Patterns of Antimicrobial Resistance and Plasmid Profiles of *Escherichia coli* Isolates Obtained from Calf, Cattle and Diarrheic Children in Mymensingh – Bangladesh

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Abstract: A total of 83 *Escherichia coli* isolated from fresh faecal samples of calf (16) and cattle (16) of Bangladesh Agricultural University (BAU) dairy farms and stool of diarrheic children (73) from S. K. Hospital (Cholera Unit), Mymensingh, Bangladesh were screened for their antibiograms and plasmid profiles. The overall recovery rate of *E. coli* from samples was 79.05%. All *E. coli* strains were analyzed to determine their susceptibility patterns to 8 commonly used antibiotics (ampicillin, cephradine, chloramphenicol, ciprofloxacin, gentamicin, streptomycin, tetracycline and sulphamethoxazole) belonging to different groups. A total of eight antibiotic resistance profiles were obtained with over 67% of the isolates showing multi-drug resistance. The overall plasmids of different size ranges were detected in 42 (50.6%) of the isolates. Some isolates with multi-drug resistance profiles were found to possess plasmids with different sizes in the range of 5.25 – 40 kb and some were not found to possess plasmids. Therefore, there was no noticeable correlation between antibiotic resistance patterns and plasmid patterns. In calves and cattle, the least resistance levels (<8%) were recorded against tetracycline and streptomycin. Very high resistance levels (>70%) were detected against sulphamethoxazole, tetracycline and ampicillin while chloramphenicol and cephradine recorded the resistance levels of 28.57% and 42.85% respectively, among the diarrheic children isolates. These data raise important questions about the potential impact of antibiotic use in animals and the possible entry of resistant pathogens into the food chain. [The Journal of American Science. 2007;3(3):78-84]. (ISSN: 1545-1003).

Keywords: *Escherichia coli*, antibiogram, plasmid profile, calf, cattle, diarrheic children

1. Introduction

Antibiotic usage is possibly the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Neu, 1992; Witte, 1998). However, the rate of development of resistance appears to have accelerated in the past decade (Smith, 1999) and today multiple resistant bacteria constitute a global problem (Obrien, 1997; Shanahan *et al.,* 1994).

It has been observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment (Hassan, 1985). This therefore demands the need for periodic screening of common bacterial pathogens for their antibiotic susceptibility profiles in different communities. According to Aibinu *et al*., (2004), *E. coli* is highly resistant to ampicillin, amoxicillin, tetracycline and trimethoprim - sulfamethoxazole. The widespread occurrence of drug resistant *E. coli* and other pathogens in our environment has necessitated the need for regular monitoring of antibiotics susceptibility trends to provide the basis for developing rational prescription programs, making policy decisions and assessing the effectiveness of both (Omigie *et al*., 2006).

There is strong evidence that the use of antimicrobial agents can lead to the emergence and dissemination of resistant *E. coli* (van den Bogaard *et al.,* 2001; Galland *et al*., 2001; Schroeder *et al.*, 2002), which can then be passed onto people via food or through direct contact with animals. Sayah *et al.,* (2005) reported that farm environmental isolates showed reduced susceptibility (as measured by disc diffusion zone sizes) compared to faecal sample isolates to most agents studied. They suggested that non-sampled sources, e.g., farm workers and wildlife with access to the farm environment, could be sources of resistance factors.

Escherichia coli is a bacterial organism that belongs to the family Enterobacteriaeceae. *E. coli* is one of the main causes of both nosocomial and community acquired infections in humans. The organism is therefore of clinical importance and can be isolated from some specimens. It is one of the organisms most frequently isolated from blood (Karlowsky, *et al*., 2004).

During recent year the wide spread use of antibiotics in the field of veterinary medicine have resulted in the development of increasing number of bacterial strains possessing resistance to many antibiotics. The property of multidrug resistance could be transferred through conjugation from resistant strains of *E. coli* to another by means of plasmid, which occur in cytoplasm of the donor bacterium and multiply independently of the chromosomal DNA. Thus a new bacterium with resistance factor emerges that is resistant to one or more antimicrobial agents (Buxton and Fraser, 1977). *E. coli* exists in large numbers in the intestinal flora, which indicates tremendous potential for plasmid dissemination in nature (Freeman *et al*., 1985).

The aim of this study was to analyze the influence of exposure to antibiotics used in Bangladesh or for veterinary in dairy farms on the resistance of faecal *E. coli* recovered from calves, cattle and human therapy on the resistance stool *E. coli* recovered from diarrheic children. So the present study was conducted to find out the correlation between antibiotic sensitivity pattern and plasmid profile of *E. coli* isolates and also to find out the effective antibiotic(s) against *E. coli*.

2. Materials and Methods

Sample collection

A total of 105 samples were collected at which, sixteen were from adult cattle, sixteen from calf, and 73 from diarrheic children. The human stool samples were collected from patients of Cholera Unit of S. K. Hospital, Mymensingh. The fresh faecal samples were collected from healthy calves and healthy cattle of BAU dairy farms. All samples (about 100 g) were collected in sterile containers. Faecal samples were collected with the help of sterile cotton bud and transferring the buds immediately to sterile nutrient broth in sterile screw capped test tubes. At each time of collection, precaution was taken to prevent or avoid cross-contamination of samples. After collection of the samples, they were transported to the laboratory as soon as possible in an insulated foam box with ice to maintain a temperature ranging from -4^0C to- 6^0C and bacteriological analyses were performed within 4 h of collection. This study was conducted during the period of July to December' 2005 in the Department of Microbiology and Hygiene and in the Central laboratory, Bangladesh Agricultural University.

Isolation of *E. coli* in pure culture

Primary culture was done in nutrient broth and then pure cultures were obtained using McConkey agar and Eosine Methylene Blue (EMB) agars. The 'pour-plate technique' was followed to get the pure culture of *E coli.* The colony characters were observed and staining was performed by Gram's methods. Isolates yielding similar biochemical tests (indole test, methyl red (MR) test, veges proskauer (VP) test, citrate test, catalase test and sugar fermentation) to the standard *E. coli* strain, ATCC 25922 were identified as *E. coli* and selected for further testing. All isolates were identified using conventional techniques (Chessbrough, 2000).

Antimicrobial susceptibility testing

Susceptibility of *E. coli* isolates to different antimicrobial agents was determined *in vitro* by employing a modified disk diffusion test of the Kirby-Bauer (Bauer *et al*., 1966) method. Antibiotics used in this study are ampicillin (10μg), cephradine (30μg), chloramphenicol (30μg), ciprofloxacin (5μg), gentamicin (10μg), streptomycin (25μg), tetracycline (30μg) and sulphamethoxazole (25μg). Cartridges of antimicrobial-containing discs were obtained from Mast Diagnostics (Merseyside, UK), stored between 4 and -20°C, and allowed to come to room temperature prior to use. The antibiotics were selected because they, or related antibiotics, have been used regularly in dairy farms on veterinary prescription and in human being and may be active against *E. coli*. Isolates were subcultured from the bank onto Miller's LB agar and incubated for 18–24 h before being transferred to 5 ml sterile 0.9% saline to match the '0.5' MacFarland standard (Remel, Kansas). A sterile cotton-tipped swab was used to streak air-dried Mueller-Hinton II plates within 15 min of adjustment of turbidity. Subsequently, antimicrobial discs were added and plates were incubated aerobically at 35 ± 2°C for 16–18 h. The diameter of the zones of inhibition surrounding the antimicrobial discs was measured to the nearest mm. Isolates were deemed resistant only when the zone of inhibition was less than or equal to the resistance breakpoint recommended by the guidelines of NCCLS (2002). Quality control was performed as recommended using *E. coli* strain ATCC 25922.

Plasmid DNA extraction, Reference marker and Agarose gel electrophoresis:

Smaller circular plasmid DNA molecules were extracted from the huge chromosomal DNA for analysis on DNA. The selected bacterial strain (single colony) was grown overnight in LB broth at 37^0C with aeration using the orbital shaker. The 1.5 ml overnight culture was taken in eppendorf tube for plasmid DNA extraction. The plasmid DNA from *E. coli* isolates was extracted through Mini alkaline lysis by SDS (Sambrook *et al*., 2001). The plasmid DNA extracted from *E. coli* isolates were compared to known molecular weight standards (Super mix DNA ladder,

ranging from 0.5 to 33.5 kbs, Bangalore Genei Pvt. Ltd. India). Electrophoresis was carried out in a horizontal gel apparatus (Max submarine, Agarose gel unit, Model He 99). The method followed for agarose gel electrophoresis was as described by Maniatis *et al*., (1983).

3. Results and Discussion

The overall prevalence of *E. coli* in samples was 79.05% (Table 1). The prevalence rate of *E. coli* was higher in the faecal samples of calf and cattle (84.37%) than the stool samples of diarrheic children (76.71%).

In nutrient broth turbid were found, in EMB agar black centered colony with metallic sheen was found. The greenish-black colonies with metallic sheen on EMB agar were presumptively identified as *E. coli* (Pelczer *et. al.,* 1998). Also in Gram's staining under microscope the organism revealed gram-negative, pink color, small rod shaped that is characteristic features of *E. coli*. Several biochemical tests were performed for confirmation of *E. coli*. They were characterized by their ability to ferment glucose, sucrose, lactose, maltose, mannitol and sorbitol to produce gas (CO_2), positive for indole test and MR test, and negative for VP and Citrate utilization test.

A total of 83 isolates of *E. coli* from faeces and stool sources were analyzed. The antibiotic sensitivity pattern and the percentage of isolates resistance and sensitivity to each antibiotic are outlined in Table 2 & 3. In the case of calves, the fifty percent (50%) isolates were resistant to sulphamethoxazole; 100% and 78.57% isolates were highly sensitive to ciprofloxacin and chloramphenicol respectively. In the same way, 71.42% *E. coli* isolates of calf were moderately sensitive and the rest (28.57%) were highly sensitive to gentamicin.

Some variation in resistance of the isolates from cattle was observed. As regards to calf, about fifty percent of the isolates of cattle (53.84%) were resistant to ampicillin. The cattle isolates were resistant and highly sensitive to sulphamethoxazole in an equal rate (30.76%). The isolates from cattle were highly sensitive (84.61%) to chloramphenicol and found to be 100% highly sensitive to ciprofloxacin.

The children isolates were 100% resistant to sulphamethoxazole, 85.71% to tetracycline and 71.42% to ampicillin. The isolates were highly sensitive to tetracycline, chloramphenicol, gentamicin and ciprofloxacin (14.28%, 42.85%, 42.85% and 28.57% respectively).

The summaries of the antibiogram profiles obtained are presented in Table 4. The results show that about 67.46% of the *E. coli* isolates are multidrug resistant, i.e. are resistant to four or more antibiotics.

Out of the 83 *E. coli* isolates, 42 (50.6%) were found to possess plasmids, which ranged in sizes from 5.25kb to 40kb. Some isolates possessed single sized plasmids while others had multiple plasmids with different sizes as shown in Table 5. Plasmid from 83 *E. coli* isolates were extracted according to the procedure described in materials and methods and analyzed by agarose gel electrophoresis. Some of the plasmids of these strains showed bigger bands that were confusing. It was uncertain whether these bands indicate large plasmid or a band of sheared chromosomal DNA.

Incase of cattle out of 13 isolates, 8 isolates did not show any plasmid bands but they showed resistant to ampicillin, sulphamethoxazole. In some cases, the isolates did not show resistant to any antibiotics that were used but they showed plasmid bands. The isolates from CF-3, CF- 8, CF- 14, CT- 1 and CT- 4 did not confer resistance to any of the antibiotics those were used but they showed plasmid bands indicating that chromosomal DNA may carry the genes that confer resistance to antibiotics. Incase of diarrheic children same pattern was also observed.

From this study, it was found that most of the isolates were resistant to some antibiotics such as ampicillin, sulphamethoxazole and tetracycline. Such high incidence of multidrug resistance may presumably be due to indiscriminate use of antibiotics at the present time, which may eventually supercede the drug sensitive microorganisms from antibiotic saturated environment (Jawetz *et al.,* 1984). From the plasmid profile analysis it was revealed that some isolates carried multiple plasmids, some carried single plasmid and some carrying no plasmids which correlates with the results of Lee *et al.,* (2000). Through the discharges of human, animal and bird faecal materials, drug resistant bacteria are distributed in the sewage and surface water where exchange of R-plasmids can occur under certain physico-chemical and biological conditions (Anonymous, 1978). The drug resistant bacteria can spread in the environment where man and animal acquire infection with bacteria carrying drug resistant plasmids (Joseph *et al.*, 1979).

The plasmids were distributed at random in the isolated *E. coli* strains. *E. coli* isolates contained single or multiple plasmids bands and showed multiple drug resistance patterns. These multiple drug resistance patterns of the *E. coli* isolates of this study might be due to drug resistance gene(s) carried out by the different plasmids (Freeman, 1985; Bakshi *et al.,* 2003). In most of the cases, strains having similar antibiotic sensitivity patterns but showed different plasmid patterns. The finding that the plasmidless strains may also be resistant to one or more antibiotics has supported this supposition. Therefore, there was no noticeable correlation between antibiotic resistance patterns and plasmid patterns.

Multi drug resistance has serious implications for the empiric therapy of infections caused by *E. coli* and for the possible co-selection of antimicrobial resistance mediated by multi drug resistance plasmids (Sherley *et al*., 2004). Multidrug resistant *E. coli* were observed to be very common in the study area as about 67.46% of isolates showed multidrug resistance. Some of the isolates that showed multiple drug resistance were found to harbour plasmids with sizes of 5.25kb to 40kb and some showed no plasmids. This is not similar to what was observed by Smith *et al*., (2003) who reported that 47 of the *E. coli* isolated from animals in Lagos harbour detectable plasmids which ranged in sizes from 0.564kb to >23kb. Danbara *et al.,* (1987) also reported plasmids of sizes between 3.9kb and 50kb in *E. coli* strains isolated from Traveller's diarrhoea. So, there is no noticeable interrelationship between the plasmid pattern and drug sensitivity or resistance pattern.

Pathogenic isolates of *E. coli* have relatively high potentials for developing resistance (Karlowsky *et al*., 2004). The significant resistance of *E. coli* isolates to ampicillin tested was observed in this study (calf 42.85%, cattle 53.84% and diarrheic children 71.42%). This is not similar to what was observed by Aibinu *et al.,* (2004) who reported 100% resistance of their *E. coli* isolates to ampicillin. Desenclos *et al*., (1988) reported 67% of their *E. coli* isolates were resistant to tetracycline. Their finding is in not harmony with the report of this study, showing 85.71% stool *E. coli* isolates from diarrheic children resistance to tetracycline. The reason for this high resistance to commonly used antibiotics may be due to widespread and indiscriminate use in our environment.

In recent years, use of fluoroquinolones has increased in many countries and emergence of resistance of bacterial isolates to fluoroquinolones has been observed. Consistent stepwise increase in *E. coli* resistance to ciprofloxacin was observed from 1995 (0.7%) to 2001 (2.5%) by Bolon *et al*., (2004). Ciprofloxacin resistance in Portugal was 25.8% and Italy 24.3% while in Germany and Netherlands it was 15.2% and 6.8% respectively (Oteo *et al*., 2005). But the percentage of ciprofloxacin sensitivity observed in this study was 28.57%, among the diarrheic children isolates. Therefore, we found that the prevalence of resistant isolates of *E. coli* in children increased against ciprofloxacin.

The reason for the high resistance to antibiotics observed in this study compared to other works may be due to increasing an irrational consumption rate, transmission of resistant isolates between people and consumption of food from animals that have received antibiotics. Self-medication and non-compliance with medication and sales of substandard drug may account for the rise in antibiotic resistance observed in this community.

Since antimicrobial resistant patterns are constantly evolving, and present global public health problem, there is the necessity for constant antimicrobial sensitivity surveillance. This will help clinicians provide safe and effective empiric therapies.

Table 1. Prevalence of *E. coli* in Various Specimens

Specimens	Number Screened	Number of Positive samples	Prevalence (%)	Prevalence overall (%)
Adult cattle and calf	32	27	84.37	79.05
Diarrheic Children	73	56	76.71	

Table 2. Antibiotic Sensitivity/Resistance Pattern of *E. coli* Strains Isolated from Various Specimens (n=83)

Sources of *E. coli* isolation	Resistance		Less sensitive		Moderate sensitive		Highly sensitive	
	Antibiotic	%	Antibiotic	%	Antibiotic	%	Antibiotic	%
Calf (14)	AMP	42.85	AMP	28.57	AMP	21.42	AMP	7.14
	SXT	50			SXT	14.28	SXT	35.71
	TE	7.14	TE	28.57	TE	21.42	TE	42.85
	S	7.14	S	35.71	S	28.57	S	21.42
					C	21.42	C	78.57

			CE	57.14	CE	28.57	CE	14.28
					GN	71.42	GN	28.57
							CIP	100
Cattle (13)	AMP	53.84	AMP	15.38	AMP	15.38	AMP	15.38
	CE	23.07	CE	30.76	CE	38.46	CE	7.69
	S	7.69	S	38.46	S	15.38	S	38.46
	SXT	30.76	SXT	15.38	SXT	23.07	SXT	30.76
					C	15.38	C	84.61
					GN	53.84	GN	46.15
	TE	7.38			TE	8.01	TE	84.61
							CIP	100
Diarrheic Children (56)	AMP	71.42	AMP	28.57	C	28.57	C	42.85
	C	28.57	CIP	42.85	CIP	28.57	CIP	28.57
	CE	42.85	CE	42.85	CE	14.28		
	S	28.57	S	42.85	S	28.57		
	SXT	100			GN	57.14	GN	42.85
	TE	85.71					TE	14.28

AMP= Ampicillin C = Chloramphenicol CE = Cephradine CIP = Ciprofloxacin
S = Streptomycin SXT = Sulphamethoxazole GN = Gentamicin TE= Tetracycline

Table 3. Antibiotic Resistance of *Escherichia coli* Isolates from Various Specimens

Antibiotic tested	**Calf (n = 14)**	**Cattle (n = 13)**	**Diarrheic Children (n = 56)**	**Total (n=83)**
Ampicillin	6(42.85%)	7(53.84%)	40(71.42%)	53
Sulphamethoxazole	7(50%)	4(30.76%)	56(100%)	67
Tetracycline	1(7.14%)	1(7.38%)	48(85.71%)	50
Gentamicin	0(0%)	0(0%)	0(0%)	0
Streptomycin	1(7.14%)	1(7.69%)	16(28.57%)	18
Chloramphenicol	0(0%)	0(0%)	16(28.57%)	16
Cephradine	0(0%)	3(23.07%)	24(42.85%)	27
Ciprofloxacin	0(0%)	0(0%)	0(0%)	0

Table 4. Summary of Antimicrobial Resistance Profiles (Antibiograms) of *E. coli* Isolated From Various Specimens

Number of antibiotics resistant to	**Number of strains showing pattern**
One antibiotic	6 (7.23%)
Two antibiotics	4 (4.82%)
Three antibiotics	17 (20.48%)
Four antibiotics	10 (12.05%)
Five antibiotics	13 (15.66%)
Six antibiotics	11 (13.25%)
Seven antibiotics	9 (10.84%)
Eight antibiotics	13 (15.66%)

Table 5. Sizes and Frequency of Plasmid Detected In *E. coli* Isolated from Different Isolates and Correlation with Resistance Profiles

Plasmid sizes (kb)	**No. (%) of isolates**	**Level of resistance profile**
≥5.25 - 10 kb	10 (23.81%)	Low
>10 – 33.50 kb	16 (38.09%)	Low to Medium
>33.50 – 40 kb	16 (38.09%)	Low to High

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