Multi-drug resistant non-typhi salmonellae in Kenya


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Two methods of plasmid characterization, restriction digest patterns and incompatibility grouping, were used to study self-transmissible multi-drug resistance among non-typhi salmonellae (NTS). Resistance to ampicillin and other commonly applied /lactams was evaluated by iso-electric focusing and disc inactivation. Of the NTS isolated from blood, 75% were Salmonella typhimurium but those included several different phage types. Over 47% of isolates were resistant to three or more of the readily available drugs including ampicillin, cefuroxime, chloramphenicol, co-trimoxazole, streptomycin and tetracycline. Self-transferable resistance plasmids (c. 100 kb) were essentially of incompatibility group incFIIA, but their restriction fragment patterns revealed a diversity in relatedness. More than half of parent strains and their transconjugants produced /-lactamases which co-electrophoresed with TEM-1 and OXA-1. This study has observed a disturbingly high prevalence of transmissible multi-drug resistance among NTS which are an important cause of morbidity in HIV-1 seropositive individuals.

Introduction

Infections with non-typhi salmonellae (NTS) are a significant cause of illness and death worldwide (Jewes 1987, Paton et al., 1991; Maiorini et al., 1994; Muñoz et al., 1993). They are of particular importance in developing countries. For example, NTS are the most frequent cause of septicaemia in children in Rwanda (Lepage et al., 1987) and Zaire (Green & Cheesbrough, 1993). HIV infection has added a new dimension to their importance, so much so that recurrent NTS bacteraemia is regarded as an early marker for AIDS in HIV infected individuals (Gruenewald, Blum & Chan, 1994). In Kenya NTS bacteraemia was detected in 11% of HIV-seropositive individuals on admission to hospital (Gilks et al., 1990). Rapid institution of antibiotics is important if significant morbidity and mortality are to be avoided. In many tropical countries including Kenya, ampicillin, chloramphenicol or co-trimoxazole are the drugs of choice for treatment of invasive NTS infection, indeed for most patients they are the only drugs available.

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Therefore, the emergence of resistance to these antibiotics would pose tremendous problems since alternatives such as fluoroquinolones are beyond the means of such patients. The aim of this study was to document the level and extent of resistance among NTS in Nairobi, to characterize plasmids encoding resistance genes and analyse the β-lactamases responsible for resistance to ampicillin and other β-lactams.

Materials and methods

Isolates

NTS were isolated from consecutive patients admitted with diarrhoea and/or bacteraemia to either the Kenyatta National Hospital, or the Kenya Medical Research Institute, Nairobi, over the 2 year period, 1993–1994. NTS were initially characterized biochemically (API 20E; bioMerieux, Basingstoke, UK), and subsequently serotyped using agglutinating antisera (Murex Diagnostics, Dartford, UK). NTS were stored at −70°C on protect beads (Technical Service Consultants Ltd, Heywood, UK) until analysed. The Salmonella typhimurium were subjected to phage typing at the Division of Enteric Pathogens, PHLS, Colindale, UK.

Antimicrobial susceptibility

Initially, antibiotic sensitivity was determined using a controlled disc diffusion method. The antibiotics (disc strength) chosen were ampicillin (10 μg), co-trimoxazole (19:1 μg), tetracycline (30 μg), chloramphenicol (10 μg), gentamicin (10 μg), co-amoxiclav (20:10 μg), ciprofloxacin (5 μg), streptomycin (10 μg), cefturoxime (30 μg) and ceftazidime (30 μg) (all from Oxoid, Basingstoke, UK). The discs were placed between lawns of the test isolate and Escherichia coli NCTC 10418 spread on DST agar (Oxoid) incorporating 5% lysed horse blood.

MIC were determined by a plate incorporation method using doubling dilutions of antibiotics (Adatabs, Mast Laboratories, Liverpool, UK) in IsoSensitest agar (Oxoid). Bacteria (10^5–10^6 cfu) were delivered using a multipoint inoculator. An IsoSensitest agar plate without antibiotics was used as a control for growth and E. coli ATCC 25922 of known MIC was included in each batch as control for antibiotic potency. For disc diffusion and MIC determination, plates were incubated at 37°C in air for 18 h. The MIC was taken as the concentration of antibiotic that resulted in the growth of five or fewer colonies.

Plasmids

Conjugation experiments were carried out in broth according to the method of Walia et al. (1987) using E. coli K12 (nal^R, lac+) as recipient. Transconjugants were selected on MacConkey agar (Oxoid) incorporating nalidixic acid (32 mg/L) and ampicillin (32 mg/L) or chloramphenicol (32 mg/L).

Plasmid DNA was extracted from NTS and transconjugants using the method of Birnboim & Doly (1979). Purified plasmid DNA was subjected to restriction endonuclease digestion using HindIII (Gibco-BRL, Paisley, UK) according to manufacturer's instructions. Digested and undigested plasmids were electrophoresed on horizontal 0.8% agarose gels and stained with ethidium bromide (Sigma, Poole, UK). DNA bands were visualized using a UV transilluminator (UVP Inc., CA, USA) and
photographed using a Polaroid MP-3 camera (Polaroid, MA, USA). Restricted plasmid DNA fragments were interpreted as previously described (O’Brien et al., 1993). Undigested plasmid molecular weights were determined by electrophoresis with plasmids of known molecular weight from E. coli strains V517 and 39R861. Those of endonuclease digested plasmids by co-electrophoresis with a HindIII digest of λ-DNA.

Incompatibility grouping (Couturier et al., 1988) was performed on purified plasmid DNA extracted from E. coli K12 transconjugants using DNA-DNA hybridization. A total of eight probes (incB/O, incFIA, incFIIA, incHI1, incK, incN, incP and incW) were labelled with biotinylated UTP by nick translation (Leary, Brigatti & Ward, 1983). Plasmid DNA was blotted into nitrocellulose membranes (Sigma) using a blotting manifold (Bio-Rad, Watford, UK). DNA-DNA hybridization was done according to Meinkoth & Wahl (1984).

Probe-target hybridization was detected using a streptavidin-alkaline phosphatase conjugate and nitroblue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate for visualization (Sigma).

**β-Lactamases**

Crude β-lactamase preparations were made by a freeze-thaw method (Corkill et al., 1991) from 61 isolates of NTS and their E. coli K12 transconjugants and from 20 NTS which did not show transferable resistance. The protein concentration of the extract was estimated by the method of Lowry et al. (1951), and an estimate of activity was made using the chromogenic cephalosporin, nitrocefin. The extracts were stored in aliquots at —70°C until used. The substrate profile of the β-lactamases were determined using a disc inactivation method (Hart & Percival, 1982). Inactivation of discs containing ampicillin (10 μg), co-amoxiclav (20:10 μg), carbenicillin (100 μg), cephalaridine (25 μg), cefuroxime (30 μg), and cefoxitin (30 μg) was detected using lawns of E. coli NCTC 10418 and of discs containing penicillin (1 μg), methicillin (5 μg), and oxacillin (1 μg) on lawns of Staphylococcus aureus (Oxford strain). Inhibition by ethylene diamine tetra-acetic acid (EDTA-Na 100 mM) and NaCl (100 mM) was detected using nitrocefin.

Analytical iso-electric focusing was carried out on polyacrylamide gels (Pharmacia, St Albans, UK) incorporating ampholytes (pH range 3.5–9.5). Proteins of known pI were electrophoresed in each run (Pharmacia). Test and controls (standard β-lactamases including TEM-1, TEM-2, OXA-1 and SHV-1) were electrophoresed on a water-cooled horizontal bed at 1500 V and 30 mA for 90 min. The gel was overlayed with nitrocefin (100 mM) and incubated for 5 min at 37°C.

**Statistical analysis**

Proportions were compared using the χ² test.

**Results**

A total of 228 stool samples and 1220 blood cultures were examined. Of the blood cultures 138 (11.3%) contained NTS and of these 18 had the same NTS isolated from stool. From 228 samples of diarrhoeic stools, 72/228 (31.6%) NTS were obtained. Thus over the 2 year period a total of 192 NTS were obtained from blood and/or stool. 240/1220 (19.7%) of those from whom blood was taken for culture were
HIV-seropositive whereas 95/138 (68.8%) of the cases of NTS bacteraemia were HIV-seropositive \((P < 0.001)\). The majority (144/192; 75%) of the NTS were \(S. typhimurium\) with smaller numbers of \(Salmonella enteritidis\) (9; 5%), \(Salmonella newport\) (8.4%) and \(Salmonella choleraesuis\) (7; 3.6%).

**Antimicrobial susceptibility**

Only 31 (16%) of NTS were sensitive to all ten antibiotics tested, 38 (20%) were resistant to one agent (usually streptomycin or tetracycline) and 33 (17%) were resistant to two agents (usually streptomycin and tetracycline or tetracycline and ampicillin). The remainder (47%) were resistant to three or more antimicrobials and the most frequent resistance pattern was to ampicillin, cefuroxime, chloramphenicol, co-trimoxazole, streptomycin and tetracycline which encompasses all the readily available antimicrobials in Kenya and most other developing countries. The MICs of these and of some additional antimicrobials are shown in Table I. Using the British Society of Antimicrobial Chemotherapy Working Party (1991) breakpoints for resistance, a large proportion of NTS were resistant to tetracycline (66%), streptomycin (49%), ampicillin (48%), co-trimoxazole (46%) and cefuroxime (35%). Ceftazidime and ciprofloxacin were the only antimicrobials to which the NTS were uniformly sensitive.

**Plasmid studies**

The 90 NTS isolates resistant to three or more antimicrobials were chosen for further study. Each isolate was found to carry a large \((c. 100 \text{ kb})\) plasmid. In addition, 65 isolates also had smaller \((3-10 \text{ kb})\) plasmids. In conjugation experiments 61/90 (67.7%) of the multi-resistant NTS transferred resistance to one or more antimicrobials to \(E. coli\) K12 (Table II). Nineteen NTS transferred their full resistance phenotype. All the NTS transferred ampicillin resistance and in 31% resistance to co-trimoxazole was transferable. Chloramphenicol resistance was transferable from 31/61 (50.8%) of the resistant NTS, tetracycline resistance from 36/61 (59%) but resistance to streptomycin was not transferable. In each case that resistance was transferred whatever the phenotype, it was associated with the large \((c. 100 \text{ kb})\) plasmid. In no case was the

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>range</th>
<th>MIC ((\text{mg/L}))</th>
<th>% Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.5–128</td>
<td>64</td>
<td>48</td>
</tr>
<tr>
<td>Co-amoxiclav</td>
<td>0.5–64</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2–128</td>
<td>8</td>
<td>35</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.125–16</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.06–16</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2–128</td>
<td>8</td>
<td>49</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>0.25–64</td>
<td>0.25</td>
<td>46</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1–32</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5–64</td>
<td>64</td>
<td>66</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1–32</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.015–0.25</td>
<td>0.03</td>
<td>0</td>
</tr>
</tbody>
</table>
smaller plasmid transferred. Resistance was not transferred by conjugation in 20 resistant NTS despite possessing the large plasmid (Table III).

The purified plasmid DNA from the 61 transconjugants shown in Table II were subjected to *HindIII* restriction endonuclease (RE) digestion and incompatibility grouping by DNA hybridization. The RE digestion resulted in three different patterns (Figure) with 6, 9 and 11 fragments, respectively. Each RE group consisted of a similar core fragment pattern differing in 1 to 2 fragments only. All of the plasmids could be assigned to incompatibility groups. IncFIIa (38; 62%) was the most frequently occurring followed by incHI1 (6; 10%) and incB/O (4; 6.6%). Some plasmids hybridized with two inc probes (Table IV). Each of the plasmid resistance phenotypes was associated with the three different RE patterns and from three to four different incompatibility groups. Similarly, plasmids of the same incompatibility group were associated with each of the resistance phenotypes and fell into the three different RE digest patterns.

*S. typhimurium* represented a large proportion of the NTS isolated. To exclude the possibility of spread of a single multi-resistant clone of *S. typhimurium*, 42 isolates which had transferable resistance and of which the plasmid had been characterized were subjected to phage typing. The most frequently encountered phage types were 56 (12 isolates), 135 (9 isolates) and 193 (4 isolates). There was one each of phage types 204, 204a and 12 and the remainder were untypable. Eleven of the phage type 56 *S. typhimurium* isolates carried incFIIa plasmids but these plasmids showed two different RE digest patterns (6 fragments; 2 isolates, nine fragments; 9 isolates). Of the nine *S. typhimurium* phage type 135 isolates five carried incFIIa plasmids (with 3 RE digest patterns), two inc B/O plasmids and one each incHI1 and incP plasmids. Although nine of the *S. typhimurium* phage type 56 carried plasmids of the same incompatibility group with the same RE digest pattern each of the four different transferable resistance phenotypes were found among those isolates. There is clearly considerable heterogeneity among the *S. typhimurium* isolates in terms of both the host bacterium and its plasmids.

### Table II. Antibiotic resistance phenotypes transferred from multi-resistant non-typhi salmonellae (NTS)

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>no. of isolates transferred to <em>E. coli</em> K12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Am, TmSu, Te, Cm</td>
<td>19 Am, TmSu, Te, Cm</td>
</tr>
<tr>
<td>Am, TmSu, Cm</td>
<td>12 Am, Cm</td>
</tr>
<tr>
<td>Am, TmSu, Cxm, St</td>
<td>17 Am, Te</td>
</tr>
<tr>
<td>Am, Te, St</td>
<td>13 Am</td>
</tr>
</tbody>
</table>

Am, Ampicillin; TmSu, co-trimoxazole; Te, tetracycline; Cxm, cefuroxime; Cm, chloramphenicol, St, streptomycin.

### Table III. NTS with non-transferable resistance

<table>
<thead>
<tr>
<th>Group</th>
<th>Parental NTS</th>
<th>no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.6, 7.0</td>
<td>8 (40)</td>
</tr>
<tr>
<td>6</td>
<td>5.4</td>
<td>12 (60)</td>
</tr>
</tbody>
</table>
**Figure 1.** HindIII restriction endonuclease digest products of the transferable 100 kb resistance plasmid from non-typhi salmonellae. Key: lane 1 (λ-ladder, lanes 2, 3 and 4, lanes 5 and 6, and lanes 7 and 9, show the three digest patterns respectively.

**β-Lactamases**

β-Lactamase preparations were made from 61 ampicillin-resistant NTS and their similarly resistant *E. coli* K12 transconjugants and from 20 ampicillin-resistant NTS from which transconjugants could not be obtained. On iso-electric focusing (IEF) the NTS and their transconjugants fell into four distinct groups (Table V). The majority (46%) were in group 1 in which the parental NTS expressed β-lactamases with pls of 5.4, 7.0 and 7.4, but transferred only the β-lactamase with a pl of 5.4. The qualitative hydrolysis (disc inactivation) and inhibition profiles indicated that the transferred enzyme was TEM-1. Group 2 parental NTS had β-lactamases of the same pl as group 1 but in this case the transconjugant *E. coli* expressed the same β-lactamases. By disc inactivation assays, the β-lactamases were able to completely hydrolyse ampicillin, penicillin, oxacillin and cefuroxime. Group 3 NTS carried TEM-1 which was

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>No. RE fragments</th>
<th>Incompatibility groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmSuTmTeCm</td>
<td>3 7 9</td>
<td>FIIA (14), FIA (2), FIA/P (2), FIIA/N (1)</td>
</tr>
<tr>
<td>AmCm</td>
<td>2 2 8</td>
<td>FIIA (6), FIIA/W (1), N (2), P (3)</td>
</tr>
<tr>
<td>AmTe</td>
<td>2 12 3</td>
<td>FIIA (13), P/W (1), W (1), H11 (2)</td>
</tr>
<tr>
<td>Am</td>
<td>1 3 9</td>
<td>FIIA (5), B/O (4), H11 (4)</td>
</tr>
</tbody>
</table>

See Table II for abbreviations.
transmissible to \( E. \ coli \) K12. The final group of NTS expressed \( \beta \)-lactamases of pl 7.0, 7.4 and 8.6 all of which were transmissible to \( E. \ coli \) K12. Of the 20 ampicillin resistant NTS that did not exhibit transmissible resistance 8 expressed TEM-2 and a \( \beta \)-lactamase of pl 7.0 and 12 expressed TEM-1 (Table III).

**Discussion**

In this 2 year survey of bacteremia in hospitalized patients in Nairobi, 971/1220 (79%) blood cultures were sterile. However, 138/1220 (11.3%) patients had NTS bacteremia and this represented over half (53%) of the episodes of bacteremia. As in other developing countries (Lepage et al., 1987; Green & Cheesbrough, 1993) the majority (75%) of our NTS isolates were \( S. \ typhimurium \). Similarly NTS were the most frequent pathogen obtained from stool representing 71% of the bacterial pathogens isolated.

Others (Gilks et al., 1990; Gruenewald et al., 1994) have noted that HIV infection is a significant risk factor for NTS bacteremia and there is urgent need for safe effective and bactericidal antibiotics to treat such infections. In Kenya, as in most developing countries, the ‘first-line’ antibiotics for treating serious infection are ampicillin, tetracycline, co-trimoxazole, streptomycin or chloramphenicol. ‘Second-line’ agents include cefuroxime, co-amoxiclav, gentamicin and piperacillin but are difficult to obtain. Antimicrobials such as ceftazidime, ciprofloxacin and cefotaxime are too expensive for most patients. It is worrying that a large proportion of the NTS isolates were resistant to ampicillin (48%), tetracycline (66%), streptomycin (49%) and co-trimoxazole (46%). Although only 26% of the isolates were resistant to chloramphenicol this proportion is too high to rely on it for empirical therapy. In an outbreak of invasive infection due to multiresistant \( S. \ typhimurium \) in Rwanda it was necessary to use a third-generation cephalosporin (Lepage et al., 1984), and in Zaire ciprofloxacin was used (Green et al., 1992). Both ciprofloxacin and ceftazidime were uniformly effective in vitro against all our isolates. Only 16% of our isolates were sensitive to all of the antibiotics tested and 47% were resistant to three or more agents. This is not an isolated phenomenon. In Argentina 85% of NTS isolated from children with gastroenteritis were resistant to ampicillin, cefuroxime, and gentamicin (Maiorini et al., 1993), and in one Spanish hospital the prevalence of resistance to chloramphenicol (11%) and ampicillin (32%) among \( S. \ typhimurium \) isolates has been rising each year (Muñoz et al., 1993).

In two-thirds of our multiresistant NTS some or all of the resistance pattern was transmissible in vitro to \( E. \ coli \) K12. In all cases this was due to transfer of a large (c. 100 kb) plasmid. Although these plasmids were of similar size and transferred only four different antibiotic resistance patterns, on further examination they proved to be diverse. Using two well recognized methods for plasmid characterization, restriction

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**Table V. Isoelectric points of \( \beta \)-lactamases from NTS and \( E. \ coli \) K12 transconjugants**

<table>
<thead>
<tr>
<th>Group</th>
<th>Parental NTS pi</th>
<th>Transconjugant ( E. \ coli ) K12 pi</th>
<th>no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.4, 7.0, 7.4</td>
<td>5.4</td>
<td>28 (46)</td>
</tr>
<tr>
<td>2</td>
<td>5.4, 7.0, 7.4</td>
<td>5.4, 7.0, 7.4</td>
<td>15 (26)</td>
</tr>
<tr>
<td>3</td>
<td>5.4</td>
<td>5.4</td>
<td>15 (26)</td>
</tr>
<tr>
<td>4</td>
<td>7.0, 7.4, 8.6</td>
<td>7.0, 7.4, 8.6</td>
<td>3 (10)</td>
</tr>
</tbody>
</table>
endonuclease digestion (Frost et al., 1984; Mamun, Shears & Hart, 1993) and incompatibility grouping (Couturier et al., 1988), over 25 different permutations of RE digest patterns and incompatibility groupings were found among the 61 plasmids examined. IncFIIA was the most frequently encountered (62%) but 11 different incompatibility groups including some plasmids that hybridized with two inc probes were seen. In previous studies of plasmid encoded multi-resistance in commensal enteric coliforms in Bangladesh (Mamun et al., 1993) and Indonesia (Levy et al., 1985), incFII plasmids were found in 46% (30/65) and 35% (8/23) of isolates. However, even among incFIIA plasmids there was considerable variability in RE digest patterns. The source of such resistance plasmids remains unclear but it is tempting to speculate that they are derived from commensal enterobacteria of other humans as has been suggested from an outbreak of cholera in Tanzania (Young & Amyes, 1986) or of shigellosis in Rwanda and Zaire (Frost et al., 1984). Another possible source of resistance plasmids is the enteric flora of domestic animals (Hunter et al., 1992). In this respect, it is noteworthy that presently Salmonella typhi isolates in Kenya do not show the same multi-resistance as the NTS nor as seen in S. typhi from the Indian sub-continent (Mirza, Beeching & Hart, 1995). Although 75% of the NTS were S. typhimurium the high prevalence of transferable resistance was not the result of spread of one or a limited number of clones. Several different phage types were encountered and even within isolates of the same phage type, several different plasmid genotypes and phenotypes were carried.

Of the 81 NTS examined for β-lactamases, 70 (86%) expressed TEM-1 β-lactamase either alone or with other β-lactamases. The TEM-1 enzyme was transferable from 58 (83%) of these. This is a little higher than the 60% of Salmonella spp. expressing TEM-1 in a study in South India (Nandivada & Amyes, 1990), but similar to the 80% reported among Enterobacteriaceae isolated at a London hospital (Liu et al., 1992). TEM-2 was found only in the NTS that did not transfer resistance and the remaining enzymes resembled OXA β-lactamases in isoelectric point and hydrolysis profiles. However, further work is needed to characterize these completely.

This study has demonstrated a high prevalence of resistance to the readily available antibiotics among non-typhi salmonellae isolated from bacteraemia patients in Nairobi. In most cases the multidrug resistance was carried on self-transmissible plasmids which showed considerable diversity.

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References


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