Antimicrobial susceptibility, auxotype and plasmid content of *Neisseria gonorrhoeae* in Northern Tanzania: emergence of high level plasmid mediated tetracycline resistance

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Abstract

***Objective*—To study the antimicrobial susceptibility, plasmid content, auxotype and serogroup of strains of *Neisseria gonorrhoeae* isolated from an urban population of STD clinic attenders in Northern Tanzania.**

***Methods*—The minimum inhibitory concentrations of nine common antimicrobial agents were measured by the agar dilution method against 130 strains of *Neisseria gonorrhoeae* isolated in a free government STD clinic in Mwanza town. The auxotype, plasmid content and serogroup of these strains were also determined by conventional techniques.**

***Results*—65 strains (50%) were penicillinase producers (PPNG), and 34 (26%) exhibited chromosomally mediated resistance to penicillin. Seven (5%) were sensitive to tetracycline; 78 (60%) showed intermediate levels of resistance, and 45 (35%) had high level plasmid mediated resistance (TRNG), all of which carried a 25·2 MDa plasmid. 79 strains (61%) showed decreased sensitivity to trimethoprim-sulphamethoxazole, and five (4%) were resistant to this agent. All isolates were fully sensitive to spectinomycin, azithromycin, cefotaxime, cefuroxime, norfloxacin and ciprofloxacin. One hundred and one strains (78%) were of type W11/111, 22 type W1, and seven cross reacting strains. The W1 strains were significantly more likely to be carrying plasmid mediated resistance to both penicillin and tetracycline. Six different auxotypes were present, the major type requiring proline. Plasmid profiles showed the presence of both the 3·2 MDa and the 4·4 MDa beta-lactamase encoding plasmids.**

***Conclusion*—a high proportion of gonococcal isolates remain resistant to penicillin in this region, and most isolates are now also resistant to tetracycline, with the emergence of plasmic mediated tetracycline resistance. Trimethoprim-sulphonamide sensitivity is also decreasing. The population of strains is heterogeneous, and both African and Asian beta-lactamase encoding plasmids are present.**

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Introduction

The incidence of gonorrhoea remains high in many African countries, and its treatment has become increasingly expensive with the rapid spread of strains resistant to the older antimicrobials.[1] The World Health Organization (WHO) has recommended a single dose of cefixime as the treatment of choice for uncomplicated gonorrhoea,[2] but this drug is unobtainable or prohibitively expensive in most developing countries.

Since patterns of antimicrobial susceptibility vary geographically, it is possible that cheaper alternatives remain effective in many areas. However, the shortage of well funded microbiology laboratories in developing countries means that there are few data on which to base sound recommendations for treatment derived from local sensitivity testing.

We report here the minimum inhibitory concentration (MIC) of nine antimicrobial agents against 130 gonococcal isolates obtained from a free municipal STD clinic in Mwanza, Tanzania. We also report on the plasmid content, auxotype and serogroup of these isolates.

Materials and methods

Bacterial isolates

Strains of *Neisseria gonorrhoeae* were isolated between June and September 1992 from patients attending a free STD clinic at the municipal hospital in Mwanza, a town in Northern Tanzania, as part of the routine investigation of all clinic attenders. After initial isolation and identification, strains were stored at −70°C in skimmed milk and transported to the London School of Hygiene and Tropical Medicine (LSHTM) for further characterisation. One hundred and thirty isolates were recovered and used in the study. The identification of all strains was confirmed by Gram stain, oxidase reaction and utilisation of glucose but not sucrose, lactose or maltose. Strains were typed using the Phadebact monoclonal antibody system (Launch Diagnostics Ltd) and were characterised as either type W1 or W11/111.

Antibiotic susceptibility

Minimum Inhibition Concentrations (MICs) were determined using an agar dilution technique. Diagnostic Sensitivity Test Agar (DST, Unipath Ltd) was used for the base medium and supplemented with 5% lysed horse blood and 1% Vitox (Unipath Ltd), except in the case of trimethoprim-sulpha-

methoxazole testing where 1% Kelloggs supplement was used to replace the Vitox. Plates were inoculated using a multipoint inoculator (Denley Instruments Ltd) with an inoculum of 10^4 colony forming units (cfu), and incubated in 5% carbon dioxide for 36 hours. The endpoint was read as the lowest concentration giving complete inhibition, or for trimethoprim-sulphamethoxazole 80% inhibition of growth. WHO reference *Neisseria gonorrhoeae* strains A and E were included for each series of antimicrobial tests.

The antibiotics tested were penicillin (0·008–8 mg/l), tetracycline (0·008–8 mg/l), trimethoprim–sulphamethoxazole (19:1) (0·5–64 mg/l), spectinomycin (2–512 mg/l), cefotaxime (0·002–0·5 mg/l), cefuroxime (0·015–0·5 mg/l), (Sigma Chemicals Ltd). Azithromycin (0·06–4 mg/l) (Pfizer Ltd), ciprofloxacin (0·002–0·06 mg/l) (Bayer UK Ltd) and norfloxacin (0·008–0·5 mg/l) (Merck Sharp and Dohme Ltd).

The breakpoint criteria used for assessing sensitivity were as recommended by the WHO manual[3] for penicillin, tetracycline, cotrimoxazole and spectinomycin and as recommended by Putnam *et al.*[4] for the remaining antibiotics.

Auxotyping

Isolates were auxotyped, using a modification of the method of Copley and Egglestone,[5] to determine the requirement for proline, arginine, hypoxanthine, uracil, histidine and methionine. Arginine requiring isolates were also tested for their ability to utilise ornithine as an alternative substrate. An inoculum of 10^4 cfu was used and plates examined for presence of colonies after incubation for 24 hours at 36°C in 5% carbon dioxide.

Plasmid analysis

Strains of *N gonorrhoeae* were grown overnight on GC agar base (Unipath) supplemented with 1% haemoglobin and 1% Vitox. Penicillinase producing strains were grown with the addition of 10mg/l of penicillin in the media, and plasmid mediated tetracycline resistant strains were grown with the addition of 10mg/l tetracycline. Strains with dual resistance were grown with both antibiotics to enhance plasmid production. The plasmid DNA was extracted by the rapid alkaline method of Birnboim and Doly.[6] Plasmid preparations were examined by agarose gel electrophoresis, using 1% agarose in tris EDTA buffer and stained with 1 mg/l ethidium bromide. Gels were examined using a transilluminator. Control strains were included with each extraction batch to ensure the technique was working correctly.

Table 1 Minimum Inhibition Concentrations (MIC) of isolates of Neisseria gonorrhoeae from Northern Tanzania (n=130)

Antimicrobial	*MIC 50 (mg/l)*	*MIC 90 (mg/l)*	*Range (mg/l)*
Penicillin	4·0	>8·0	0·125->8·0
Tetracycline	4·0	>8·0	0·25->8·0
Trimethoprim-sulphamethoxazole 19:1	16·0	16·0	1·0-32·0
Spectinomycin	16·0	32·0	4·0–32·0
Azithromycin	0·5	1·0	0·06–2·0
Cefotaxime	0·008	0·015	0·004–0·06
Cefuroxime	0·06	0·25	0·008–1·0
Norfloxacin	0·06	0·125	0·008–0·25
Ciprofloxacin	0·008	0·015	0·002–0·03

Table 2 Distribution of penicillin and tetracycline MICs in isolates of Neisseria gonorrhoeae from Northern tanzania

Tet MIC	*>8mg/l TRNG*	*8mg/l*	*4mg/l*	*2mg/l*	*1mg/l*	*0·5mg/l*	*0·25mg/l*
Pen MIC							
>8mg/l PPNG	33	8	15	5	3		1
4mg/l CMRNG		1	2	1	1		
2mg/l CMRNG	6	2	3				1
1mg/l CMRNG	1	5	4	6	1		
0·5mg/l		4	2	5	2		1
0·25mg/l	5		1	6	1	2	1
0·125mg/l							1

Results

Of the 130 isolates of *N gonorrhoeae* tested only 31 (24%) remained susceptible to treatment with penicillin, with MICs of less than 1mg/l, 34 isolates (26%) showed intermediate chromosomal resistance, CMRNG, with MICs in the range 1–4 mg/l, and 65 (50%) showed high level beta-lactamase mediated resistance, PPNG, with MIC of 8 mg/l or greater and B lactamase positive. Only seven (5%) of strains were sensitive to tetracycline, with MICs less than 1mg/l, 78 strains (60%) showed intermediate chromosomal resistance, MICs in the range 1–8 mg/l, and 45 (35%) showed high level plasmid mediated tetracycline resistance, TRNG, (MIC > 8 mg/l). All these strains grew on media containing 10 mg/l tetracycline.

Trimethoprim-sulphamethoxazole susceptibility testing showed that 45 (35%) of isolates were fully sensitive, with MIC less than 8 mg/l, and 79 (61%) exhibited intermediate level resistance, with MICs of 8–16 mg/l, five isolates (4%) had MICs of greater than 32 mg/l and were fully resistant. All isolates were sensitive to spectinomycin, azithromycin, cefotaxime, cefuroxime, norfloxacin and ciprofloxacin. MIC 50s, MIC 90s and ranges of MICs are shown in table 1. The MICs of individual isolates to penicillin and tetracycline are shown in table 2.

Isolates were typed using the Phadebact system, which is based on monoclonal antibodies to outer membrane protein 1. Of the 130 isolates, 22 typed W1, containing protein 1A, 101 typed W11/111 containing protein 1B, and 7 isolates cross reacted with both monoclonals. A correlation between Phadebact type and PPNG showed that 20 of the 22 W1 strains were PPNG compared with 41 of the 101 W11/11 type ($\chi^2 = 16{\cdot}3$; $p < 0{\cdot}001$). Similarly when Phadebact type was correlated with TRNG 18 of W1 were resistant compared with 22 of type W11/11. This difference was also highly significant ($\chi^2 = 27{\cdot}0$; $p < 0{\cdot}001$).

Auxotyping results showed that the majority 85 (65%) of strains were proline requiring, with 29 (22%) non requiring and 12 (9%) proline-arginine requiring. Four isolates were of more demanding auxotypes; two requiring proline arginine and hypoxanthine,

Table 3 Plasmid profiles of isolates of Neisseria gonorrhoeae from Northern Tanzani (n=130)

Plasmid mediated resistance	*Plasmids (MDa)*	*Number of strains (%)*
None	nil	8 (6·2)
None	2·6	36 (27·7)
None	2·6, 24·5	9 (6·9)
PPNG	2·5, 3·2	8 (6·2)
PPNG	2·6, 3·2, 24·5	8 (6·2)
PPNG	2·6, 4·4	14 (10·8)
PPNG	2·6, 4·4, 24·5	2 (1·5)
TRNG	2·6, 25·2	12 (9·2)
TRNG/PPNG	2·6, 3·2, 25·2	30 (23·1)
TRNG/PPNG	2·6, 4·4, 25·2	3 (2·3)

one requiring proline and histidine and one requiring proline, histidine and hypoxanthine, these were all type W1 and both PPNG and TRNG. Additionally 24 of the 29 non requiring strains were type W11/11 with only three typing W1, and 11 of the 12 proline arginine dependent strains were also W11/111.

The plasmid profiles of the isolates are shown in table 3. 53 strains contained no antibiotic resistance plasmids. Of these eight had no plasmids at all, 36 carried the 2·6 MDa cryptic plasmid and nine carried the 2·6 MDa cryptic plasmid and the 24·5 MDa conjugative plasmid. The 12 strains with plasmid mediated resistance to tetracycline only all carried the 2·6 and 25·2 MDa plasmids.

The PPNG strains were heterogeneous; 46 carried the 3·2 MDa plasmid and 19 the 4·4 MDa plasmid. Ten of these strains also carried the conjugative plasmid (24·5 MDa), and the 34 strains which also carried plasmid mediated tetracycline resistance all contained the 25·2 MDa plasmid. Three penicillin and tetracycline resistant strains had additional plasmids, of nine MDa and 23 MDa, but the function of these plasmids was not investigated.

The characteristics of the group of TRNG strains isolated are shown in table 4. Eighteen of the 22 W1 type strains isolated are included in this group, 13 are proline requiring auxotypes and one nonrequiring; the reverse occurs in the TRNG W11/111 group where the majority of strains are nonrequiring; this difference is highly significant ($\chi^2 = 14{\cdot}8$; $p < 0{\cdot}001$), and does not occur for the PPNG strains. Seventeen of the 18 W1 group are PPNG/TRNG and 13 of the W11/111 group; however, nine of the 12 TRNG (non PPNG) strains are in W11/111 compared with 1 in W1.

Discussion

There has been a remarkable increase in antimicrobial resistance among strains of *N gonorrhoeae* in many developing countries in recent years. In 1980, the expected treatment failure rates for conventional doses of aqueous procaine penicillin and oral tetracycline were only 5·6% and 6·9% respectively in Nairobi, Kenya.[7] By the mid-1980s, penicillinase-producing strains comprised 30-50% of isolates in this and other African cities.[8] Chromosomally mediated resistance to many of the cheaper antimicrobials, such as tetracycline, cotrimoxazole and thiamphenicol, has also become prevalent in other developing countries,[9-11] and the emergence of high level tetracycline resistance, coded for by the plasmid-borne tetM determinant, was documented in Kinshasa, Zaire in 1992 in 10% of isolates.[12]

The present study documents the rapid spread of plasmid-mediated tetracycline resistance among strains of *N gonorrhoeae* in Africa; the high prevalence of such strains in Mwanza, which is on a major truck route passing from Mombasa on the Indian Ocean via Nairobi to the Central African countries of Rwanda and Burundi, is likely to be reflected in other towns on the route. Presumably the widespread availability of tetracycline in the informal health sector in developing countries has played an important role in selecting for these strains.

The fact that the isolates tested in this study were taken from patients attending a free clinic suggests that they are reasonably representative of gonococcal strains in the community. However, many patients had received treatment in the informal sector before attending the clinic, so it is possible that the proportion of resistant strains in clinic attenders is higher than in the general population. Another possible source of bias is the fact that the 130 isolates studied represented only 60% of the total obtained during the period of the study. Most of the remaining 40% were lost because of contamination, which, on examination of records, appeared to be a random event.

Plasmid analysis showed that penicillinase producing strains carried both the 3·2 MDa "African" and the 4·4 MDa "Asian" plasmids, although the 3·2 MDa plasmid was more prevalent. Both plasmids have also been found in Rwanda, Nigeria, Zimbabwe and in Senegal,[13-16] suggesting that the strains are not all indigenous, but have been imported. All high level tetracycline resistant strains carried the 25·2 MDa plasmid described by Morse *et al* in 1985[17] and also isolated by Van Dyck *et al* in Zaire.[12] In the present study a high proportion (74%) of the plasmid carrying tetracycline resistant strains also carried a penicillinase-encoding plasmid; this was also noted in the study in Zaire, but was not found in the first tetracycline resistant strains isolated in the USA.[18]

Current treatment for urethritis in males in our clinic is trimethoprim (800 mg) + sulphamethoxazole (4000 mg), two single oral daily doses, and doxycycline 100 mg twice daily for one week. WHO guidelines recommend that this regimen should only be used in

Table 4 Characteristics of plasmid mediated tetracycline resistant Neisseria gonorrhoeae from Northern Tanzania (n=45)

^Phadebact type	*Number of strains*	*Auxotype*			*Plasmids*		
		Pro	*Non-req.*	*Other*	*2·6/25·2*	*2·6/3·2/25·2*	*2·6/4·4/25·2*
W1	18	13	1	4	1	16	1
W11/111	22	6	16	—	9	11	2
W1/11/111	5	4	1	—	2	3	—
Total	45	23	18	4	12	30	3

regions where its efficacy has been proven and can be regularly monitored.[2] In the present study, for strains isolated in 1992, susceptibility to trimethoprim/sulphamethoxazole was reduced in 61% of isolates (MIC 8–16 mg/l), but only five strains (4%) were resistant, suggesting that the current high dose regimen should have been effective in over 95% of cases. During 1993, however, an increasing number of treatment failures were seen[19] emphasising the importance of continued monitoring of both *in vitro* and *in vivo* antimicrobial susceptibility of local strains of *N gonorrhoeae*.

Unfortunately the other drugs recommended by the WHO for the treatment of gonorrhoea (cefixime, ceftriaxone and ciprofloxacin) are all very much more expensive than trimethoprim/sulphamethoxazole, and are not on the essential drug list of Tanzania or other African countries; moreover, in the case of the quinolones, reduced sensitivity has already been reported among gonococcal isolates from Rwanda.[20] The effective treatment of gonorrhoea seems likely to become increasingly expensive in those developing countries with the lowest health budgets, and the help of international donors will be required if control is to be seriously attempted.

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