Antimicrobial susceptibility of *Neisseria gonorrhoeae* in Zaire: high level plasmid-mediated tetracycline resistance in Central Africa

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Abstract

Objective—To determine the in vitro antimicrobial susceptibility of gonococcal strains isolated in 1988 among female prostitutes in Kinshasa, Zaire and to characterise strains with high level tetracycline resistance.

Methods—Minimal inhibitory concentrations of 8 antimicrobials were measured by agar dilution technique. Plasmidprofiles and serovars were determined.

Results—Two hundred and thirteen strains of *Neisseria gonorrhoeae* were tested of which 59% were β-lactamase producers and an additional 21% showed intermediate or chromosomal resistance to penicillin (MIC = 0·5–8 mg/l). Eleven percent of the strains were resistant to the combination sulfamethoxazole-trimethoprim (MIC > 8 mg/l) and 57% of the isolates showed decreased susceptibility to thiamphenicol (MIC = 1–4 mg/l). All strains were sensitive to spectinomycin, norfloxacin and ceftriaxone and moderately sensitive to kanamycin. Chromosomal resistance to tetracycline was observed in 45% of strains (MIC = 2–8 mg/l). Ten percent were highly resistant to tetracycline (TRNG, MIC = 16–128 mg/l) and were shown to carry a plasmid borne Tet M determinant; such strains were not found in Kinshasa in 1985. TRNG belonged to 4 different serovars, which were also the dominant serovars in non-TRNG.

Conclusion—These findings illustrate the high frequency of multiresistant gonococci in Zaire and suggest that high level tetracycline resistant strains of *N. gonorrhoeae* have become endemic in Central Africa.

Introduction

Gonorrhoea is a major health problem in Africa and female prostitutes appear an important reservoir of gonococcal infection.[1–3] Tetracycline is the most widely available antibiotic in many African countries and is still one of the common drugs for treating gonococcal infections. In recent years increased chromosomal resistance to tetracycline (minimal inhibitory concentration (MICs) of 1·0 to 8·0 mg/l) has been found in *Neisseria gonorrhoeae* strains in Africa, and such strains account now for more than 50% of all isolates in some African countries.[4,5]

Plasmid-mediated high level resistance to tetracycline was first observed in *N. gonorrhoeae* (TRNG), (MIC 16–64 mg/l) in 1985 in the US.[6] Since then TRNG strains have also been isolated in Canada, the Netherlands, England and France.[7–10] Tetracycline resistance in these isolates is due to the presence of a 25·2 megadalton (MDa) conjugative plasmid carrying the Tet M determinant.[11]

We report here on the in vitro susceptibility of 213 *N. gonorrhoeae* strains isolated in Kinshasa, Zaire, to eight antimicrobials. About 10% of these isolates exhibited high level resistance to tetracycline. Plasmid profiles and serovars of these strains were determined.

Materials and methods

Strains and susceptibility testing

From May to December 1988, 800 female prostitutes were enrolled in a study on the prevalence and incidence of sexually transmitted diseases (STD) in Kinshasa, Zaire. During the study period, a total number of 238 isolates of *N. gonorrhoeae* were obtained using modified Thayer Martin medium. The strains were stored in skimmed milk at −70°C and sent to the Institute of Tropical Medicine, Antwerp, Belgium, where 226 strains (95%) could be recovered. The identification of all strains was confirmed by Gram strain, oxidase reaction and acidification of glucose, but not sucrose, lactose or maltose. The production of β-lactamase was determined by the chromogenic cephalosporin test (Nitrocefin, Oxoid, Basingstoke, UK).

MICs were determined on 213 strains by an agar dilution technique using a Steers replicator. The final bacterial inoculum was 10^4 colony forming units (CFU). Penicillin, tetracycline, kanamycin and thiamphenicol were provided by the Laboratory of Standards, Ministry of Health, Brussels (Belgium); norfloxacin by Merck Sharp and Dohme (Brussels, Belgium); ceftriaxone, sulfamethoxazole and trimethoprim by Roche (Brussels, Belgium) and spectinomycin by Upjohn (Puurs, Belgium). The antimicrobial combination sulfamethoxazole—trimethoprim (19:1) was tested on Diagnostic Sensitivity Test agar (DST, Oxoid), supplemented with 5% lysed (freezing—thawing) horse blood and 1% Kellogg's supplement. All other antimicrobials were tested on GC agar base (Difco, Detroit, Michigan, USA) with 1% haemoglobin (Difco) and 1% IsoVitaleX (BBL,

Cockeysville, Maryland, USA). Culture plates containing twofold dilutions of antibiotics were incubated for 24 h at 36°C in a 5% CO_2 incubator. The MIC was defined as the lowest concentration of an antimicrobial agent that yielded no growth, no more than 2 colonies or a fine barely visible haze. WHO reference *N. gonorrhoeae* strains A, B, C, D and E (received from I. Lind, Statens Seruminstitut, Copenhagen, Denmark) were included for each antimicrobial assay.

DNA preparation, DNA fixation and restriction digests

Whole-cell DNA was prepared using a small-scale protocol described below. About one quadrant of the cell-yield of a 90 mm diameter blood agar plate was scraped from the plate and suspended in 500 μl 0·15 M NaCl plus 0·01 M EDTA (pH 8·0) in a 2·5 ml Eppendorf tube. Fifty μl 10% SDS and 25 μl (1 mg/ml) Proteinase K were added and the mixture was incubated at 50°C for 1/2 to 1 h. Five hundred μl phenol/chloroform (1:1) was added and the mixture was rotated for 15 min and centrifuged for 10 min at 13000 rpm. The phenol/chloroform extraction was repeated followed by precipitation of the DNA with two volumes of ethanol for 2 h at −20°C. After pelleting and drying, the DNA was dissolved in 100 μl of 0·01 M Tris-Cl pH 7·0 plus 0·001 M EDTA.

The DNA (about 1 μg/spot) was fixed on nitrocellulose membranes (BA85, Schleicher & Schüll, Dassel, FRG) as described previously.[12] Restriction enzymes were purchased from Boehringer Mannheim GmbH (Mannheim, FRG) and used as recommended by the suppliers. About 1 μg DNA per digest was used.

Agarose-gelelectrophoresis and Southern blotting

The electrophoresis was done in 0·7% agarose (Type III, Sigma, St Louis, Missouri, USA) gels at approximately 8V/cm for 1 to 3 h in 40 mM Tris-Cl pH 7·8, 20 mM NaOAc and 2 mM EDTA. After staining in ethidium bromide (1 mg/ml), Southern blotting to a nitrocellulose membrane (BA85) was performed using a standard protocol.[13]

Probe synthesis, purification and digoxigenin-labelling

A 24-mer oligonucleotide, with a sequence complementary to a randomly chosen region in the Tet M gene, was chemically synthesised and purified as described earlier.[12]

Purified oligonucleotide (8 μmol) was labelled using 1 nmol digoxigenin-11-dUTP (Boehringer Mannheim GmbH) and 12·5 units of terminal transferase (Boehringer Mannheim GmbH) in 50 μl 0·1 M sodium cacodylate pH 7·0, 1 mM $CoCl_2$, 0·1 mM dithiothreitol and 50 μg/ml bovine serum albumin for 1/2 h at 37°C. The labelled oligonucleotide was used without further purification.

Hybridisation

The membranes were prehybridised and hybridised following the recommendations provided with the non-radioactive DNA labelling and detection kit (Boehringer Mannheim GmbH) except that 3 X SSC (1 X SSC is: 0·15 M NaCl plus 0·015 M sodium citrate, pH 7·0) instead of 5 X SSC was used. The prehybridisation was performed at 60°C for about 1 h, the hybridisation was done at the same temperature for 3 h using approximately 1 μmol/ml digoxigenin-labelled probe. Subsequently the membranes were washed at 60°C for 30 min in 3 X SSC and for 10 min in 1·5 X SSC at room temperature. The colorimetric detection was performed as recommended by the supplier of the kit mentioned above.

Serological classification

Serological classification into serogroups (W I and W II/III) and serovars was performed by coagglutination as described by Tam et al[14] using reagents with both the Ph set of monoclonal antibodies (Pharmacia Diagnostics AB, Uppsala, Sweden)[15 16] and the GS set of monoclonal antibodies (Syva, Palo Alto, California, USA).[17]

Results

Antimicrobial susceptibility

Of the 213 *N. gonorrhoeae* strains tested, 126 (59%) were producing β-lactamase (PPNG) showing MICs for penicillin of 8 to ≥64 mg/l. Thirty four of the 87 non-PPNG strains had MICs for penicillin of 0·5 to 1 mg/l and 12 had MICs of ≥2 mg/l. Thus only 19% of the total number of isolates were sensitive to penicillin. Resistance to tetracycline was also common: 45% of strains had MICs of 2 to 8 mg/l and 10% were highly resistant showing MICs of 16 to 128 mg/l. All strains were moderately sensitive to kanamycin. A decreased sensitivity to thiamphenicol was seen in 58% of the isolates (MIC 1–4 mg/l) and 11% of the strains were resistant to the combination sulfamethoxazole: trimethoprim (19:1) with MICs of more than 8 mg/l. All of the isolates were susceptible to spectinomycin, norfloxacin and ceftriaxone.

During an earlier survey performed in Kinshasa in 1985, the sensitivities of 93 consecutive isolates of *N. gonorrhoeae* to six antimicrobials were tested. The results of MIC 50, MIC 90 and MIC ranges for all antimicrobials tested on strains from 1988 and 1985 are shown in table 1. For penicillin the results for non-PPNG strains only are compared. In general the MIC 50 and MIC 90 values of 1988 are equal or one dilution higher than those of 1985. The only important difference between both studies is the sensitivity pattern for tetracycline with a MIC range of 0·5–8 mg/l in 1985 and of 0·5–128 in 1988.

Twenty one isolates from 1988 were highly resistant to tetracycline (table 2) including 20 strains with a MIC of 16 mg/l, and 1 strain with a MIC of 128 mg/l. This latter strain was the only TRNG with a MIC for thiamphenicol of 4 mg/l, all others showed MICs of 0·25 mg/l for that antimicrobial while 64% of nonTRNG strains showed decreased susceptibility to thiamphenicol (MICS 1–4 mg/l), (Chi square, Yates corrected; p < 0·00001). Seventeen

Table 1   Minimum inhibitory concentrations (MICs) of clinical isolates of Neisseria gonorrhoeae from Kinshasa in 1988 (n = 213) and 1985 (n = 93)

Antimicrobial		MIC 50 and MIC 90 (mg/liter)		
		MIC 50	MIC 90	MIC range
Penicillin*	1988	0·5	2	0·015–8
	1985	0·25	1	0·015–2
Tetracycline	1988	2	8	0·5–128
	1985	2	4	0·5–8
Thiamphenicol	1988	1	2	0·12–4
	1985	1	1	0·12–2
Kanamycin	1988	16	32	8–32
	1985	8	16	4–32
Spectinomycin	1988	32	32	16–32
	1985	16	16	8–64
Norfloxacin	1988	0·03	0·12	0·015–0·25
	1985	0·03	0·06	0·015–0·12
Ceftriaxone†	1988	≤0·004	0·008	≤0·004–0·03
Sulfamethoxazole/trimethoprim† (19/1)	1988	4	16	0·5–64

*non PPNG strains only: 1988, n = 87; 1985, n = 38.
†not tested in 1985.

TRNG strains (81%) were PPNG, the remaining four strains were sensitive to penicillin (MICS 0·06–0·25 mg/l).

Plasmid content
The TRNG isolates were examined for their plasmid content by agarose gelelectrophoresis. The plasmid profiles of some representative TRNG are shown in fig 1 (lanes 2 to 5). For comparison plasmid profiles of some non-TRNG strains are also shown (fig 1, lanes 6 to 9). All TRNG strains harboured a large plasmid with an electrophoretic mobility higher than the chromosomal material (fig 1); this is most probably the 25·2 MDa plasmid also identified in TRNG strains by other investigators.[4 8 16 19]

Among the TRNG isolates three different plasmid profiles (table 2 and fig 1) could be distinguished. Seventeen of the 21 TRNG strains contained a 3·2 MDa plasmid (lanes 2 and 3 of fig 1) and produced a β-lactamase, with the exception of one strain, (ITM 4451; MIC

for penicillin: 0·25 mg/l). Three TRNG strains did not harbour the 3·2 MDa plasmid (lane 5 of fig 1) and lacked β-lactamase activity. In strain ITM 4446 an additional plasmid band at the 6·2 Mda level was clearly visible (fig 1, lane 4). Six *N. gonorrhoeae* isolates moderately resistant or susceptible to tetracycline as well as three Belgian reference isolates (ITM 3939, 4401 and 4437) were also examined for the presence of the 25·2 MDa plasmid). Four of them harboured a large plasmid, slightly smaller in size than the plasmid found in the TRNG strains (fig 1, lanes 6 and 7). This is most probably the 24·5 MDa conjugative plasmid. Plasmids of that size were not observed in the other five isolates.

Plasmid characterisation
The large plasmid of TRNG strain ITM 4448 was further characterised by restriction digest analysis. The DNA fragments of restriction endonuclease digested whole-cell DNA of this strain were separated on an agarose gel, blotted and hybridised with the Tet M probe. From fig 2 (lane 1) it can be inferred that the Tet M determinant is located on the extra-chromosomal element visible above the chromosomal DNA band. The Southern blot revealed that the Tet M determinant is found on *Sma*I, *Hind*II (*Hinc*II) and *Hind*III fragments with estimated sizes of respectively about 17000 bp (11·0 MDa), about 9000 bp (5·8 MDa) and about 25000 bp (16·2 MDa) (fig 2).

Dot-spot hybridisations
Dot-spotted purified whole-cell DNA of 30 *N. gonorrhoeae* strains was hybridised with the digoxigenin-labelled Tet M probe. The results are shown in fig 3. All TRNG isolates produced a clearcut signal, whereas no significant reaction could be detected with nine *N. gonorrhoeae* strains moderately resistant or susceptible to

Table 2   Characteristics of highly tetracylcine resistant Neusseria gonorrhoeae strains and selected isolates moderately resistant or susceptible to tetracycline

Isolate number	β-lactamase	MIC		Plasmids (MDa)*				Hybridisation with tet M probe	Serovar	
		Penicillin	Tetracycline	3·2	4·5	24·5	25·2		Ph panel	GS panel
4444	+	>32	16	+	0	0	+	+	Arst	IA–6
4445	+	>32	16	+	0	0	+	+	Arst	IA–6
4446	+	>32	16	+	0	0	+	+	Arts	IA–6
4448	+	>32	16	+	0	0	+	+	Arst	IA–6
4449	+	>32	16	+	0	0	+	+	Arst	IA–6
4452	+	>32	16	+	0	0	+	+	Arst	IA–6
4454	+	>32	16	+	≤0·004	0	+	+	Arst	IA–6
4456	+	>32	16	+	0	0	+	+	Arst	IA–6
4457	+	>32	16	+	0	0	+	+	Arst	IA–6
4458	+	>32	16	+	0	0	+	+	Arst	IA–6
4460	+	>32	16	+	0	0	+	+	Arst	IA–6
4461	+	>32	16	+	0	0	+	+	Arst	IA–6
4462	+	>32	16	+	0	0	+	+	Arst	IA–6
4463	+	>32	16	+	0	0	+	+	Arst	IA–6
4464	+	>32	16	+	0	0	+	+	Arst	IA–6
4451	0	0·25	16	+	0	0	+	+	Arst	IA–6
4455	+	>32	16	+	0	0	+	+	Ars	IA–8
4447	0	0·03	16	0	0	0	+	+	Av	IA–4
4450	0	0·06	16	0	0	0	+	+	Av	IA–4
4459	0	0·125	16	0	0	0	+	+	Av	IA–4
4453	+	>32	128	+	0	0	+	+	Bropt	IB–1
4465	+	32	1	+	0	+	0	0	Bx	NR†
4466	0	1	4	0	0	0	0	0	Bopyt	IB–3
4467	+	16	0·5	0	0	+	0	0	Arst	IA–6
4468	+	>32	4	+	0	0	0	0	Bropt	IB–1
4469	0	0·5	4	0	0	0	0	0	Bropt	IB–1
4470	+	>32	0·5	0	+	+	0	0	Arost	IA–2

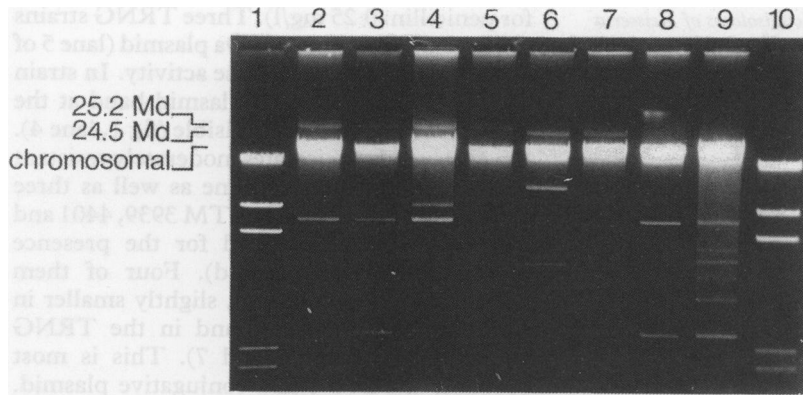*All strains contained the 2·6 MDa (cryptic) plasmid.
†Non reactive.

Fig 1   Ethidium-bromide stained agarose gel of whole-cell DNA of N. gonorrhoeae strains. Lanes 1 and 10: bacteriophage lambda DNA cut with HindIII. Lanes 2 to 5: TRNG strains (lane 2, ITM 4448; lane 3, ITM 4451, lane 4, ITM 4446; and lane 5 ITM 4447). Lanes 6 to 9: non-TRNG strains (lane 6, ITM 4470, lane 7, ITM 3939, lane 8, ITM 4468; and lane 9, ITM 4437).
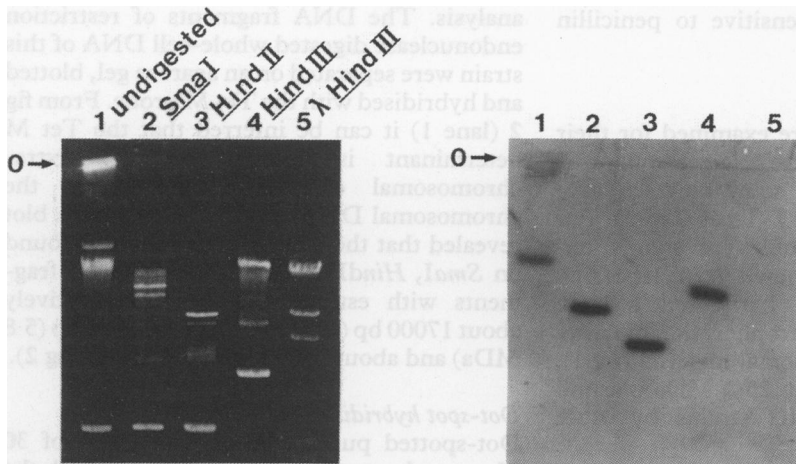

Fig 2   Ethidium-bromide stained agarose gel (panel A) and Southern blot hybridised with a digoxigenin-labeled Tet M olignucleotide probe (panel B) of whole-cell DNA of N. gonorrhoeae ITM 4448. Lane 1: undigested DNA; lane 2: SmaI digest; lane 3: HindII digest; lane 4: HindIII digest; lane 5: bacteriophage lambda DNA cut with HindIII.

Fig 3   Dot-spots of whole-cell DNA of 30 N. gonorrhoeae isolates hybridised with the digoxigenin-labelled Tet M probe. About 1 µg DNA of each strain was applied to a nitrocellulose membrane. From A1 to D3: 21 TRNG strains from ITM 4444 to ITM 4464 in order; from D4 to E6: 9 non-TRNG strains: ITM 4465, 4467, 4470, 3939, 4401, 4466, 4468, 4469 and 4437.
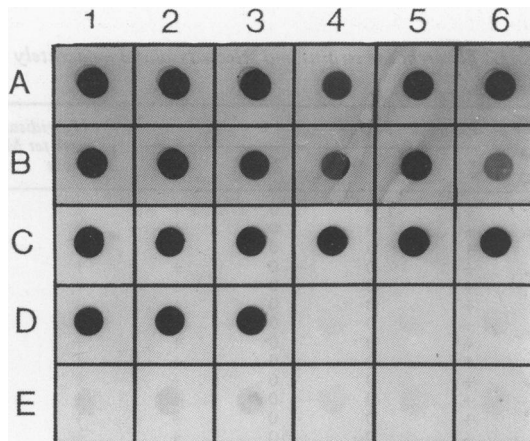
tetracycline. DNA of two strains each of *Neisseria meningitidis*, *Kingella denitrificans*, *Eikenella corrodens*, and *Haemophilus ducreyi* was also found negative with the Tet M probe (results not shown).

Serological classification

The serovars of the TRNG strains and some reference strains are listed in table 2. All 20

TRNG strains with tetracycline MIC of 16 mg/l belonged to serogroup W I. Only one TRNG strain (tetracycline MIC 128 mg/l) was a W II/III strain, serovar Bropt/IB-1. Sixteen of the 20 W I TRNG strains belonged to serovar Arst/IA-6 and contained the 3·2 MDa plasmid. The 17th W I TRNG strain with the 3·2 MDa plasmid belonged to serovar Ars/IA-8. The remaining three W I TRNG strains which were non-PPNG and harboured only the cryptic and the 25·2 MDa plasmid were of serovar Av/IA-4.

Among the non-TRNG isolates of 1988, 18 different serovars were found. Fifty two percent of those strains belonged to serogroup WI and the most common serovars observed were Arst/IA-6 (24%), Av/IA-4 (18%) and Bropt/IB-1 (15%).

Discussion

In this study the antimicrobial susceptiblity of *Neisseria gonorrhoeae* strains isolated from prostitutes in Kinshasa (Zaire) in 1988 was determined and compared with the results of a study performed in 1985. In concordance with studies performed in other African countries[4 18] we found for both surveys (1985 and 1988) in Kinshasa a high prevalence of PPNG (59%).

Among the non-PPNG strains we observed an increasing number of isolates with chromosomal resistańce to penicillin. Thus only 63% and 47% of non PPNG strains isolated in 1985 and 1988 respectively showed MICs to penicillin of ≤0·25 mg/l. in Dakar, Senegal (1988) as many as 96% of non-PPNG isolates showed MICs of ≤0·25 mg/l.[19]

Antimicrobials recommended by the World Health Organisation for the treatment of gonococcal infections include spectinomycin, quinolones and ceftriaxone.[20] No in vitro resistance was detected to those products among the isolates tested. Less expensive drugs such as kanamycin and thiamphenicol are recommended by WHO as alternative treatment of uncomplicated gonorrhoea.[20] Our in vitro results indicate that kanamycin may be a suitable alternative and that thiamphenicol cannot be recommended for the treatment of gonorrhoea in Kinshasa, since thiamphenicol treatment failures have been correlated with MICs of >1 mg/l.[21] MICs of 1 to 4 mg/l were found in respectively 57% and 58% of strains isolated in 1985 and 1988. Those figures are much higher than the 26% and 17% found respectively in Rwanda (1985) and Senegal (1988).[4 19]

Although tetracycline is not recommended for the treatment of gonorrhoea, its use is extremely common in the developing world. It is cheap, and active against *Chlamydia trachomatis*. Therefore it is frequently used to treat urethritis and cervicitis, especially when diagnostic laboratory facilities are lacking. Resistance to tetracycline was observed in well over half of the isolates examined. This is comparable with data from Rwanda,[4] whereas in Senegal only 7% of strains showed a MIC of ≥2 mg/l.[19] However, highly tetracycline resistant *N. gonorrhoeae* isolates have not been

reported as yet from Africa, and seem to be a new phenomenon. This prompted us to study these strains in more detail.

Gelelectrophoretic analysis revealed the presence of a large plasmid in the DNA preparations of all TRNG isolates (fig 1). This plasmid is somewhat larger than the 24·5 MDa plasmid found in some non-TRNG strains and was not observed together with the 24·5 MDa plasmid in the same strains. Hybridisation experiments demonstrated that all TRNG isolates specifically hybridised with a Tet M oligonucleotide probe derived from the Tet M gene sequence (fig 3). It was also shown that the target sequence of this Tet M probe is located on the large plasmid (fig 2 lane 1). This suggests that the high tetracycline resistance in these African isolates is due to the presence of the 25·2 MDa plasmid carrying the Tet M gene described earlier.[7 10 11] In support of this conclusion is the fact that the plasmid has the same electrophoretic mobility as the 25·2 MDa plasmids described by Morse et al [11] and Casin et al[7] and that the length of the restriction fragments carrying the Tet M gene coincide with those reported by Gascoyne et al.[23] The observation that the size of the *Hinc*II and *Sma*I fragments found in this study and reported by Gascoyne et al[23] differ from those reported by Morse et al[11] indicates that different types of plasmids harbouring the Tet M determinant might exist in *N. gonorrhoeae* strains. Whether or not the 25·2 MDa is derived from the 24·5 MDa by insertion of the Tet M gene as proposed by Morse et al[11] and recently disputed by Gascoyne et al[23] cannot be inferred from our data.

The patterns of plasmid profile, serovar and antimicrobial resistance suggest that the TRNG strains in Kinshasa do not represent a single clone, but that multiple strains are involved. However, 76% of the TRNG isolates were PPNG of the Arst/IA-6 serovar and did appear to represent a single clone. Strain ITM 4455 differs from organisms of the former group in serovar (Ars/IA-8) but is very similar in all other features examined. Another group of TRNG strains encompasses three non-PPNG strains, consequently lacking the 3·2 MDa plasmid, which are of the Av/IA-4 serovar.

Strain ITM 4453 has the same plasmid profile as strains of the first main group but is the only TRNG strain belonging to serogroup W II/III, serovar Bropt/IB-1. This strain shows a much higher resistance level to tetracycline (MIC = 128 mg/l) and is also resistant to thiamphenicol (MIC = 4 mg/l), whereas all other TRNG are susceptible to this antimicrobial (MIC = 0·25 mg/l).

It is noteworthy that most (81%) African TRNG strains are PPNG. In contrast less than 1% of TRNG isolated in the US between 1985 and 1989 were PPNG; but the frequency of TRNG/PPNG increased significantly between 1988 and 1989 in some states and accounted for 9·7% of all *N. gonorrhoeae* isolates from Philadelphia in 1989.[24] Among the first 99 TRNG isolates studied in the US only three were PPNG and they belonged to serovars IA-1 and IA-2.[25]

In the Netherlands 12 cases of TRNG were seen in 1985, all isolates carried the 3·2 MDa plasmid and belonged to serovar IB-4.[19] Since 1987 an increasing number of β-lactamase positive and β-lactamase negative TRNG have been isolated. Among PPNG the prevalence of TRNG has increased to 42% during 1989, these isolates predominantly belonged to serovars IB-6, IA-3 and IB-3.[26]

In France one case of TRNG/PPNG was found in 1989; the isolate carried the 3·2 MDa plasmid and belonged to serovar IB-6.[7] In the UK the first cases of TRNG were isolated in 1988: the eight isolates were susceptible to penicillin and all belonged to serovar IB-2.[8]

Based on these data it is difficult to understand the probable association between outbreaks of TRNG in different continents.

Our findings illustrate the high frequency of multiresistant gonococci in Zaire and suggest that tetracycline resistant strains of *N. gonorrhoeae* have become endemic in Central Africa. Blind therapy with tetracyline used for simultaneous eradication of *N. gonorrhoeae* and *C. trachomatis* should be discouraged. The further spread of these strains over Africa seems probable.

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