies were transferred to Nutrient broth and incubated at 37˚C for 24 hours. Mixing of equal amount of broth culture and PBS in test tubes. Then centrifuged at 10,000 RPM for 10 minutes. After centrifugation discarding the supernatant and repeated this process three times. The supernatant are decanted and precipitated mass of toxins were collected. These cellular extracts were used in cell toxicity assays (Nobutoshi et al., 2000; Schwartz et al., 1963).

2.5. Detection of lethal effect of toxin

A group of 30 specific-pathogen-free (SPF) day-old-chicks were taken. Out of them, 10 chicks were inoculated subcutaneously with toxin extracted from field isolate, 10 inoculated orally and 10 received no bacteria and acted as control group. After 1 or 2 days of inoculation determination of lethal effect of extracted toxin from field isolate was performed by observing both live and dead chicks. The postmortem changes were observed in different visceral organs of infected live and dead chicks (Shah *et al.,* 2003).

3. Results

The results of isolation and identification of *Staphylococcus aureus* from suspected birds by using staining, cultural and biochemical tests are summarized in Tables 1, 2, 3 and 4. A total of 131 samples (pus/fluid) were collected from suspected broiler flocks belonging to Group A, B and C and comprising 92, 24 and 15 birds respectively. The suspected samples were inoculated into different bacteriological media. It was observed that the highest percentage of prevalence of *Staphylococcus aureus* (52.17%) was obtained from group A followed by group B (41.67%) and group C (20.0%) respectively. The isolated *Staphylococcus aureus* produced golden yellow mannitol fermenting colony in mannitol salt base agar, golden yellow colony in Staphylococcus Agar No110 and smooth, shiny, round and convex colony with haemolysis in blood agar media. The non-motility of *Staphylococcus* aureus was confirmed by hanging drop method. The isolated *Staphylococcus aureus* fermented glucose, sucrose, fructose and mannitol. Furthermore, the isolated *Staphylococcus aureus* showed positive reaction in TSI agar slant, MR test, catalase test and coagulase test. However, the isolated *Staphylococcus aureus* were negative in VP test and indole test.

The results of antimicrobial susceptibility of the isolated *Staphylococcus aureus* are summarized in Table 5. Out of 20 *Staphylococcus aureus,* 14 isolates were susceptible to ciprofloxacin, gentamicin, nitrofurantion, penicillin and levofloxacin. On the other hand, out of 20 *Staphylococcus aureus,* 100% isolates were resistant to cephradine, ampicillin, bacitracin, amoxacillin and sulphamethoxazole-trimethoprim. However, a few isolates were intermediate resistant to kanamycin, tetracycline and doxycycline.

Determination of lethal effects of toxin and average toxicity in experimental birds are presented in Tables 6 and 7. After 2 to 3 days of inoculation of extracted toxin from field isolate both live and dead chicks were observed. The birds infected with *Staphylococcus aureus* seemed weak, huddled together and had a watery diarrhea. Infected live chicks were lameness, swollen above the hock and around the hocks and feet (Figure 1). The birds were off-feed and water also. Some deaths from acute septicemia due to very heavy challenge. In postmortem examination it was found that tenosynovitis, most commonly in the plantar area of the foot or just above the hock joint. This was progress to abscess formation in these areas. Infected joints had clear exudates with fibrin clots (Figure 2). The postmortem changes of normal and infected birds are shown in Figures 3, 4, 5 and 6. The umbilicus was open, infected and discolored to bluish black. There was pungent odour from the chicks. The abdomen felt soft, mushy, flabby and enlarged. Lesions associated with heavy challenge of infection generally consist of congestion of internal organs, including the liver, spleen, kidneys, and lungs, accompanied by areas of tissue death. All chicks in control group maintained a normal healthy appearance and feed consumption and no mortality was recorded in this group. Prevalence of toxicity in case of day old chick those were given toxin orally was 30% and in case of chicks those were inoculated subcutaneously was 10%. Out of 30 day old chicks 11 infected, 04 died and average prevalence of toxicity is 20%.

Table 1
Isolation and identification of *Staphylococcus aureus* from suspected birds by using staining, cultural and biochemical tests.

Group of birds	Name of the sample	Total sample tested	Grand total of sample	Positive case	% of Positive case	Total no. of positive isolates
A (0-10 days)	fluid/ pus	92	131	48	52.17	61
B (11-20 days)		24		10	41.67	
C (Above 20 days)		15		3	20.0	

Table 2
Characterization of *Staphylococcus aureus* by using different bacteriological culture media.

Name of the media	Colony characteristics	Remarks
General cultural media		
Nutrient broth	Uniform turbidity	*Staphylococcus species*
Nutrient agar	Circular, small smooth, convex colonies.	*Staphylococcus species*
Specific cultural media		
Mannitol salt base agar	Golden yellow mannitol fermenting colony.	*Staphylococcus species*
Staphylococcus Agar No110	Golden yellow colony.	*Staphylococcus species*
Blood agar media	Smooth, shiny, round and convex colony with haemolysis.	*Staphylococcus species*

Table 3
Morphological characterization of *Staphylococcus* isolate by microscopic examination.

Test performed	observation	Remarks
Microscopic examination by grams staining	Showing gram positive, cocci shape, grape-like cluster	*Staphylococcus species*
Motility test by hanging drop slide	Absence of swinging movement of bacteria.	*Staphylococcus species*

Table 4
Characterization of *Staphylococcus aureus* by using different biochemical techniques.

Different biochemical test	Result	Remarks
Glucose	+	*Staphylococcus species*
Sucrose	+	*Staphylococcus species*
Fructose	+	*Staphylococcus species*
Mannitol	+	*Staphylococcus species*
TSI agar slant	+	*Staphylococcus species*
MIU	-	*Staphylococcus species*
MR	+	*Staphylococcus species*
VP	-	*Staphylococcus species*
Indole	-	*Staphylococcus species*
Catalase test	+	*Staphylococcus species*
Coagulage test	+	*Staphylococcus aureus*

(+) = positive, (-) =Negative, MR= Methyl red, VP= Voges-Proskauer, MIU= Motility, Indole, Urea

Table 5
Results of antimicrobial susceptibility test of the isolated bacteria (n = 20).

Antimicrobial agent	No (%) of *Staphylococcus aureus*		
	Susceptible	Intermediate	Resistant
Amoxycillin	0 (0)	0 (0)	20 (100)
Cephradine	0 (0)	0 (0)	20 (100)
Ciprofloxacin	14 (70)	0 (0)	6 (30)
Gentamicin	14 (70)	0 (0)	6 (30)
Kanamycin	9 (45)	6 (30)	5 (25)
Nitrofurantion	14 (70)	0 (0)	6 (30)
Penicillin	14 (70)	0 (0)	6 (30)
Levofloxacin	14 (70)	0 (0)	6 (30)
Tetracycline	9 (45)	5 (25)	6 (30)
Doxycycline	10 (50)	5 (25)	5 (25)
Azithromycin	8 (40)	0 (0)	12 (60)
Sulphamethoxazole-Trimethoprim	0 (0)	0 (0)	20 (100)
Ampicillin	0 (0)	0 (0)	20 (100)
Bacitracin	0 (0)	0 (0)	20 (100)

Table 6
Determination of lethal effects of toxin by gross postmortem lesion in different visceral organ of experimental bird.

Postmortem changes	Observation Experimental bird					
	Yolk sac	Liver	Spleen	Kidney	Lung	Heart
Discoloration	++	++	++	++	++	++
Offensive odor	+++	+	+	+	+	+
Consistency Watery	+++	-	-	-	-	-
Congestion	-	++	++	++	++	++
Joint infection	Pododermatitis, tenosynovitis most commonly in the plantar area of the foot or just above the hock joint. This was progress to abscess formation in these areas. Infected joints had clear exudates with fibrin clots.					

Here, +++: High, ++: Moderate, +: Less, -: Absent

Table 7
Determination of toxicity (Average %).

Route of inoculation	No. of day-old-chicks	Total no of day old birds	No of healthy chicks	No of infected chicks	No of dead chicks (%)	Average toxicity (%)
Orally	10	30	2	5	3 (30%)	20%
Subcutaneously	10		3	6	1 (10%)	
Control	10		10	0	0%	0%

4. Discussion

The infectious disease mainly caused by bacteria and virus have been recognized as outbreak forms associated with high morbidity and mortality in commercial poultry worldwide including Bangladesh (Calnek et al., 1997; Samad 2000). The probability of the infection is increased by any injury that's provides the bacteria with

route of entry. Arthritis is most commonly caused by *Staphylococcus aureus* in association with other organism (Rashed 2011).

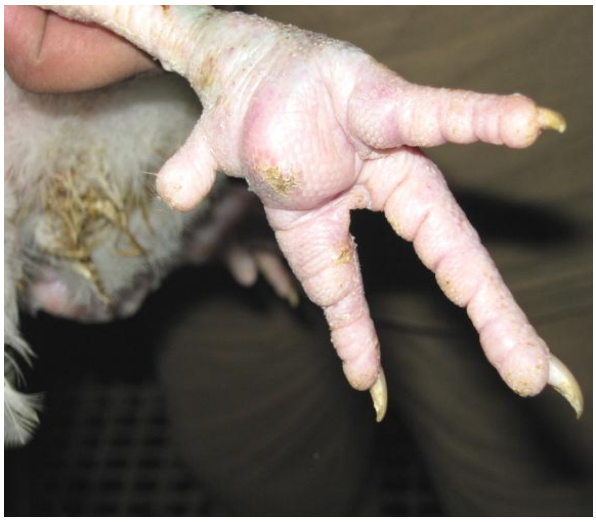

Fig. 1. Leg, foot pad affected with Staphylococcal infection.

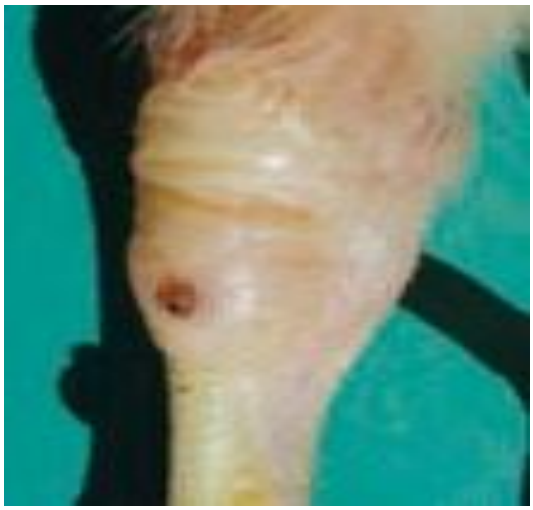

Fig. 2. Joint affected with Staphylococcal infection.

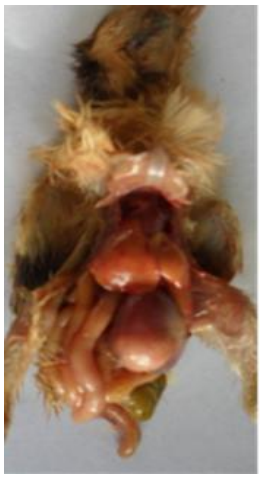

Fig. 3. Post-mortem of control birds showing normal appearance

Fig. 4. Infected birds showing congestion of livers.

Fig. 5. Yalk sac infected with Staphylococcal infection showing large yellowish brown colour.

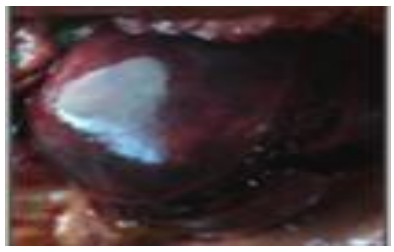

Fig. 6. Enlargement and congestion of liver.

Bones, tendon sheaths and joints especially tibiotarsal and stifle joints were the most frequent sites of *Staphylococcus aureus* infection in poultry. In the present study, specific culture media and biochemical tests which were used for the detection of *staphylococcus sp*, were also used by a number of scientists (Shareef et al., 2009; Rashed 2001). In the present study *Staphylococcus aureus* was isolated at the percentage of 52.17% from broiler similar to the findings of other authors (Rashed, 2011; Shareef *et al*. 2009). The results of this study were more or less in agreed with the findings of previous workers who also conducted research investigation on staphylococcus. But in this present study higher percentage of *Staphylococcus aureus* were recorded from group A birds. The difference of prevalance percentage of bacteria is probably due to the differences of age, breed and different environmental factors.

Out of 20 *Staphylococcus aureus,* 14 isolates were susceptible to ciprofloxacin, gentamicin, nitrofurantion, penicillin and levofloxacin in this study. On the other hand, out of 20 *Staphylococcus aureus,* 100% isolates were resistant to cephradine, ampicillin, bacitracin, amoxacillin and sulphamethoxazole-trimethoprim. However, a few isolates were intermediate resistant to kanamycin, tetracycline and doxycycline. The study was more or less similar to Miranda et al. (2008); Nemati et al. (2008) ; Zhou Q et al. (2007) ; White et al. (2003).

Prevalence of toxicity in case of day old chick those were given toxin orally was 30% and in case of chicks those were inoculated subcutaneously was 10%. Out of 30 day old chicks 11 infected, 04 died and average prevalence of toxicity is 20%. In postmortem examination it was found that tenosynovitis, most commonly in the plantar area of the foot or just above the hock joint. This was progress to abscess formation in these areas. Infected joints had clear exudate with fibrin clots. The abdomen felt soft, mushy, flabby and enlarged. Lesions associated with heavy challenge of infection generally consist of congestion of internal organs, including the liver, spleen, kidneys, and lungs, accompanied by areas of tissue death. The study is more or less similar to Shah et al. (2003). The infected yolks were large in size. Having yellowish brown and green to yellowish red appearances, offensive pungent smell and watery to caseous consistency. Jordan (1990), Skeeles (1991), Sainsbury (1992) and Anjum (1997) reported similar observations.

5. Conclusion

Staphylococcosis is the most devastating diseases in Bangladesh causing continuous infection and high economic loss in broiler. It may be concluded from this study that Staphylococcosis has emerged as one of the most serious problems having adverse effects on poultry. In future for the control of Staphylococcal infection in poultry, molecular characterization and development of toxoid from the field isolate need to be performed in Bangladesh to save the poultry industry.

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