Characterisation of *penA* and *tetM* resistance genes of *Neisseria gonorrhoeae* isolated in southern Africa — epidemiological monitoring and resistance development

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Objective. To investigate *penA* and *tetM* resistance gene variation of *Neisseria gonorrhoeae* in order to define gene types for epidemiological monitoring and resistance development.

Design. Isolates of *N. gonorrhoeae* which were susceptible and resistant to penicillin and/or tetracycline were selected. Strains comprised South African isolates (22 from Bloemfontein, 13 from Transvaal, 20 from the Cape) and 15 Botswana and 4 Namibia isolates. The *penA* genes (2 kb) of all strains and *tetM* genes (765 bp) of 11 high-level tetracycline-resistant strains were amplified and restricted with *Hpa*II.

Results and conclusions. Twelve different *Hpa*II fingerprint patterns were obtained from the 74 isolates analysed for penicillin-binding protein (PBP) 2 gene (*penA*) alterations. Focusing on the transpeptidase domain, 25 isolates (3 whole gene patterns, minimal inhibitory concentrations (MICs) ≤ 0,03 - 0,125 µg/ml) had restriction sites equivalent to those previously described for a susceptible strain. Of the remaining 9 PBP 2 gene groups, 25 strains fell into a designated group E. Penicillin/penicillin + clavulanic acid MICs determined on these group E isolates gave a range of 0,125 - 2,0 µg/ml, although MICs against 4 strains were ≤ 0,03 µg/ml. MICs of penicillin/penicillin + clavulanic acid for the 24 isolates that contained altered PBP 2 transpeptidase gene regions not designated group E were only ≤ 0,03 - 0,125 µg/ml. The lack of a *Hpa*II restriction site at nucleotide 1934 in

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the PBP 2 gene of group E strains was indicative of a small terminal region of *N. cinerea* DNA. This gene block, which was found in all the southern African areas studied, appears to predispose isolates to increased penicillin resistance. The 25,2 MDa conjugative plasmid carrying the *tetM* resistance determinant was readily demonstrated in 11 Botswana/ Namibia isolates exhibiting high-level resistance to tetracycline (MICs ⩾ 16 µg/ml). The *tetM* gene was shown to be of the American type.

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Gonorrhoea is one of the most prevalent sexually transmitted diseases, and causes serious complications if it goes undetected or is incorrectly treated. Continued surveillance of antibiotic resistance patterns and the specific resistance determinants involved is required, as effective treatment is often dependent on all isolates of *Neisseria gonorrhoeae*'s being susceptible to proposed antibiotic regimens in a particular geographical area. Chromosomally mediated β-lactam resistance in *N. gonorrhoeae* is due to alterations in binding affinity of penicillin-binding proteins (PBPs) 1 and 2.[1,2] Studies have shown that alterations to PBP 2 genes as a result of an Asp-345A insertion combined with mutations and/or acquisition of resistant gene blocks from commensal *Neisseria* species are responsible for increased penicillin resistance.[3,4] Because *N. gonorrhoeae* possess mosaic PBP 2 genes, there may be sufficient gene variability to apply restriction fragment length polymorphism (RFLP) analysis to group isolates for epidemiological purposes as recently applied to group clinical isolates of *N. meningitidis.*[5-7] In addition, via the direct study of PBP 2 genes, RFLP grouping may correlate directly with increased resistance to other β-lactam antibiotics. Although tetracycline is not the antibiotic of choice for the treatment of *N. gonorrhoeae* in South Africa it is used throughout central Africa as it is relatively inexpensive and is effective against *Chlamydia trachomatis.* Tetracycline resistance in *N. gonorrhoeae* can be chromosomally or plasmid-mediated.[8] Chromosomal resistance gives rise to low-level resistance while *N. gonorrhoeae* that have acquired a 25,2 MDa conjugative plasmid carrying the *tetM* determinant exhibit high levels of resistance minimal inhibitory concentrations (MICs) ⩾ 16 µg/ml).[9,10] High-level tetracycline-resistant strains were first reported in the USA in 1985[11] and subsequently spread throughout Europe and central Africa.[12-15] Two *tetM* genes have been described — the American and the Dutch types, which are thought to have different origins.[16]

This study investigates penicillin- and tetracycline-resistant genes in *N. gonorrhoeae* isolates from South Africa, Botswana and Namibia.

Material and methods

Bacterial strains

Isolates of *N. gonorrhoeae* that were susceptible and resistant to penicillin and/or tetracycline were selected from areas in southern Africa. The strains comprised the following South African isolates: 22 from Bloemfontein, 1994, 13 from Transvaal, 1993 and 20 from the Cape, 1994, as well as 15 strains from Gaborone, Botswana and 4 from Windhoek, Namibia, 1993. All isolates were identified as *N. gonorrhoeae* by Gram stain and conventional biochemical methods — oxidase positivity and ability to produce acid from glucose but not maltose, sucrose or lactose.[17,18] Beta-lactamase production was detected by means of the chromogenic cephalosporin (Nitrocefin, Oxoid) method.[19] The Bloemfontein, Cape and Transvaal strains were selected mainly form β-lactamase-negative isolates so as not to complicate detection of chromosomally mediated penicillin resistance. The Botswana and Namibia isolates were selected to provide a range of tetracycline-resistant strains.

Antibiotic susceptibility tests

Standard reference powders were obtained from the following sources: penicillin (Glaxo), tetracycline (Upjohn) and clavulanic acid (Beecham). MICs were determined by the agar dilution method,[20] using dextrose starch agar (Oxoid) supplemented with 1% (v/v) IsoVitaleX (BBL) and 5% (v/v) laked horse blood. Plates were inoculated with 1 x 10^4 colony-forming units per millilitre using a Steers Multipoint replicator (Mast Laboratories), and were incubated at 35°C for 24 hours in an atmosphere containing 5 - 10% CO_2. Fixed clavulanic acid concentrations of 4 µg/ml in combination with serial dilutions of penicillin were tested on β-lactamase-positive strains. The World Health Organisation reference strains A - E were used as controls.

DNA fingerprinting of PBP 2 genes

Cells from half a chocolate agar plate showing confluent growth were resuspended in 0,5 ml buffer (0,5 mM tris-HCl, 20 mM EDTA, pH 7,4) containing 25 µg of lysozyme. After 10 minutes at room temperature 0,5 ml of 2% Triton-X 100 and 12,5 µg of Proteinase K (Boehringer) were added. The *penA* genes were amplified using 5 µl of crude cell extract in a final reaction volume of 100 µl. Amplification of the entire PBP 2 gene (2 kb) was performed by polymerase chain reaction (PCR) using previously described primers GC11 and GCdown3.[5,21] For amplification of the transpeptidase domain of the *penA* gene (1,4 kb), primer GC11 was replaced with primer GCup2.[21] Amplification was carried out for 20 cycles; 95°C, 1 minute; 52°C, 2 minutes; 72°C, 3 minutes. PCR products were restricted with *Hpa*II and fragments end-labelled with [^{32}P] dCTP (Amersham) as described previously.[5] Samples were run on a 6% nondenaturing polyacrylamide gel and autoradiographed. After *Hpa*II digestion of pBR322 DNA, fragments were end-labelled with [^{32}P] dCTP and used as a size marker.

Plasmid analysis

High-level tetracycline-resistant strains were grown on GC agar base (Difco) supplemented with 1% (v/v) IsoVitaleX that contained 10 µg/ml tetracycline. Cells harvested from 1 - 3 plates (approximately 1 x 10^9 CFU/ml) were suspended in cell resuspension solution provided in the Magic Miniprep DNA Purification System (Promega). Plasmids were isolated according to the Magic Miniprep (Promega) protocol with the exception that 4 µg of mutanolysin (Sigma) and 10 µg lysozyme (Sigma) were added to aid lysis. Plasmid

preparations were electrophoresed on 0,7% agarose gels and visualised by staining with ethidium bromide. The β-lactamase plasmids were sized using a supercoiled DNA ladder (Promega).

TetM *gene analysis*

Cells were harvested from confluent growth on half an IsoVitaleX GC agar base plate containing 10 µg/ml tetracycline. Cells were resuspended and lysed as described above for PBP 2 gene amplification. The *tetM* genes were amplified with 5 µl of crude cell extract in a final reaction volume of 100 µl. PCR conditions using primers A and B with thermal cycling: 30 cycles; 94°C, 30 seconds; 50°C, 20 seconds; 72°C, 20 seconds were employed as previously described.[10] PCR products were restricted with *Hpa*II, and the fragments generated were separated on a 2% agarose gel and visualised using ethidium bromide. λDNA was used as a *Hpa*II restriction enzyme control and DNA MWM V (Boehringer) as a size marker. American (strain 6418) and Dutch (strain 2903) type *tetM* genes were amplified and restricted for comparative purposes.

Results

The MICs of penicillin for the southern African isolates studied are shown in Tables I and II. As the majority of Botswana and Namibia isolates were resistant to penicillin because of β-lactamases, MICs of penicillin + clavulanic acid were also determined for β-lactamase-producing isolates in order to detect reduced susceptibility mediated by underlying PBP alterations. All the isolates were screened for high-level tetracycline resistance with plates containing 10 µg/ml tetracycline. Only Botswana and Namibia strains exhibited high-level tetracycline resistance (Table II).

Representative *Hpa*II-restricted PBP 2 gene fingerprint profiles of isolates from the different areas studied are shown in Fig. 1. Major groups were designated A - E with some subgroups defined in groups A, B and C. The PBP 2 fingerprint groups assigned to all the strains studied are shown in Tables I and II. The A1 fingerprint group contains the same restriction fragments that would be generated from the reported sequence of a susceptible isolate. *N. gonorrhoeae* LM306.[21] Fingerprint groups A2 and A3 showed alterations upstream of the transpeptidase domain; penicillin MICs of these 25 strains were ⩽ 0,03 - 0,125 µg/ml. Penicillin/penicillin + clavulanic acid MICs of strains in groups B, C and D also showed MICs in the range of ⩽ 0,03 - 0,125 µg/ml whereas 20 of 24 group E strains on which penicillin/penicillin + clavulanic acid MICs were performed showed MICs of 0,125 - 2,0 µg/ml. It should be noted that four group E strains showed penicillin MICs ⩽ 0,03 µg/ml. Analysing the *Hpa*II restriction sites within the transpeptidase domain of the PBP 2 gene with published sequences of penicillin-susceptible and resistant *N. gonorrhoeae* isolates,[4,21] the major alterations in restriction sites for groups C, D and E were present in the terminal 450 bp region and those of group B upstream of the terminal 450 bp area. Fingerprint patterns specific to the transpeptidase domain of groups C1, D and E are shown in Fig. 1. Group D patterns appeared comparable to strain USACDC84060418,[4] which contains a *N. flavescens* and a *N. cinerea* insertion. Group E patterns were comparable with strain USACDC77-124615,[4] and lacked a *Hpa*II restriction site at nucleotide 1934. This suggests a small terminal

Table I. Details of the *N. gonorrhoeae* isolates from South Africa

Bloemfontein (*N* = 22)					Cape (*N* = 20)					Transvaal (*N* = 13)			
Strain	β-lact.	Pen MIC (µg/ml)	Pen + clav*	PBP 2 pattern	Strain	β-lact.	Pen MIC (µg/ml)	Pen + clav*	PBP 2 pattern	Strain	β-lact.	Pen MIC (µg/ml)	PBP 2 pattern
P4	–	⩽ 0,03		C3	C1	–	0,125		E	T1	–	⩽ 0,03	A1
P6	+	4,0	0,25	E	C2	–	0,125		E	T2	–	⩽ 0,03	A1
P7	–	⩽ 0,03		A1	C3	–	⩽ 0,03		B3	T3	–	⩽ 0,03	A1
P8	–	0,03		B2	C4	–	⩽ 0,03		A2	T4	–	⩽ 0,03	A1
P9	–	0,03		A3	C5	–	0,125		C2	T5	–	⩽ 0,03	A1
P10	–	0,5		E	C6	–	0,125		C2	T6	–	0,125	B1
P11	–	0,5		E	C7	–	⩽ 0,03		A2	T7	–	0,125	A2
P12	–	⩽ 0,03		A3	C9	–	⩽ 0,03		E	T8	–	0,125	C1
P13	–	⩽ 0,03		A1	C10	+	2,0	⩽ 0,03	C4	T9	–	0,125	A1
P14	–	⩽ 0,03		A1	C11	–	0,06		B3	T10	–	0,125	D
P15	–	⩽ 0,03		A1	C14	–	⩽ 0,03		A1	T11	–	0,125	C1
P16	–	⩽ 0,03		B2	C15	–	0,5		E	T12	–	1,0	E
P17	–	⩽ 0,03		B2	C16	–	⩽ 0,03		A2	T13	–	1,0	E
P19	–	⩽ 0,03		E	C17	–	⩽ 0,03		A2				
P20	+	2,0	⩽ 0,03	C2	C18	+	16,0	⩽ 0,03	C2				
P21	+	32,0	ND	E	C19	+	8,0	⩽ 0,03	C1				
P22	–	⩽ 0,03		E	C20	+	64,0	⩽ 0,03	E				
P23	–	⩽ 0,03		A3	C21	–	0,06		A1				
P24	–	⩽ 0,03		B2	C22	–	⩽ 0,03		A1				
P25	–	⩽ 0,03		A1	C23	–	⩽ 0,03		A1				
P26	–	⩽ 0,03		A1									
P27	–	⩽ 0,03		D									

* Penicillin was combined with clavulanic acid (4 µg/ml) for isolates producing β-lactamases.
β-lact. = β-lactamase production; Pen = penicillin; ND = not determined, strain P21 was no longer viable.

Table II. Details of the *N. gonorrhoeae* isolates from Botswana and Namibia

Botswana (*N* = 15)							Namibia (*N* = 4)						
Strain	β-lact.	Pen MIC (μg/ml)	Pen + clav*	PBP 2 pattern	Tet MIC (μg/ml)	*tetM* gene†	Strain	β-lact.	Pen MIC (μg/ml)	Pen + clav*	PBP 2 pattern	Tet MIC (μg/ml)	*tetM* gene†
B1	+	> 128	2,0	E	4,0	–	N1	+	32	0,5	E	> 128	+
B2	+	> 128	≤ 0,06	C1	0,5	–	N2	+	4	0,125	E	16	+
B3	+	> 128	2,0	E	4,0	–	N3	+	> 4	≤ 0,03	C1	32	+
B4	+	> 128	1,0	E	128	+	N4	+	16	0,5	E	128	+
B5	+	> 128	≤ 0,06	D	4,0	–							
B6	+	> 128	1,0	E	> 128	+							
B7	+	128	0,125	D	8,0	–							
B8	–	1,0	–	E	128	+							
B9	+	64	0,125	D	4,0	–							
B10	+	64	≤ 0,06	C2	1,0	–							
B11	–	0,125		A1	4,0	–							
B12	–	1,0		E	> 128	+							
B13	–	1,0		E	> 128	+							
B14	–	1,0		E	64	+							
B15	–	2,0		E	> 128	+							

* Penicillin was combined with clavulanic acid (4 μg/ml) for isolates producing β-lactamases.
† American-type *tetM* gene.
β-lact. = β-lactamase production; Pen = penicillin; Tet = tetracycline.

N. cinerea gene block (Fig. 2). The group C1 pattern lacks the 309 bp fragment because of an extra *Hpa*II restriction site at nucleotide 1594, C1 appears to have a small block of *N. flavescens* DNA, while C2 and C3 had fewer restriction sites over the terminal region (Fig. 2); pattern C4 found with one strain only is similar to C1 with additional bands of questionable significance. The plasmid profiles of the high-level tetracycline-resistant isolates (MIC ≥ 16 μg/ml) from Botswana are shown in Fig. 3. All the strains possessed the 25,2 MDa conjugative plasmid as did the four Namibian isolates (not shown). In some isolates (6/11) the *tetM* conjugative plasmid was seen in association with 3,4 MDa β-lactamase plasmids. All the isolates harboured the cryptic 2,6 MDa plasmid; however, the 24,5 MDa conjugative plasmid was not observed. PCR products of the *tetM* gene of the predicted size 765 bp,[10] were obtained from all the isolates with tetracycline MICs of ≥ 16 μg/ml. A PCR product was not demonstrable with DNA from a tetracycline-susceptible strain (MIC 0,25 μg/ml), or with the DNA from 3 Botswana isolates where the tetracycline MICs ranged between 4 and 8 μg/ml. *Hpa*II restriction patterns of *tetM* genes are shown in Fig. 4 and can be seen to be of the American type. All the strains from Botswana and Namibia with tetracycline MICs ≥ 16 μg/ml were found to contain the American-type *tetM* resistance gene (Table II).

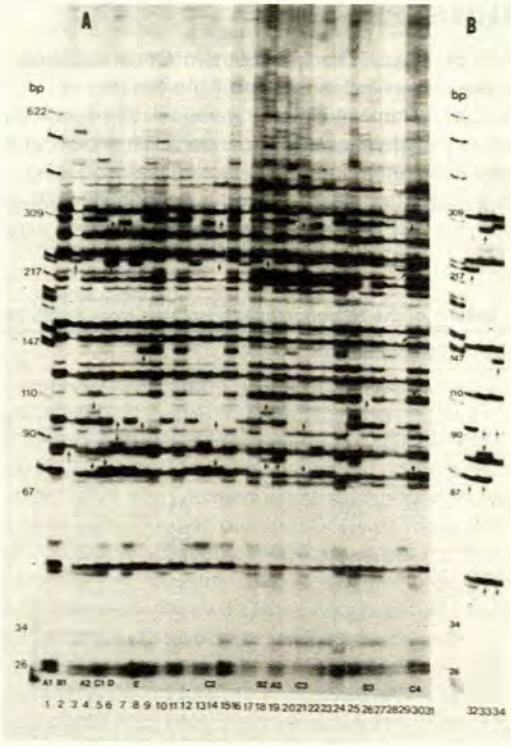

Fig. 1. *Hpa*II PBP 2 gene fingerprint groups. (A) complete PBP 2 gene fingerprints; (B) transpeptidase domain fingerprints. Fingerprint groups are given in brackets after each strain. (A) LANES: 1 - 9 Transvaal strains, LANES: 10 - 14 Botswana strains, LANES: 15 - 16 Namibia strains, LANES: 17 - 24 Bloemfontein strains, LANES: 25 - 31 Cape strains. LANES: 1, T5 (A1); 2, T6 (B1); 3, T2 (A1); 4, T7 (A2); 5, T8 (C1); 6, T10 (D); 7, T11 (C1); 8, T12 (E); 9, T13 (E); 10, B11 (A1); 11, B14 (E); 12, B2 (C1); 13, B5 (D); 14, B10 (C2); 15, N4 (E); 16, N3 (C1); 17, P7 (A1); 18, P16 (B2); 19, P12 (A3); 20, P19 (E); 21, P4 (C3); 22, P27 (D); 23, P20 (C2); 24, P6 (E); 25, C16 (A2); 26, C3 (B3); 27, C14 (A1); 28, C9 (E); 29, C19 (C1); 30, C10 (C4); 31, C18 (C2). (B) LANES: 32, T8 group C1; 33, T10 group D; 34, T12 group E. Arrows indicate fragments that differ (either absent or new) from those of group A1.

Discussion

*Hpa*II *penA* gene fingerprinting and *tetM* gene restriction analysis appears to be useful in characterising these genes in *N. gonorrhoeae* isolates. The *penA* fingerprints of groups D and E are of interest because of their similarity to previously described sequences,[4,21] and groups B and C appear more individual, especially in respect of restriction site alterations upstream of the terminal 450 bp area. However, whether any clonal relationships exist between strains assigned to a specific group requires further studies.

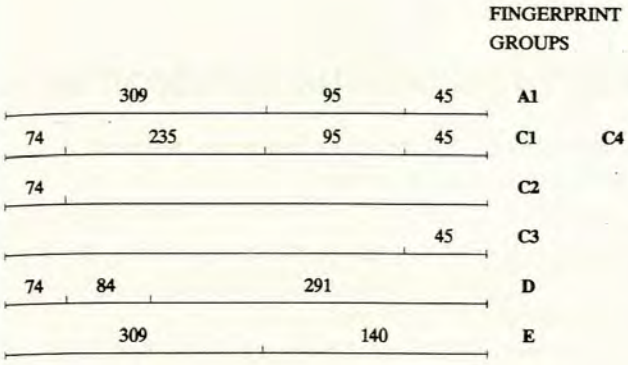

Fig. 2. *Hpa*II-restriction sites in the terminal 450 bp region of the transpeptidase domain of the *penA* gene, based on published sequences.[4,21]

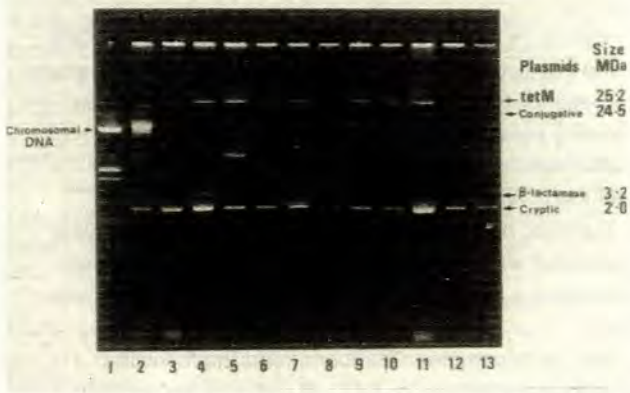

Fig. 3. Plasmid profiles of high-level tetracycline-resistant strains isolated in Botswana. LANES: 1, MWM III; 2, strain B2 tetracycline-susceptible; 3 and 11, American control strain 6418 containing *tetM* conjugative plasmid; 4, B4; 5, B13; 6, B15; 7, B6; 8, B8; 9, B12; 10, B14; 12, control strain E β-lactamase-positive tetracycline-susceptible; 13, control WHO strain A β-lactamase-negative tetracycline-susceptible.

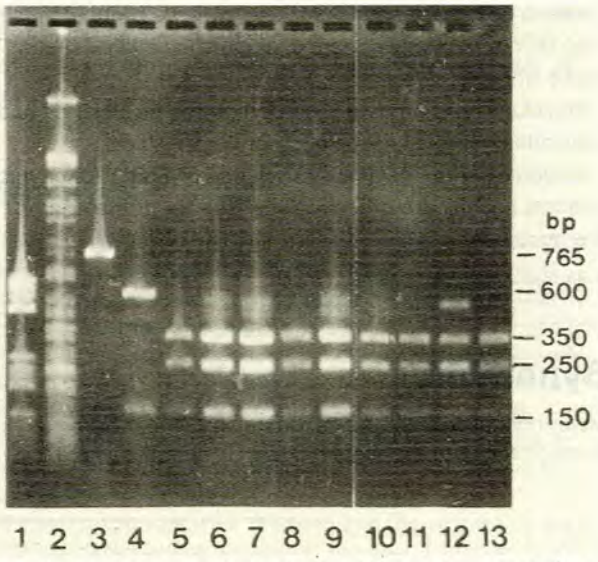

Fig. 4. *Hpa*II restriction patterns of the *tetM* gene of representative Botswana and Namibia strains. LANES: 1, MWM V; 2, λDNA; 3, PCR product unrestricted; 4, Dutch control type *tetM* gene; 5, American control type *tetM* gene; 6, B4; 7, B8; 8, B14; 9, B12; 10, N1; 11, B6; 12, N2; 13, B15.

Identification of the American type *tetM* gene indicates that the PCR-RFLP method proposed by Ison *et al.*[10] is of value for epidemiological typing of *N. gonorrhoeae* isolates that exhibit high-level tetracycline resistance. Given that the *tetM* gene currently present in Botswana and Namibia has now been identified as the American type, it should prove interesting to see if the Dutch-type gene is introduced into the southern African environment or whether the American-type gene continues to predominate and spread.

The findings of isolates with penicillin MICs 0,125 - 2,0 μg/ml that possessed only *Hpa*II fingerprint pattern E tends to suggest that pattern E predisposes *N. gonorrhoeae* strains to increased resistance. However, four strains with group E fingerprint patterns were susceptible (MICs ⩽ 0,03 μg/ml). This type of result is unfortunately consistent with *Neisseria* PBP 2 gene analysis: introduction of recognised gene blocks into *N. gonorrhoeae* and *N. meningitidis penA* genes has occurred, but the full implications of their presence for development of penicillin resistance are not completely understood.[4,7,22] The Asp-345A insert has been demonstrated in all *N. gonorrhoeae* strains with MICs ⩾ 0,03/0,06 μg/ml studied to date.[3,23] Contributions of PBP 2 insertions and mutations in conjunction with PBP 1 and permeability alterations have to be evaluated in order to explain MICs of ⩾ 0,125 μg/ml in the absence of β-lactamase production. Decreased susceptibility to cefoxitin was also seen, with 12 out of 15 strains from Botswana being associated with *Hpa*II PBP 2 fingerprint groups D and E. Initial stages of reduced susceptibility to ceftriaxone (MIC range ⩽ 0,002 - 0,06 μg/ml) was also evident in the *N. gonorrhoeae* isolates studied, and has been observed in Johannesburg strains.[24]

The results of this study of the high-level tetracycline-resistant isolates as regards combined penicillin resistance (chromosomally or plasmid-mediated) are disturbing. The American type *tetM* gene has been described in Gabon, Kenya and Zaire,[15] and now appears to have spread to southern Africa. The presence of the *tetM* conjugative plasmid in neighbouring countries is cause for concern in South Africa as the usage of tetracycline to treat *C. trachomatis* may still select for high-level tetracycline-resistant *N. gonorrhoeae* strains in the future. The presence of conjugative plasmids are a threat as they are capable of mobilising nonconjugative resistance plasmids and rapidly disseminating them throughout populations. In the present study, in 6 of the 11 high-level tetracycline-resistant isolates the *tetM* conjugative plasmid was seen to coexist with the African-type (3,4 MDa) β-lactamase plasmids. Of even greater concern, the presence of *tetM* genes may facilitate the establishment of additional resistance determinants as *N. gonorrhoeae* is naturally transformable and the 25,2 MDa plasmid has been shown to have a wider host range than the 24,5 MDa conjugative plasmid.[25]

The spread of penicillin and tetracycline resistance genes (Namibia and Botswana) has recently lead to the introduction of more expensive antibiotics to treat gonococcal infections. Treatment costs per patient have escalated and as exposure increases, continued monitoring of resistance development to ceftriaxone and ciprofloxacin is vital, since these drugs are likely to become ineffective. This would inevitably result in further rises in antibiotic cost, problems in obtaining effective/non-toxic antibiotics and, with the loss of empirical antibiotic cover to treat

gonococcal infections, the added expense of culture and susceptibility testing.

The study emphasises the importance of determining the location (chromosomal or plasmid) of antibiotic resistance genes and the need to characterise these genes individually. Numerous genetic techniques are available to subtype bacterial species. The cost-effectiveness of each method and its relevance need to be established before such techniques are used in epidemiological studies. By applying genetic techniques, resistance genes can be characterised, origins of new resistance determinants can be identified and, hopefully, dissemination can be contained.

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