Characterization of a novel β-lactamase-producing plasmid in *Neisseria gonorrhoeae*: sequence analysis and molecular typing of host gonococci

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Objectives: To determine the complete nucleotide sequence of the novel Johannesburg β-lactamase-encoding gonococcal plasmid (pEM1) and to determine the strain relatedness of Johannesburg plasmid-containing penicillinase-producing *Neisseria gonorrhoeae* (PPNG) by molecular typing.

Methods: Eleven PPNG isolates containing the Johannesburg β-lactamase-encoding plasmid were previously identified among gonococci isolated from men with urethral discharge attending a clinic in Alexandra (Johannesburg) using a PCR assay. DNA sequence-based characterization of one such plasmid was performed to determine its relatedness to the prototype Asia plasmid. The 11 PPNG isolates containing the Johannesburg plasmid and 105 other clinical gonococci isolates were typed using *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST).

Results: Plasmid pEM1 was determined to comprise 4865 bp and to be a deletion derivative of the prototype Asia plasmid with a unique 2560 bp deletion in the non-TnA region. NG-MAST genotyping demonstrated a significant association between sequence type (ST) 502, or other closely related STs, and the Johannesburg plasmid-containing PPNG (P < 0.0001).

Conclusions: Sequencing of a novel β-lactamase-encoding plasmid (pEM1) found in PPNG isolates in Johannesburg shows it to be a deletion derivative of the prototype Asia plasmid, the deletion most likely arising as a result of DNA rearrangements. The majority of Johannesburg plasmid-containing PPNG isolates were, or were very closely related to, ST502.

Keywords: antibiotic resistance, penicillin, NG-MAST, Johannesburg

Introduction

Penicillinase-producing *Neisseria gonorrhoeae* (PPNG) isolates were first described in the UK and North America in 1976, and subsequently 1 year later in South Africa.¹⁻³ These first PPNG isolates contained TEM-1-type β-lactamase plasmids, termed ‘Africas’ and ‘Asias’, encoded by the TnA transposon (Tn2), which are responsible for the transfer and dissemination of high-level penicillin resistance among gonococci.¹⁴ These gonococcal plasmids appear to have been created by the direct acquisition of plasmids from other Gram-negative bacteria. To date, six gonococcal β-lactamase-producing plasmid types, named according to their epidemiological origin, have been described in *N. gonorrhoeae*.¹⁵ We previously reported the presence of a novel ‘Johannesburg’ plasmid in 11 clinical PPNG isolates (MICs >32 mg/L).⁶ This plasmid produces a 2350 bp amplification product in a multiplex PCR assay.⁶ In this study, we describe the DNA sequence relationship between the Johannesburg plasmid (pEM1) and the Asia (pJD4) β-lactamase-producing prototype plasmid. Additionally, the 11 PPNG isolates with Johannesburg plasmids, along with 105 clinical isolates isolated in the same time period in the same survey, were further characterized by *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST).⁷

Methods

Patients

Gonococcal isolates collected from 116 men with urethral discharge as part of national microbiological surveillance activities were used in this
A novel gonococcal β-lactamase-producing plasmid

study. The sample collection took place at Alexandra Health Centre, a public facility in Johannesburg within South Africa’s Gauteng Province, in 2008. Although data on ethnicity and sexual orientation were not routinely collected in the 2008 survey, nurses taking the survey specimens reported that almost all men presenting to this clinic were heterosexual black Africans living in Alexandra Township. Data were available on the geographical location of the female partner(s) for 106 (90%) of these men; although 38 (33%) men reported partners in South African provinces other than Gauteng and 8 (7%) men reported partners in neighbouring countries, no statistically significant geographical associations existed regarding detection of the Johannesburg plasmid in men’s urethral discharge and the residence of their female partner(s) (D. A. L.).

**Gonococcal isolates**

Frozen stock cultures of 116 gonococcal isolates, representing 42% of all gonococci cultured in the 2008 Alexandra Health Centre survey, and comprising 11 Johannesburg plasmid-containing PPNG isolates, 13 other plasmid-containing PPNG isolates (8 with Toronto plasmids and 5 with Africa plasmids) and 92 non-PPNG isolates, were freshly subcultured onto selective modified New York City agar and incubated in 5%–10% CO2 at 35°C in a humidified environment for 48 h. 6 Suspect colonies were sub-cultured onto chocolate agar to ensure purity and reconfirmed as PPNG by Gram stain, oxidase and nitrocefin tests (Mast Group Ltd, Merseyside, UK).

**Plasmid molecular analyses**

Three β-lactamase-producing control plasmids, namely pJD4 (4.4 MDa, 7426 bp Asia plasmid), pJD5 (3.2 MDa, 5598 bp Africa plasmid) and pJD7 (3.05 MDa, 5154 bp Toronto plasmid), were used to transform the 11 PPNG isolates containing the Johannesburg plasmid. Associations described. 7 Neighbour-joining cluster analysis was performed using the NG-MAST technique, as previously described. 7

NG-MAST typing

The internal fragments of two highly polymorphic antigen-encoding loci, the por and tbpB genes, were amplified from DNA extracted from 116 gonococci and sequenced using the NG-MAST technique, as previously described. 7 Neighbour-joining cluster analysis was performed using MEGA 4 (Center for Evolutionary Medicine and Informatics, The Biodisgn Institute, Tempe, AZ, USA) in order to show similarities of the por alleles in the 11 PPNG isolates containing the Johannesburg plasmid. Associations between N. gonorrhoeae isolates (PPNG and non-PPNG) and NG-MAST sequence types (STs) were assessed using the χ2 test with the level of significance set at $P=0.05$.

**Ethics approval**

All men had signed consent forms for further research to be undertaken on their specimens subject to approval, which was granted, from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg, South Africa (protocol no. M081046).

**Results and discussion**

The DNA sequences of the 11 Johannesburg plasmid-derived 2350 bp amplicons were almost all identical, with the exception that one plasmid’s amplicon possessed a single silent base substitution (guanine to adenine). The entire sequence of one of these β-lactamase-producing plasmids (pEM1) was determined to consist of 4865 bp (Figure 1). Plasmid pEM1 was aligned with the prototype Asia plasmid (pJD4) for comparison. A map was constructed to demonstrate the deletion fragment of pEM1 in relation to pJD4, pJD5 and pJD7 (Figure 1). The pJD4 plasmid is thought to have originated from an independent deletion within an ancestral Haemophilus plasmid, resulting in the loss of the left inverted repeat, the whole trnA gene and part of the trnP gene. Plasmid pEM1 is homologous to the prototype plasmid pJD4 except for a 2560 bp deletion in the non-TnA region (between coordinates 1927 and 1928 of pEM1, corresponding to coordinates 1928–4487 of pJD4) and the absence of one nucleotide in the TnA sequence. This nucleotide is also missing from the Africa and Nmès plasmids, although the exact cause of this truncation remains unclear. 9

Direct repeated sequences on β-lactamase-encoding plasmids can lead to recombination and rearrangements, and a number of such repeated sequences in gonococcal β-lactamase-producing plasmids have been described previously. 9 The 2560 bp deletion in pEM1 could possibly be linked to a larger 509 bp direct repeat on pJD4. Direct repeat DR-30, which is present as two copies (DR-30A and DR-30B) in pJD4, is absent in pEM1. 9 DR-30A has also been implicated in the 1826 bp deletion in the African plasmid (pJD5). Although the site of deletion closest to the proximal end of DR-30A in pEM1 and pJD5 is similar (coordinates 1918 in pJD5 and 1928 in pEM1; separated by 10 nucleotides, AAAATTACAA), the whole DR-30B repeat is also missing in pEM1. A smaller 92 bp direct repeat (DR-2), which is present as two identical copies in pJD4, is also proposed to be implicated in the formation of deletion derivatives of pJD4. 9 This DR-2 repeat is present as one copy in pEM1 (coordinates 502–593 of pEM1 and pJD4). The distal DR-2 repeat (coordinates 2386–2477 of pJD4) is absent in pEM1.

To establish whether the 11 gonococci containing Johannesburg plasmids were related, we determined their NG-MAST STs and compared them with those of 105 other gonococcal strains isolated and typed during the same 2008 survey. Most Johannesburg plasmid-containing PPNG (nine isolates, 82%) were either ST502 (por 251; tpb8 165) (three isolates) or closely related to ST502 (six isolates), as defined by possession of either the por 251 allele without the tpb8 165 allele (two isolates; ST4367 and ST4371) or the tpb8 165 allele without the por 251 allele (four isolates; ST4366, ST4368, ST4373 and ST4374) (Table 1). These six isolates (closely related to ST502) were all new STs and have not been described before. In comparison, none of the other 13 PPNG isolates, containing Toronto and
Africa plasmids, were ST502 or closely related to ST502 (Table 1). Only 9 (10%) of the 92 non-PPNG isolates were either ST502 or closely related to ST502 (Table 1). The most common gonococcal genomovar among non-PPNG isolates was ST217 (por158; tbpB4) and its closely related ST variants (23 isolates, 25%). The presence of the Johannesburg plasmid was significantly associated with an NG-MAST profile of either ST502 alone (P = 0.0183) or a combination of ST502 and closely related STs (P < 0.0001). Of relevance to this observation, PPNG isolates from Scotland were previously associated with ST502, but plasmid types were not determined.10 The remaining Johannesburg plasmid-containing PPNG isolates (two isolates, ST4369 and ST4370) did not contain either the por 251 allele or the tbpB 165 allele and were new ST submissions. With the exception of one isolate with a por 361 allele that was geographically linked to Alexandra, the por alleles of the remaining 10 Johannesburg plasmid-containing PPNG isolates were >98% similar (Figure 2).

In conclusion, we have identified a novel 4865 bp Johannesburg β-lactamase-producing plasmid among PPNG isolates in Johannesburg, which appears to be a deletion derivative of the prototype Asia (pJD4) plasmid. The majority of Johannesburg plasmid-containing PPNG isolates were, or were very closely related to, ST502.

**Acknowledgements**

We wish to thank Professor Jo-Anne Dillon (University of Saskatchewan, Canada) for providing the β-lactamase plasmid controls used in this study and Ms Lindy Gurnede (STI Reference Centre, NICD/NHLS) for preparing, culturing and undertaking antimicrobial susceptibility testing of the N. gonorrhoeae isolates.

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**Table 1.** Distribution of gonococcal strains possessing NG-MAST ST502, or closely related STs defined by having either the por 251 allele or the tbpB 165 allele, among 24 PPNG and 92 non-PPNG isolates

<table>
<thead>
<tr>
<th>NG-MAST data</th>
<th>Johannesburg (n = 11), n (%)</th>
<th>Toronto/Africa (n = 13), n (%)</th>
<th>Non-PPNG (n = 92), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>por 251 allele</td>
<td>5 (45)</td>
<td>0 (0)</td>
<td>5 (5)</td>
</tr>
<tr>
<td>tbpB 165 allele</td>
<td>7 (64)</td>
<td>0 (0)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>ST502</td>
<td>3 (27)</td>
<td>0 (0)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>ST502 or other STs closely related to ST502</td>
<td>9 (82)</td>
<td>0 (0)</td>
<td>9 (10)</td>
</tr>
</tbody>
</table>

Figure 1. A linear map of β-lactamase-encoding plasmids demonstrating the deletion fragment of pEM1 in relation to pJD4, pJD5 and pJD7. The JDA and JDB primer pair, selected restriction sites and Tn2 transposon components [partial trnR, bla and right inverted repeat IR(R)] are indicated to aid orientation. Coordinates are based on the complete sequence of plasmid pJD4 and spaces indicate deletion sites. GenBank accession numbers of plasmids described in this paper are as follows: Asia, U20421; Africa, U20375; Toronto, U20419; and Johannesburg, HM756641.1.

Figure 2. Neighbour-joining clustering showing similarity of por alleles in PPNG isolates containing the Johannesburg plasmid. The por alleles in bold font represent STs with the tbpB 165 allele.

Africa plasmids, were ST502 or closely related to ST502 (Table 1). Only 9 (10%) of the 92 non-PPNG isolates were either ST502 or closely related to ST502 (Table 1). The most common gonococcal genomovar among non-PPNG isolates was ST217 (por 158; tbpB 4) and its closely related ST variants (23 isolates, 25%). The presence of the Johannesburg plasmid was significantly associated with an NG-MAST profile of either ST502 alone (P = 0.0183) or a combination of ST502 and closely related STs (P < 0.0001). Of relevance to this observation, PPNG isolates from Scotland were previously associated with ST502, but plasmid types were not determined.10 The remaining Johannesburg plasmid-containing PPNG isolates (two isolates, ST4369 and ST4370) did not contain either the por 251 allele or the tbpB 165 allele and were new ST submissions. With the exception of one isolate with a por 361 allele that was geographically linked to Alexandra, the por alleles of the remaining 10
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Transparency declarations
None to declare.

References