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ESCHERICHIA COLI FROM HORSES REARED IN AND AROUND BANGLADESH AGRICULTURAL UNIVERSITY CAMPUS- A STUDY ON ISOLATION AND CHARACTERIZATION

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ABSTRACT

The present study was conducted with a view to isolate and characterize *Escherichia coli* (*E. coli*) from horses reared in and around Bangladesh Agricultural University (BAU) campus and to know the clonal relationship of the isolates with *E. coli* of cattle, goat and chicken. It also focused on the determination of antimicrobial sensitivity and resistant pattern of the isolated horse *E. coli.* A total of 10 faecal samples, comprising 4 from diarrhoeic horses and 6 from apparently healthy horses were collected. The overall prevalence of *E. coli* was recorded as 60%. All isolates fermented dextrose, maltose, sucrose, lactose and mannitol with the production of both acid and gas. The isolates were positive to MR and Indole tests, but negative to VP test. DNA fingerprinting analysis using PFGE of *XbaI*-digested genomic DNA revealed that the strains of *E. coli* from different areas seem to be same clonal lineage, although no genetic clue could be found related to cattle, goat and chicken *E. coli* strains. The antimicrobial sensitivity and resistance pattern showed that the isolates of horse *E. coli* were highly sensitive to ciprofloxacin and chloramphenicol but to the amoxicillin and cephalexin, the isolates were highly resistant

Key Words: *E. coli*, Horse, Isolation, Characterization

INTRODUCTION

E. coli is a Gram-negative, rod shaped, motile, capsulated, flagellated and non-spore forming facultative anaerobes under the family Enterobacteriaceae. Some *E. coli* strains can cause clinical symptoms in humans and animals, among which enteritis is common. *E. coli* infection is a problem in horse rearing industries and it causes septicemia, enteric infections, chronic arthritis, lethargy, depression, anorexia and sudden death. Endotoxemia, septicemia and enteric infections are the most severe problems affecting neonatal foals, often culminating in death (Hung *et al.*, 2006). Approximately 25 percent of all septicemias in foals are caused by *E. coli.* Invasive strains of these bacteria enter the foal's bloodstream either through the intestinal tract, the respiratory tract, or the umbilical cord. Bacteria begin to circulate throughout the body and releases toxins. Septicemia in

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foals often results in chronic arthritis, since the bacteria tend to take refuge in the joint cavities where they are difficult to reach with antibiotics. Foals with *E. coli* infection may also show diarrhea. The most common factor predisposing foals to developing *E. coli* septicemia is hypogammaglobulinemia, i.e., inadequate levels of passive antibody protection from the mare's colostrums. This is caused by several factors like inadequate nursing, poor quality colostrums in the mare, loss of colostrums prior to foaling, etc. Since a foal is often not recognized as being hypogammaglobulinemic until it becomes sick, treatment is often ineffective.

There is randomly use of antibiotic for the treatment of diarrhoeal diseases in Bangladesh. Multi-drug resistant *E. coli* are continuously increasing due to indiscriminate use of antibiotics in Bangladesh (Hussain *et al.*, 1982 and Kabir, 2010). Incomplete course of treatment of diarrhea or urinary tract infection might have influenced to produce a new generation of virulent and resistant type of *E. coli* (Kaura *et al.*, 1988; Marshall *et al.*, 1990). Bacterial resistance to antibiotics is mainly due to the presence of plasmid and chromosomal DNA, which contains resistance factor. Thus, bacteria become more and more resistant to antibiotics and new generation of antibiotics becomes necessary. Different parameters including the isolation, identification, vaccination, plasmid profiling, antibiotic sensitivity and epidemiological investigation of *E. coli* of different species have been studied in Bangladesh. However, *E. coli* isolation from horse and their clonal relation with other *E. coli* strain of cattle, goat, and chickens by pulsed field gel electrophoresis (PFGE) with specific objectives are yet to be in the context of Bangladesh. Considering this fact, the present research work was undertaken with the objectives of (i) to isolate and identify *E. coli* from horse, (ii) to characterize the isolated *E. coli* using cultural, biochemical, and molecular techniques and (iii) to study the antibacterial sensitivity of the isolated *E. coli.*

MATERIALS AND METHODS

The study was conducted in the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh and Enteric Microbiology Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka during the period of May 2009 to May 2010. Diarrhoeic horses and apparently healthy horses were selected for the experimental study. A total number of 10 field samples comprising rectal swabs were aseptically collected into nutrient broth (NB) from BAU campus, i.e., BAU Sheep & Goat Farm (BAUSGF) and BAU Veterinary Clinic (BAUVC) and around the campus, i.e., Boyra Union Parishad (BUP). Then the samples were carried to the laboratory for the characterization of *E. coli.* Out of 10 samples 4 were collected from diarrhoeic horses and 6 were from apparently healthy horses. Nutrient broth (NB) was used to grow the *E. coil* organisms from the collected samples before performing hanging drop slide test and biochemical test. Nutrient agar (NA) Blood agar (BA) media were used to grow the *E. coli* organisms from the collected samples. Eosin methylene blue agar (EMBA) medium was used as a selective medium for the identification of *E. coli* organisms and antibiotic sensitivity study. McConkey agar (MCA) medium was used for enhancing

the growth of the organisms under the family Enterobacteriaceae and characteristic colonies were produced which helps in differentiating lactose fermenting organisms from lactose non fermenting organisms (Cheesbrough, 1984). Salmonella-Shigella agar (SSA) and Brilliant green agar (BGA) were also used for the differential growth of the organisms.

In order to identify *E. coli*, sugar media (dextrose, maltose, lactose, mannitol and sucrose) were used for biochemical tests. Chemicals and reagents used for conducting the biochemical tests were Gram's stain, Methyl red solution, Methyl-Red Voges- Proskaure (MR-VP) broth, Kovac's reagent, Alpha-nepthanol solution, Potassium hydroxide solution, Phosphate buffered saline (PBS) solution, Phenol red, 20% glycerine, liquid paraffin, wax, alcohol and spirit. Antibiotic sensitivity test was performed using disc diffusion or Kirby-Bauer test method (Bauer *et al.* 1996). Nine different antimicrobial discs with their concentration were used for the test. The antimicrobial agents were tetracycline, streptomycin, amoxicillin, chloramphenicol, cephalexin, ciprofloxacin, gentamicin, trimethoprim and cephradine with concentration of 30, 10, 10, 30, 30, 5, 120, 5 and 30 µg. The isolated *E. coli* of horse and previously isolated *E. coli* of cattle, goat and chicken were preserved in 20% glycerine. Then the isolated *E. coli* were inoculated into EMBA and kept at 37°C for overnight incubation. The organisms on EMBA produced greenish colonies with metallic sheen. Then the isolated *E. coli* on EMBA were placed in icebox and transported to the Enteric Microbiology Laboratory of ICDDR,B for molecular characterization by Pulsed-Field Gel Electrophoresis (PFGE). PFGE involves embedding organism in agarose, lysing the organism in situ and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently. The DNA restriction patterns of the isolates are compared with one another to determine their relatedness.

RESULTS AND DISCUSSION

NB inoculated separately with the swab samples revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically and was indicated by the presence of turbidity. In the same way, separately streaked NA plates revealed the growth of bacteria and was indicated by the growth of circular smooth, opaque, colorless colonies. BA was discolored around the growth of the organism and there was hemolysis. The organisms of the faecal samples produced slight pinkish colony and those produced green colonies were tentatively chosen to be *E. coli*. *E. coli* was presumptively selected and tentatively confirmed by bright pink colored colonies on MCA and greenish colonies with metallic sheen on EMBA produced by the organisms after overnight incubation. In Gram's staining under microscope the organism revealed Gram-negative, pink color, small rod shaped arranged as single or paired. All the isolates were found to be motile in hanging drop slide (Table 1).

Presumptively selected 2 to 3 mm colonies were repeatedly streaked on EMBA to check and confirm their purity. A series of biochemical tests were performed with the culture positive and Gram-negative rod shaped cells. All the isolates fermented five basic sugars (dextrose, sucrose, lactose, maltose and mannitol) produced acid and gas. Acid production

was indicated by the color change from reddish to yellow and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tubes. All the isolates were catalase positive, MR positive, VP test negative and Indole test positive. Isolates giving such pattern of biochemical reactions were regarded as *E. coli* (Table 2).

Table 1. Cultural, morphological and motility characteristics of the isolated *E. coli*

Sources of isolates	Colony characteristics					Staining character	Motility
	EMBA	MCA	BGA	SSA	BA		
BAUSGF	Greenish black colony with metallic sheen	Bright pink color smooth transparent raised colonies	Green color colony	Slight pinkish smooth colonies	Colorless colony with hemolysis	Pink short rod, Gram negative bacilli	+
BAUVC	do	do	do	do	do	do	+
BUP	do	do	do	do	do	do	+

Legend: + = positive

Table 2. Biochemical characteristics of *E. coli*

Different Biochemical tests	Result
Fermentation reaction with five basic sugars:	
Dextrose	+
Sucrose	+
Fructose	+
Maltose	+
Mannitol	+
Fermentation reaction with dulcitol:	
Indole	+
MR	+
VP	-

Legend: + = positive, - = negative

Out of 4 and 6 faecal samples of diarrhoeic and apparently healthy horses respectively, 3 samples each were found to be positive for *E. coli* infection. So, the rate of infection was greater in diarrhoeic horses compared to that of apparently healthy ones and the overall prevalence was recorded as 60% (Table 3).

PFGE analysis of the *XbaI* digested chromosomal DNA of the *E. coli* strains yielded 16 to 23 reproducible DNA fragments ranging in size approximately from <20 to <668.9 Kbp (Fig. 1). The fingerprint pattern in the gel was analyzed using computer software package (Quantity One Version 3.0) and after background subtraction and gel normalization, the

fingerprint patterns were subjected to cluster analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The enzyme (*XbaI*) restriction digestion restricted chromosomal genome into 14-23 fragments. For cluster analysis, only fragments having a molecular weight of 30 kb and above were considered. PFGE analysis revealed that, the strains isolated from the horse from different areas displayed more or less similar restriction fingerprint pattern while the strains of horse, cattle, goat and chicken *E. coli* yielded diverse and heterogeneous banding pattern (Table 4, Fig. 1). Thus, major differences in band patterns were observed among the strains of different species.

Table 3. Prevalence of *E. coli* in diarrhoeic and apparently healthy horses

Sources of samples	Samples examined (no.)	Culture Examination		Biochemical Examination		Prevalence	
		Positive for *E. coli*	Negative for *E. coli*	Positive for *E. coli*	Negative for *E. coli*	Sample (no.)	%
Diarrhoeic horses	4	3	1	3	1	3	75
Apparently healthy horses	6	3	3	3	3	3	50
Total	10	6	4	6	4	6	60

Table 4. Approximate number of band of *E. coli* from horse, cattle, goat and chicken after PFGE analysis

Source	Lab ID	Restriction Enzyme	Approximate restriction fragment (no.)
Horse	H1F	*Xbal*	15-16
Horse	H2VC	*Xbal*	15-16
Cattle	C1VC	*Xba1*	18-19
Cattle	C2VC	*Xba1*	18-19
Cattle	C3VC	*Xba1*	18-19
Goat	G1VC	*Xba1*	15-16
Goat	G2VC	*Xba1*	15-16
Goat	G3VC	*Xba1*	17-18
Goat	G5VC	*Xba1*	17-18
Chicken	P1PF	*Xba1*	15-16
Chicken	P3PF	*Xba1*	16-17

Legend

H1F, H2VC = isolates of horse from BAUSGF
C1VC, C2VC, C3VC = isolates of cattle from BAUVC
G1VC, G2VC, G2VC, G5VC = isolates of goat from BAUVC
P1PF, P3PF = isolates of chicken from BAU Poultry Farm

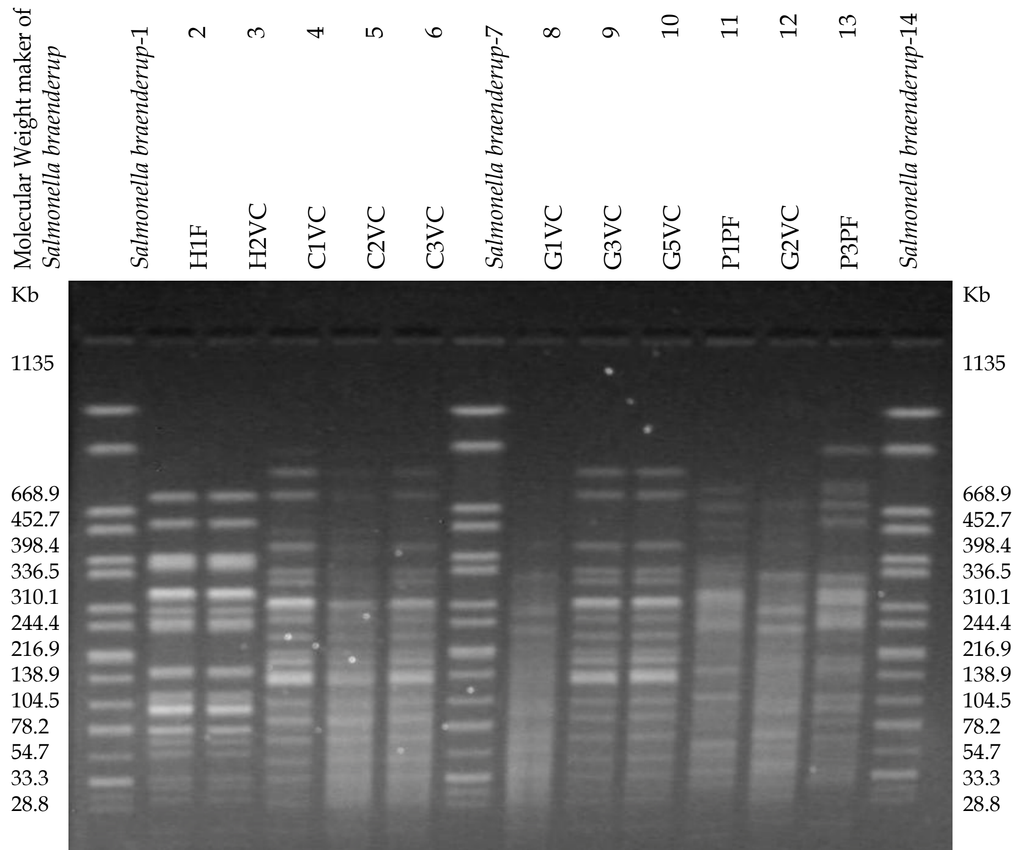

Fig. 1. PFGE pattern of representative E. coli isolated from horse, cattle, goat and chicken with Xba1

Legend
Lane 1, 7, 14 = Genomic organization of the *Salmonella* braenderup (Marker)
Lane 2 = Genomic organization of the isolates of horse from BAUSGF
Lane 3 = Genomic organization of the isolates of horse from BAUVC
Lane 4, Lane 5, Lane 6 = Genomic organization of the isolates of cattle from BAUVC
Lane 8, Lane 9, Lane 10, Lane 12 = Genomic organization of the isolates of goat from BAUVC
Lane 11, Lane 13 = Genomic organization of the isolates of chicken from BAU Poultry Farm

From the antibiogram study, it was revealed that among the isolates of BAUSGF, 100% were highly sensitive to ciprofloxacin, chloramphenicol and trimethoprim; 66.67% were highly sensitive and 33.33% were resistant to tetracycline; 100% were intermediately sensitive to streptomycin; 66.67% were highly sensitive and 33.33% were intermediately sensitive to gentamicin and 100% of the isolates were resistant to amoxicillin, cephalexin and cephradine. The isolates of horse from BAUVC (100%) were highly sensitive to ciprofloxacin, chloramphenicol and cephradine; 66.67% were intermediately sensitive and 33.33% were resistant to trimethoprim; 33.33% were highly sensitive and 66.67% were intermediately sensitive to tetracycline; 66.67% were highly sensitive and 33.33% were

intermediately sensitive to gentamicin and 100% of the isolates were resistant to amoxicillin, cephalexin and streptomycin. Among the isolates of BUP, 100% were highly sensitive to ciprofloxacin and chloramphenicol; 50% of the isolates were highly sensitive and 50% were resistant to tetracycline; 50% were highly sensitive and 50% were resistant to streptomycin; 50% were highly sensitive and 50% were intermediately sensitive to gentamicin and 100% of the isolates were resistant to amoxicillin, cephalexin and cephradine (Table 5).

Table 5. Antibiotic sensitivity pattern of the isolated *E. coli*

E. coli isolates	CIP	C	AML	CL	W	CE	TE	ST	GN
H1F	+++	+++	-	-	+++	-	+++	++	+++
H2F	+++	+++	-	-	+++	-	+++	++	+++
H3F	+++	+++	-	-	+++	-	-	++	++
H1VC	+++	+++	-	-	++	+++	++	-	+++
H2VC	+++	+++	-	-	++	+++	+++	-	+++
H3VC	+++	+++	-	-	_	+++	++	-	++
H1B	+++	+++	-	-	+++	-	+++	+++	++
H1B	+++	+++	-	-	+++	-	++	-	+++

Legend

H1F, H2F, H3F = isolates of horse from BAUSGF
H1VC, H2VC, H3VC = isolates of horse from BAUVC
H1B, H2B = isolates of horse from BUP
+++ = highly sensitive, ++ = intermediately sensitive
CIP = ciprofloxacin, C = chloramphenicol, AML = amoxicillin, CL = cephalexin, W = trimethoprim
CE = cephradine, TE = tetracycline, ST = streptomycin, GN = gentamicin

In this study, colony characteristics of *E. coli* observed on EMBA, MCA, SSA, BA and BGA were similar to the findings of other authors (Buxton and Fraser, 1977; Hasina, 2006 and Nazir, 2004). Differences in colony morphology manifested by the isolates may be due to loosing or acquiring some properties by the transfer of host or choice of host tissue. In Gram's staining the morphology of the isolated bacteria exhibited pink; small rod shaped Gram negative bacilli and in the hanging drops technique all the isolates revealed motility. These findings were agreed with several authors such as Buxton and Fraser (1987), Freeman (1985), Jones *et al.* (1987). Faecal isolates revealed a complete fermentation and all the isolates fermented dextrose, sucrose, fructose, maltose and mannitol with the production of acid and gas within 24h-48h of incubation. These results are positive as reported by Buxton and Fraser 1977 and Honda *et al.*, 1982. The isolates also revealed positive reaction in MR test and Indole test but negative reaction in V-P test which was supported by several authors (Buxton and Fraser 1977; Honda *et al.*, 1982).

In PFGE method chromosomal DNA was digested with a restriction endonuclease that generates large fragments. The restriction fragments were resolved in a pattern of discrete bands. Choice of restriction enzyme is an important factor to obtain a reproducible and well discriminatory banding pattern in PFGE. Genomic organization of the strains of horse *E. coli* from different sources showed that they were same clonal origin but there was no clonal relationship with cattle, goat and chicken *E. coli*. These findings are supported by other studies like Bolton *et al*., 2007; Tenover *et al*., 1995. It was found that most of the *E. coli* isolates were resistant to amoxicillin and cephalexin. Such high prevalence of resistance may presumably be due to indiscriminate use of antibiotics at the present time, which may eventually supersede the drug sensitivity of microorganism from antibiotic saturated environment (Jawetz *et al.,* 1984). These drug resistant bacteria can spread in the environment where man and animal acquire infection resulting in the problem of treatment of infection caused by these *E. coli* (Joseph *et al.,* 1979). Almost all of the strains of horse *E. coli* were showed resistance pattern to amoxicillin and cephalexin. The prevalence of multi-drug resistant *E. coli* observed here appeared to be analogous to earlier predication made by many previous studies (Khan *et al*., 2005 and Nazir *et al*., 2005).

CONCLUSION

The development and use of antibiotics has been one of the most important steps towards controlling of infectious bacterial diseases in the 21st century. However, the subsequent appearance and spread of antibiotic resistance in pathogenic organisms have made many currently available antibiotics ineffective. To successfully fight the increasing numbers of drug resistant and multi drug-resistant bacteria, extensive knowledge of the molecular mechanisms of acquiring antibiotic resistance and updated information regarding current distribution of resistance pattern is required. Prudent use of antibacterial drugs using the appropriate drug at the appropriate dosage and for the appropriate duration is one important means of reducing the selective pressure that helps resistant organisms emerge. The other vital aspect of controlling the spread of multi-drug resistant organisms is providing sufficient hygienic measures and proper care of animal for infection control in Bangladesh. Use of ciprofloxacin and chloramphenicol might be of first choice of treatment against *E. coli* infection in horses. Sufficient efforts in using antimicrobial agents wisely and strict attention to infection may prevent the emergence of resistant organisms in a great extent.

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