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Study of five penicillinase producing *Neisseria gonorrhoeae* isolated in Italy

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SUMMARY

Five penicillinase producing *Neisseria gonorrhoeae* (PPNG) were isolated from urethral specimens of men admitted to the "Santa Chiara" Hospital (Trento, Italy). All strains proved to be resistant to penicillin and ampicillin, and sensitive to cefuroxime, erythromycin, tetracycline, spectinomycin, nalidixic acid and ciprofloxacin. PPNG plasmid profiles showed that four of the isolates carried the 3.2 MDa "Africa" plasmid and one the 4.5 MDa "Asia" plasmid, the two well-known phenotypes reported in the USA and Europe as well as in Asian and African countries. Membrane matings were performed using *N. gonorrhoeae* carrying the 24.5 MDa conjugative plasmid as donors and *E. coli* K12 J 53 as recipient. The transfer of β-lactamic antibiotic resistance was supported by the presence of 4.5 or 3.2 MDa plasmid bands and by β-lactamase production in the transconjugants. Restriction analysis of Asian and African plasmids is reported.

KEY WORDS: PPNG, *Neisseria gonorrhoeae*, plasmids, conjugation

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INTRODUCTION

Gonorrhoea is a common transmissible bacterial infection, and WHO (1999) estimates place the yearly number of new gonococcal infections worldwide at nearly 60 million. Gonorrhoea begins as an infection of the superficial mucous membranes (Brown, 1999), such as those of the endocervix, urethra, pharynx or rectum (LiMaye, 2003). Approximately 10-20% of infected women develop salpingitis, with resultant tubal scarring and either infertility or ectopic pregnancy (Westrom and Eschenbach, 1999; Zhu *et al*., 2004). Many cases are asymptomatic and therefore remain undiagnosed. Untreated or inadequately treated gonococcal infections may have repercussions such as blood dissemination (LiMaye, 2003) and spread to other people.

Neisseria gonorrhoeae has developed chromosomal and plasmid resistance to several antibiotics, and the number of penicillinase-producing *N. gonorrhoeae* (PPNG) increased rapidly from 1987 to 1991 (Fox *et al*., 1997), slightly falling off when penicillin therapy against gonorrhoea was discontinued (Lind, 1997). It has been suggested that the decrease of PPNGs is due to the therapeutic use of active drugs other than penicillins and to the use of fluoroquinolones, which can attack β-lactamase plasmids (Kam *et al*., 1995).

Penicillinase-producing *N. gonorrhoeae* carrying the Asian plasmid (4.4-4.7 MDa) were first isolated in the USA in 1976 (Ashford *et al*., 1976) from United States military servicemen who had relocated from Southeast Asia, and these agents are named according to their epidemiological origin in the Far East. The first strains carrying the African plasmid phenotype (3.2-3.4 MDa) were instead isolated in the UK in 1976 (Phillips, 1976).

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Two other deletion derivatives were detected in 1984 by separate research groups and designated Toronto (3.05 MDa) (Yeung *et al*., 1986) and Rio plasmids (2.9 MDa) (van Embden *et al*., 1985). Other plasmids have been reported, including Nimes (4.0 MDa) (Gouby *et al*., 1986), New Zealand (6.0 MDa) (Brett, 1989) and an unnamed variant (3.9 MDa) isolated in the Philippines (Knapp *et al.*, 1997), but global dissemination of these plasmids has not as yet followed their emergence (Pagotto *et al*., 2000, Palmer *et al*., 2000). Studies, based on restriction endonuclease, hybridisation and heteroduplex analysis by electron microscopy have shown that the African, Toronto, Nimes, Rio, and New Zealand-type plasmids are deletion or insertion derivatives of the prototype Asian plasmid (Yeung *et al*., 1986; Dillon *et al*.,1989). In particular, the African plasmid presents a 2.1 Kb deletion as compared to the Asian plasmid.

Penicillinase production in these plasmids is mediated by a TEM1-type β-lactamase encoded by the Tn*2* transposon carried on several related plasmids (Palmer *et al*., 2000) that is cut and includes 84% of tnp*R* noncoding sequences, the entire *bla* gene, and the right inverted repeat (IR-R) (Fayet *et al*., 1982; Pagotto *et al*., 2000).

In spite of a worldwide prevalence (Lee *et al*., 2000), PPNG strains have rarely been reported in Italy, probably due to the limited use of penicillin in gonococcal urethritis therapy. The isolation of *N. gonorrhoeae* less sensitive to penicillin was first reported up to 1980 (Coppini *et al*., 1980). Subsequently, we isolated two PPNG strains carrying the Asian and African plasmids (Bondi *et al*., 1984; Manicardi *et al*., 1992). Considering the lack of new reports on PPNG in Italy, the present work aims to study the plasmid phenotypes of five penicillinase-producing *Neisseria gonorrhoeae* isolated at the "Santa Chiara" Hospital of Trento.

MATERIALS AND METHODS

Bacterial strains

The penicillinase producing *Neisseria gonorrhoeae* (PPNG) used in this study are listed in Table 1. The strains were isolated from urethral specimen of men admitted to the "Santa Chiara" Hospital (Trento, Italy) and identified by standard biochemical methods (API NH, bioMeriéux, France). β-lactamase production was assayed using the chromogenic cephalosporin test (Nitrocefin, Oxoid, Milano, Italy) (O'Callaghan *et al*., 1972). All *N. gonorrhoeae* were grown on GC agar base (Oxoid) supplemented with 1% IsoVitalex (Oxoid) and ampicillin (10 mg/ml, Sigma Chemical Co. St. Louis, MO, USA), and incubated for up to 24h at 37°C in a humid atmosphere supplemented with 5% CO_2.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined in GC agar base supplemented with 1% IsoVitalex by breakpoint criteria, as defined by the National Committee for Clinical

TABLE 1 - *MIC of the five PPNG strains*

	MIC (μg/ml)							
	P	*Ap*	*Cf*	*E*	*Sp*	*T*	*Na*	*Cip*
N. gonorrhoeae 57	>128	>128	0,004	0,008	2	1	1	0.25
N. gonorrhoeae 98	>128	>128	0,004	0,016	8	0.06	0.5	0.03
N. gonorrhoeae 157	>128	>128	0,008	0,016	4	1	0.06	0.015
N. gonorrhoeae 237	>128	>128	0,016	0,008	16	0.06	0.5	0.125
N. gonorrhoeae 243	>128	>128	0,008	0,008	1	0.06	0.25	0.03

P = penicillin; Ap = ampicillin; Cf = cefuroxime; E = erythromycin; T = tetracycline; Sp = spectinomycin; Na = nalidixic acid; Cip = ciprofloxacin.

Laboratory Standards (NCCLS, 2000). Twofold serial dilutions of the following antibiotic powders were tested: ampicillin (Ap), penicillin (P), cefuroxim (Cf), erythromycin (E), tetracycline (T), spectinomycin (St), nalidixic acid (Na), ciprofloxacin (Cip) (all from Sigma).

PPNG plasmid profiles

Plasmid DNA was isolated by the lyses method of Birnboim and Doly (1979). Electrophoresis was conducted on 0.7% agarose, horizontal slab gels, in TRIS acetate buffer at pH 8.0, using a steady voltage of 75 V for 120 min. Purified plasmids of *E. coli* V517 (Macrina *et al.*, 1978) were used as size reference plasmid for molecular weight determinations.

Conjugation experiments

Matings were performed by a modified Roberts and Falkow (1977) method, using the PPNGs carrying the 24.5 MDa conjugative plasmid as donors and *E. coli* K12 J 53 Na^R, Ap^S as recipient. For each conjugation, equal volumes of donor and recipient suspensions were inoculated in 10 ml of GC broth (Oxoid) with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ added. After overnight incubation at 37°C, matings were performed on a membrane filter (0.45 µm-pore-size filter; Millipore Corp., Bedford, Mass.) then placed in a GC-agar base containing Kellogg's supplement (Difco, Detroit, MI). After 6 h of further incubation the cells were resuspended in 2 ml GC broth by vigorous shaking and spread on McConkey agar (Oxoid) supplemented with 10 µg/ml ampicillin and 50 µg/ml nalidixic acid. The developed colonies were tested for 4.5 and 3.2 MDa plasmid bands and for β-lactamase activity.

Asian and African plasmids restriction analysis

Asian and African plasmids were recovered from agarose gel by the Maniatis technique (Maniatis, 1982). Plasmids were digested with *Bam*HI, *Hin*dIII, *Pst*I according to the manufacture's instructions (Promega, Madison, WI). In all analysis lambda phage DNA (Roche Applied Science, Indianapolis, IN) digested with *Bam*HI, *Hin*dIII and *Eco*RI (Boehringer, Mannheim, Germany) was used as molecular size marker. Digests were separated by electrophoresis on 1% (w/v) agarose gel in TRIS acetate buffer at pH 8 at 30V for 16h.

RESULTS

Minimum inhibitory concentration

MIC values of PPNGs are reported in Table 1. All strains were resistant to penicillin and ampicillin (MIC >128 mg/ml), whereas they were sensitive to other antibiotics tested at the following MIC ranges: cefuroxime 0.004-0.016 mg/ml, erythromycin 0.008-0.016 mg/ml, tetracycline 0.06-1 mg/ml, spectinomycin 1-16 mg/ml, nalidixic acid 0.06 -1 mg/ml, ciprofloxacin 0.0015-0.25 mg/ml.

PPNG and E. coli transconjugant plasmid profiles

N. gonorrhoeae 343, 237, 157 and 98 presented the 3.2 MDa African plasmid and *N. gonorrhoeae*

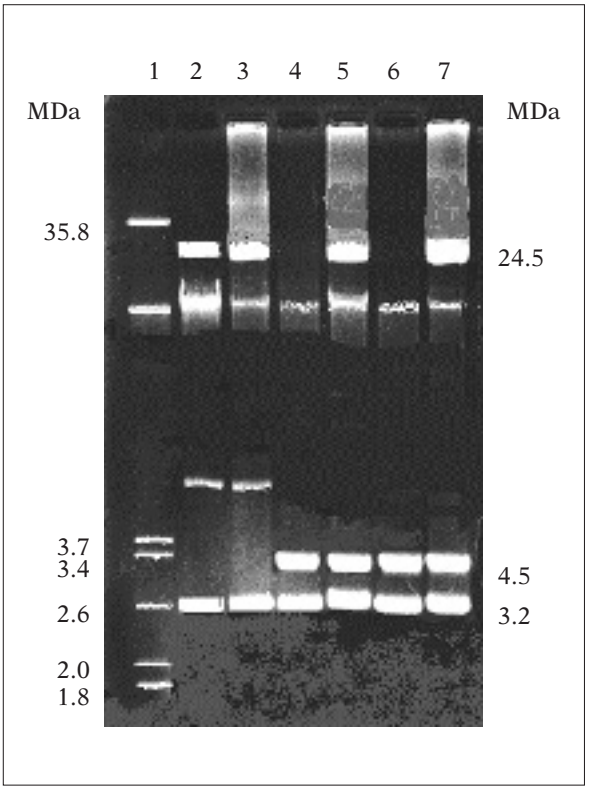

FIGURE 1 - *PPNG strains and E. coli NGC Asian transconjugant plasmid profiles. Lane 1: molecular size markers prepared from* E. coli *V517 (35.8, 3.7, 3.4, 2.6, 2.0, 1.8 MDa); lane 2:* E. coli *NGC 57 Asian transconjugant; lane 3:* N. gonorrhoeae *57 with Asian profile; lanes 4, 5, 6, 7:* N. gonorrhoeae *237,* N. gonorrhoeae *157,* N. gonorrhoeae *343,* N. gonorrhoeae *98, all with African profile.*

57 showed the "Asia" configuration, characterized by the 4.5 MDa plasmid. *N. gonorrhoeae* 157, 98 and 57 harboured the 24.5 MDa conjugative plasmid. All strains had the 2.6 MDa cryptic plasmid.

African and Asian PPNG strains carrying the 24.5 MDa conjugative plasmid (*N. gonorrhoeae* 157, 98 and 57) transferred β-lactamic resistance to *E. coli* K12 J 53. This transfer was supported by 4.5 or 3.2 MDa plasmid bands observed in the transconjugants and confirmed by β-lactamase production. Plasmid profiles of PPNG strains and *E. coli* K12 NGC Asia transconjugant are shown in Figure 1.

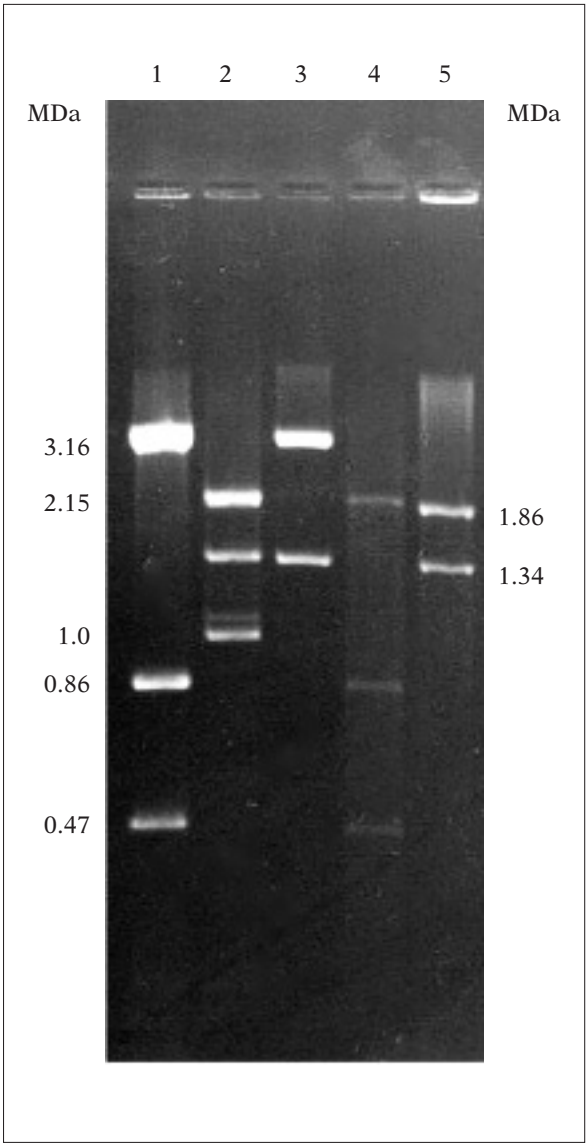

FIGURE 2 - *Restriction analysis of* N. gonorrhoeae *57 Asian plasmid and* N. gonorrhoeae *237 African plasmid. Lanes 1, 2, 3: Asian plasmid digest with* Bam*HI and* Pst*I (lane 1), with* Bam*HI and* Hind*III (lane 2), with* Bam*HI (lane 3); lanes 4, 5: African plasmid digested with* Bam*HI and* Pst*I (lane 4) and with* Bam*HI (lane 5*).

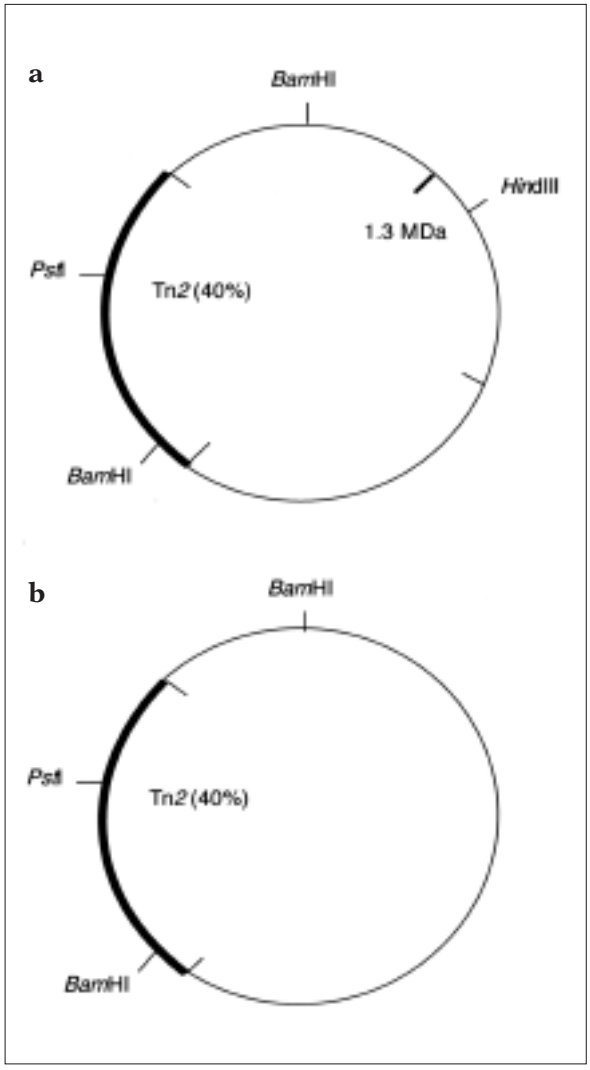

FIGURE 3 (a, b) - *Restriction endonuclease map of the* N. gonorrhoeae *57 Asian plasmid (a) and* N. gonorrhoeae *237 African plasmid (b). In both plasmids the* Pst*I site is located in the common region containing the portion of the Tn2 transposon between the two* Bam*HI sites, and this explains why two bands with equal molecular weight are formed. The Asian plasmid, which shares a region containing a* Hind *III site was also cleaved with* Bam*HI and* Hind*III into three fragments.*

Asian and African plasmids restriction analysis

Figure 2 illustrates the restriction analysis of 4.5 and 3.2 MDa plasmids. *Bam*HI cleaves the plasmids into two characteristic bands (3.16, 1.34 MDa for Asian plasmid and 1.86, 1.34 MDa for African plasmid), as reported by McNicol *et al*., (1983). Using both *Bam*HI and *Pst*I enzymes, each plasmid is cleaved into three fragments (1.86, 0.86, 0.47 MDa for African plasmid and 3.16, 0.86, 0.47 for Asian plasmid). In both plasmids, the *Pst*I site is located in the common region containing the portion of the Tn*2* transposon between the two *Bam*HI sites, and this explains why two bands with equal molecular weight are formed. The Asian plasmid, which shares a region containing a *Hin*dIII site with another R factor carried by *Haemophilus ducrey* (Brunton *et al*., 1981), was also cleaved with *Bam*HI and *Hin*dIII into three fragments (2.16, 1.34 and 1.00 MDa) (McNicol *et al*., 1983). The plasmidial maps are shown in Figure 3 (a, b).

DISCUSSION

Gonorrhoea remains a major cause of bacterial sexually transmitted disease (STD) worldwide. In Africa, for example, *N. gonorrhoeae* is the most important cause of male urethritis, and the prevalence of gonococcal infection among women is high, in particular among prostitutes and workers in the sex industry (Germain *et al*., 1997). The high prevalence of *N. gonorrhoeae* in developing countries is largely related to poor accessibility to diagnostic tests and appropriate treatments (Alary *et al*., 1996). The control of gonococcal infection is important considering the high incidence of complications. Moreover, gonorrhoea may play a role in facilitating HIV acquisition and transmission, due to mucosal inflammation and greater release of the virus in semen (Wasserheit, 1992; Bhuiyan *et al*., 1999).

Antimicrobial resistance among *N. gonorrhoeae* isolates in many developing countries has increased remarkably (Nissinen *et al*., 1997). Surveys by the WHO Western Pacific Region in 1997 (WHO, 1998) showed that PPNG was widely distributed throughout the region. PPNG is particularly prevalent in the Philippines (81.8%), Korea (79.3%), Singapore (61%), Vietnam (64%) and Malaysia (41%). Interestingly, rates are very low in other countries, such as Japan (2.3%), Australia (6.4%) and New Zeland (7.4%). The literature reports high levels of penicillin resistance that may be a consequence of therapy for unrelated illnesses or self-medication with penicillin or penicillin-related drugs. For this reason the Centres for Disease Control have recommended a first-line therapeutic regimen based on fluoroquinolones and cephalosporin (CDC, 1993).

Although in some parts of Europe penicillins are still used effectively to treat gonorrhoeae, resistance is frequent in imported cases (Tapsall, 2001). Even in our work the gonococci studied had high MIC values for penicillin and ampicillin, and considering the lack of PPNG reports in Italy, these strains were probably imported. Resistance was not detected against cefuroxime, erythromycin, tetracycline or nalidixic acid. Our isolates were also susceptible to spectinomycin and ciprofloxacin, which remain valuable alternatives for treating gonorrhoea.

In our isolates, plasmid analysis showed that four strains carried the 3.2 MDa "Africa" and only one the 4.5 MDa "Asia" plasmids, which are responsible for the two well-known phenotypes reported in the USA and Europe as well as in Asian and African countries. The presence of the 24.5 MDa conjugative plasmid in both phenotypes suggests the possibility of a horizontal spread of plasmid-mediated penicillin resistance. For example, the host range of an Asia-type plasmid was shown to include gonococci, members of the family *Enterobacteriaceae* and *Haemophilus influenzae* (Pagotto and Dillon, 2001). This occurrence is supported by the transferability of β-lactamic resistance from PPNG to bacteria belonging to different genera, as observed for *E. coli* in our study.

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