Evaluation of commercial kits for the identification of Neisseria gonorrhoeae

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Eight identification methods were evaluated against 100 isolates of Neisseria gonorrhoeae and 21 non-gonococcal Neisseria strains. The methods examined included four commercial biochemical kits, API NH, RapID NH, Gonocheck II and Neisseria Preformed Enzyme Test (PET), three immunological kits, Phadebact Monoclonal GC test, GonoGen II and MicroTrak, and one in-house carbohydrate-utilization method, cystine trypticase agar (CTA) sugars. The percentage of isolates unambiguously identified as N. gonorrhoeae by each of the methods was as follows: API NH, 66 %; RapID NH, 64 %; GonoChek II, 66 %; Neisseria PET, 66 %; Phadebact Monoclonal GC OMNI test, 99 %; GonoGen II, 100 %; MicroTrak, 100 %; and CTA sugars, 96 %. The low sensitivity of the biochemical kits for the identification of N. gonorrhoeae was due to a lack of the enzyme proline iminopeptidase (Pip) in 34 % of the isolates examined. All the biochemical kits utilized the presence of this enzyme as a marker for N. gonorrhoeae. The Phadebact Monoclonal GC kit, GonoGen II, MicroTrak, CTA sugars and the API NH kit all exhibited high specificity, but non-gonococcal Neisseria strains were misidentified as N. gonorrhoeae using RapID NH (two strains), Gonocheck II (11 strains) and Neisseria PET (11 strains). Whilst the isolates examined in this study may not be truly representative, they do indicate that N. gonorrhoeae isolates lacking the enzyme Pip can give anomalous results when using commercially available biochemical tests and that some non-pathogenic Neisseria species are still being misidentified using some biochemical kits. This further reinforces the recommendation that any dubious biochemical result should be confirmed with an immunological test.

INTRODUCTION

The rapid and accurate identification of Neisseria gonorrhoeae from culture is essential for both medical and legal reasons. The traditional method of identification of this pathogen relies on its unique pattern of carbohydrate utilization in cystine trypticase agar (CTA) sugars (Kellogg & Turner, 1973). N. gonorrhoeae can be differentiated from other oxidase-positive Gram-negative diplococci as it produces acid from glucose alone, whereas other members of the Neisseria genus such as Neisseria lactamica and Neisseria meningitidis produce acid from both glucose and maltose. Whilst CTA sugars are a cost-effective method of identification they do require a heavy inoculum and 24 h incubation, and can be difficult to interpret. Additionally, glucose-negative N. gonorrhoeae isolates have been reported, raising concerns about false-negative results (Knapp, 1988).

For a more rapid identification of this pathogen there are a variety of commercial test kits available. The three most widely used immunological kits are the MicroTrak N. gonorrhoeae culture confirmation test (an immunofluorescence test), the Phadebact Monoclonal GC test (a coagglutination test) and GonoGen II (a membrane immunoassay), all of which employ mAbs raised to specific epitopes on the two types of the major outer-membrane protein, PIA and PIB (Tam et al., 1982). Whilst the specificity of these kits has been shown to be very high, N. gonorrhoeae strains giving negative results have been reported (Dillon et al., 1988).

A range of commercially available biochemical kits are also widely used, including the Neisseria Preformed Enzyme Test (PET), Gonocheck II, RapID NH and API NH. The Neisseria PET and Gonocheck II are both single-use tubes containing chromogenic substrates that detect the presence of three preformed enzymes, each of which is indicative of a pathogenic Neisseria species, namely N. lactamica, N. meningitidis and N. gonorrhoeae. The API NH and RapID NH kits employ a battery of tests, combining carbohydrate utilization and preformed enzymes. However, biochemical tests that detect the presence of preformed enzymes should be interpreted with caution because proline iminopeptidase (Pip)-negative N. gonorrhoeae have been reported (HPA, 2001).

As taxonomic differences between members of the Neisseria genus remain small, the identification of this pathogen can be problematic and as such no single method is currently
recommended for a definitive identification. This study aimed to evaluate the performance of the following eight detection methods: the Neisseria PET, Gonocheck II, CTA sugars, API NH, RapID NH, the Phadebact Monoclonal GC OMNI test, GonoGen II and the MicroTrak kit.

**METHODS**

**Bacterial strains and growth conditions.** All isolates tested in this study were referred to the Sexually Transmitted Bacteria Reference Laboratory (STBRL) during 2003 from laboratories throughout England and Wales because of problems with identification. Isolates were initially identified using CTA sugars, the Phadebact Monoclonal GC test and where necessary the Gonocheck II test. A total of 121 isolates of Neisseria species were examined in this study, including N. gonorrhoeae (100 isolates) and 21 non-gonococcal Neisseria species as controls. All isolates were recovered from glycerol stocks onto chocolate agar and the eight identification tests were carried out where possible on the same day using growth from the second subculture. Gram stain and oxidase tests were performed on all cultures to ensure purity. In instances where isolates failed to give a consistent result the test was repeated.

**Identification methods.** All tests were carried out where possible according to manufacturer’s instructions and are detailed briefly below.

**MicroTrak N. gonorrhoeae culture confirmation test (Trinity Biotech).** Four or five colonies were emulsified in distilled water on a microscope slide. Following fixing, the smear was covered with 30 μl of MicroTrak reagent and incubated at 37 °C. Slides were washed thoroughly and examined for the presence of fluorescent green diplococci under a ×100 objective.

**GonoGen II test (Key Scientific).** A McFarlands 1 suspension of test organism was made up in the solubilizing buffer provided, and then mixed with mAbs linked to a metal sol carrier. Following a 10 min incubation the suspension was passed through a membrane filtration device, and a clear red dot was recorded as a positive N. gonorrhoeae result.

**Phadebact monoclonal GC test (Boule).** A light suspension of the test organism prepared in 0.9 % saline was boiled for 10 min before being mixed with one drop of the two pools of murine mAbs. Following gentle agitation for 2 min, visible agglutination was recorded as a positive N. gonorrhoeae result.

**Cystine trypticase agar (CTA) carbohydrate test.** A heavy inoculation of test organism was transferred onto spots of CTA agar impregnated with glucose, maltose and sucrose (final concentration 1 %). Carbohydrate degradation patterns were recorded following 24 h incubation.

**Gonocheck II (E-Y Laboratories).** The chromogenic substrates were rehydrated with five drops of PBS into which five to seven colonies were emulsified. Tubes were incubated at 37 °C for 30 min. Following incubation a colour change to either blue (hydrolysis of 5-bromo-4-chloro-3-indolyl β-D-galactoside) or yellow (hydrolysis of γ-glutamyl-p-nitroanilide) was recorded. In the absence of a colour change the primary lid was removed, the secondary lid replaced and the tube inverted. If a red colour (hydrolysis of L-proline naphthylamide) or blue/purple was recorded.

**RapID NH (Remel).** A McFarlands 3 suspension of test organism was made up in 1 ml of inoculation fluid. The entire suspension was transferred into the chamber and inoculated into the test cavities, which included two carbohydrate cavities (glucose and sucrose) and eleven biochemical wells [proline p-nitroanilide, γ-glutamyl-p-nitroanilide (5-γ-glutamyl aminopeptidase), α-nitrophenyl β-D-galactoside (β-D-galactosidase), fatty acid ester, resazurin (hydrolysis of resazurin), β-nitrophenyl phosphate (phosphatase activity), ornithine (ornithine decarboxylation), urea (urea hydrolysis), nitrite, nitrate and trypthphan (utilization of the enzyme tryptophan)]. Following 4 h incubation at 35 °C the profile was recorded.

**API NH (bioMérieux).** A heavy suspension (McFarlands 4) of test organism was prepared in 0.9 % saline. The suspension was inoculated into the 13 test wells, which included a penicillinase well, four carbohydrate utilization wells (glucose, fructose, maltose and sucrose) and eight biochemical wells [β-glucuronidase, 4-nitrophenyl-β-D-glucopyranoside, alkaline phosphatase, 4-nitrophenyl-phosphatase (phosphatase activity), ornithine (ornithine decarboxylation), urea (urea hydrolysis), nitrite, nitrate and tryptophan (utilization of the enzyme tryptophan)]. Following 24 h incubation at 35 °C the profile was recorded.

**RESULTS**

**Identification of N. gonorrhoeae isolates**

The immunological kits were found to be more sensitive for the identification of N. gonorrhoeae than the biochemical kits (Table 1), with the Microtrak, Phadebact Monoclonal GC OMNI test and GonoGen II kits correctly identifying 100 %, 99 % and 100 % of N. gonorrhoeae isolates, respectively. Initially it was thought that the GonoGen II kit was the least sensitive of the mAb kits, with 6 % of isolates examined being negative twice when using this test. However, using an extended extraction method, which involved boiling (as detailed in kit instructions), these six strains were positively identified.

The CTA sugar technique produced the characteristic glucose-positive, maltose-negative, sucrose-negative (G−M−S−) pattern of carbohydrate degradation in 96 % of the N. gonorrhoeae isolates, with 17 % of isolates examined being negative on primary testing and requiring retesting on a fresh culture in order to obtain a positive reaction. However, 4 % of isolates failed to be identified by CTA sugars even on repeat testing, despite being shown to be glucose-positive using the API NH and RapID NH test systems.

The four commercially available biochemical kits Neisseria PET, Gonocheck II, API NH and RapID NH all showed a greatly reduced sensitivity, generating unambiguous N. gonorrhoeae identifications in 66 %, 66 %, 66 % and 64 % of isolates, respectively. The low sensitivity of the four
biochemical kits was due to the lack of the Pip enzyme in 34% of the *N. gonorrhoeae* isolates. Complete concordance was observed between the RapID NH, API NH and Gonochek II as to which *N. gonorrhoeae* isolates lacked the Pip enzyme and which isolates possessed it. However, the Neisseria PET, which detects the presence of the same enzyme, was found to be more variable. Two *N. gonorrhoeae* isolates repeatedly produced results using the Neisseria PET that were inconsistent with the other biochemical tests; one isolate shown to be Pip positive using the RapID NH, API NH and Gonochek II kits was negative using the Neisseria PET, and another showed the reverse situation. It is also noteworthy that 21 isolates required repeat testing with the Neisseria PET in order to generate a comparable result.

There was also a discrepancy between the number of *N. gonorrhoeae* isolates being unambiguously identified using API NH as compared with the RapID NH kit (Table 1). This was due to the API NH kit being more sensitive at detecting glucose degradation than the RapID NH kit. The predicted identification of the Pip-negative *N. gonorrhoeae* isolates using the biochemical kits varied considerably. The API NH kit identified these isolates as 'doubtful *N. gonorrhoeae*', followed by a recommendation to confirm identification using an alternative method. The Gonochek II and the Neisseria PET misidentified these isolates as 'presumptive *Moraxella catarrhalis*', although in the kit instructions of both tests the phenomena of Pip-negative *N. gonorrhoeae* strains is mentioned. The ERIC software provided with the RapID NH kits predicted the identification of these isolates as 'presumptive *Kingella kingae*', with no indication in the manufacturer’s instructions of the possibility of *N. gonorrhoeae*.

**Misidentification of Neisseria strains as N. gonorrhoeae**

The specificity of the immunological kits was high, with none of the 21 non-gonococcal control strains examined producing a false-positive result with the MicroTrak, Phadebact Monoclonal GC OMNI test or GonoGen II kits (Table 2). The biochemical tests investigated, however, produced more variable results. When examined using the CTA sugar method all 21 strains exhibited non-gonococcal patterns of carbohydrate degradation (although one isolate required repeat testing).

The API test correctly identified all 21 non-gonococcal strains (albeit only to genus level in many cases), with none of the strains being misidentified as *N. gonorrhoeae*. The RapID NH test misidentified two of the 21 strains examined as gonococci. The misidentification of these two strains can be attributed to the lack of a maltose well and a lack of sensitivity in detecting sucrose degradation. The incorporation of both nitrite and nitrate wells within the RapID NH tests enabled species-level identification of both *Neisseria cinerea* and *Neisseria mucosa*.

The specificity of both the Gonochek II and the Neisseria PET was low. Of the total 21 isolates examined, eleven strains were misidentified as *N. gonorrhoeae* using the Gonochek II and Neisseria PET. Interestingly one of these was actually a

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<th>Table 1. A comparison of eight different test systems for the identification of <em>N. gonorrhoeae</em></th>
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<td><strong>Test</strong></td>
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*Six isolates were found to be positive using the GonoGen II kit when the extended extraction method was performed.*
N. lactamica strain that had been shown to be β-galactosidase positive using both the API NH test and the RapID NH test kits.

DISCUSSION

The taxonomy of the Neisseria genus is complex. Although the clinically important members N. gonorrhoeae, N. lactamica and N. meningitidis are relatively straightforward to identify, the differences between many of the non-pathogenic strains remain small and the speciation of these strains within a diagnostic setting is not always possible. Ultimately the accurate identification of N. gonorrhoeae can be problematic but is critical for successful patient management. Whilst the identification of non-gonococcal Neisseria strains to species level is generally not required, the mis-identification of these strains as N. gonorrhoeae can have serious legal and social consequences.

This study evaluated the performance of eight different methods for their ability to correctly identify 100 referred N. gonorrhoeae isolates. The three immunological tests investigated were found to have both higher sensitivities and specificities than the biochemical tests, with the MicroTrak, Phadebact Monoclonal GC OMNI test and GonoGen II kits correctly identifying 100 %, 99 % and 100 % of isolates, respectively. This is in good agreement with previous studies, where the sensitivities of both MicroTrak and the Phadebact Monoclonal GC OMNI test were high (Boehm et al., 1990; Laughon et al., 1987; Welch & Cartwright, 1988). Although marginally more sensitive, the MicroTrak test was found to be more labour intensive than the Phadebact Monoclonal GC OMNI test and required access to a fluorescence microscope.

Technical problems were encountered when using the GonoGen II kit, with six isolates requiring repeat testing in order to generate positive N. gonorrhoeae identification. In this instance the detergent-based extraction buffer did not adequately solubilize six of the N. gonorrhoeae isolates examined, and consequently a reaction with the labelled antibody did not occur and a false-negative result was repeatedly generated. In order to overcome this problem the manufacturer of this test included an additional method for those N. gonorrhoeae isolates that form ‘mucoid colonies’. Although the isolates in this study did not appear overtly ‘mucoid’, when this extended extraction method was performed positive N. gonorrhoeae identifications were produced for all six isolates examined. However, the reality of repeating all negative strains within a clinical microbiology setting is questionable.

The CTA sugar technique was found to lack sensitivity, with only 79 % of isolates examined producing an unambiguous identification of N. gonorrhoeae on the first attempt, despite all isolates examined in the study being glucose positive. Additionally one strain of N. mucosa was initially misidentified because the method was not sensitive enough to detect both maltose and sucrose fermentation. Therefore this strain would have been misidentified as N. gonorrhoeae if repeat testing had not been performed. Clearly the lack of sensitivity of CTA sugars in detecting carbohydrate degradation compromises the integrity of the test both in terms of sensitivity and specificity. The lack of sensitivity and the prolonged incubation time required for this test makes it undesirable for the identification of clinical isolates, where timely identifications are essential for optimum patient care.

The four commercially available biochemical kits Neisseria PET, Gonocheck II, API NH and RapID NH all showed a greatly reduced sensitivity, generating unambiguous N. gonorrhoeae identifications in 66 %, 66 %, 66 % and 64 % of isolates, respectively. The low sensitivity of the four biochemical kits can be explained by 34 % of the isolates examined lacking the enzyme Pip. The excellent agreement observed between API NH, RapID NH and Gonocheck II as to which isolates possessed or lacked the Pip enzyme in gonococci was reassuring; however, the performance of the Neisseria PET was poor. When using the Neisseria PET 21 isolates required repeat testing in order to generate a result consistent with the remaining biochemical kits and two isolates even with repeat testing failed to generate a result consistent with the other biochemical kits. The poor performance of the Neisseria PET may partly be explained by difficulty in detecting a colour change. When performing this test, in the absence of an initial colour change, which indicates either N. meningitidis or N. lactamica, a drop of PET reagent is added and the presence of an orange or red colour indicates the presence of Pip and a presumptive N. gonorrhoeae identification is generated. However, following the addition of the PET reagent the test is yellow in colour and the observation of a colour change from a yellow to orange colour over a 2 min period can be difficult to determine, which may account for the lack of agreement with the other tests.

The API NH system was found to be more robust than the RapID NH system, generating unambiguous N. gonorrhoeae profiles in 66 % as compared to 64 % of the isolates examined. Additionally eight isolates had to be repeat tested when using the RapID NH test in order to generate the correct N. gonorrhoeae profile, compared with only two isolates when using the API NH test. The difference in sensitivity between these two tests may be explained by the API NH test requiring a denser inoculum than the RapID NH kit. Additionally the criteria for judging a positive reaction are more flexible in the API NH kit, in which either an orange or a yellow colour in the carbohydrate wells can be scored as a positive reaction. This is in contrast to the RapID NH kit, in which according to the manufacturer’s instructions an orange well must be scored as negative and only a distinct yellow, gold or yellow-orange colour is positive. In addition to greater sensitivity and specificity the API NH kit also has the advantage over the RapID NH kit of having a shorter incubation time (2 h as compared to 4 h).

The failure to adequately describe Pip-negative N. gonorrhoeae in the majority of commercially available biochemical kits is unsatisfactory. The API NH kit is the only commercial
kit to correctly identify these isolates as ‘doubtful \textit{N. gonorrhoeae}'. Although the Gonocheck II and the \textit{Neisseria} PET do describe the possibility of Pip-negative \textit{N. gonorrhoeae}, the final identification of isolates that fail to generate a colour change is still referred to as ‘presumptive \textit{Moraxella catarrhalis}'. The \textit{ERIC} software provided with the RapID NH kits predicts the identification of these isolates as ‘presumptive \textit{Kingella kingae}', with no indication in the kit instructions of the possibility of \textit{N. gonorrhoeae}. This highlights the responsibility of manufacturers to regularly update their databases to ensure that significant pathogens such as \textit{N. gonorrhoeae} are correctly identified.

In the current study, a small number of non-gonococci were included as controls and the immunological tests examined displayed a high degree of specificity, with none of the non-gonococcal isolates examined cross-reacting to produce false-positive results. The specificity of the commercial biochemical tests, however, was found to be lower, with the API NH test being the only kit to correctly identify all non-pathogenic \textit{Neisseria} species, albeit only to genus level. Two non-pathogenic \textit{Neisseria} species were misidentified as \textit{N. gonorrhoeae} using the RapID NH system. This has also been reported in another study, where one strain of \textit{Neisseria subflava} was erroneously identified as \textit{N. gonorrhoeae} (Robinson & Oberhofer, 1983). Gonocheck II and \textit{Neisseria} PET misidentified 11 of the non-gonococcal strains as presumptive \textit{N. gonorrhoeae}. The misidentification of \textit{Neisseria} species as \textit{N. gonorrhoeae} using rapid tests such as the \textit{Neisseria} PET has also been widely reported (Dillon et al., 1988; Boyce & Mitchell, 1985). This is particularly significant when Pip-positive non-pathogenic \textit{Neisseria} species have been reported to grow well on selective media (Knapp et al., 1984).

Whilst previous studies have highlighted that non-pathogenic \textit{Neisseria} species can be misidentified as \textit{N. gonorrhoeae} when using commercially available biochemical kits, this is the first study to determine that these kits may also give false-negative or ambiguous results when used to identify some gonococcal isolates. Although the isolates examined in this study are not truly representative as they were referred because of identification problems, currently the prevalence of Pip-negative \textit{N. gonorrhoeae} within the UK is unknown. In order to establish the possible extent of this problem and indeed to determine the reliability of tests that rely solely on the presence or absence of preformed enzymes in clinical microbiology settings, prevalence data are essential. Such tests are currently used widely, particularly for the differentiation of Gram-negative diplococci isolated from sites, such as the pharynx, where \textit{N. meningitidis} is a common commensal. It is important that users of these tests are informed as to the presence of Pip-negative \textit{N. gonorrhoeae} isolates and encouraged to investigate all Gram-negative diplococci that are either \(\gamma\)-glutamyl transferase- or \(\beta\)-galactosidase-negative irrespective of the result of the Pip reaction, ideally with an immunological test.

The results of this study have shown that \textit{N. gonorrhoeae} isolates lacking the enzyme Pip can give anomalous results when using commercially available biochemical tests and that some non-pathogenic \textit{Neisseria} species are still being misidentified using some biochemical kits. This further reinforces the current recommendation that all clinical isolates of \textit{N. gonorrhoeae} be identified using two methods preferably biochemical and immunological.

\section*{Acknowledgements}

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\section*{References}


