The prevalence and genetics of resistance to commonly used antimicrobial agents in faecal Enterobacteriaceae from children in Bangladesh

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SUMMARY

The present study was undertaken to investigate the occurrence of antibiotic resistance in enteric flora in 64 children in rural Bangladesh over a 12-month period. The antibiotic resistance pattern of the isolates varied throughout the year and multiple resistance was highest during the post monsoon period. Seventy-three percent of children had isolates resistant to more than three antibiotics throughout the year. Resistance to streptomycin was highest (78%), followed closely by ampicillin (72%). Of 82 multiply resistant isolates, plasmid DNA was demonstrated in 75%. Plasmid sizes ranged between 3·7 and 110 MDa, the commonest plasmids were of 70, 98 and 110 MDa. Complete or partial resistance was transferred by conjugation from 52% of the isolates, most frequently by single plasmids. The commonest plasmid incompatibility group was F11-A (46%) followed by incompatibility group P (22%). Plasmids of molecular weight 98 MDa most often hybridized with F11-A probes and those of 110 MDa with H11 probes. Plasmids from 10 transconjugants were digested with restriction enzymes and digest patterns demonstrated the presence of common plasmids. The findings show that there is a diverse, and mobile, genetic pool of resistance genes in this rural community. This genetic reservoir is potentially transferable to enteric pathogens, with major implications for public health and diarrhoeal disease control.

INTRODUCTION

The widespread use of antimicrobial agents in the treatment of infections in the tropics has led to serious problems of antimicrobial resistance. The emergence and spread of antibiotic resistance in bacteria of medical importance imposes serious constraints on the options available for the treatment of many infections [1]. This problem has been brought into prominence by the recent widespread outbreaks of enteric diseases caused by drug resistant organisms [2]. Among enteric pathogens, major epidemics of infection with resistant shigellae have occurred in Latin

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America [3], Central Africa [4] and south Asia [5]. An extensive epidemic of typhoid fever in Mexico with over 10000 cases was due to multi-drug resistant strains of *Salmonella typhi* [6]. Similar multiresistant strains of *S. typhi* have also been reported from other countries [7–9]. *Vibrio cholerae* resistant to commonly used antibiotics have been reported from Philippines [10], Thailand [11], Tanzania [12] and Kenya [13]. In Bangladesh several studies have demonstrated the extensive problems of multiple resistance in shigellae [14–16]. Some strains have been isolated that are resistant to all commonly available oral antimicrobials [17].

A number of studies have indicated that multiple resistance in enteric pathogens occurs by *in-vivo* transfer of resistance plasmids from normal gut flora [18]. Resistance in gut flora may arise from the wide-spread, indiscriminate use of antimicrobials [19] and from environmental spread of resistant enteric flora [20]. Several reports have indicated a relatively high prevalence of antimicrobial resistance in enteric flora in children in tropical countries [21–22]. Despite the extensive data on antibiotic resistance in enteric pathogens in Bangladesh, little information on resistance in normal enteric flora, which is likely to be the source of resistance in enteric pathogens, has been published.

The present study was undertaken to investigate the prevalence and genetics of resistance in enteric flora from children in a rural area of Bangladesh over a year.

MATERIALS AND METHODS

Study population

Rajbari district is situated 100 km to the west of Dhaka, the capital of Bangladesh. The study was undertaken in two neighbouring villages in Rajbari district, Matipara and Kazibandha. Both villages are typical of the rural area of Bangladesh. Sixty-four healthy children between 1 and 5 years were selected from the two villages. At least one child was selected from each 'bari' in the villages. A bari is a compound comprising a few houses belonging to different members of the same extended family. On five occasions two children from the same family were included. Consent was obtained from parents to include their child in the study. Only the children whose parents agreed to complete the study period of 1 year were included.

Bacterial isolates

Faecal specimens were collected in sterile plastic containers from each child once every month. A portion of the specimen was transferred with a dry sterile swab in to Amies transport medium (Vi-pack Transport Swab System, Exogen, UK) and these were kept in a cooling box till they were brought back to the Institute of Public Health (IPH), Dhaka. The specimens were serially plated onto three media; MacConkey agar, MacConkey agar containing chloramphenicol (30 µg/ml), and Direct Sensitivity Test agar (+5% lysed blood) with trimethoprim (5 µg/ml). Coliforms for sensitivity testing were isolated from the plate containing chloramphenicol, from the trimethoprim plate if there were no coliforms on the chloramphenicol plate, or from the MacConkey plate if there were no coliforms on the antibiotic containing plates. A single representative colony was selected for each child. Coliform bacteria from the plates were tested for sensitivity to the

following antibiotics using the Stokes comparative method [23]: ampicillin (10 µg), trimethoprim (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), sulphonamide (300 µg), streptomycin (10 µg), and nalidixic acid (30 µg). A fully sensitive *Escherichia coli* (NCTC 10418) was used as the control. Multiply resistant (resistant to six antibiotics) isolates were stored on Amies transport medium and kept at +4 °C for further studies.

Genetic studies were done on 82 isolates sensitive to nalidixic acid but resistant to the other six antibiotics. Each of these isolates was identified biochemically using the API-10 system (API, Basingstoke, UK).

Resistance transfer

Conjugation experiments were done by broth mating for each of the 82 isolates. The recipient in each case was the laboratory strain *E. coli* K-12 (14R525 Lac⁺, F⁻, Na^r). This strain is sensitive to each of the six antibiotics used in this study, contains no plasmid and has chromosomally encoded resistance to nalidixic acid. Both the donor and recipient were grown to log phase in 5 ml fresh warm nutrient broth at 37 °C in a shaker at 250 rev/min. The donor and recipient were mixed in 5 ml of fresh nutrient broth at a ratio of 1:4 and incubated overnight at 37 °C without agitation. MacConkey plates incorporating chloramphenicol (30 µg/ml) and nalidixic acid (30 µg/ml) were used for selection of transconjugants. Antibiotic sensitivity tests with the same six antibiotics were performed for all the transconjugants to determine the resistance pattern transferred.

Isolation of DNA

Plasmid DNA was extracted from all the 82 multiple resistant isolates and transconjugants using the method of Birnboim and Doly [24]. Plasmid DNA was demonstrated by gel electrophoresis. Electrophoresis was performed with a horizontal electrophoresis system (Model H5, BRL, USA) using 0.7% agar gel. Electrophoresis was carried out at 120 V for 2.5 h. After electrophoresis, the gel was stained for 30 min in ethidium bromide (2 µg/ml). A vertical polaroid camera was used to take pictures, using UV light for illumination.

Ten selected transconjugants were digested with restriction enzyme, *Bam* H1. For restriction enzyme digest the method of Sambrook [25] was followed. Digested plasmid DNA was demonstrated as previously described.

DNA hybridization

Strains of *E. coli* containing incompatibility 'rep' probes on plasmids were obtained from the Department of Molecular Biology, University Libre de Bruxelles, Belgium [26]. They were grown overnight in 10 ml nutrient broth, and then plated onto nutrient agar plates and tested for appropriate antibiotic sensitivity. Bulk plasmid preparations were made for individual incompatibility probes [25]. Colony hybridization was done for all the 82 multiply resistant strains. Labelling of probes was performed as follows: 13.5 µl of probe DNA was placed in an Eppendorf tube and boiled for 5 min and then kept at 37 °C for 10 min. This was then added to reagents from Boehringer Random Primer DNA labelling kit (2 µl Reaction mixture, 1 µl each ATP, GTP and TTP and 1 µl

Klenow enzyme) and 2.5 μ l alpha 2 p-d CTP. The labelling reaction was kept at room temperature for a minimum of 5 h. The labelled probe was then ready for hybridization experiments. Single colonies of the strains to be hybridized were transferred to nitrocellulose paper and denatured with 0.5 M NaOH and washed in 1M Tris HCl. The filter was then air dried and baked at 80 °C for 2 h.

The labelled probe was boiled for 5 min and added to 20 ml of the hybridization solution containing 5 $\times$ Denhardt's solution (0.1 % each of bovine serum albumin, polyvinylpyrrolidone, and ficoll), 6 $\times$ SSC buffer, 0.5 % SDS, 0.01 M-EDTA, 50 % deionized formamide, and 100 μ g/ml denatured calf thymus DNA. The baked nitrocellulose paper was covered with the above solution, and incubated at 42 °C overnight. After hybridization, the filter was washed with 2 $\times$ SSC containing 0.5 % SDS and air-dried. Hybrids were detected by exposure to X-ray film for 12 h at -70 °C.

RESULTS

Sixty-four children from the two villages were included in this study. The age of the children ranged from 1-5 years. Out of the 64 children, 33 (52 %) were boys and 31 (48 %) were girls. Thirty-three children were from Matipara and 31 from Kazibandha.

Specimens were obtained from each child every month. Difficulties in storage of specimens and delays in transport to Dhaka resulted in failure to isolate coliform bacteria from some specimens. The greatest difficulty occurred in March when only 45 specimens were culture positive. The numbers of children with isolates resistant to individual antibiotics throughout the year is shown in Table 1. In January most of the children (over 90 %) had strains resistant to ampicillin, chloramphenicol, tetracycline and sulphonamide. From March onwards the resistance to streptomycin increased in comparison to other antimicrobials, and from August to December resistance was over 91 % on all occasions. In March and June the numbers of resistant isolates were low in relation to other months of the year, but the percentage resistance to most of the antimicrobials remained above 25 %. Overall, the highest prevalence of resistance was to streptomycin, followed by ampicillin and sulphonamide. In comparison to other antibiotics trimethoprim resistance remained low throughout the year ranging from 70 % in January to 17 % in May. In this study all the isolates were sensitive to nalidixic acid.

Table 2 shows the distribution of multiple resistance throughout the year. In all but two of the months, over 50 % of children had coliforms resistant to three or more antibiotics. Resistance to six antibiotics ranged between 9-45 %, however, in most of the months it was above 15 %. There was some seasonal pattern to the occurrence of multiple resistance, occurring least during the drier months of the year (March-June) and most during and following the monsoon (July-October). A total of 82 coliforms each resistant to six antibiotics, were selected for plasmid analysis. These were selected to ensure that isolates for each month and each village were included. Of these 65 were *E. coli*, 12 were *Klebsiella* spp and the remaining 5 were *Citrobacter freundii*. Plasmid DNA was demonstrated in 62 (75 %) of these isolates. The number of plasmids per isolate ranged from 1-4. The majority of isolates (63 %) had a single plasmid, but 26 % had 2, 10 % had 3 and 1 isolate had 4 plasmids. Plasmid sizes ranged from 3.7 MDa to 110 MDa. The

Table 1. *Monthly prevalence of antibiotic resistance (percentage of children carrying coliforms resistant to the different antibiotics)*

Month	*	Am	Tm	Cm	Tc	Su	Sm
Jan.	52*	94	70	94	92	87	77
Feb.	49*	71	53	63	45	76	47
Mar.	45*	58	31	29	29	20	57
Apr.	54*	74	41	59	65	59	87
May	48*	71	17	52	65	38	48
June	53*	45	26	32	34	32	45
July	51*	84	55	88	82	67	88
Aug.	55*	76	51	65	73	73	96
Sep.	58*	67	62	65	63	75	91
Oct.	60*	80	51	62	80	82	93
Nov.	62*	76	45	76	81	69	98
Dec.	62*	66	40	69	66	69	98

* Represents number of children from whom coliform bacteria were isolated in each particular month.

Table 2. *The monthly distribution of multiple antibiotic resistance (percentage of children with isolates resistant to 3–5 or 6 antibiotics)*

No. of anti-biotics*	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.
3–5	98	69	31	65	56	34	94	80	86	90	85	73
6	45	14	9	26	13	15	41	31	26	28	29	29

* 3–5, Resistance to 3 to 5 antibiotics; 6, resistance to all six antibiotics (Ap Tm Cm Tc Su Sm).

most commonly occurring plasmids were in the size range 96–104 MDa and occurred in 35 (56%) of the isolates, the second most common was in the size range of 61–95 MDa and occurred in 19 (30%) of isolates. Plasmids of size 98 MDa and 70 MDa were present throughout the year. Plasmids of size 110 MDa were present only after September.

Among the 62 isolates, there were 22 different plasmid profiles. The most commonly occurring profile was that of a single plasmid of size 98 MDa, which occurred in 35% of the isolates.

Conjugation experiments were done on each of the 82 isolates that were resistant to six antibiotics. In 43 (52%) strains there was successful transfer of some or all of the resistance determinants.

DNA was extracted from each of these transconjugants. In seven donor isolates even after repeated attempts it was not possible to demonstrate any plasmids despite being able to transfer the resistance. However, in four of these, plasmid DNA was demonstrated in the transconjugant. In the other three it was not possible to demonstrate plasmids in the donors or transconjugants. In another three isolates, plasmid DNA was demonstrated in donor and antibiotic resistance was transferred but no plasmid DNA was visible in the transconjugant. In the remainder, the molecular weight of transconjugant plasmids corresponded with the molecular weight of plasmids in the respective donor. The commonest

Table 3. *Resistance patterns transferred by plasmids of different molecular weight*

Transconjugant plasmid size (Md)	Resistance pattern(s) transferred*
110	Ap Tm Cm Tc Su Sm
98	{ Ap Tm Cm Tc Su Sm Ap Tm Cm Tc Su Ap Tm Cm Tm Cm Tc Ap Cm
80	Ap Tm Cm Tc Su Sm
70	Ap Tm Cm Tc Su Sm
60	{ Ap Tm Cm Tc Su Sm Ap Tm Cm Tc
24	Ap Cm Tc Su

* Ap, ampicillin; Tm, trimethoprim; Cm, chloramphenicol; Tc, tetracycline; Su, sulphonamide; Sm, streptomycin.

resistance pattern to be transferred was Ap Tm Cm Tc Su Sm, from 28 isolates followed by Ap Cm in 9 strains.

Table 3 shows the resistance patterns transferred by different plasmids.

The commonest size of plasmid to be transferred was 98 MDa. In 17 strains it was transferred alone and in three it was transferred with another plasmid. The next commonest plasmid size was 110 MDa found in seven transconjugants. Only one plasmid below 60 MDa was transferred alone (23.9 MDa) and this encoded resistant to Tm Cm Tc Su.

Chloramphenicol was used in the selective medium so all transconjugant strains were resistant to chloramphenicol. Ampicillin was the next most common resistance to be transferred (95%). The lowest transfer frequency for individual antibiotics was found for sulphonamide and streptomycin which occurred in 70%.

Plasmid DNA from 10 transconjugants were digested with *Bam* HI (Fig. 1). *Lamda* digested with *Hind* III was used as a molecular weight marker.

Three 98 MDa plasmids were digested (lanes 1, 7 and 9). Two of these (lanes 1 and 9) transferred the same resistance determinants and appeared to have the same digest pattern. The third 98 MDa plasmid had a different resistance and digest pattern. Of the three 110 MDa plasmids, two (lanes 4 and 10) have similar digest pattern and the third (lane 3) had an indistinct pattern. Among the four remaining plasmids of sizes 80, 70, 60 and 24 Md, there was no obvious similarity.

Incompatibility testing by colony hybridization of the 82 isolates resistant to 6 antibiotics was done using 11 incompatibility group probes (Table 4). Fifty-eight isolates hybridized with one or more than one incompatibility group probe. The most commonly occurring incompatibility groups were FII-A, hybridizing with 38 (46%) of isolates, and Inc P hybridizing with 18 (22%). Three Inc group probes (N, W and X) did not hybridize with any of the isolates. The results showed some association between plasmid size and incompatibility groups. Eighteen of 35 strains containing plasmids of size 98 MDa hybridized with Inc FII probe. Four of 8 strains containing plasmids of molecular weight 110 MDa hybridized with Inc HI probe.

Fig. 1. Photograph of gel showing restriction digests

Lane	Isolate number	Plasmid size (MDa) in transconjugant	Resistance in transconjugant	Plasmid Inc gr in donor
1	48 1	98	ApCm	—
2	76 2	70	APTmCmTcSuSm	FII P
3	5 10	110	ApTmCmTcSuSm	—
4	6 10	110	ApTmCmTcSuSm	FII
5	23 7	80	ApTmCmTcSuSm	FII P
6	Lamda			
7	40 8	98	ApTmCmTcSuSm	FII
8	81 8	23.9	TmCmTcSu	—
9	78 9	98	ApCm	FII
10	48 10	110	ApTmCmTcSuSm	—
11	3 11	60	ApTmCmTc	FII P T

The longitudinal carriage of multiply resistant strains and the properties of their plasmids is shown for children from the two villages in Table 5. For each child the isolates were *Escherichia coli*.

In some cases, strains with a common plasmid profile were isolated in different months for a given child, suggesting persistent carriage of a single strain. For most

Table 4. *Incompatibility groups of different plasmids*

Incompatibility group	No. of strains positive	Plasmid sizes in positive strains*
FIA	4	98, 70:60
FIIA	38	98, 80, 70, 60, 32
III	16	110, 98, 80:4.6
B	7	98:4.6, 70
N	0	
P	18	110, 98, 80:4.6, 70
T	5	110, 98, 80:4.6, 70
U	2	70:60
W	0	
X	0	

* Indicates more than one plasmid in the isolate.

Table 5. *Longitudinal carriage of multiply resistant strains: differentiation by plasmid profiles (plasmid sizes in Md)*

	Child	Winter	Hot season	Monsoon	Post monsoon	
Matipara	3	98	—	98/4.6	110	98/60
	23	—	98	70/60	70/30 98	
	28	98	98/44	98/4.6	110	98/4.6
	31	98	98	70 98/50	70/60	
Kazibandha	46	70	32	98 70/60	110	60
	47	98	98	—	110	
	48	—	98	98	98	
	55	—	98	70/60	98	70

children, different strains, on the basis of plasmid profiles, occurred in different months, suggesting loss and gain of different resistant strains. In some months, strains were isolated from a given child in which one plasmid size persisted, but which varied in the presence or absence of other plasmids. These findings may have arisen from the acquisition of different strains, or from plasmid transfer into existing strains. The table shows the probable introduction of strains containing plasmids of 110 Md occurring in both villages after September.

DISCUSSION

The study has shown a high prevalence of resistance to individual antibiotics, and of multiple resistance within the community. The overall occurrence of resistant isolates ranged from 78% resistant to streptomycin to 45% resistant to trimethoprim. There was some seasonal pattern to the occurrence of multiple resistance with the lowest prevalence during the drier months (March–June) and highest during and following the monsoon (July–October). In all but 2 of the months, over 50% of children had faecal coliforms resistant to three or more antibiotics. The seasonal pattern of resistance follows the pattern seen in environmental pollution and enteric diseases, being more common during and after the monsoon.

Plasmid DNA was demonstrated in 62 of the 82 multiply resistant isolates tested. The three most frequently occurring plasmids were of 70 MDa, 98 MDa and 110 MDa size. While plasmids of molecular weights 70 and 98 MDa occurred throughout the year, plasmids of molecular weight 110 MDa appeared only after September suggesting that they were introduced from outside. Among strains resistant to all antibiotics, 22 different plasmid profiles occurred indicating that the widespread occurrence of resistance was not due to a single, multiply resistant strain. However, common profiles occurred in different children, and at different times of the year, indicating certain strains were widely distributed.

The number of different plasmid sizes was not large and it is possible that different strains arise from genetic re-arrangements within a limited number of strains in the community, rather than a wide range of strains being introduced separately. Among individual children, in some cases strains with a common plasmid profile were maintained in different months, in other children different profiles were obtained at different months showing that individual children gained and lost strains throughout the year.

Resistance to each of the six antimicrobials was transferable *in vitro*. In most cases, multiple resistance was transferred by single plasmids, most commonly of size 98 MDa. However, plasmids of this molecular weight transferred different resistance patterns, and so were not all genetically homogenous. This was confirmed by the restriction digests which demonstrated common patterns in plasmids of the same molecular weight and resistance pattern, and different digest patterns in plasmids with different resistance patterns, though of the same molecular weights.

Incompatibility group testing showed a limited range of incompatibility groups. In particular, plasmids of molecular weight 98 MDa hybridized with FII-A probes and those of molecular weight 110 MDa with HII probes. Some of the 110 MDa plasmids that were isolated from different children had common incompatibility groups, resistance transfer pattern and restriction digest patterns, indicating these plasmids were widely disseminated in the community. The introduction of the 110 MDa plasmid of a different incompatibility group to the 98 MDa plasmid, which was also capable of transferring all six antimicrobials, demonstrated how multiple resistance could rapidly be introduced into a community. Among plasmids with common antimicrobial resistance patterns, most showed some degree of DNA homology in belonging to the same incompatibility group. However, some of the same sized plasmids belonged to different incompatibility groups and different sized plasmids belonged to the same incompatibility groups. This suggests that similar resistant determinants have spread to different plasmids of the same and different incompatibility groups probably in the form of transposons.

A relatively low level of personal hygiene among children, and close contact between children is likely to result in an easy spread of resistant enteric flora. This resistant gut flora might be a reservoir for the dissemination of resistant 'epidemic' plasmids into pathogenic enteric bacteria, particularly as the peak occurrence of resistance coincides with seasonal peaks for diarrhoeal diseases. The increase in resistance in gut flora coincides with the time when there is usually an increase in number of shigella and cholera cases in Bangladesh [27-28]. There is

thus an increased opportunity for acquisition and spread of resistance genes in enteric pathogens from normal flora, by both *in-vivo* transfer and person-to-person spread. The poor environmental conditions for much of the rural population of Bangladesh are unlikely to improve significantly in the next decade, and indeed, are likely to be worsened by natural disasters. Unless the increasing spread of resistance to antimicrobial agents can be controlled, morbidity and mortality due to enteric and other pathogens will continue to affect these populations.

There is evidence from studies in other countries in the tropics that demonstrate a high prevalence of multiple antibiotic resistance in normal bowel flora and suggesting that they may act as a reservoir of resistance, available for transfer to enteric pathogens. A study of commensal gut flora in children in Sudan [29] found that 39% of children had strains resistant to 6 antibiotics and over 70% of the children had strains resistant to at least 4 of the 6 antibiotics commonly used in the country. Of 40 isolates tested 19 were able to transfer all or part of their resistance pattern, most commonly by plasmids of 62 MDa. Enterobacteriaceae collected from children with diarrhoea in Indonesia showed that all children carried multiply resistant strains, and many strains were resistant to 6–9 antibiotics [30]. On incompatibility grouping plasmids of FII, N, B, FI, I₁, H2 and T group occurred, the commonest were FII, B and N. The possibility of acquisition of resistance from normal flora was demonstrated in a shigella epidemic in Zaire and Rwanda [31]. Most of these strains were resistant to commonly used antibiotics and contained plasmids belonging to incompatibility group X. Tetracycline was replaced by cotrimoxazole for treating the cases [32]. A few months later a new plasmid appeared belonging to incompatibility group I₁ which encoded trimethoprim resistance. The development of trimethoprim resistance during treatment of cases and the appearance of a new plasmid of different incompatibility group suggests that this was acquired locally, probably from some indigenous bowel bacterium.

Acquisition of resistance from normal flora has probably occurred in *V. cholerae*. In Tanzania following widespread use of tetracycline to treat sensitive *V. cholerae*, tetracycline resistant strains appeared, containing incompatibility group C plasmids [33]. When tetracycline was replaced by trimethoprim, the strains soon became resistant to this drug [34]. This resistance was mediated by DHFR type I enzyme which is usually transposon encoded. It is likely that the strains acquired the transposon from gut flora.

The increasing spread of antimicrobial resistance is a major constraint to improved health care in many tropical countries. In order to develop rational drug policies it is necessary to obtain information on the prevalence and mechanisms of drug resistance at the community level. Prospective studies on antibiotic use at district and village level would provide important data. Surveillance studies of antimicrobial resistance, and molecular epidemiology as described here would enable the detection of the occurrence and spread of epidemic plasmids.

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