GONORRHOEA

Global transmission of prolyliminopeptidase-negative *Neisseria gonorrhoeae* strains: implications for changes in diagnostic strategies

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Background: Species confirmation of *Neisseria gonorrhoeae* is commonly performed with biochemical kits, rely on the activity of the enzyme prolyliminopeptidase (PIP). This enzyme has previously been considered to be almost universally present in *N gonorrhoeae*. However, increasing numbers of *N gonorrhoeae* isolates lacking PIP activity have been identified.

Objectives: To investigate the possibility of a widespread transmission of one or several *N gonorrhoeae* PIP-negative strains among several countries worldwide.

Methods: PIP-negative *N gonorrhoeae* isolates cultured from 2001 to 2004 in Australia, New Zealand and Scotland were comprehensively characterised and compared with previous data from England and Denmark. All isolates were characterised by antibiotic susceptibility testing, serovar determination, pulsed-field gel electrophoresis (PFGE), opa-typing, sequencing of the entire *porB* gene and *N gonorrhoeae* multiantigen sequence typing (NG-MAST).

Results: Most (83%) of the viable Australian isolates, and all the New Zealand and Scottish isolates were assigned serovar IB-4, with similar antibiograms, nearly identical *porB1b* gene sequences, identical (ST210) or highly related (ST292, ST1259) NG-MAST STs, and indistinguishable or related PFGE fingerprints as well as opa-types. The isolates showed characteristics indistinguishable or highly related to the previously described English and Danish outbreak strain.

Conclusions: A comprehensive characterisation indicates a widespread dissemination, mainly among men who have sex with men (MSM), of indistinguishable and highly related genotypes that have evolved from a single *N gonorrhoeae* PIP-negative serovar IB-4 strain among several countries worldwide. An increased awareness of PIP-negative *N gonorrhoeae* strains is crucial and changes in the diagnostic strategies may need to be considered.

A highly sensitive and specific laboratory diagnosis of *Neisseria gonorrhoeae* is crucial for case management of gonorrhoea and disease surveillance. Despite the development of numerous molecular methods for diagnosis of *N gonorrhoeae*, bacterial culture remains the gold standard for diagnosis. This is primarily because thorough antibiotic susceptibility testing can be performed only after culture, and the antibiotic resistance of *N gonorrhoeae* is increasing worldwide.^{1–5} Subsequent species confirmation is commonly performed using commercial biochemical kits including API NH, RapID NH, Gonocheck II, Bactocard *Neisseria* and *Neisseria* Preformed Enzyme Test. These kits are used worldwide and rely entirely or in part on evidence of prolyliminopeptidase (PIP; also referred to as hydroxyproline aminopeptidase, proline aminopeptidase or proline arylamidase) activity. This preformed hydrolase was previously considered to be almost universally present in *N gonorrhoeae*.^{6–9} However, *N gonorrhoeae* strains lacking the expression of this enzyme as a result of point mutations or deletions in the encoding single-copy *pip* gene^{7 10} have increasingly been identified, potentially resulting in incorrect, doubtful or delayed identification of the correct species.^{7 9 11} Further, *N meningitidis* strains may also produce PIP.^{12–14} However, PIP expression does not seem to be essential for viability of *N gonorrhoeae*, because PIP-negative strains seem, at least in vitro, to grow normally (present study).^{6 7}

Between 2000 and 2003, highly related isolates of a PIP-negative *N gonorrhoeae* strain were transmitted, predominantly among men who have sex with men (MSM), in England (where data on sexual orientation were available, 24/30

patients were MSM, 1/30 was a bisexual man and 3/30 were women; data were unavailable for 23 other patients)¹⁰ and in Denmark (23/24 patients were men and 16 of these had known homosexual contacts).⁹ Recently, the number of PIP-negative isolates has increased in Australia,¹⁵ New Zealand⁷ and Scotland (Helen Palmer, personal communication 2006), and continues to increase in England and Wales.¹⁶

The aim of this study was to investigate the possibility of a widespread transmission of one or several *N gonorrhoeae* PIP-negative strains among several countries worldwide. PIP-negative *N gonorrhoeae* isolates from Australia, New Zealand and Scotland were comprehensively characterised and the data compared with those from previously documented clusters in England and Denmark.

MATERIALS AND METHODS

N gonorrhoeae isolates

The *N gonorrhoeae* isolates (41 PIP-negative and 3 PIP-positive isolates) were grown as described previously.¹⁷ The PIP-negative isolates were from different patients from diverse geographical areas of Australia (n = 26; cultured from 2001 to 2004), New Zealand (n = 4; cultured in 2004) and Scotland (n = 11; from a total of 34 PIP-negative isolates identified in 2004). A selection of PIP-negative isolates from the relevant laboratories in the different countries was necessary because of logistics and

Abbreviations: MSM, men who have sex with men; NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing; PFGE, pulsed-field gel electrophoresis; PIP, prolyliminopeptidase

increasing regulatory constraints on shipping of live organisms. The selection of isolates from Australia¹⁵ and New Zealand⁷ was made on the basis of PIP negativity and phenotypic characterisation. The selection of isolates from Scotland was additionally based on molecular characterisation (opa-typing) and sex of the patient. In every case, the isolates were from a wider collection and were selected to represent the maximum diversity in the PIP-negative strains from each country. The three PIP-positive strains from Denmark (IB-4 reference strain CCUG 41811),⁹ Australia (cultured in 2003) and Scotland (cultured in 2005) were included for comparison.

PIP-negative isolates from the previously identified outbreaks in Denmark (n = 26) and England (n = 3)⁹ were typed using *N gonorrhoeae* multiantigen sequence typing (NG-MAST)¹⁸ as part of this study.

Patient sexual preference and contact tracing information were sought retrospectively for all PIP-negative isolates.

Phenotypic and genotypic characterisation

Using the 44 selected isolates, serovar determination, antibiotic susceptibility testing, pulsed-field gel electrophoresis (PFGE; restriction endonucleases *SpeI* and *BglII*), opa-typing (restriction enzymes *TaqI* and *HhaII*) and full-length *porB* gene sequencing together with subsequent sequence and phylogenetic analysis were performed as described previously.⁹ In the PFGE, isolates were considered indistinguishable if no band differed in any of the fingerprints (identical PFGE type, eg, A,a); closely or possibly related if 1–6 bands differed in any of the fingerprints (variant of the same PFGE type, eg, A1,a); and different if a difference in ≥ 7 bands was documented in any of the fingerprints (eg, C,c).¹⁹ In the opa-typing, isolates were considered to have indistinguishable opa-types if they differed by ≤ 1 band. NG-MAST was performed on the 44 selected isolates and on the 29 isolates from the earlier study⁹ as described previously.²⁰

The characterisation profiles of the Danish and English IB-4 isolates from the previous study⁹ were included in the phylogenetic analysis to enable a comprehensive comparison of the whole collection of PIP-negative isolates.

RESULTS

Phenotypic characterisation

Two of the Australian isolates were not viable and, consequently, were subjected only to the genetic analyses. The majority (n = 20, 83%) of the viable PIP-negative Australian isolates, all the New Zealand isolates (n = 4) and all the Scottish isolates (n = 11) were serovar IB-4—that is, serovar identical as the English and Danish outbreak strain. The remaining four Australian isolates were identified as other serovars: IA-6, IB-1, IB-6 and IB-26, whereas the three PIP-positive isolates were identified as serovars IB-4 (Danish and Scottish isolates) and IB-3 (Australian isolate; fig 1). All the PIP-negative serovar IB-4 isolates (n = 35) were fully susceptible to cefixime (minimum inhibitory concentration (MIC) range <0.016–0.023 mg/l), ceftriaxone (MIC 0.004–0.012 mg/l), azithromycin (MIC 0.032–0.125 mg/l), ciprofloxacin (MIC <0.002–0.006 mg/l), and spectinomycin (MIC 8–12 mg/l), displayed a reduced susceptibility to ampicillin (0.19–0.38 mg/l) and did not produce β -lactamase. In comparison with the English and Danish outbreak strain,⁹ similar MIC values—that is, with differences within $\pm 1 \log_2$ for all antibiotics and isolates—were identified. The Australian PIP-negative isolates of other serovars (n = 4) and the three PIP-positive isolates showed distinct antibiograms.

Genotypic characterisation

The PIP-negative IB-4 isolates (n = 35) and non-viable isolates (n = 2) from Australia, New Zealand and Scotland comprised

eight slightly different *porB1b* gene sequences (fig 1). A comparison including the previously characterised English IB-4 isolates (n = 3) and Danish IB-4 isolates (n = 25)⁹ showed a total of nine slightly divergent *porB1b* gene sequences, which differed by 0–3 nucleotides (fig 1). Phylogenetic tree analysis of the *porB1b* sequences suggested that this only represented the ongoing evolution of the *porB1b* gene sequence of the same strain (fig 1). In all, 39 of these 65 (60%) isolates were assigned NG-MAST ST210 (*porB* allele 59 and *thpB* allele 4) and 25 were designated as ST292 (*porB* allele 28 and *thpB* allele 4), which differs from ST210 by only one nucleotide in the *porB1b* gene segment; the remaining isolate was assigned ST1259 (*porB* allele 811 and *thpB* allele 4), which differs from ST292 and ST210 by only one and two nucleotides, respectively, in the *porB1b* gene segment (fig 1). The PIP-negative IB-4 isolates showed 13 distinguishable opa-types. All the isolates differed by ≤ 5 bands from each other and they were considered to be related but not indistinguishable (fig 1). Further, among the viable PIP-negative IB-4 isolates (n = 63), 10 distinguishable PFGE fingerprints by using *BglII* and eight with *SpeI* were identified, which resulted in a total of 16 distinguishable PFGE profiles. However, in both the fingerprints, all the isolates showed differences of ≤ 6 bands from each other and thus they were considered to be closely or possibly related (fig 1).

The PIP-negative isolates of other *PorB1b* serovars (n = 3) and the PIP-positive isolates (n = 3) showed highly divergent PFGE fingerprints and opa-types (with >10 band differences) as well as NG-MAST sequence types and *porB* gene sequences, with the exception of the Scottish PIP-positive *N gonorrhoeae* ST210 strain (fig 1).

Epidemiological data

Of the 41 patients, most from Australia (25/26), New Zealand (4/4) and Scotland (9/11) were men. Most of those selected from Scotland were heterosexual (8/11, two women and six men), although of all the Scottish patients with PIP-negative isolates in 2004, 44% (n = 15) were heterosexual, 29% (n = 10) were MSM, 3% (n = 1) were bisexual and for 24% (n = 8) the sexual preference was unknown. The sexual preference of all the patients from Australia and New Zealand was not known. Contact tracing data showed that one male Australian patient (the suspected index case) had a sexual contact with a man in Belgium.

DISCUSSION

The results of the present study taken together with the data from Denmark and England⁹ indicate a widespread dissemination of indistinguishable and highly related genotypes that have evolved from a single *N gonorrhoeae* PIP-negative serovar IB-4 strain among several countries worldwide. The transmission has caused outbreaks in England between 2000 and 2002,¹⁰ and in Denmark between 2002 and 2003,⁹ and the strain, including its genetically highly related subtypes, has now also been identified in Australia (2001–4), New Zealand (2004) and Scotland (2004). Previously,⁷ the *pip* gene of the New Zealand isolates (n = 4), included in the present study, was sequenced. All these isolates possessed a deletion of thymidine at nucleotide position 110, which introduced a frame shift with major codon changes, and ultimately a truncated protein that explained the PIP negativity. Circulation between 2000 and 2003 was mainly among MSM but also identified in women (present study).^{9, 10} By 2004, at least in Scotland, a greater proportion of patients were heterosexuals. Contact tracing yielded a link between Denmark and the UK,⁹ and between Australia and Belgium; MSM in both instances. Conventional epidemiological surveillance and routine phenotyping indicate that the Danish outbreak identified in 2002–3⁹ might still be in

Figure 1 Neighbor-joining phylogenetic tree describing the evolutionary relationships of entire *porB1b* sequences (987 unambiguously aligned nucleotides) encoding the mature PorB of prolyliminopeptidase (PIP)-negative *Neisseria gonorrhoeae* isolates ($n = 68$). These isolates were cultured in Australia in 2001–4 ($n = 25$), and in New Zealand ($n = 4$) and Scotland ($n = 11$) in 2004. In addition, English and Danish IB-4 isolates from a previous study (indicated in red),⁹ were included to enable a comprehensive comparison of the whole collection of PIP-negative isolates. A PIP-positive *N gonorrhoeae* IB-4 reference strain from Denmark (CCUG 41811), one Australian PIP-positive *N gonorrhoeae* IB-3 isolated in 2004 and one Scottish PIP-positive *N gonorrhoeae* IB-4 strain isolated in 2005 that comprised the same *Neisseria gonorrhoeae* multiantigen sequence typing (NG-MAST) sequence type—that is, ST210—as most of the PIP-negative isolates were included to root the tree and for comparison (indicated in italics). Origin and characterisation profile is denoted for each isolate as follows: serovar; pulsed-field gel electrophoresis types or variants of the same type using *SpeI* (in capital letters), *BglII* (in lower case letters); *opa*-type; NG-MAST sequence type (ST). ND, not determined; *not previously identified ST.

progress.²¹ However, hopefully, the widespread transmission of this PIP-negative IB-4 strain, including its genetically highly related subtypes, is on the decline. Some phenotypic subtypes of PIP-negative strains appeared in Australia, expanded, peaked regarding their prevalence in 2003 and then declined.¹⁵ These strains followed an “epidemic curve”, and such temporal changes regarding predominance of individual strains may

usually be expected with time. Further, although the isolates of the PIP-negative IB-4 strain and the evolved subtypes in our study showed a high level of genetic conservation, they did provide evidence of some increasing diversity over time and in different countries. It would be prudent to continue monitoring this aspect of *N gonorrhoeae*, especially wherever reliance is placed on PIP reactions for species confirmation of the bacteria.

The limited differences shown by full-length *porB* gene sequencing, NG-MAST and fragment-based typing methods probably represent minor changes in the genome of some isolates of the same strain—that is, reflecting only ongoing evolution and resulting in genetically highly related subtypes, and are remarkably few during the 5 years of circulation in widely disseminated geographical areas worldwide. Previous studies have shown that some strains of *N gonorrhoeae* can persist locally or nationally over time.^{9 17 22–24} Our study shows that some strains can also persist for several years when geographically dispersed among several countries worldwide. Although *N gonorrhoeae* is competent for genetic exchange throughout its entire growth phase, the opportunity for and extent to which this occurs in vivo has not been established. Apparently, for some strains at least, there is little genetic exchange and reasonably low mutation rate resulting in stable genotypes over several years. Overall, the genetic stability and successful long-term transmission of certain strains of *N gonorrhoeae* definitively deserves further investigation, and has only recently become possible with the advent of molecular typing tools such as NG-MAST that are appropriate to such longitudinal surveillance. However, also in our study, one PIP-positive strain from Scotland was assigned ST210, but by other high-resolution molecular tests and by the antibiogram, it was shown to be unrelated. This indicates that, occasionally, isolates may be coincidentally assigned a common sequence type, and confirms the value of using supplementary typing tools when other available data indicate a possible discordance (in this case, the antibiogram and PIP status were discordant).

Other PIP-negative strains have been sporadically isolated—for example, four in the present study from Australia, one in the study from Denmark⁹ and five from England over 5 years.¹⁰ These strains have not resulted in worldwide spread, although it is interesting that the Danish isolate (serovar IA-6) from the previous study⁹ was highly related to the Australian IA-6 isolate in the present study. These isolates were both assigned ST556 (*porB* allele 90 and *tlpB* allele 122), differed by only one nucleotide in the entire *porB* gene, comprised similar MIC values for all antibiotics except ciprofloxacin and showed related PFGE profiles and opa-types. Fortunately, the PIP-negative strains identified so far do not seem to comprise any resistance to antibiotics presently recommended in most countries for the treatment of gonorrhoea.

Case management of gonorrhoea, epidemiological surveillance, and the medicolegal, social and public health implications of the diagnosis of gonorrhoea render it crucial that a rapid, highly sensitive and specific confirmation of *N gonorrhoeae* is provided. The frequent transmission of PIP-negative *N gonorrhoeae* strains in several countries has highlighted that many biochemical diagnostic kits used in conjunction with culture methods are suboptimal. To avoid errors in laboratory diagnosis of *N gonorrhoeae*, selective culture medium, conventional presumptive diagnosis and, subsequently, at least two different assays for species confirmation should be used and should be based on different principles. For instance, a combination of two sensitive and specific assays, such as carbohydrate utilisation test and the current *N gonorrhoeae* antigen detection tests (eg, Phadebact Monoclonal GC test), accurately performed and interpreted, seems highly suitable.^{11 25–28} Alternatively, molecular diagnostic systems exist, which have been reported to, so far, lack cross-reaction with other *Neisseria* species.²⁹ Also, in some geographical areas, separate rapid screening for PIP production by using proline aminopeptidase discs or other methods can be used.¹⁵ Our study also highlights the importance of regularly updating databases, which are used for interpretation of the results of biochemical assays. This is crucial to provide a correct diagnosis and is also a

responsibility of the manufacturer of the commercial diagnostic assays.

In conclusion, a comprehensive phenotypic and genotypic characterisation indicates a widespread dissemination among several countries of indistinguishable and highly related genotypes that have evolved from a single *N gonorrhoeae* PIP-negative serovar IB-4 strain, initially among MSM but now also among heterosexual networks in some countries. An increased awareness of the existence of PIP-negative *N gonorrhoeae* strains is crucial and, in several geographical areas worldwide, changes in the diagnostic strategies may need to be considered. For example, the use of at least two different assays for species confirmation, which are based on different principles, is fundamental for highly sensitive and specific laboratory diagnosis of *N gonorrhoeae*.

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