Characterization of Prolyl Iminopeptidase-Deficient *Neisseria gonorrhoeae*

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Received 22 March 2005/Returned for modification 7 May 2005/Accepted 22 May 2005

Prolyl iminopeptidase (PIP) is an enzyme produced by *Neisseria gonorrhoeae*, the detection of which is incorporated into several commercial test panels. In this report we describe two distinct mutations in the *pip* gene which account for the loss of PIP activity.

Many commercial biochemical test panels used to identify gram-negative cocci include substrates for the enzymes prolyl iminopeptidase (PIP) and δ-glutamyl aminopeptidase (GGT), which are produced by *Neisseria gonorrhoeae* and *Neisseria meningitidis*, respectively (3, 4). A number of *N. gonorrhoeae* isolates have been identified in New Zealand which lacked PIP activity, which resulted in delayed or misidentification of the organism in some cases. In this report we describe two mutations in the *pip* gene, which encodes the PIP enzyme, which were found to be responsible for the lack of PIP activity. Laboratories should be aware that not all strains *N. gonorrhoeae* possess PIP activity.

The first isolate came from a man presenting to a sexual health clinic with acute dysuria and urethral discharge. The urethral exudate was plated in the clinic onto New York City medium and a Gram stain performed, which demonstrated numerous intracellular gram-negative diplococci. He was treated empirically with a single dose of ciprofloxacin. The isolate was indeed PIP-negative. The isolate was nonreactive onKS), which detects preformed enzymes rapid utilization of carbohydrates detected with API NH (Bio-Merieux Inc.), MicroTrak direct fluorescent antibody test (Syva, Palo Alto, Calif.), GGT, and PIP. The isolate was nonreactive onall tests, and hence a negative report was issued for the presence of *N. gonorrhoeae*. The clinic nurse questioned the result, and further identification methods subsequently showed that the isolate was indeed *N. gonorrhoeae*. The clinic nurse questioned the result, and further identification methods subsequently showed that the isolate was indeed *N. gonorrhoeae*. These methods included rapid utilization of carbohydrates detected with API NH (Bio-Merieux Inc.), MicroTrak direct fluorescent antibody test (Syva, Palo Alto, Calif.), and analysis of the 16S rRNA gene sequence. PIP activity was not detected with the Gonocheck II (E-Y Laboratories Inc, San Mateo, Calif.), API NH, and RapID NH (Remel) test kits.

We then requested that other New Zealand laboratories refer PIP-negative isolates to us. Seventeen PIP-negative isolates were referred between June 2002 and August 2004. In comparison, approximately 1,200 cases of culture-confirmed gonorrhea were identified over the same period (2% PIP-negative). It is possible that not all PIP-negative isolates were referred to us.

The first cases occurred shortly after the publication of a report by Takahashi et al. describing a GGT-deficient strain of *N. meningitidis* (9). The *ggt* gene of this strain was disrupted by an insertion sequence which was readily identified by PCR and agarose gel electrophoresis. This prompted us to determine a whether a similar mechanism was responsible for the PIP-deficient *N. gonorrhoeae* we had observed.

The *pip* gene from *N. gonorrhoeae* was amplified by PCR, using primers Pro-F (5'-GTC GGG ATG TTC GGA TTG T-3') and Pro-R (5'-CAA AAC AAA AGC AGC TTC CA-3'), based on the sequence published by Albertson and Koomey (1). There was no distinguishable difference in size of the product obtained from either PIP-negative or -positive strains (data not shown).

We then sequenced the entire 933 nucleotides (nt) of the *pip* gene open reading frame, using the primers described above and the internal primers innerPro-F (5'-GTC GGG ATG TTC GGA TTG T-3') and innerPro-R (5'-CAA AAC AAA AGC AGC TTC CA-3'). The BigDye Terminator cycle sequencing ready reaction kit on an ABI Prism 3100 was used (Applied Biosystems, Foster City, CA). The sequences of the *pip* genes of 12 PIP-positive isolates produced a derived amino acid sequence of 310 amino acids (aa) as expected. Seventeen PIP-negative isolates were sequenced. Fourteen possessed a thymidine deletion mutation at nt 110, which introduced a frameshift with significant codon changes and ultimately a truncated protein of 123 aa. Three isolates, including the case isolate, contained a thymidine insertion at nt 157, resulting in a truncated protein of 55 aa. In contrast to isolates containing the deletion mutation, these three isolates all originated from one city. Noncoding base substitutions were present in 25 of 29 isolates but these 1 or 2 contiguous nucleotide changes on their own did not affect the derived size of the protein. (These substitutions occurred at nt 159 and nt 160.)

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All isolates were typed by macrorestriction digestion with XbaI and pulsed-field gel electrophoresis (PFGE). Representative strains are shown in Fig. 1. PIP-negative isolates with the deletion at nt 110 exhibited one banding pattern, and the nt 157 isolates exhibited a different pattern. Most of the PIP-positive strains tested were of a similar, but different, pattern consistent with a relatively short time period of collection. There were no unusual or unique antibiotic resistance patterns (data not shown).

This is the first published report describing in detail PIP-negative N. gonorrhoeae. We found that PIP-negative isolates possessed either of two unique mutations which produced truncated, presumably nonfunctional proteins, as deduced from the derived amino acid sequence. These two mutations also were associated with two different strains detected by PFGE using macrorestriction digestion with only one enzyme. PFGE with another restriction enzyme may demonstrate further different strains of the organism, but the PFGE results and the sequence analysis are clearly consistent with the presence of two strains of N. gonorrhoeae. It is perhaps surprising that we did not find any previously published reports describing PIP-negative isolates. The well-known genetic variability of N. gonorrhoeae (10) and other Neisseria spp. (6) is demonstrated by the presence of noncoding mutations in the pip genes of most of the isolates tested. PIP does not appear to be essential for viability because naturally occurring N. gonorrhoeae that we have observed and a cloned strain lacking PIP grew normally in vitro (1). In particular, it is surprising to find two different mutations at the time of discovering what appears to be a recent emergence of PIP-negative strains. The discovery of two unique mutations causing the PIP-negative phenotype at the same time is most interesting and may facilitate further research into the evolution and epidemiology of N. gonorrhoeae.

The combination of PIP, GGT, and butyrate esterase has until recently been considered a reliable test panel for the identification of pathogenic Neisseria spp. (3, 8). PIP-negative N. gonorrhoeae isolates have now been identified in New Zealand, Australia, the United Kingdom, and Denmark (J. Tappendall, M. Unemo, and C. Ison, personal communication). Their emergence suggests that panels which rely entirely on the detection of preformed enzymes should not be completely relied upon. Moreover, some strains of N. gonorrhoeae are not detected by direct immunofluorescence (2, 7). The medicolegal and public health implications of the diagnosis of gonorrhea make it essential that diagnostic laboratories are aware of potential shortcomings of routine biochemical test panels. The case described in this report illustrates the importance of reconciling Gram stain results of genital specimens with the results of bacterial culture. The diagnosis of gonorrhea may not have been made if the clinic nurse had not challenged the laboratory results, which relied on PIP detection for identifying suspicious colonies. These observations reinforce the need to use two or more identification methods for the identification of gram-negative cocci. The Manual of Clinical Microbiology recommends that three levels of certainty be used when a laboratory identifies and reports gonorrhea: suggestive, presumptive, and definitive. Combinations of carbohydrate utilization, enzyme detection, and serological or nucleic acid-based methods should be used to definitively identify gram-negative, oxidase-positive cocci (5).

REFERENCES


FIG. 1. PFGE after restriction with XbaI of representative gonococcal isolates. Lanes 1 to 3, PIP-negative strains with nt 157 insertion; lane 4, PIP-positive strain; lanes 5 to 7, PIP-negative strains with nt 110 deletion; lanes 8 to 10, PIP-positive strains.