Characterisation of *Branhamella catarrhalis* and differentiation from *Neisseria* species in a diagnostic laboratory

F AHMAD,* H YOUNG,† D T MCLEOD,‡ M J CROUGHAN,* M A CALDER*

From the Departments of Bacteriology, *City Hospital, Edinburgh, the †University Medical School, University of Edinburgh, and the ‡Chest Unit, City Hospital, Edinburgh, Scotland

SUMMARY To distinguish *Branhamella catarrhalis* from *Neisseria* species a study of 140 strains was made on simple laboratory media, with particular reference to deoxyribonuclease (DNase) production, superoxol reaction, and growth characteristics. All 97 clinical isolates of *B catarrhalis* (58 of which were β -lactamase positive) and eight strains of *B catarrhalis* from the National Collection of Type Cultures were DNase positive and superoxol positive. None grew on modified New York City medium, modified Thayer Martin medium, MacConkey agar, crystal violet blood agar, nor under anaerobic conditions. Of the 16 different non-pathogenic *Neisseria* species tested, all were DNase negative, eight (50%) were superoxol reaction negative, and 13 (81%) grew on crystal violet blood agar.

Using simple laboratory media, DNase, and superoxol tests, it was possible to identify *B catarrhalis* and to distinguish it from pathogenic and non-pathogenic *Neisseria* species.

The unresolved problem of classification and differentiation of the family Neisseriaceae has a long history. In 1906 Von Lingelsheim¹ first described the identification system for Gram negative cocci using microscopic and macroscopic features and carbohydrate reactions. The same year Kutscher² and subsequently Arkwright in 1907³ extended the identification system of *Neisseria* spp and discussed their possible role in clinical disease. By 1909 Elser and Huntoon⁴ had overcome some of the difficulties in the classification of *Neisseria* spp by comparing their cultural and biochemical features. In 1921 Gordon⁵ reclassified the known *Neisseria* spp on the basis of carbohydrate fermentation reactions. By the late 1960's it was clear that *Neisseria catarrhalis* was quite different from the other *Neisseria* spp in its biochemical reactions and fatty acid and DNA base composition. In 1970 Catlin⁶ proposed a new genus, *Branhamella*, for *N catarrhalis* on the basis of biochemical and DNA base differences. Members of the genus *Neisseria* and the genus *Branhamella* are now classified in the family Neisseriaceae, along with the genera *Kingella*, *Moraxella*, and *Acinetobacter*.⁷ The

distinction between pathogenic and non-pathogenic strains of *Neisseria* spp and *B catarrhalis* has become important because of the changed perception of their pathogenicity. It is well known that pathogenic *Neisseria* spp are not restricted to their classical anatomical sites: pharyngeal infection with *N gonorrhoeae* and anogenital tract infection with *N meningitidis* are increasingly being recognised. Arko *et al*⁸ found that *B catarrhalis* and *N meningitidis* were the two organisms most often confused with *N gonorrhoeae*. Knapp *et al*⁹ discussed the difficulties in differentiating *B catarrhalis* and *N cinerea*. Conventional methods using carbohydrate utilisation tests require careful control of physical and biochemical conditions. The reports by Catlin in 1961¹⁰ and 1970⁶ and subsequently by others¹¹ that *B catarrhalis* was unique among Neisseriaceae in producing a DNase, and more recently, work by Young *et al*¹² on the superoxol test to differentiate gonococcal from non-gonococcal species, could offer useful means of differentiating *Neisseria* spp and *B catarrhalis*. We therefore examined a large number of *B catarrhalis* strains and various non-pathogenic *Neisseria* spp to compare and contrast DNase production, superoxol reactions, and growth characteristics on different media.

Material and methods

A total of 140 strains of *B. catarrhalis* and *Neisseria* spp were studied. Clinical isolates were used as test strains and known reference strains were used for control and comparison. The clinical isolates comprised 97 strains of *B. catarrhalis* and 13 strains of *N. perflava*, isolated from sputum specimens (bacteriology laboratory, City Hospital, Edinburgh) from patients with bronchopulmonary infection, and considered to be clinically important, and eight strains of *N. lactamica* isolated from throat cultures (department of bacteriology, University of Edinburgh). The following bacteria used as controls were obtained from the National Collection of Type Cultures (NCTC), Colindale, London: eight strains of *B. catarrhalis* (all are non- β -lactamase producers) (NCTC numbers 3622, 3623, 3625, 4103, 11015, 11016, 11017, and 11020), and the following 14 recognised strains of *Neisseria* spp: *N. caviae* (10293), *N. mucosa* var *heidelbergensis* (10777), *N. species* (11049), *N. animalis* (10212), *N. elongata* subspecies *glycolytica* (11050), *N. canis* (10296), *N. pharyngis* (4590), *N. mucosa* var *mucosa* (10774), *N. cuniculi* (10297), *N. denitrificans* (10295), *N. flavescens* (8263), *N. ovis* (11018), *N. elongata* (10660), *N. cinerea* (10294).

All the clinical isolates of *B. catarrhalis*, *N. perflava*, and *N. lactamica* were primarily identified by using previously described laboratory criteria,¹³ comprising morphology, oxidase and catalase reactions, and rapid carbohydrate utilisation tests (RCUT).¹⁴

All the test strains and NCTC strains were examined for DNase and superoxol activity. A gonococcus coagulination test (Phadebact) was performed to determine possible cross reactivity with the gonococcal antigen.

DNASE TEST¹¹

Oxoid DNase agar (Code CM321, Oxoid Ltd, Basingstoke, England) was used. Freshly prepared plates were divided into four to six sections. Each section of the plate was inoculated heavily with the strain to be tested and spread to form a circle of about 6 mm in diameter. The Oxford strain of *Staphylococcus aureus* (NCTC 6571) was used as a positive control. A known strain of *S. epidermidis* served as a negative control. After 24 hours of incubation the plates were flooded with 0.1 M hydrochloric acid—the appearance of a clear zone around the inoculum in three to four minutes was taken as a “positive” test. A known strain of *B. catarrhalis* was also used as an additional positive control because the test gives a much weaker reaction with *B. catarrhalis* than it does with *S. aureus*.

SUPEROXOL TEST^{1 2}

A few colonies of the culture to be tested were picked

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from the primary isolation plate with a plastic loop and emulsified in a drop of 30% H₂O₂ placed on a clean glass slide. A known strain of *N. gonorrhoeae* and of *N. perflava* were similarly tested as positive and negative controls, respectively. A positive superoxol test was defined as abundant production of bubbles within two to three seconds. Weak or delayed bubbling after three seconds indicated a negative reaction.

All isolates were tested by the Phadebact Gonococcus test (Phadebact Diagnostics, AB Sweden) according to the manufacturer's instructions. Beta lactamase production was detected using chromogenic cephalosporin.¹⁵

CULTURE CHARACTERISTICS

To study the effect of various commonly used media on the growth characteristics of *B. catarrhalis* and *Neisseria* spp an inoculum containing 10⁴–10⁵ colony forming units was applied to the surface of plates containing the following media:

1 Modified New York City medium (MNYC)¹⁶ containing the selective agents trimethoprim lactate 6.5 mg/l, amphotericin 1 mg/l, lincomycin 1 mg/l, and colistin 4 mg/l.

2 Modified Thayer Martin medium (MTM) containing vancomycin 3 mg/l, colistin 7.5 mg/l, trimethoprim 5 mg/l and nystatin 12500 U/l (Difco Laboratories).

3 MacConkey agar (Oxoid code No CM7).

4 Crystal violet blood agar, bi-layer plate: lower layer, columbia agar (Oxoid code CM331); upper layer, Columbia blood agar with crystal violet at final concentration 0.0002%.

5 Nutrient agar (Oxoid code CM3).

6 Bacitracin agar—heated blood agar containing 1 × 10⁴ U/l of bacitracin.

7 Neomycin sulphate blood agar—Columbia agar base + 7% defibrinated horse blood + 5 mg/l of menadione and 1 ml/l of 7000 mg/l neomycin sulphate.

Nutrient agar plates were incubated at 22°C and 37°C aerobically and 37°C anaerobically. A nutrient agar plate seeded with a known culture of *Pseudomonas aeruginosa* was included in each anaerobic jar as a control. All other plates were incubated at 37°C in air and 8% carbon dioxide. Cultures were read after overnight incubation and growth was recorded as +, ++, +++, representing light, moderate, or heavy growth.

Results

DNase activity was exhibited by all 97 clinical isolates of *B. catarrhalis* and by the eight NCTC *B. catarrhalis*

Table Characterisation of *B. catarrhalis* and *Neisseria* spp

		Characterisation tests			Growth on					
Organisms	(No of isolates)	DNase	Superoxol	Phadebact	MNYC and MTM media agar	MacConkey	Crystal violet agar	Nutrient agar 22°C	Nutrient agar 37°C	Nutrient agar 37°C (anaerobic)
<i>Branhamella catarrhalis</i>										
Clinical isolates	97									
β-lactamase positive	58	+	+	—	—	—	—	++	+++	—
β-lactamase negative	39	+	+	—	—	—	—	++	+++	—
NCTC strains	8	+	+	—	—	—	—	++	+++	—
<i>Neisseria perflava</i>	13	—	—	—	—	++	++	+++	+++	++
<i>Neisseria lactamica</i>	8	—	—	25% +	+++	—	++	—	++	+
<i>Neisseria caviae</i> (NCTC 10293)		—	+	—	—	—	+	—	+	—
<i>Neisseria mucosa</i> var <i>heidelbergensis</i> (NCTC 10777)		—	—	—	—	+	+	—	+	+
<i>Neisseria species</i> (NCTC 11049)		—	—	—	—	—	—	+	+	+
<i>Neisseria animalis</i> (NCTC 10212)		—	+	—	—	—	+	—	+	+
<i>Neisseria elongata</i> subspecies <i>glycolytica</i> (NCTC 11050)		—	+	+	—	—	+	+	+	—
<i>Neisseria canis</i> (NCTC 10296)		—	+	—	—	—	+	+	+	+
<i>Neisseria pharyngis</i> (NCTC 4590)		—	—	—	—	—	—	+	+	+
<i>Neisseria mucosa</i> var <i>mucosa</i> (NCTC 10774)		—	—	—	+	+	+	—	+	+
<i>Neisseria cuniculi</i> (NCTC 10297)		—	+	—	+	+	+	—	+	—
<i>Neisseria denitrificans</i> (NCTC 10295)		—	+	—	+	+	+	+	+	+
<i>Neisseria flavescens</i> (NCTC 8263)		—	+	—	—	—	+	—	+	+
<i>Neisseria ovis</i> (NCTC 11018)		—	+	—	—	—	+	—	+	+
<i>Neisseria elongata</i> (NCTC 10660)		—	—	—	—	—	—	—	+	+
<i>Neisseria cinerea</i> (NCTC 10294)		—	—	—	+	—	+	—	+	+

strains; it was not detected in the clinical isolates of *N. perflava* nor *N. lactamica* nor in the 14 NCTC strains of *Neisseria* spp. All 105 (100%) strains of *B. catarrhalis* were positive in the superoxol test as were some uncommon non-pathogenic *Neisseria* spp—namely, *N. caviae*, *N. animalis*, *N. elongata* subspecies *glycolytica*, *N. canis*, *N. cuniculi*, *N. denitrificans*, *N. flavescens* and *N. ovis* A334/72. All clinical isolates of *N. perflava* and of *N. lactamica* and the following NCTC strains—*N. mucosa* var *heidelbergensis*, *N. mucosa* var *mucosa*, *N. elongata* and *N. cinerea* were negative in the superoxol test.

N. elongata subspecies *glycolytica* and two of the eight (25%) isolates of *N. lactamica* were positive in the Phadebact Gonococcus test. Beta lactamase production was detected in 58 (60%) of the clinical isolates of *B. catarrhalis*. None of the remaining strains of *B. catarrhalis* produced β-lactamase.

GROWTH CHARACTERISTICS

None of the *B. catarrhalis* strains was able to grow on MNYC or MTM media, whereas *N. lactamica*, *N. mucosa* var *mucosa*, *N. cuniculi*, *N. denitrificans* and *N. cinerea* did grow on these media (table). All strains of *B. catarrhalis* failed to grow on MacConkey and crystal violet blood agar media, whereas *N. perflava* grew on both, and *N. lactamica* grew on the crystal violet blood agar but not on MacConkey agar. The growth of the remaining reference (NCTC) strains of *Neisseria* spp was variable on MacConkey agar, although 13 (81%) produced growth on crystal violet blood agar.

All the strains of *B. catarrhalis* and *N. perflava* grew on nutrient agar at 22°C and 37°C. All strains of *N. lactamica* failed to grow on nutrient agar at 22°C. The growth of the other *Neisseria* spp on nutrient agar at 22°C was variable (table). No difference was detected in any of the tests between β lactamase producing and non-β-lactamase producing strains of *B. catarrhalis*. All the 140 strains tested grew on blood agar, chocolate agar, and nutrient agar media at 37°C.

None of the 140 strains studied grew on neomycin or bacitracin containing media. None of the *B. catarrhalis* strains grew under anaerobic conditions, whereas all the strains of *N. perflava*, *N. lactamica*, and other non-pathogenic *Neisseria* spp, with the exception of *N. caviae*, *N. elongata* as subspecies and *N. cuniculi* grew anaerobically.

Discussion

Increasing evidence that *B. catarrhalis* is an important pathogen in diseases such as bronchopulmonary infections,^{13 17 18} otitis media,¹⁹ maxillary sinusitis,²⁰ and conjunctivitis,²¹ warrants its proper identification and differentiation from non-pathogenic *Neisseria* spp. Morphological similarities and biochemical variations among different *Neisseria* spp and *B. catarrhalis* may cause confusion and result in an error or delay in their recognition.

Results of our study indicate that the DNase test is reliable and simple to perform and because of its high specificity, it can be used as a confirmatory test in the

identification of *B catarrhalis*. The superoxol test is a simple and economical test which can be performed on colonies from primary culture plates. It can differentiate *B catarrhalis* from *N perflava*, *N pharyngis* and several other non-pathogenic *Neisseria* spp (table). In a previous study Young *et al*¹² found that 128 of 133 isolates of *N meningitidis* were superoxol negative. The superoxol test cannot be used to differentiate between *N gonorrhoeae* and *B catarrhalis* as all but one of 596 gonococci tested were positive in the test.¹² The Phadebact gonococcus test, however, is highly specific for *N gonorrhoeae*. In our study all the isolates of *B catarrhalis* were negative in the Phadebact gonococcus test. Although some non-gonococcal strains of *Neisseria* spp reacted in the Phadebact test, such cross reactions can be eliminated by use of the currently available Phadebact gonococcus test which uses monoclonal antibodies (H Young, unpublished data). Thus superoxol positive isolates may be differentiated using the Phadebact test into *B catarrhalis* and *N gonorrhoeae* on the same day. Together, the superoxol and DNase tests constitute reliable and cost effective means of differentiating *B catarrhalis* from other *Neisseria* spp.

Differences in growth characteristics of *Neisseria* spp and *B catarrhalis* on various media may also be used advantageously in presumptive identification. *B catarrhalis* and *N perflava* (the most commonly isolated *Neisseria* spp) do not grow on MNYC and MTM media as the minimum inhibitory concentrations of colistin for these strains is lower than the concentration used in these media.²² Additional characteristics that distinguish *B catarrhalis* from *N perflava* are inability of *B catarrhalis* to grow on MacConkey crystal violet, and on nutrient agars under anaerobic conditions.

Growth at 22°C on basic media has been considered to be a property of non-pathogenic *Neisseria* spp.²³ It has been suggested that β -lactamase producing strains of *B catarrhalis* do not grow at 22°C.^{23 24} In this study, however, all the isolates of *B catarrhalis* did grow on nutrient agar at 22°C (table). All the isolates of *B catarrhalis* and *Neisseria* spp failed to grow on media containing bacitracin or neomycin. Therefore, a non-selective medium should be added where *B catarrhalis* is likely to be a significant isolate. *N cinerea* may colonise the oropharynx and less commonly the genital tract. Difficulties have been experienced in differentiating *N cinerea* from *N gonorrhoeae* and *B catarrhalis* in several rapid systems used for the identification of pathogenic *Neisseria* spp.^{9 25} Knapp *et al*⁹ used DNA hybridisation to identify *N cinerea*, but this test is not readily available in most laboratories. We feel that DNase production, the superoxol test, and growth characteristics on various media are sufficient (table).

We conclude that simple laboratory tests like DNase production, superoxol reaction, anaerobic culture and growth characteristics on routinely used laboratory media such as MNYC, MTM, MacConkey and crystal violet blood agars are enough to distinguish *B catarrhalis* from pathogenic and non-pathogenic *Neisseria* spp.

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Requests for reprints to: Dr Fareeduddin Ahmad, Department of Microbiology, Central Middlesex Hospital, Park Royal, Acton Lane, London NW10 7NS, England.