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ISOLATION AND CHARACTERIZATION OF BACTERIA FROM KERATOCONJUNCTIVITIS AFFECTED CATTLE WITH THEIR PATHOGENICITY AND *IN VITRO* ANTIBIOTIC SENSITIVITY

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ABSTRACT

Isolation and characterization with antibiotic sensitivity of the bacteria in the eye swab of 34 keratoconjunctivitis affected cattle were carried out on the basis of their morphology, staining, cultural and biochemical properties during the period from October 1999 to March 2000. *Staphylococcus* spp. (76.5%), *Streptococcus* spp. (38.2%), *E. coli* (52.9%), *Bacillus* spp. (70.6%), unidentified Gram positive cocci (5.9%) and unidentified Gram negative rods (20.6%) were identified as a single or mixed infection. Pathogenicity study of these isolated organisms showed conjunctivitis associated with *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. and *E. coli* in experimentally inoculated mice whereas unidentified Gram positive cocci did not show any conjunctivitis in mice. The mixed intraocular inoculation of these isolated bacteria produced severe keratoconjunctivitis within 24 hours both in mice and calves. Results of antibiotic sensitivity test showed that all types of bacterial isolates were found highly sensitive to oxytetracyclin (80-100%) and chloramphenicol (70-100%). *Staphylococcus* spp., *Streptococcus* spp. and *Bacillus* spp. were moderately susceptible to streptomycin (69.56%), trisulfa (60%) and trisulfa (80%) respectively. It may be concluded that among the tested antibiotics, oxytetracyclin may have the preference in clinical therapy of keratoconjunctivitis in cattle caused by bacteria in Bangladesh.

Key words: Isolation, characterization, bacteria, keratoconjunctivitis, pathogenicity, antibiotic sensitivity

INTRODUCTION

Various forms of eye infection in cattle of Bangladesh, which are characterized by mild or severe watery exudate followed by purulent lacrymal discharge, conjunctivitis and keratitis commonly known as infectious bovine keratoconjunctivitis (IBK). Infectious bovine keratoconjunctivitis occurs in five different forms such as acute form, subacute form, chronic form, fulminating form and carrier form (Jackson, 1953). The disease occurs predominantly in the warmer months of the year often in association with the onset of humid conditions (Adinarayanan and Singh, 1961). There are multiple predisposing factors of IBK leading to natural outbreak. The strong dry wind prevailing at the times when the mean ambient temperature various from 27^0C to 35^0C, the environmental dust enters into the eyes and produces partial corneal desiccation leads to eye infection (Lepper and Barton, 1987). If the disease appears in eyes of cattle, it becomes highly infectious and spreads rapidly among the susceptible herds. The incidence of IBK in young animals may exceed upto 90% of the infected herds. Among the affected herds more than 20% of infected animals get atleast temporary blindness (Spradbrow, 1967). In Bangladesh, there are no reports of extensive research on bacterial causes of keratoconjunctivitis in cattle and their sensitivity to antibiotic. Therefore, this study was undertaken for the isolation and identification of bacterial pathogens from clinical cases of keratoconjunctivitis in cattle and to characterize their pathogenicity in mice and calves and *in vitro* sensitivity to antibiotics.

MATERIALS AND METHODS

Eye swab samples

An eye swab sample from each of 34 randomly selected severely infected different aged groups of cattle was collected by using sterile cotton and screw capped tube from Central Cattle Breeding Station and Dairy Farm (CCBSDF), Savar, Dhaka; Bangladesh Agricultural University (BAU) Veterinary Clinic, Bangladesh Agricultural University Dairy Farm and also from some adjacent villages around the BAU Campus during the period from October 1999 to March 2000.

Culture of swab samples

Each swab sample was divided and inoculated separately in nutrient agar (NA) and blood agar (BA) media to promote the growth of bacteria. The aerobic culture plates were incubated at 37^{0}C for 72 hours and the anaerobic culture plates were incubated at 37^{0}C for 2-7 days. The colonies on primary cultures were repeatedly subcultured by streak plate method (Cheesbrough, 1985) until the pure culture with homogenous colonies were obtained. Media such as nutrient agar (NA), sheep blood agar (SBA), eosin methylene blue (EMB) agar, MacConkey agar (MA) and Chocolate agar (CA) were used for subcultures. Haemolytic patterns of the bacteria were categorized according to the type of haemolysis they produced on SBA (Carter, 1986).

Identification of bacterial isolates

The bacterial isolates were identified by their cultural, morphological and biochemical characters. For the cultural characteristics, discrete colonies on the agar surface were observed. Their shape, size, consistency and colour were observed. Gram stained slides of the isolates were examined microscopically to study their cellular morphology. The biochemical tests were performed as fermentation with five basic sugars (dextrose, maltose, sucrose, lactose and mannitol) and thereby production of acid or acid and gas, IMViC utilization test, catalase and coagulase tests. Individually isolated colonies of the same morphology were selected from appropriate agar plates, cloned and checked for purity of growth prior to characterization of the respective genera and species. Characterization into respective genera and species were done on the basis of morphological, cultural and biochemical reactions. The classification and specification of organisms were based on the scheme presented in Bergey's Manual of Systemic Bacteriology (Halt *et al.*, 1985).

Catalase test

The test was performed as described by Cowan (1985). To perform the test a 3.0 ml of 3% hydrogen peroxide solution was poured into a test tube. A confluent growth of test organism was immersed into the solution by mixing with a sterile glass rod. Bobbles of oxygen will be seen by the accumulation on the wall of the glass rods if the organisms are catalase producer.

Coagulase test

The test was performed as described by Carter (1986). For this test 1-2 drops of diluted rabbit plasma was mixed with an equal volume of freshly cultured broth of a particular organism on a slide and examined under microscope for the occurrence of any coagulation.

IMViC utilization test

Motility indole urea medium (MIU), Bacto MR-VP medium, Koser's citrate medium are commonly used for this test following the procedures described by Cowan (1985). The IMViC bacteria must reveal the following characteristics such as indole production from tryptose, methyl red for acid production from glucose, and Voges-Proskauer for the production of acetone from glucose, and ability of the positive bacteria to utilize citrate as the sole source of carbon.

Carbohydrate fermentation test

The production of acid or acid and gas was tested for *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, *Bacillus* spp., unidentified Gram positive cocci and unidentified Gram negative rods using five basic sugars namely dextrose, sucrose, maltose, lactose and mannitol. The carbohydrate test was performed by inoculating a loop-full organisms into the tubes containing different sugar media and incubated for 24 hours at 37^{0}C. Acid production was indicated by the change of the phenol red to yellow colour and accumulation of gas in the inverted Durham's tube indicated gas production.

Maintenance of stock culture

The stock culture was maintained following the procedures of Carter (1979). Nutrient agar slants used for the maintenance of stock culture for each of the bacterial isolate. After growth of the organism in the slant, the sterile mineral oil was overlaid and the culture was kept at room temperature for use as seed.

Experimental infection in mice and calves

Mice: A total of 264 adult mice of both sexes were divided into 88 groups, consisting of three mice in each, of which 80 experimental groups of mice were inoculated with individual isolate of each species of bacteria e.g., *Staphylococcus* spp. (23 isolates), *Streptococcus* spp. (10 isolates), *E. coli* (18 isolates), *Bacillus* spp. (20 isolates), unidentified Gram positive cocci (2 isolates) and unidentified Gram negative rods (7 isolates) and each mouse of another one group was inoculated with mixed infection of above mentioned bacterial species combindly. Remaining seven groups were served as control, of which six groups for individual species of bacteria and one group for mixed infection of the species combindly. Inoculum of each isolate was prepared by culturing the organisms in nutrient broth and harvesting 24 hours old culture. The viable counts of the bacteria in these preparations ranged from 5×10^6 to 1×10^9 CFU / ml. Inoculation was made by intraocularly one drop of bacterial suspension singly and combindly in each mouse of different experimental groups. The mice were observed for every 6 hours interval for 7 days for the manifestation of clinical signs of keratoconjunctivitis.

Calves: Six calves of six months of age were divided into two groups. One group of calves was inoculated with mixed infection of *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, *Bacillus* spp., unidentified Gram positive cocci and unidentified Gram negative rods combindly. The viable counts of the bacteria in the preparation was ranged from 5×10^6 to 1×10^9 CFU / ml. Inoculation was made by intraocularly combindly. The calves were observed for every 6 hours interval for 7 days for the manifestation of clinical signs of conjunctivitis.

Antibiotic sensitivity test

In vitro antibiotic sensitivity test of the isolated bacteria was performed with the standardized commercial sensitivity discs as per method described by Tripathi and Soni (1982) and Joshi *et al.* (1986). Sensitivity to antibiotic was studied mostly on blood agar plates with penicillin-G (PG), ampicillin (AM), kanamycin (KA), oxytetracycline (OT), chloramphenicol (CH), streptomycin (ST) and trisulfa (TS). The concentration of each antimicrobial sensitivity disc was 100 µg. An amount of 0.5 ml freshly grown pure culture of the bacteria was poured on BA plates and allowed to spread gently over the entire surface with a glass rod spreader. After 1 to 2 minutes the antibacterial discs were placed on the inoculated plates keeping a distance of about 1 cm apart and incubated at 37^0C for overnight. The inhibitory effect of the antibacterial to the growth of the culture was recorded.

RESULTS AND DISCUSSION

Cultural and staining characteristics of the bacteria isolated from the cattle affected with keratoconjunctivitis are presented in Table 1. The presumptive *Streptococci* produced small, circular, discrete, translucent convex colonies on nutrient agar, pin-point colonies surrounded by clear zones of haemolysis on blood agar. All the isolates of presumptive *Streptococci* were Gram positive and arranged in chains or pairs. The presumptive *Staphylococci* produced gray white or yellowish colony on nutrient agar, white to golden yellow coloured colony on blood agar. All the isolates of presumptive *Staphylococci* were Gram positive and arranged in cluster. The presumptive *Bacilli* produced thick grayish white or cream coloured colony with uneven surface on nutrient agar and creamy yellow coloured colony on blood agar media. The presumptive *Bacilli* were rod shaped and spore forming bacteria. They were Gram positive, arranged in single, pair or also in long chain. The rod shaped Gram negative organisms arranged singly, pairs or in short chain indicating *E. coli* which produced smooth, circular, white to grayish white colonies with peculiar foetid odour on nutrient agar, rose pink colour colony on MacConkey agar and metallic sheen on EMB agar. The organism was found as Gram positive cocci arranged singly or in pairs indicating unidentified Gram positive cocci and Gram negative rod arranged in singly or in pairs indicating unidentified Gram negative rods.

Frequency distribution of the bacteria

The results of frequency distribution of different bacterial isolates are presented in Table 2. A total number of 34 eye swab samples of keratoconjunctivitis affected cattle were examined for the isolation of bacteria of which 26 (76.5%) samples were found positive for *Staphylococcus* spp., 13 (38.2%) for *Streptococcus* spp., 18 (52.9%) for *E. coli*, 24 (70.6%) for *Bacillus* spp., 2 (5.9%) for unidentified Gram positive cocci and 7 (20.6%) for unidentified Gram negative rods. The different types of bacteria isolated in the present study correlate with the findings of Atyabi and Barin (1997), Seham *et al.* (1995) and Liao *et al.* (1997) with slight variation. In addition to these organisms they also isolated *Mycoplasma bovis* and *Pseudomonas aeruginosa* and other bacteria from cattle suffering from conjunctivitis.

Table 1. Cultural colony characteristics and Gram's staining reaction of the bacteria isolated from keratoconjunctivitis affected cattle

Staining characters			Cultural characters				Identified organisms
Shape	Arrangement	Gram's staining reaction	Nutrient agar	Blood agar	MacConkey agar	EMB agar	
Small cocci	Chains or pairs	Gram +ve	Small, circular discrete, translucent convex colonies	Pin-point colonies with β-haemolysis	No growth	No growth	*Streptococcus* spp.
Cocci	Clusters	Gram +ve	Gray white or yellowish colonies	White to golden yellow colonies	Small colonies	No growth	*Staphylococcus* spp.
Rod with square ends	Single, paired or in long chain	Gram +ve	Thick grayish white or cream coloured colonies with an uneven surface	Abundant growth, creamy yellow coloured colonies with hemolysis	No growth	No growth	*Bacillus* spp.
Short, plump rods	Single, pairedor in long chain	Gram –ve	Smooth, circular, white to grayish white colonies, peculiar foetid odour	Small colonies with haemolysis	Rose pink lactose fermented colonies	Moist circular colonies with dark centers yellow green metallic sheen	*E. coli*
Cocci, oval	Singly or in pairs	Gram +ve	Scanty growth	Small, smooth, discrete glistening translucent colonies	No growth	No growth	Gram positive cocci
Rod shaped	Singly or in pairs	Gram –ve	Poor growth	Abundant growth on 10% sheep blood agar	No growth	Poor growth	Gram negative rods

Table 2. Status of bacteria isolated from the eyes of cattle affected with keratoconjunctivitis

Isolated bacteria	No. of samples	Positive sample	
		No.	%
Staphylococcus spp.	34	26	76.5
Streptococcus spp.	34	13	38.2
E. coli	34	18	52.9
Bacillus spp.	34	24	70.6
Unidentified gram positive cocci	34	02	5.90
Unidentified gram negative rods	34	07	20.6

Biochemical tests

The biochemical characteristics of the bacteria isolated from the cattle affected with keratoconjunctivitis are presented in Table 3. The isolates of *Staphylococci* were able to ferment all the sugars completely with the production of acid to their corresponding sugars without gas. The isolated *Streptococci* produced only acid from dextrose, maltose and sucrose, but no fermentation was observed with lactose and mannitol. The isolates of *E. coli* were found to ferment all the five basic sugars and produced both acid and gas. The isolated *Bacilli* produced acid from dextrose, maltose, sucrose and mannitol but no fermentation was observed with lactose. The isolates of *Staphylococci* were catalase, coagulase positive and that of *E. coli* were found positive in catalase, indole production and methyl-red positive, and *Bacilli* were catalase positive.

The haemolytic activity of 26, 13, 24 and 18 isolates of *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. and *E.* coli were tested for haemolysin production, of which 23 (88.46%), 10 (76.92%), 20 (83.33%) and 15 (83.33%) isolates were found for haemolysin production respectively (Table 3).

Table 3. Biochemical reactivity pattern of the isolated organisms from cattle affected with keratoconjunctivitis

Isolated bacteria	No. of isolates tested	Fermentation properties with carbohydrates					Catalase test	Coagulase test	Indole production	Methyl red test	VP reaction	Citrate utilization	Haemolysin production No. (%)
		D	ML	S	L	MN							
Staphylococcus spp.	26	+A	+A	+A	+A	+A	+	+	x	x	x	x	+ 23 (88.46)
Streptococcus spp.	13	+A	+A	+A	–	–	–	x	x	x	x	x	+ 10 (76.92)
E. coli	18	+AG	+AG	+AG	+AG	+AG	+	x	+	+	–	–	+ 15 (83.33)
Bacillus spp.	24	+A	+A	+A	–	+A	+	x	x	x	x	x	+ 20 (83.33)

D = Dextrose, ML = Maltose, S = Sucrose, MN = Mannitol, L = Lactose, VP = Voges-Proskauer, + = Positive reaction, – = Negative reaction, x = Test was not performed, A = Production of acid only, AG = Production of acid and gas.

The different isolates of *Staphylococcus* spp., *Streptococcus* spp., *E. coli* and *Bacillus* spp. showed identical results in different biochemical tests including sugar fermentation, catalase and haemolytic activity. The actual causes for which the manifestation of an identical result in biochemical tests by the four groups of known identified isolates were not clear.

Pathogenicity study in mice and calves

The isolates of unidentified Gram positive cocci did not produce conjunctivitis within 24 hours of experimental infection. Of the 23 isolates of *Staphylococcus* spp., 20 (86.96%) isolates, of 10 isolates of *Streptococcus* spp., 7 (70%) isolates, of 18 isolates of *E. coli*, 16 (88.89%) isolates, of 20 isolates of *Bacillus* spp., 14 (70%) isolates and of 7 isolates of Unidentified Gram negative rods, 6 (85.71%) isolates showed conjunctivitis in experimental mice after 24 hours of infection. Mixed bacterial isolates developed severe keratoconjunctivitis with lachrymal and white purulent pus both in mice and calves at 72 hours of post-infection (Table 4). Variation in the ability of the individual and mixed isolates to produce different kinds of eye lesions is not very clear yet. It may be thought that more than one predisposing factors such as nutrition, stress or ultraviolet irradiation might help in the production of serious infection along with the presence of more than one type of bacteria. Similar observations were also recorded by Dietz *et al.* (1983).

Antibiotic sensitivity test

The results of antibiotic sensitivity of bacteria isolated from cattle affected with keratoconjunctivitis are presented in Table 5. All the isolated bacteria were highly sensitive to oxytetracycline (80-100%) and chloramphenicol (70-100%). *E. coli* were highly sensitive to oxytetracycline (88.89%) followed by chloramphenicol (83.33%). *Staphylococci* were highly sensitive to oxytetracycline (86.96%) followed by chloramphenicol (78.26%), streptomycin (69.56%), trisulfa (65.22%) and penicillin-G (65.22%). The isolates of *Bacilli* were highly sensitive to oxytetracycline (95%) followed by chloramphenicol (90%), trisulfa (80%), ampicillin (75%) and penicillin -G (65%). *Streptococci* showed high

Table 4. Experimental production of conjunctivitis in mice and calves with the isolated bacteria from keratoconjunctivitis affected cattle

Isolated bacteria	No. of isolates tested	No. of isolates positive for infection		Experimental animal (n = 3)	Time elapsed for the appearance of infection/lesions in eyes			
		No.	%		24 hrs	48 hrs	72 hrs	96 hrs
Staphylococcus spp.	23	20	86.96	Mice	+	+	++	++
Streptococcus spp.	10	07	70.00	Mice	+	+	+	++
E. coli	18	16	88.89	Mice	+	++	++	++
Bacillus spp.	20	14	70.00	Mice	+	+	+	++
Unidentified Gram positive cocci	02	00	00.00	Mice	–	–	–	–
Unidentified Gram negative rods	07	06	85.71	Mice	+	++	++	+++
All the isolates	Mixed			Mice	+	++	+++	+++
All the isolates	Mixed			Calves	+	++	+++	+++

Mixed = Includes all six types of isolated bacteria, – = Negative eye infection, + = Positive eye infection / mild conjunctivitis, ++ = Conjunctivitis with lacrymal discharge, +++ = Conjunctivitis with lacrymal discharge and white purulent pus.

Table 5. *In vitro* antibiotic sensitivity of the isolated bacteria to different antibiotics

Isolated bacteria	No. of Isolates tested	Antibiotic sensitivity No. (%)						
		PG	AM	KA	CH	ST	TS	OT
Staphylococcus spp.	23	15 (65.22)	11 (47.83)	13 (56.52)	18 (78.26)	16 (69.56)	15 (65.22)	20 (86.96)
Streptococcus spp.	10	06 (60)	05 (50)	05 (50)	07 (70)	04 (40)	06 (60)	08 (80)
E. coli	18	03 (16.67)	02 (11.11)	04 (22.22)	15 (83.33)	04 (22.22)	05 (27.78)	16 (88.89)
Bacillus spp.	20	13 (65)	15 (75)	09 (45)	18 (90)	11 (55)	16 (80)	19 (95)
Unidentified Gram positive cocci	02	01 (50)	01 (50)	01 (50)	02 (100)	01 (50)	01 (50)	02 (100)
Unidentified Gram negative rods	07	03 (42.86)	01 (14.28)	02 (28.57)	06 (85.71)	02 (28.57)	05 (71.43)	06 (85.71)

PG = Penicillin-G, AM = Ampicillin, KA = Kanamycin, ST = Streptomycin, CH = Chloramphenicol, OT = Oxytetracycline, TS = Trisulfa (sulfadiazine, sulfadimidine and sulfamethazine).

sensitivity to oxytetracycline (80%) followed by chloramphenicol (70%), trisulfa (60%) and penicillin-G (60%). These findings are in agreement with the results of Khot and Ajinkya (1980) and Seham *et al.* (1995) who reported that most of the bacteria responsible for IBK were highly sensitive to chloramphenicol and oxytetracycline and less sensitive to penicillin and other antimicrobial agents.

The results of isolation, identification, biochemical test, frequency distribution, pathogenicity and antibiotic sensitivity of the bacteria isolated from cattle affected with keratoconjunctivitis in the present study indicated that five different types of bacteria are responsible for keratoconjunctivitis in cattle of Bangladesh. This experiment also suggests that there might be some mutual exchange of virulent factors between the organisms which ultimately helps to aggravate the infection in cattle.

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