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Isolation of *Clostridium perfringens*, Causal agents of necrotic enteritis in chickens

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Abstract

The present study was conducted to isolate the causal agent of necrotic enteritis (NE) from broiler chickens. A total of 50 jejunal samples from necropsied broiler chickens were collected for isolation of *Clostridium perfringens,* the causal agent of NE. The novel method for isolation of *Clostridium perfringens* was stab culture in association with pouring of olive oil on the surface of the stab culture. The collected samples in nutrient broth were kept in a candle jar and incubated for 24 hrs at 37°C. For maintenance of anaerobic condition olive oil (2-3 cm) were poured on surface of culture broth in test tube. In Gram's staining, the morphology of the isolated bacteria was Gram-positive, rod-shaped, anaerobic, spore-forming single or paired in arrangement. The addition of olive oil on the surface of stab culture was effective for the growth of *Clostridium perfringens*. The characterization of *Clostridium perfringens* was then performed by other routine cultural study, staining procedure and different types of biochemical tests. The occurrence of necrotic enteritis was 8%.

Keywords: *Clostridium perfringens*, Stab culture, Olive oil, Broiler chickens

Introduction

Poultry industry is an emerging agribusiness started practically during 1980's in Bangladesh (Huque, 2001). There are a lot of constrains in the development of poultry industry in Bangladesh. In the country, among different constraints of poultry industries, outbreak of several devastating diseases is one of the major constraints causing economic loss and discouraging poultry rearing (Das *et al*., 2005). Diseases are causing about 30% mortality of chickens per year. Incidence of the diseases varies depending on the geo-climatic condition, season, breed and age of birds. A survey report on both breeding flocks of commercial broiler and layer in major poultry raising belt in and around Dhaka and Gazipur districts in Bangladesh was conducted by Saleque *et al.*(2003) and reported bacterial, viral, mycoplasmal, protozoal, paracitic, fungal and noninfectious diseases as 45%, 17%, 12.4%, 6.6%, 4.5%, 1.5% and 12.4%, respectively. Among bacterial diseases, necrotic enteritis (NE) is one of the most important diseases in poultry that destroys the intestinal lining of the digestive tract, outbreaks occurring in broilers from 2-5 weeks of age. Mortality is usually between 2-10% but can be as high as 40-50%, symptoms can also resemble coccidiosis and may be mis-diagnosed. The incidence of necrotic enteritis in Mymensingh district of Bangladesh is 0.60% (Islam *et al.,* 1998) and 0.52% (Talha *et al.,* 2001). The incidence of necrotic enteritis in Sylhet and Rajshahi Region of Bangladesh is 0.44% (Islam *et al.,* 2003) and 0.91% (Hossain *et al.,* 2002), respectively.

Necrotic enteritis is reported by postmortem examination in our country (Islam *et al.,* 1998), (Talha *et al.,* 2001), (Hossain *et al.,* 2002) and (Islam *et al.,* 2003) but isolation and identification of causal agent in Bangladesh have not been performed. Therefore, the study was undertaken for the isolation and identification of *Clostridium perfringens* from dead or sick bids.

Materials and Methods

Collection of samples

A total of 50 samples from SK Diagnostic Centre, Mymensingh were collected from jejunum of chickens suffering from diarrhoea as well as showing typical postmortem lesions of enteritis. Intestinal contents from jejunum were collected aseptically in nutrient broth and transported in ice box. For the isolation of causal agent, impression smears were also taken from jejunum of intestine.

Gram's staining of impression smear

Impression smears from 50 samples prepared from jejunum of intestine were stained with Gram's staining method. The stained slides were examined according to the procedure described by Rahman (1995). Among these 8 samples revealed the presence of *Clostridium* organisms.

Culture in broth

All the suspected *Clostridium* organisms were inoculated into nutrient broth and kept in a candle jar and incubated for 24 hours at 37^0C. For maintenance of anaerobic condition 5 ml of olive oil (2-3 cm) was poured on surface of culture broth in 10 ml size test tube (Eyre, 2009).

Stab culture

A stab culture was prepared from 8 suspected organisms in agar containing a deep column of medium, then thrusted the inoculating loop to the bottom of the tube.1 or 2 cm layer of sterilized oil (Olive oil) was poured on the surface of the medium and incubated for 24 hrs at 37^0 C. (Eyre, 2009). Repeated passages in stab culture were performed until the culture became pure (Eyre, 2009).

Carbohydrate fermentation test

Carbohydrate fermentation test was conducted with the suspected *Clostridium* isolates.The carbohydrate fermentation test was performed by inoculating 5 ml of nutrient broth culture of the organisms into the tubes containing different sugar media and incubated for 72 hours at 37°C (Eyre, 2009).

Methyl red (MR) test

After incubation of 8 isolates at 37^0 C, 2 - 4 drops of methyl red solution were added to the test tube which was incubated for 5 days for MR test (Eyre, 2009).

Voges-Proskauer (V-P) test

The V-P test of 8 isolates was performed by adding 6 ml of VP reagent-1 and 0.2 ml of VP reagent-2 for each ml of culture. The ingredients were mixed thoroughly and allowed to stand for 2 minutes (Eyre, 2009).

Indole test

All 8 isolates were tested for indole positivity. Two ml of peptone water was inoculated with *5* ml of bacterial culture and incubated for 48 hours at 37^0C. 0.5 ml Kovac's reagent was added, shaken well and examined after 1 minute (Eyre, 2009).

Dulcitol fermentation test

Dulcitol test was performed with 8 isolates. This test was performed according to the method described by Eyre (2009).

Catalase test

This test was performed as described by Cowen *et al*. (1987). To perform the test a 3 ml of 3% hydrogen peroxide solution was poured into a test tube containing 2 ml of cultured broth. A confluent growth of test organism was immersed into the solution by mixing with a sterile glass rod (Eyre, 2009).

Blood agar for hemolytic activity

Eight isolates from stab culture were spread on blood agar media (sheep blood) and were incubated anaerobically for 24 hours at 37^0C (Eyre, 2009).

Egg yolk agar for lecithinase test

All 8 isolates were used for this test. Egg yolk emulsion (0.5 ml) was mixed with nutrient broth (10 ml) and added 1% NaCl for clearance of media. The inoculating loop was thrusted with sample to the egg yolk media and was incubated anaerobically for five days at 37^0C (Eyre, 2009).

TSI agar slant reaction

From 8 samples, colonies of each isolate were inoculated by stab or streak method into TSI agar slants (Eyre, 2009).

Results and Discussion

Staining characteristics in impression smear

Fifty selected samples showed different staining characters in impression smears (Table 1). Among these 8 selected samples showed numerous large, thick, gram-positive rods were observed in impression smears prepared from jejunum. In smears prepared from infected tissues, the rod shaped bacteria were observed with an arrangement of single, pair or in a group (Fig. 1). These were suspected as *Clostridium* organisms. Thirty seven samples showed mixed type of bacteria, but dominantly gram negative rod and different shaped and sized gram positive cocci were also observed in the Gram's stained smears prepared from intestinal contents. Five samples did not show any bacteria in smears.

Table 1. The prevalence of bacteria determined by smears in intestines of 50 broiler chickens

Morphological characters of isolated bacteria	Number of birds	Tentatively identified organisms	Percentage (%)
Numerous large, thick and gram-positive rod	8	*Clostridium* sp.	16
Gram negative, long, short, slender and slightly rounded end, rod shaped bacteria plus Gram positive cocci	37	Mixed bacteria	74
No rod or cocci were found	5	No bacteria	10

Cultural characteristics of *Clostridium perfringens*

Eight isolates that were tentatively identified as *Clostridium sp.* were used for culture. Tubular cylindrical colonies were found in stab culture (Fig. 2). Blood agar plates were inoculated with suspected sample from stab culture and incubated anaerobically at 37°C, which showed the growth of bacterial colonies. The colonies were round, smooth, circular, gray-white color and surrounded by a typical zone of haemolysis (β-haemolysis) (Fig. 5) which resembled to the colony characteristics of *Clostridium pefringens.* In Egg Yolk Media, showing a typical oplascent growth (Fig. 6). In Triple Sugar Iron (TSI) slant agar, the slant was turned yellow due to the increased level of acid production indicating carbohydrate fermentation. H_2S was also produced due to the reaction of sulphur containing compounds. Hydrogen sulphide reacted with the ferrous sulphate of the medium producing ferric sulphide giving a black precipitate to the upper layer of the slant (Fig. 7).

Enzymatic activity test

Bubbles of oxygen were not seen on the wall of the glass rods indicating catalase negative (Table 2). In this test, 4 samples were tentatively identified as *Clostridium* organism and other 4 samples were identified as unclassified bacteria.

Carbohydrate fermentation test

All of the 8 isolates fermented glucose and produced acid and gas or only acid but did not fermented mannitol. Some fermented maltose and dulcitol; some did not (Fig. 3). Acid production was indicated by the color change from red to yellow and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tubes.

Table 2. Catalase test for 8 isolates

Number of sample	Test result	Tentatively identified organism
Sample 7	Positive	Unclassified*
Sample 16	Positive	Unclassified*
Sample 22	Negative	*Clostridium perfringens*
Sample 27	Positive	Unclassified*
Sample 32	Negative	*Clostridium perfringens*
Sample 39	Negative	*Clostridium perfringens*
Sample 40	Positive	Unclassified*
Sample 45	Negative	*Clostridium perfringens*

***=**Unclassified Gram positive short rod

Different biochemical test

Table 3 describes the biochemical test for *Clostridium perfringens.* Different biochemical test of 8 isolates were used to identify the *Clostridium* organisms. All isolates of clostridia were MR and VP negative (Fig.4A and 4B). Indole test was also negative (Fig.4C). Above these isolates, there were 4 isolates that considered as *Clostridium perfringens* and other 4 isolates were unclassified.

Table 3. Shows carbohydrate fermentation test and biochemical test

Sample No.	Dex	Lac	Suc	Fru	Mal	Man	Dul	Ind	MR	VP	Identified organism
Sample22	AG	AG	AG	AG	AG	-	-	-	-	-	*Clostridium perfringens*
Sample7	A	-	-	-	-	AG	A	-	+	+	Unclassified*
Sample16	A/AG	-	-	A	-	A	A/AG	+	-	+	Unclassified*
Sample32	AG	AG	AG	AG	AG	-	-	-	-	-	*Clostridium perfringens*
Sample39	AG	AG	AG	AG	AG	-	-	-	-	-	*Clostridium perfringens*
Sample27	A	A	A	A	A	-	AG	-	+	-	Unclassified*
Sample45	AG	AG	AG	AG	AG	-	-	-	-	-	*Clostridium perfringens*
Sample40	AG	A	-	A	A	AG	AG	+	-	+	Unclassified*

*= Unclassified Gram positive short rod

Dex = Dextrose, Lac = Lactose, Suc = Sucrose, Fru = Fructose, Mal = Maltose, Man = Mannitol, Dul = Dulcitol, Ind = Indole, MR = Methyl Red, VP = Voges Proskauer, A = Acid, AG = Acid and Gas.

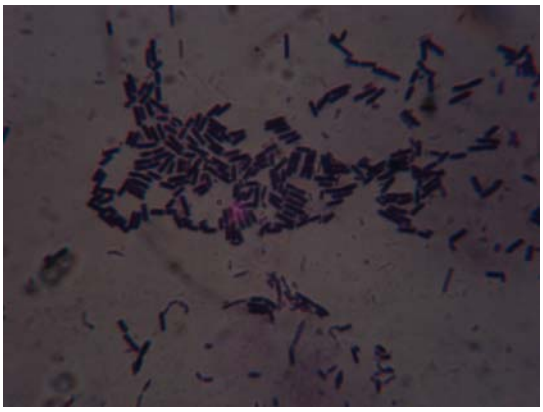

Fig.1. *Clostridium* shows numerous large, thick, gram-positive rod shaped bacilli in Gram's staining (x825)

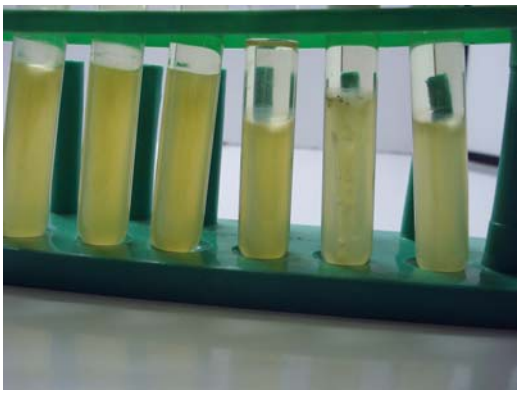

Fig. 2. Culture of *Clostrdium* shows tubular cylindrical colonies in stab culture.

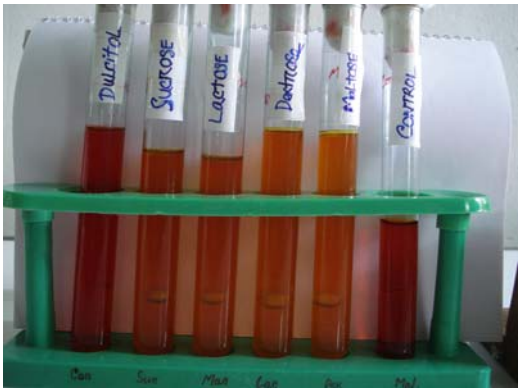

Fig. 3. Carbohydrate fermentation test with *Clostridium Perfringens* produces acid and gas in sucrose, lactose, dextrose and maltose but no change in others.

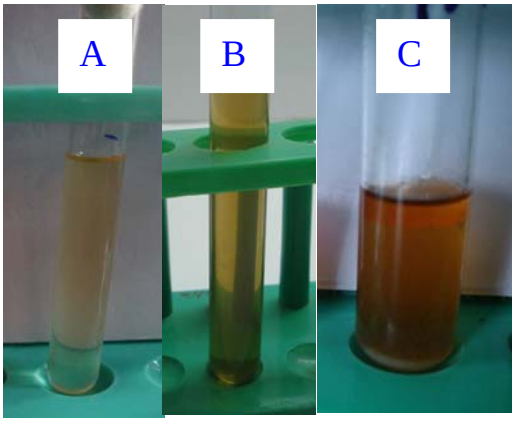

Fig. 4A, 4B& 4C: Shows M-R, V-P & indole test negative

Fig. 5. Culture of *Clostridium* shows a typical zone of haemolysis (β-haemolysis)

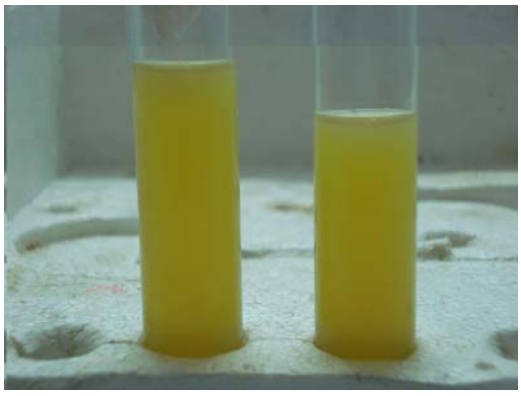

Fig.6. Shows a typical oplascent in egg yolk media

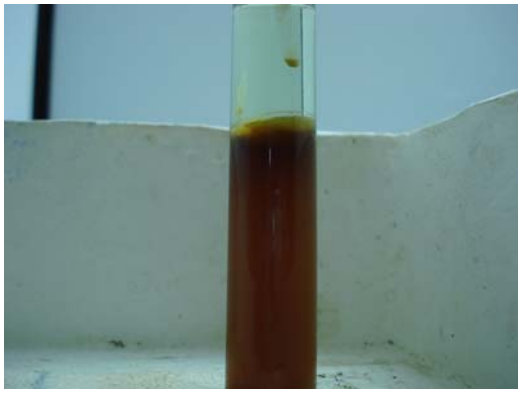

Fig.7. Culture of *Clostridium* in TSI slant agar shows black color colony

The present research work was undertaken to identify the causal agent of necrotic enteritis, which has been hindering the poultry development in Bangladesh and causing economic losses. Routine methods of bacterial cultures in different media, specific colony characters, microscopic examination, different staining techniques and different types of biochemical tests were used for the isolation and identification of *Clostridium perfringens.* The collected 50 sample in nutrient broth were kept in a candle jar and incubated for 24 hours at 37°C. For maintenance of anaerobic condition olive oil (2-3 cm) were poured on

surface of culture broth in test tube and this technique was reported by Eyre (2009). In Gram's staining, the morphology of the isolated bacteria was Gram-positive, rod-shaped, anaerobic, spore-forming single or paired in arrangement which was supported by several authors (Rhodehamel *et al.,* 1998 and Shamimuzzaman, 1999).

In this study, the colony characters of *Clostridium,* 4 samples produced hydrogen sulfide gas with black color colonies on TSI agar were corresponded with the findings of others (Rhodehamel *et al.,*1998*).* In the present study, 4 samples showed a typical opalescent, whilst on the egg yolk media, their colonies were surrounded by zones of opacity which was supported by author Rhodehamel *et al.* (1998)*.* Based on the colony characteristics mentioned in blood agar media, colonies showed a typical double zone of haemolysis (β -hemolysis) formed in each plate that indicated the presence of *Clostridium perfringens* which corresponded the results of Ellner *et al.* (1966)*.* In the present study, specific biochemical media were used for the detection of *Clostridium perfringens.* All of the 4 isolates fermented dextrose, sucrose, lactose and maltose but did not ferment dulcitol and mannitol, and all of the isolates were Indole, M-R and V-P negative which were previously suggested by a number of scientists (Sacks and Olson, 1979*;* Chattopadyay and Harbola, 1988*;* Rahman *et al.,* 1997 and Rhodehamel *et al.,* 1998*).* The occurrence of necrotic enteritis in present study was *8%* which were relatively higher than the reports by other authors (Islam *et al.,* 2003*;* Hossain *et al.,* 2002*;.* Talha *et al.,* 2001 and Islam *et al.,* 1998*).* This is probably due to the fact that other authors diagnosed the diseases mostly based on postmortem examination.

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