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β-Lactam resistance in normal faecal flora from South Africa

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SUMMARY

The genetic and biochemical basis of ampicillin resistance amongst the aerobic Gram-negative commensal faecal flora of healthy volunteers in South Africa has been determined. Amongst 608 ampicillin resistant strains isolated from 320 of the participants, 158 were able to transfer their ampicillin resistant determinants into *Escherichia coli* K-12 J62-2. Iso-electric focusing of the β-lactamases, extracted from the transconjugants, demonstrated that ampicillin resistance resulted from the presence of the TEM-1, TEM-2 and SHV-1 β-lactamases in 94·3%, 2·5% and 3·2% of isolates respectively. Endonuclease restriction digests of the plasmids isolated from the transconjugants showed that the β-lactamase genes were present on a wide variety of plasmid types; 101 distinct plasmid endonuclease restriction patterns were identified. Transferable ampicillin resistance was associated with resistance to other antibiotics at the following frequencies: trimethoprim (48·7%), streptomycin (35·4%), tetracycline (27·2%), spectinomycin (9·5%), chloramphenicol (3·2%) and gentamicin (1·3%). One antibiotic resistance pattern, ampicillin and trimethoprim, predominated (28%). In total, 77·9% of the plasmids conferred resistance to other antibiotics raising the possibility that use of any of these agents, not simply ampicillin, may contribute to the maintenance of resistance genes.

INTRODUCTION

The normal commensal aerobic flora, in particular *Escherichia coli*, of healthy populations is increasingly recognized as an important reservoir of antibiotic resistance genes [1–6]. This is of considerable concern firstly, because commensal gut bacteria may themselves cause endogenous infections and secondly, because the resistance determinants may be transferred to any incoming pathogens complicating the treatment of the subsequent infection [7].

Although a number of studies have elucidated the carriage of antibiotic resistance determinants in the normal commensal flora, few have proceeded to examine the genetic and biochemical mechanisms of such resistance in this specific population. In contrast, the mechanisms of antibiotic resistance in clinical isolates have been well investigated. The most important mechanism of resistance identified to the most widely used class of antimicrobials, the β-lactam agents [8, 9], is the production of β-lactamases [10]. Amongst clinical isolates, TEM-1 is

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the most frequently isolated β-lactamase [11, 12]. The ability of the TEM-1 gene to mutate to both extended-spectrum β-lactamases and inhibitor-resistant β-lactamases is well documented raising the possibility that the normal commensal flora may act not only as a reservoir of antibiotic resistance determinants to current β-lactam agents but also of new TEM-derived β-lactamases as newer agents are introduced into clinical practice. Indeed the implication that epidemic TEM-1 plasmids in the community provide a source of new β-lactamases has recently been demonstrated in Edinburgh where TRC-1, a novel TEM-derived β-lactamase with increased resistance to β-lactamase inhibitors, was found to be present on an identical plasmid to a TEM-1 containing plasmid widespread within the normal flora of the healthy community [13].

The plasmids on which resistance genes reside effect the levels of antibiotic resistance in a given population; rapid rises in levels of resistance have been shown to result from the spread of epidemic plasmids [14]. As a result, the need has been emphasized for effective surveillance and classification of antibiotic resistance plasmids in order to predict future resistance problems [14, 15]. There is, however, little information currently available on genetic transfer of antibiotic resistance in normal enteric flora which, as has been suggested previously, is likely to be the source of resistance in enteric pathogens.

Amongst both clinical isolates and commensal isolates from healthy populations, the problems of antibiotic resistance in developing countries are major, with much higher levels of resistance than found in Europe or the USA [16]. In 1992, a study of the prevalence of resistance to antimicrobials in faecal Enterobacteriaceae of the healthy population in South Africa was conducted [5]. The survey examined eight separate population groups comprising infants (0–5 years), children (6–11 years), teenagers (12–19 years) and adults (> 19 years) from four study areas. A high carriage rate of ampicillin resistance (88·6%) amongst this total population was revealed. In the current study, we investigated the biochemical and genetic basis for transferable β-lactam resistance amongst the 608 ampicillin resistant strains obtained.

METHODS

Identification of bacterial isolates

Bacterial colonies were identified by standard biochemical tests. Strains were allocated into one of four groups: *E. coli*, *Klebsiella* spp., *Enterobacter/Citrobacter* spp., or other Enterobacteria.

Conjugation studies

All the isolates were tested for the ability to transfer their resistance determinants by the method of Amyes and Gould [17]. The conjugative experiments employed the rifampicin-resistant *E. coli* K-12 J62-2 as the recipient.

Plasmid analysis

Plasmid DNA was extracted from 4·5 ml of overnight broth culture by a modification of the procedure described by Takahashi and Nagano [18]. Restriction endonuclease digestion was performed with *Eco*R I for 4 h at 37 °C; an

Table 1. *Identification of transferable bacterial isolates*

Species	Total number	Transferable number	Transferable percentage
E. coli	445	127	80·4
Klebsiella spp.	104	20	12·6
Enterobacter/Citrobacter spp.	35	3	1·9
Other	24	8	50·0

appropriate buffer was used according to the manufacturer's instructions (Gibco BRL). Resultant DNA fragments were fractionated by electrophoresis at 60 V for 18 h on horizontal agarose gels (0·7 %), stained with ethidium bromide (50 mg/l), visualized and photographed under ultraviolet light. A *Hin*d III digest of phage λ was employed for gel calibration.

Antimicrobial sensitivity testing

The antibiogram of each transconjugant was determined by testing the strain's sensitivity to a range of antimicrobials including amoxycillin (8 mg/l) and amoxycillin plus clavulanic acid (8:4 mg/l) from SmithKline Beecham Pharmaceuticals, Surrey, UK, trimethoprim (10 mg/l) (from Wellcome Medical Division, Crewe, UK), gentamicin (4 mg/l), spectinomycin (10 mg/l), streptomycin (10 mg/l) and nalidixic acid (10 mg/l) all from Sigma Chemical Co. Ltd, Poole, UK, cephaloridine (10 mg/l), cefuroxime (4 mg/l) and ceftazidime (2 mg/l) all from Glaxo Group Research, Greenford, UK, cefotaxime (1 mg/l) (Roussel Laboratories Limited, Uxbridge, UK), ciprofloxacin (1 mg/l) (Bayer, Newbury, UK), tetracyline (10 mg/l) (Lederle Laboratories, Gosport, UK) and chloramphenicol (8 mg/l) (Boehringer Mannheim, East Sussex, UK). Serial dilutions were made in single strength Davis and Mingioli medium (DM) [19] for each overnight Isosensitest broth culture to give a 10^{-4} dilution. Of this bacterial suspension, 2 μl was spotted onto a range of Isosensitest agar (Oxoid) plates containing a single antibiotic at a given breakpoint value as recommended in the BSAC guidelines [20]. All plates were incubated at 37 °C for 18 h. The control strains employed were *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* NCTC 10418. MICs were determined by following the same method as for the antibiogram except that the test plates contained serial doubling concentrations for the antibiotic as recommended in the BSAC guidelines [20]. The MIC was defined as the lowest concentration on which there was no visible growth after overnight incubation at 37 °C.

Preparation and analysis of β-lactamases

β-lactamases for iso-electric focusing were prepared from bacterial colonies grown overnight on 10 ml nutrient agar slopes and resuspended in 1 ml sodium phosphate buffer (50 mM, pH 7·0). The cells were disrupted by ultrasonication (8 μm, 2 × 15 s separated with a 30 s break, Soniprep). Cell lysate was removed by centrifugation for 10 min at 4 °C at high speed (MSE Micro Centaur). Iso-electric focusing was performed by the method of Matthew and colleagues [21]; the ampholine range used was pH 3·5–10. Iso-electric focusing was carried out at 4 °C

at 500 V and 20 mA, limited by constant power set at 1 W for 18 h (LKB 2197 Power pack). The standard β-lactamase producing strains used as controls were *E. coli* K12 J62-2 containing either plasmid R1 (TEM-1), plasmid RP4 (TEM-2), plasmid R1010 (SHV-1) or plasmid R455 (OXA-1).

Specific activity of β-lactamases

β-lactamase production by strains was quantified with reference to the protein concentration which was measured by the method of Waddell [22]. Nitrocephin (50 μg/ml) was employed as the substrate.

RESULTS

Bacterial identification

The incidence of bacterial species amongst the ampicillin resistant isolates was as follows: *E. coli* (73%), *Klebsiella* spp. (17%), *Enterobacter/Citrobacter* spp. (6%) and other Enterobacteria (4%).

Transfer of ampicillin resistance determinants

Amongst the 608 isolates 158 (26%) were shown to contain self-transmissible plasmids in conjugation studies [5]. The transferable strains were not restricted to one species although *E. coli* was found to predominate (80·4%) (Table 1). As shown in Table 2, a particularly high carriage rate of transferable strains (74%) was identified amongst the infants in study area 1.

Transconjugant antibiograms

In addition to ampicillin, resistance to other antibiotics occurred at the following frequencies: trimethoprim (48·7%), streptomycin (35·4%), tetracycline (27·2%), spectinomycin (9·5%), chloramphenicol (3·2%) and gentamicin (1·3%). A total of 17 different antibiogram patterns was identified amongst all transconjugants (Table 3). The most frequently identified antibiotic resistance profile to be transferred was that of ampicillin and trimethoprim which accounted for 45 (28·5%) of the antibiograms. Ampicillin alone was also a very common resistance profile being found in 35 (22·2%) of the transconjugants. Three other resistance patterns, those of ampicillin and tetracycline, ampicillin and streptomycin, and ampicillin, trimethoprim and streptomycin were repeatedly recognized in 14, 15 and 16 of the transconjugants respectively. The 12 other resistance patterns shown in Table 3 were isolated much less frequently, between one and six occasions. None of the transconjugants could confer resistance to amoxycillin plus clavulanic acid, cephaloridine, cefuroxime, cefotaxime, ceftazidime, nalidixic acid or ciprofloxacin.

Plasmid analysis

The plasmids were extracted from the transconjugants and investigated by endonuclease restriction analysis with *Eco*R I. Plasmids were assigned to a

Table 2. *Numbers and proportions of gram-negative isolates from different population groups which transferred antibiotic resistance genes*

Study area/ population group	Total number of isolates	Total number of transconjugants	Percentage
Study area 1: Shongwe			
Infants	81	60	74
Adults	112	12	10
Study area 2: Kagiso			
Children	54	17	31
Teenagers	82	9	11
Study area 3: Soweto			
Infants	80	22	27
Adults	80	11	14
Study area 4: Hekpoort			
Children	66	16	24
Teenagers	53	11	20

Table 3. *Prevalence of different antibiogram profiles amongst the transconjugants*

Antibiogram*	Number of transconjugants (percentage)	Number of different restriction patterns
Ap	35 (22·2)	25
ApSm	15 (9·5)	10
ApTc	14 (8·9)	5
ApTp	45 (28·5)	20
ApSmSp	2 (1·2)	2
ApSmTc	6 (3·8)	5
ApSmTp	16 (10·1)	10
ApSpTc	4 (2·5)	4
ApSpTp	2 (1·2)	2
ApCmSmTc	1 (0·6)	1
ApSmSpTc	3 (1·8)	3
ApSmTcTp	3 (1·8)	3
ApSpTcTp	5 (3·2)	5
ApCmGmTcTp	2 (1·2)	1
ApCmSmSpTc	1 (0·6)	1
ApCmSmTcTp	1 (0·6)	1
ApSmSpTcTp	3 (1·8)	3

* Ap, ampicillin; Sm, streptomycin; Tc, tetracycline; Tp, trimethoprim; Sp, spectinomycin; Cm, chloramphenicol; Gm, gentamicin.

number of groups based on the restriction digest pattern. Plasmid profiles were considered closely related and assigned to the same group if they differed by no more than two bands. If the plasmids differed by three bands or more, they were assigned to a separate group. Among the 158 ampicillin resistant transconjugants, a total of 101 different plasmid profiles was identified. For each plasmid the plasmid size (kb) was determined. In addition the number of occasions a single plasmid type was isolated was recorded. Ten plasmids types were isolated on three or more occasions and were subsequently termed 'common' plasmids (Table 4; Fig. 1). Five of these 'common' plasmids originated in the isolates obtained from

Table 4. *Profile of the 'common' plasmids isolated from the transconjugants*

pUK number	Number of transconjugants	Plasmid size (kb)	Antibiogram	Study area/ population group
2400	14	64	Ap	1-Infants
2401	9	134	ApTc	1-Infants
2402	7	47	ApTp	1-Infants
2403	5	60	ApSmTp	3-Infants
2404	4	67	Ap	2-Children
2405	4	41	ApSm	4-Children
2406	4	48	ApSmTp	3-Infants/Children
2407	3	79	ApTp	1-Infants
2408	3	48	Ap	2-Children
2409	3	63	ApTp	1-Infants

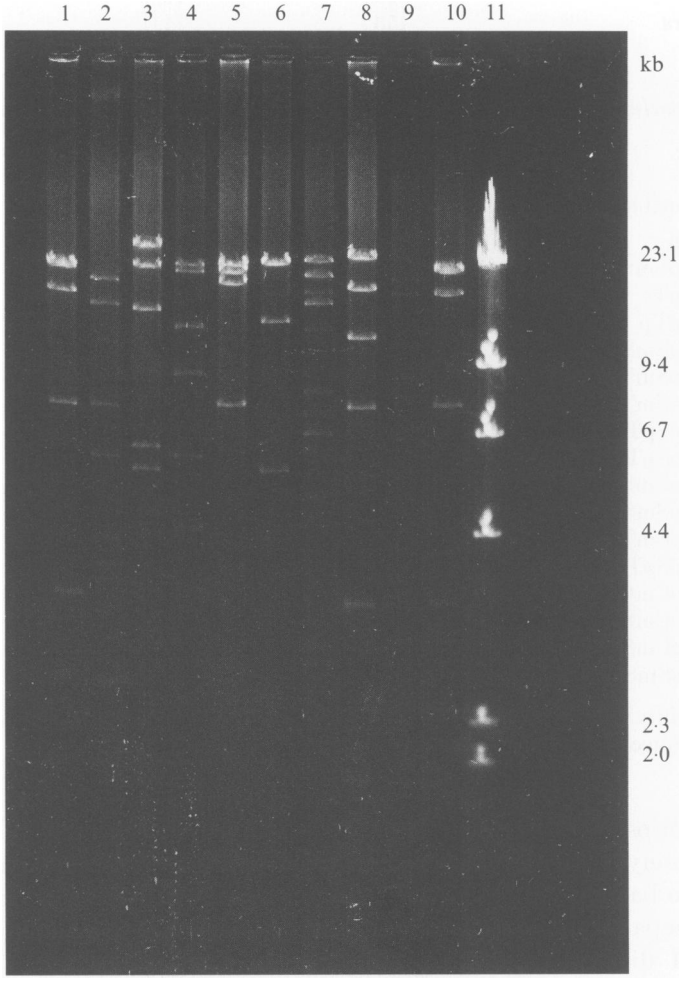

Fig. 1. Endonuclease restriction of 'common' plasmids in the transconjugants. Lane 1, pUK2409; lane 2, pUK2403; lane 3, pUK2404; lane 4, pUK2407; lane 5, pUK2406; lane 6, pUK2405; lane 7, pUK2401; lane 8, pUK2400; lane 9, pUK2408; lane 10, pUK2402; lane 11, λ pre-cut with *Hin*d III.

infants in study area 1 (Fig. 1, lanes 4, 5, 7, 8 10). The maximum number of occasions a single plasmid type was identified was 14 (8·9%). This plasmid, also identified in infants from study area 1, is represented by pUK2400 (Fig. 1, lane 8), was estimated as being 64 kb in size and was found to confer resistance to ampicillin alone (Table 4). The second most common plasmid, again from infants from study area 1, occurred in nine (5·7%) isolates and is represented by pUK2401 (Fig. 1, lane 7). This plasmid was identified as being 134 kb in size but in addition to ampicillin, was found to mediate resistance to tetracycline (Table 4). The eight other 'common' plasmids ranged in size from 41 kb to 79 kb. Only one 'common' plasmid, represented by pUK2406 (Fig. 1, lane 5), resided in more than one population group. This plasmid was identified in both infants and children from study area 3.

There was a wide diversity in endonuclease restriction profile amongst those plasmids isolated on less than three occasions; plasmids ranged in size from 12 kb to 178 b. There was no correlation observed between the size of the plasmid and the number of resistance determinants that it carried; 20 plasmids were larger than 100 kb, yet the number of resistance determinants carried by them ranged from one to five. While 74 plasmids encoded two resistance determinants, 35, 30 and 19 plasmids were found to encode 1, 3 and > 3 resistance determinants respectively.

β-lactamase analysis and distribution

The different β-lactamases responsible for mediating resistance to ampicillin amongst the transconjugants were separated by iso-electric focusing. The TEM-1 β-lactamase was the most frequently isolated β-lactamase accounting for 94·3% of the plasmid-encoded β-lactamases. TEM-2 β-lactamase was found in four (2·5%) of the transconjugants while SHV-1 was identified in five (3·2%) of the transconjugants. While it is recognized that some extended-spectrum β-lactamases focus at or near the same pI value as TEM-1, TEM-2 or SHV-1, the antibiotic sensitivity testing results revealed these enzymes lacked any extended-spectrum activity. The plasmids encoding TEM-2 β-lactamase all conferred resistance to ampicillin alone. Two of these plasmids were identical in endonuclease restriction profile, a third plasmid exhibited a similar profile while the fourth plasmid was completely unrelated in endonuclease restriction profile. In contrast, the five transconjugants containing plasmids encoding the SHV-1 β-lactamase exhibited a complete diversity of antibiogram and restriction profiles.

Specific activity of the TEM-1 β-lactamase and resistance to clavulanic acid

There has been concern over the emergence of strains resistant to β-lactam/β-lactamase inhibitor combinations as a result of increased production of TEM-1. The specific activity of the TEM-1 β-lactamase isolated from the transconjugants was determined and was found to range from 0·001–0·729 μmol nitrocephin hydrolysed/min/mg protein. The MICs of amoxycillin and amoxycillin plus clavulanic acid in the transconjugants, were also ascertained. In the transconjugants the MIC of amoxycillin varied between 256 mg/l and > 1024 mg/l while the MIC of amoxycillin plus clavulanic acid varied between 2 mg/l and

8 mg/l. No significant correlation was found, between the MIC of amoxycillin or the MIC of amoxycillin plus clavulanic acid and the specific activity of the β-lactamase.

DISCUSSION

Increasing evidence suggests that the normal commensal flora of the healthy individual, in particular *E. coli*, may act as the largest reservoir of antibiotic resistance determinants. A number of studies have demonstrated that in this specific bacterial population, as with clinical isolates, the carriage of antibiotic resistant determinants is higher in developing than developed countries [23]. Until now there have been few investigations into the genetic and biochemical basis of the development and spread of resistance amongst the non-pathogenic commensal flora of healthy populations. The value of such studies is obvious if we wish to assess whether the resistance genes and plasmids in the normal flora are the progenitors of those encountered in clinical isolates.

In a previous study, the prevalence of antibiotic resistance in commensal isolates from healthy black populations in South Africa was assessed [5]. In the current study these bacterial strains were investigated further; the β-lactamases responsible for transferable ampicillin resistance and the genetic carrier for this resistance were identified. In particular, we wished to establish whether the genetics of the ampicillin resistance genes differed from those found in commensal bacteria in Scotland [6]. This was because the black populations studied in South Africa form part of a different social structure from that of northern Europe, probably living in conditions more closely resembling those of a developing country except that water supplies were generally pure [5].

Amongst the 158 transconjugants, restriction endonuclease fingerprinting identified 101 different plasmid types. Interestingly, five of 10 'common' plasmids, that is plasmids occurring on three or more occasions, were identified in one specific population group (infants in study area 1). Perhaps surprising was the lack of more 'common' plasmids in infants from study area 3. As described in some detail in the previous study [5], 20 of the specimens from this group were supplied from infants attending one childminder. Previous surveys have indicated an association between the presence of a common plasmid and children attending day care centres [24]. Apart from a single plasmid type represented by pUK2406 which was identified in two population groups, that is amongst both infants and children from study area 3, the different plasmid types appeared to be restricted to specific population groups. Analysis of ampicillin-resistant isolates from the healthy community in Scotland identified a completely different situation, showing an epidemic plasmid widely dispersed across various population groups [6]. Antibiotic resistance determinants may disseminate as a result either of the clonal spread of epidemic strains or the spread of epidemic plasmids, as in Scotland, or, alternatively resistance genes may migrate to may different strains and plasmids by transposition [8]. The latter appears to be happening in South Africa because in this study the discovery of the β-lactamase gene on such a wide variety of plasmids suggests that ampicillin resistance is not simply the result of the spread of one particular strain or plasmid. As the TEM-1 β-lactamase gene resides on transposons, the gene is able to spread and be independently acquired through a

series of transposition and integration events. Why it should be different from the situation in Scotland is unclear. Limited hygiene and sanitation are conditions in developing countries which may be more conducive to the spread of individual genes rather than epidemic strains [25].

The predominance of TEM-1 as the determinant of β-lactam resistance amongst clinical isolates has been extensively reported [26, 27]. From this and other studies, it is now apparent, and perhaps not surprising, that the TEM-1 β-lactamase is largely responsible for β-lactam resistance amongst the commensal faecal flora of healthy populations [6, 25]. The clinical implications of the widespread presence of TEM-1 amongst isolates from the healthy population is of concern. In recent years the TEM-1 β-lactamase has shown a remarkable capacity to cope with changes in antibiotic selective pressure. Indeed TEM-1 is recognized as the progenitor of many extended-spectrum and inhibitor resistant β-lactamases which, from clinical experience have been shown to be selected in the presence of newer cephalosporins and β-lactam/β-lactamase inhibitor combinations [13, 28, 29]. The potential exists for extended-spectrum β-lactamases to appear amongst the healthy community as oral later generation cephalosporins are introduced into general practice. Although it has been argued that oral cephalosporins are no more likely to select out extended-spectrum β-lactamases than their parenteral counterparts [30], the threat of these new compounds lies in their increased usage.

Previous studies have shown that the MICs of amoxycillin and amoxycillin plus clavulanic acid are dependent on the amount of TEM-1 β-lactamase synthesized by clinical isolates [26, 31]. It has been suggested that this is not the only factor determining the degree of resistance and that permeability, affinity for penicillin binding proteins and overproduction of the chromosomal enzyme may each contribute [31]. In order to overcome these influences in one study, the TEM-1 gene was transferred into an isogenic background. A clear correlation between the specific activity of the β-lactamase and the level of resistance was identified [26]. In contrast, in the current study, analysis of the transconjugants revealed no obvious relationship between the specific activity of the TEM-1 β-lactamase and the level of resistance to amoxycillin and amoxycillin plus clavulanic acid.

This study has demonstrated for the first time the presence of SHV-1 and TEM-2 β-lactamases in the commensal faecal flora of healthy populations. TEM-2 was, however, found on a restricted plasmid range. As the occurrence of these β-lactamases was rare, it is not clear if they will persist in the community or if TEM-1 β-lactamase will replace them. This study has also confirmed the successful nature of the TEM-1 β-lactamase in this specific bacterial population. The detrimental impact that TEM-1 may have on the healthy community is twofold. Not only will current therapy be complicated by the presence of this enzyme but in addition, as newer cephalosporins and β-lactam/β-lactamase inhibitor combination agents are introduced into general practice, the presence of TEM-1 provides the opportunity for the selection of extended-spectrum and inhibitor-resistant β-lactamases that are able to render these newer agents ineffective.

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