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Characterization of a *Moraxella* species that causes epistaxis in macaques

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Abstract

Bacteria of the genus *Moraxella* have been isolated from a variety of mammalian hosts. In a prior survey of bacteria that colonize the rhesus macaque nasopharynx, performed at the Tulane National Primate Research Center, organisms of the *Moraxella* genus were isolated from animals with epistaxis, or “bloody nose syndrome.” They were biochemically identified as *Moraxella catarrhalis*, and cryopreserved. Another isolate was obtained from an epistatic cynomolgus macaque at the U.S. Army Medical Research Institute of Infectious Diseases. Based on differences in colony and cell morphologies between rhesus and human *M. catarrhalis* isolates, we hypothesized that the nonhuman primate *Moraxella* might instead be a different species. Despite morphological differences, the rhesus isolates, by several biochemical tests, were indistinguishable from *M. catarrhalis*. Analysis of the cynomolgus isolate by Vitek 2 Compact indicated that it belonged to a *Moraxella* group, but could not differentiate among species. However, sequencing of the 16S ribosomal RNA gene from four representative rhesus isolates and the cynomolgus isolate showed closest homology to *Moraxella lincolni*, a human respiratory tract inhabitant, with 90.16% identity. To examine rhesus macaques as potential hosts for *M. catarrhalis*, eight animals were inoculated with human *M. catarrhalis* isolates. Only one of the animals was colonized and showed disease, whereas four of four macaques became epistatic after inoculation with the rhesus *Moraxella* isolate. The nasopharyngeal isolates in this study appear uniquely adapted to a macaque host and, though they share many of the phenotypic characteristics of *M. catarrhalis*, appear to form a genotypically distinct species.

Keywords

Moraxella; Epistaxis; Nonhuman primate; *Macaca*

1. Introduction

Members of the genus *Moraxella* are Gram-negative, aerobic, asaccharolytic bacteria that can be pleomorphic, resistant to Gram-stain decolorization, and occur predominantly in pairs

or short chains. Those of the subgenus *Branhamella* (e.g. *Moraxella catarrhalis*) are small (0.6–1.0 µm) cocci, whereas organisms of the subgenus *Moraxella* are larger (1.0–1.5 × 1.5–2.5 µm) plump rods (Holt et al., 2000). To date, the genus contains at least 14 different species, having been isolated from a variety of mammalian hosts, both terrestrial and aquatic (Hernández-Castro et al., 2005; Kodjo et al., 1994; Shotts Eb et al., 1990; Tan and Grewal, 2001). Despite the few species that have been characterized, the breadth of *Moraxella* subgenus *Moraxella* variants, organ tropism, and host range may well be quite extensive. The species most studied of this subgenus is *Moraxella bovis*, which is the cause of infectious kerato-conjunctivitis in cattle and horses (Hughes and Pugh, 1970; Huntington et al., 1987; Kodjo et al., 1994).

M. catarrhalis, the human pathogenic species, comprises a subgenus. Originally thought to be a commensal inhabitant of the upper respiratory tract (Enright and McKenzie, 1997), more current work has shown that this organism is also a pathogen of medical importance (Enright and McKenzie, 1997; Karalus and Campagnari, 2000). *M. catarrhalis* is one of the prominent species found in middle ear infections, or otitis media (OM) and is also a cause of sinusitis, along with *Streptococcus pneumoniae* and nontypeable *Haemophilus influenza* (Karus and Campagnari, 2000; Murphy and Parameswaran, 2009). In adults, infection with *M. catarrhalis* may manifest as exacerbation of chronic obstructive pulmonary disease (Murphy and Parameswaran, 2009). Other *Moraxella* spp. are commensal organisms of human epithelia rarely associated with disease and include *Moraxella osloensis*, *Moraxella non-liquefaciens*, *Moraxella lacunata*, *Moraxella atlantae* and *Moraxella lincolni*.

A study published in 1991 described an outbreak of nasal discharge/epistaxis among 25 cynomolgus macaques housed in the same room (VandeWoude and Luzarraga, 1991). *Branhamella catarrhalis* was implicated, and isolates were subsequently used to transfer disease to five out of eight test animals. The goals of our study were to determine the degree of similarity between nonhuman primate and human isolates by examination of a number of genotypic and phenotypic characteristics, and to ascertain the infectious capacity of both types in rhesus macaques.

2. Materials and methods

2.1. Clinical samples

The Tulane National Primate Research Center (TNPRC) is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AALAC) and all procedures were approved by the Tulane University Institutional Animal Care and Use Committee (IACUC). At the TNPRC, nasal swabs were taken from 11 rhesus macaques (*Macaca mulatta*) of various ages with BBL CultureSwabs™ (Becton Dickinson & Co., Franklin Lakes, NJ) inserted into the nares at presentation with epistaxis. At the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), cynomolgus macaques (*Macaca fascicularis*) that also demonstrated sneezing with clear to serosanguinous (bloody) nasal discharge were sampled by deep nasopharyngeal insertion of a Dacron swab onto the nares of anesthetized animals and four isolates were obtained. Human nasopharyngeal (KSA) and middle ear (7169) *M. catarrhalis* isolates were provided by Drs. A. Campagnari and N. Luke, University of Buffalo, Buffalo, NY.

2.2. Growth conditions

All nasal swabs were plated onto 5% sheep blood agar plates, streaked for isolation and incubated at 37 °C, 5% CO₂ for growth of *Moraxella*. Those colonies that tested positive by the Remel™ Catarrhalis test disk (Perez et al., 1990) were used to inoculate a liquid culture of Trypticase™ soy broth (Becton Dickinson & Co.), grown for 48 h at 37 °C, and frozen in

30% glycerol at -80°C . The glycerol stocks were used to plate bacteria for colony morphology, Gram stain, and biochemical tests.

The human *M. catarrhalis* isolates were grown in TrypticaseTM soy broth (Becton Dickinson & Co.) for 48 h or in BactoTM Brain Heart Infusion media (Becton Dickinson & Co.) for 28–34 h in order to achieve mid- to late-log phase growth. Both human isolates were β -lactamase positive/penicillin resistant.

2.3. Biochemical tests

Two specific biochemical tests and two commercial panels were used to aid in identifying the organisms. Isolated colonies from growth on BD BBLTM Chocolate II agar plates (Becton Dickinson & Co.), were used for the butyrate esterase Catarrhalis disk test (Remel) and the Oxidase test (Becton Dickinson & Co.). Manufacturer's protocols were followed, and as such, a pronounced color change within 2 min was scored as positive. Additionally, two commercially available test systems were used for biochemical identification. The API NH panel (BioMérieux, Marcy l'Etoile, France) was used to distinguish our isolates from *Neisseria* and *Haemophilus* species. The RapIDTM NH System (Remel) was also used to aid discrimination within the *Moraxella* genus. Each test was run, according to the manufacturer's protocol, three times for each isolate, by two separate investigators. The cynomolgus macaque isolates were analyzed instead on the Vitek[®] 2 Compact identification system (BioMérieux).

2.4. Sequencing

For DNA sequencing of the 16S ribosomal RNA genes from seven rhesus isolates, PCR amplifications were performed as described (Harmsen et al., 2001) using genomic DNA purified with QuickExtract DNA Extraction Solution (EPICENTRE Biotechnologies, Madison, WI) and Platinum *Taq* High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA). Amplicons were purified with the Qiagen MinElute Kit (Santa Clarita, CA), subjected to automated DNA sequencing (RPCI Biopolymer Facility, Roswell Park Cancer Institute, Buffalo, NY) and analyzed with MacVector version 10.0 and the Wisconsin sequence analysis package (Genetics Computer Group, Madison, WI). Primer binding sites were removed from the sequence files prior to submission for nucleotide BLAST analyses at the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/>) and to the RIDOM reference database for microbial identification (<http://rdna.ridom.de/>) as described (Harmsen et al., 2001). For sequencing of the 16S rRNA gene from a single cynomolgus isolate, approximately 10 ng of purified amplicon DNA was used as template in BigDye[®] Terminator v1.1 Cycle Ready reactions and sequenced on an ABI PrismTM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

For sequencing of the housekeeping genes, degenerate primers that were previously described (Angelos et al., 2007) were used in PCR reactions to amplify sequences using *Taq* DNA polymerase kit (Qiagen Corp., Valencia, CA) and the appropriate thermal cycler conditions (Angelos et al., 2007). The amplified products of correct size were obtained when primers for RNA polymerase subunit B and ATP synthase genes were used on the nonhuman primate isolate CI24 genomic DNA. These products, run on a 1% agarose gel, were cut from the gel and purified using the Qiaex II Gel Extraction Kit (Qiagen). Purified amplicons were then cloned into the PCR Cloning vector pDrive (Qiagen) using the Qiagen PCR Cloning^{plus} Kit (Qiagen). The Sp6 and T7 promoter primers outlined in the manufacturer's handbook were supplied for sequencing by the DNA Sequencing Core facility at Tulane University Health Sciences Center.

2.5. Nonhuman primate inoculations and surveillance

Prior to the inception of animal studies, all procedures were approved by the Tulane University IACUC. The infectivity studies in nonhuman primates consisted of three phases in which: (1) animals were inoculated with either human nasopharyngeal (KSA) or middle ear (7169) *M. catarrhalis* isolates; (2) animals were inoculated with isolate 7169 only; and (3) animals were inoculated with a nonhuman primate *Moraxella* isolate (CI24). All animals were housed in separate cages after inoculation. Upon conclusion of the study, animals were treated with amoxicillin/clavulanic acid (7.5 mg/kg 3× daily) for five days and returned to the colony.

In phase I, two animals per *M. catarrhalis* isolate were inoculated by intranasal instillation (see below) with 6.5×10^7 organisms. A fifth animal was sham-inoculated with PBS and housed in the same room. In phase II, a total of four animals (three naïve and the control from phase I) were inoculated with 1×10^8 *M. catarrhalis* isolate 7169. This organism was grown to log-phase in Bacto™ Brain Heart Infusion media (Becton Dickinson & Co). The third phase was an inoculation of four animals (from phase I that had been antibiotic-treated) with the nonhuman primate epistaxis-causing isolate CI24, grown in Trypticase™ soy broth. Each animal received 7.8×10^6 organisms.

For all inoculations, bacteria suspended in a total of 0.5 mL PBS were added drop-wise to both nares of the anesthetized animal. At one, two, three, four, six and eight weeks post-inoculation, nasal-pharyngeal swabs were collected and otoscopic examinations were performed with a 1.9 mm rigid videoscope (Karl Storz GmbH & Co. KG, Tuttlingen, Germany) for detection of middle ear effusion. For tympanocentesis, a 22-gauge 1.5 in.-long needle with a short bevel was inserted into a 3.5 French red-rubber feeding tube cut to approximately the same length as the needle. The needle and feeding tube were then inserted alongside the videoscope until the feeding tube was visualized just beyond the edge of the scope. The needle was advanced through the feeding tube into the medial ventral aspect of the TM. Once the bevel was completely through the TM, the pus was aspirated. Photographic images of the tympanic membrane were taken at all time points. In an attempt to re-isolate *Moraxella*, the swabs were placed in 0.5 mL Trypticase™ soy broth (Becton Dickinson & Co.) and mixed thoroughly to dislodge bacteria. Swabs and 100 µL from the original suspension were spread onto three types of media, – BD BBL™ chocolate II agar plates, 5% sheep blood agar (SBA) + 1 or 4 µg/mL penicillin plates (Fisher Scientific, USA), where appropriate. Glycerol stock medium was added to the remaining 200 µL and samples were stored at –70 °C. Plates were grown ~48 h at 37 °C, 5% CO₂ for growth of *Moraxella*. All colonies with similar morphology (off-white, medium-large, non-hemolytic) were tested by Catarrhalis disk test (Remel, Lenexa, KS) and examined by Gram stain if positive. For phases I and II, any presumed positive re-isolates were tested by PCR to confirm the identity.

2.6. In vitro growth inhibition assay

To test the growth of *M. catarrhalis* in the presence of rhesus normal flora in vitro, 20 µL from a glycerol stock of each of the following were added to 1 mL of Trypticase™ soy broth: (a) *M. catarrhalis*, strain 7169; (b) HB57 (control animal) week 4 nasal swab stock; and (c) HB57 nasal plus 7169. Broth cultures were inoculated in duplicate and placed in either 37 °C, 5% CO₂ without shaking or in 37 °C with shaking at 225 rpm. These cultures were grown overnight (18–20 h), then plated onto chocolate enrichment agar, SBA with 1 µg/mL penicillin (SBAp 1 µg/ml), and SBA with 4 µg/mL penicillin (SBAp 4 µg/ml). The plates were incubated overnight at 37 °C, 5% CO₂.

3. Results

In a previous survey (Bowers et al., 2002), bacteria from epistatic macaques were tentatively identified as *M. catarrhalis* based on colony morphology and Gram stain, and then more specifically with the butyrate esterase disk test (Remel). Eleven cryopreserved isolates from that survey were subjected to a series of biochemical tests for further characterization and distinction.

3.1. Phenotypic tests

3.1.1. Colonial morphology and Gram stain—Based on the morphology of colonies following 24–48 h growth at 37 °C in 5% CO₂ on nutrient-rich chocolate agar plates, the isolates could be placed into one of two categories: (1) mucoid (thick, glossy); or (2) dry, round, colony morphologies. The sizes of the colonies varied slightly, but generally the mucoid type grew larger (~2–3 mm diameter) and the dry type gave rise to smaller (~1 mm) colonies after 48 h growth. The positive control *M. catarrhalis* strain 7169 conformed to the “dry” phenotype when grown on chocolate agar. Interestingly, the dry isolates did not grow well on chocolate agar in a non-CO₂ incubator.

Gram stains were performed on each isolate after 48 h growth on chocolate agar. The positive control strain 7169 was provided on Mueller-Hinton agar and was grown for 24 h following receipt. This control strain was also examined after 48 h growth on chocolate agar, as the others were grown.

The positive control strain 7169 stained pink/Gram-negative and appeared as small (~1 µm) cocci, which also exhibited pleomorphism in shape and size (Fig. 1A). Most of the rhesus isolates appeared as Gram-negative diplococci of larger (~2–2.5 µm) size and exhibited some resistance to decolorization, especially the larger cocci. Some appeared, rather, as plump rods in pairs and in chains (Fig. 1B). The morphology and Gram reactions of the rhesus isolates were similar to one another, and the predominant difference from the *M. catarrhalis* strain 7169 was size.

3.1.2. Biochemical tests—All isolates and the positive control were oxidase and butyrate esterase positive. With the exception of the beta-lactamase test, all but one isolate exhibited identical reactivity to that of the *M. catarrhalis* control strain 7169 in the biochemical tests of the API NH panel (BioMérieux). Isolates were beta-lactamase negative, whereas the *M. catarrhalis* strain 7169 was beta-lactamase positive. None produced acid from glucose, fructose, maltose or saccharose. All were positive for lipase, and negative for ornithine decarboxylase, urease, alkaline phosphatase, galactosidase, proline arylamidase, and indole. All but one isolate were negative for γ-glutamyltransferase. Although the reactions were consistent with that of *Moraxella (Branhamella) catarrhalis*, this panel does not distinguish between *Moraxella (Branhamella) catarrhalis* and other subspecies' of the genus *Moraxella* (Table 1a).

Due to the variety of tests and potential for distinction among *Moraxella* species, the RapID™ NH System (Remel) panel was also performed. These results placed the isolates into one of four different categories (Table 1b). The “dry” type colony morphology isolates (AA73, AJ30, V756, AA24 and AC73) did not show identity with *Moraxella*, but with CDC M-5, which is now called *Neisseria weaveri* (Andersen et al., 1993). As part of normal canine oral flora, this species has been associated with dog bite wounds in humans (Panagea et al., 2002). The “mucoid” strains (CI24, M900, CB66, AA61, CH45 and 0408225) biochemically resembled *M. catarrhalis*, and V840 appeared to be a unique variant. These isolates thus fell into three different categories based on morphology and biochemistry: (1) mucoid, *M. catarrhalis*-type; (2) mucoid, *M. catarrhalis* rare variant (V840); and (3) dry, *N.*

weaveri type. At least one isolate from each category was characterized by 16S ribosomal DNA sequencing (Table 2). All of those isolates that were scored in the *M. catarrhalis* family with 99.9% probability were of the mucoid type of colony morphology and were considered to be representative of the nonhuman primate *Moraxella* species. CI24 was selected for further genetic analysis and fulfillment of Koch's postulates in nonhuman primates. The cynomolgus isolates were identified by the Vitek 2 Compact system as *Moraxella* group. The Vitek was unable to differentiate between *M. osloensis*, *M. nonliquefaciens* and *M. lacunata*.

3.2. Genotypic tests

3.2.1. PCR using *M. catarrhalis*-specific primers—*M. catarrhalis*-specific primers for *kdsA* (Luke et al., 2003) and *ssb* genes were used for PCR on all of the 11 nonhuman primate isolates, three *M. catarrhalis* strains (7169, 073HF and 39P33), *M. bovis* 39503, *M. nonliquefaciens* 17953, *M. osloensis* 15276 and *M. caviae* 14607. Amplicons were only obtained from *M. catarrhalis* strains with each primer set (data not shown). This result serves to further indicate that the nonhuman primate isolates are not *M. catarrhalis*.

3.2.2. 16S rDNA sequencing—Diagnostic identification of *Moraxella* species has been accomplished with sequencing of ribosomal RNA genes (Harmsen et al., 2001; Pettersson et al., 1998). Broad-range 16S rRNA primers (described in Harmsen et al., 2001) were used in PCR reactions with template DNA from all of the mucoid *M. catarrhalis*-type Tulane isolates, the rare variant, and *M. catarrhalis* positive controls. Amplicons were purified, subjected to DNA sequence analyses, and submitted to the ribosomal differentiation web-based service (RIDOM) at <http://rdna.ridom.de/> (Harmsen et al., 2002). Results are shown in Table 2. A region of the 16S sequence was also obtained for the cynomolgus isolate (GenBank accession # HM037355) and compared to the Tulane rhesus isolate CI24 (Fig. 3). This section of 16S demonstrated 99% identity, with 442/443 base pairs of matching sequence.

3.2.3. Sequencing of housekeeping genes—Using degenerate primers described previously (Angelos et al., 2007), two housekeeping genes from CI24 that encode the RNA polymerase subunit B and ATP synthase F1, epsilon subunit were amplified, inserted into a cloning vector, and sequenced. Sequences were obtained from the genes. The sequences were subjected to a nucleotide BLAST, available in the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). While sequences of these genes were not available in the database for any *Moraxella* species, the closest identities phylogenetically placed our new species near *Psychrobacter* and *Acinetobacter*, just as with other moraxellae (Hays, 2006; Pettersson et al., 1998). For RNA polymerase B subunit (partial sequence; GenBank accession # HM037356), the *Acinetobacter baylyi* strain ADP1 was 80% identical to CI24. For the ATPase gene (partial sequence; GenBank accession # HM037357), *Psychrobacter articus* possessed the highest level of identity (of those available in the database) at 69%.

3.3. Colonization and infection of nonhuman primates

3.3.1. Inoculation with *M. catarrhalis*—Infection of nonhuman primates with *Moraxella* isolates was carried out essentially for two purposes: (1) to determine if the human *M. catarrhalis* could colonize and produce disease in the nonhuman primate; and (2) to use the nonhuman primate isolate to reproduce disease (i.e. epistaxis) in fulfillment of Koch's postulates. The infection scheme is depicted in Fig. 2.

In the first phase, two animals each were inoculated with one of the two human *M. catarrhalis* isolates. Otoscopy with a 1.9 mm rigid videoscope was conducted, and nasopharyngeal swabs were taken at one, two, three, four, six and eight weeks post-

inoculation. During videoscopic examination of the inner ear, cloudiness or lack of translucence in the tympanic membrane (TM) was considered as evidence for possible otitis media. While this was observed in two animals, the *M. catarrhalis* used for inoculation was only re-isolated from the nasopharyngeal swabs in one of these animals (Table 3a). This animal was inoculated with the human middle ear isolate 7169, so this was used for subsequent inoculations that comprised phase 2. Here, four rhesus macaques were inoculated with isolate 7169. If otitis media was suspected, tympanocentesis was performed. This only occurred in one animal and subsequent culture of the inner ear fluid yielded no growth. Thus, colonization and disease by *M. catarrhalis* in nonhuman primates was very infrequent, as it occurred in only one of six macaques that were inoculated with the inner ear isolate and in one of eight animals that were exposed to either isolate.

3.3.2. Growth inhibition assay—To test the probable explanatory hypothesis that growth of *M. catarrhalis* is inhibited by normal rhesus nasopharyngeal flora, the human isolate 7169 was grown in vitro in the presence or absence of organisms collected from the nasal swab of a control animal. Because this isolate is resistant to penicillin, this was used for selection and growth of *M. catarrhalis* from the mixed culture. The results are presented in Table 4. Strain 7169 grew well in Trypticase soy broth, independent of shaking and CO₂ concentration. This isolate could also grow in 4 µg/mL penicillin, which inhibited growth of the organisms within rhesus normal flora. However, growth of 7169, in the presence of normal flora, appeared to be inhibited.

3.3.3. Inoculation with the macaque isolate—The third set of nonhuman primate inoculations was performed using antibiotic-treated monkeys from the phase I experiments. Each of the animals was tested prior to exposure for the presence of residual *Moraxella* growth and all were negative. Four animals were inoculated with the nonhuman primate isolate (CI24) and subjected to periodic screening by otoscopic examination and nasopharyngeal swabbing. All four animals showed signs of disease (epistaxis) at some point and were colonized by this bacterial isolate (Table 3b) for up to four weeks. The epistaxis that was observed consisted of nose bleeding without significant discharge that was noted on physical exam. For all animals but HG81, epistaxis was bilateral and all presented with some nose bleeding within four weeks of the inoculation. Additionally, the nonhuman primate *Moraxella* sp. was the predominant organism recovered from nasal swabs in most instances.

4. Discussion

In 1991, VandeWoude and Luzarraga reported an outbreak of nasal discharge/epistaxis among nonhuman primates housed in the same room. They identified “*B. catarrhalis*” in symptomatic animals, derived isolates, and later inoculated healthy animals with an isolate. The “bloody nose syndrome” was observed in five of eight test animals. In a survey of bacteria that colonize the rhesus nasopharynx, isolates of “*M. catarrhalis*” were observed in animals at the TNPRC that presented with epistaxis. A total of 33 animals from a colony of 968 rhesus macaques showed the “bloody nose” sign. All of these animals were shown to be infected with “*M. catarrhalis*” (Bowers et al., 2002). In this study, the organisms were identified as *M. catarrhalis* based on colony morphology, Gram stain, and the Remel™ Catarrhalis disk test.

The genus *Moraxella* is divided into the subgenus *Moraxella*, which includes all the rod-shaped species, and the subgenus *Branhamella*, which contains the cocci (Holt et al., 2000). *Moraxella* subgenus *Moraxella* rods are often very short and plump, approaching a coccus shape. Cells usually occur in pairs or short chains with one plane of division. Pleomorphism is enhanced by lack of oxygen and by incubation at temperatures above the optimum. The

medically important species are *M. atlantae*, *M. lacunata*, *M. nonliquefaciens* and *M. osloensis*. *Moraxella* subgenus *Branhamella* cocci are about 0.6–1.0 µm in diameter, occur singly or in pairs with adjacent sides flattened, and sometimes form tetrads. This subgenus contains one medically important species, *M. catarrhalis*.

In this study, the mucoid type isolates derived from macaques fall into the *Moraxella* subgenus *Moraxella* category by Gram stain morphology. These isolates, for instance, morphologically mirror the description of a *Moraxella* subgenus *Moraxella* species, *M. nonliquefaciens*, which was isolated from three patients of Aboriginal descent that presented with chronic lung disease (Davis et al., 2004). As probable members of this subgenus, the rhesus isolates and cynomolgus isolate were predicted to have exhibited biochemically different results from *M. catarrhalis* by the RapID™ NH System (Remel). Our isolates, however, did not. According to the manufacturer's protocol, the intended use for this product is "to definitively identify *N. gonorrhoeae*, *N. meningitidis* and *M. catarrhalis* and to differentiate these organisms from other species of *Neisseria*, *Moraxella*, CDC M-groups and *Kingella*." The test is also intended for use on human clinical samples. We learned that the nonhuman primate isolates differ enough from human medically important species for this test to be informative. These test results presented a conundrum as to the actual identity of both the mucoid and dry colony types of isolates. Insofar as the mucoid isolates were concerned, the biochemical test results were consistent with *Moraxella* (*Branhamella*) *catarrhalis*. However, the colony morphology and Gram stain morphology clearly differed from that of the human isolate 7169.

With regard to the dry colony type isolates, their classification by the RapID™ NH System (Remel) as CDC M-5 (*N. weaveri*) contradicted previous tests such as the API strip and the butyrate esterase test, which are intended to distinguish a *Moraxella* species from its *Neisseria* and *Haemophilus* counterparts (Perez et al., 1990). Thus, their categorization as a *Moraxella* species cannot be made without further supportive evidence.

Therefore, while all biochemical tests commonly used to identify *M. catarrhalis* produced identical results for our prototype isolate (CI24), we have shown that the rhesus *Moraxella*, which is the etiologic agent of epistaxis, is not *M. catarrhalis*. The organisms appear different than *M. catarrhalis* in a Gram stain, but similar to other members of the *Moraxella* subgenus *Moraxella* group of human and animal pathogens such as *M. osloensis* and *M. bovis*.

The distinction of our isolates as a newly defined species can be made by the 16S ribosomal RNA sequence, which is unique from all previously reported moraxellae. The rhesus isolate 16S sequence was essentially identical to that of the cynomolgus macaque isolate. Moreover, *M. catarrhalis* isolates from humans, with one exception, were not able to colonize rhesus macaques, whereas the nonhuman primate isolate readily colonized and caused disease in naïve animals. We put forth evidence that the nasopharyngeal flora of rhesus macaques may in fact inhibit the growth of *M. catarrhalis*. This could be due to competition for limiting nutrients or by direct inhibition of *M. catarrhalis* growth by organisms within this milieu. It is unlikely that the colonization with CI24 in phase III may have been influenced by prior antimicrobial therapy for three specific reasons: (1) the macaque-specific *Moraxella* has been isolated from numerous animals and *M. catarrhalis* has not; (2) the aforementioned inhibition of *M. catarrhalis* growth by rhesus normal flora and (3) 4.5 months elapsed between antibiotic treatment and the subsequent (phase III) inoculation, so the nasal flora should have had sufficient time to re-establish colonization.

Sequencing of two housekeeping genes from the macaque isolate and comparison with available sequences in the most comprehensive database provided an inexact, but reasonable

approximation of its taxonomic placement. The gene sequences from organisms with closest identities to that of our isolate phylogenetically placed our new species near *Psychrobacter* and *Acinetobacter*, just as other moraxellae (Hays, 2006; Pettersson et al., 1998). Thus, our described species should fall into the Phylum *Proteobacteria*, Class *Gammaproteobacteria*, Order *Pseudomonales*, Family *Moraxellaceae*, Genus I. *Moraxella*, where Genera II and III are *Acinetobacter* and *Psychrobacter*, respectively (Hays, 2006). This species was identified in both rhesus macaques (*M. mulatta*) and cynomolgus macaques (*M. fascicularis*). We therefore propose that this new species be named “*Moraxella macacae*.”

In conclusion, we have shown that: (1) the macaque *Moraxella* isolate is distinct from *M. catarrhalis* and other *Moraxella* species isolated from humans; (2) *M. catarrhalis* generally does not colonize or cause disease in rhesus macaques; and (3) the nonhuman primate *Moraxella* species colonizes the nasopharynx of both rhesus and cynomolgus macaques and causes epistaxis, but likely not otitis media.

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Fig. 1. Morphology of the human *Moraxella catarrhalis* isolate 7169 (A) and the rhesus macaque isolate CI24 (B). Single colonies from cultures grown on agar were Gram stained. The image magnification is 100× in both panels.

Fig. 2.
Diagram of nonhuman primate inoculation sequence.

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CI24      ACGATGATTATCCAGCTTGCTGGATAAGATTAGTGGCGAACGGGTGAGTAACATTTAGGA 98
          |||
0408225   ACGATGATTATCCAGCTTGCTGGATAAGATTAGTGGCGAACGGGTGAGTAACATTTAGGA 60
          |||

          ATCTGCCTCATAATGGGGGATAGCTTGGGGAAACCCAAATTAATACCGCATACGTCCTAC 158
          |||
          ATCTGCCTCATAATGGGGGATAGCTTGGGGAAACCCAAATTAATACCGCATACGTCCTAC 120
          |||

          GGGAGAAAGATGGCGCAAGCTGTCGTTATGAGATGAGCCTAAACCAGATTAGCTAGTTGG 218
          |||
          GGGAGAAAGATGGCGCAAGCTGTCGTTATGAGATGAGCCTAAACCAGATTAGCTAGTTGG 180
          |||

          TGGGGTAAAAGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACA 278
          |||
          TGGGGTAAAAGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACA 240
          |||

          CTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAT 338
          |||
          CTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAT 300
          |||

          GGGGGCAACCTTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCA 398
          |||
          GGGGGCAACCTTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCA 360
          |||

          CTTTAAGTGGGAGGAACAATTATCAATTAATACCTGATAATCTTGACGTTACCCACAGA 458
          |||
          CTTTAAGTGGGAGGAACAATTATCAATTAATACCTGATAATCTTGACGTTACCCACAGA 420
          |||

          ATAAGCACCGGCTAACTCTGTGC 481
          |||
          ATAAGCACCGGCTAACTCTGTGC 443
          |||

Identities = 442/443 (99%),

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Fig. 3.
Comparison of 16S rRNA sequence from rhesus (CI24) and cynomolgus (0408225) Isolates.

Table 1a

Comparison of *Moraxella catarrhalis* with nonhuman primate *Moraxella* isolates.

	<i>Moraxella catarrhalis</i>	Nonhuman primate <i>Moraxella</i>
Gram reaction	Negative	Negative; resistance to decolorization
Cell morphology	Small (0.6–1.0 µm) cocci	Larger (1.0–1.5 × 1.5–2.5 µm) plump rods
Colony morphology	Round, convex, greyish-white, “hockey puck”	Round, large, mucoid
Hemolysis	No	No
Oxidase	Yes	Yes
Butyrate esterase	Yes	Yes
Acid from simple sugars	No	No
Reduction of nitrate/nitrite	Yes	Yes
Penicillin resistance	Yes	No

Table 1b

RapID™ NH System (Remel) panel results for 11 nonhuman primate *Moraxella* isolates.

Numerical profile	System identification	Probability	Isolates in this category
1010	CDC M-5	57.9%	AA73, AJ30, V756, AA24, AC73
	<i>N. cinerea</i>	41.3%	
	CDC M-6	0.6%	
1400	<i>M. catarrhalis</i>	99.9%	+ control (M.cat7169), CI24
1402	<i>M. catarrhalis</i>	99.9%	M900, CB66, AA61, CH45, 0408225 (Cyno isolate)
3412	<i>K. denitrificans</i>	0.1%	V840
	Questionable <i>M. catarrhalis</i> (very rare)	99.9%	

Table 2

16S ribosomal sequence identification for nonhuman primate isolates.

Input DNA sequence of 16S rRNA	GenBank accession number	RIDOM result	BLAST-N (NCBI)
<i>M. Catarrhalis</i> 7169		<i>Moraxella catarrhalis</i> 100%	
AA24	HM037353	No close relative; highest ID: <i>Moraxella bovis</i> 93.07%	
AA61	HM037352	No close relative; highest ID: <i>Moraxella lincolnii</i> 90.16%	<i>Moraxella boevrei</i> 93%
CB66	HM037351	No close relative; highest ID: <i>Moraxella lincolnii</i> 90.16%	<i>Moraxella boevrei</i> 93%
CH45	HM037350	No close relative; highest ID: <i>Moraxella lincolnii</i> 90.16%	<i>Moraxella boevrei</i> 93%
CI24	HM037349	No close relative; highest ID: <i>Moraxella lincolnii</i> 90.16%	<i>Moraxella boevrei</i> 93%
M900	HM037348	No close relative; highest ID: <i>Moraxella lincolnii</i> 90.16%	<i>Moraxella boevrei</i> 93%
V840	HM037354	No close relative; highest ID: <i>Moraxella osloensis</i> 91.3%	

Table 3aResults from inoculation of nonhuman primates with human *M. catarrhalis* isolates.

Animal	Inoculation strain/isolate	Colonization	Disease ^a
HB57	Control (PBS)	–	–
HB78	<i>M. catarrhalis</i> /KSA	–	–
HG81	<i>M. catarrhalis</i> /KSA	–	Possible OM, week 3
HD29	<i>M. catarrhalis</i> /7169	–	
HC79	<i>M. catarrhalis</i> /7169	+ weeks 1–6	E, weeks 4–6; possible OM weeks 2, 3
HB57	<i>M. catarrhalis</i> /7169	–	–
HJ60	<i>M. catarrhalis</i> /7169	–	Possible OM week 3, culture-neg.
HG89	<i>M. catarrhalis</i> /7169	–	–
HG67	<i>M. catarrhalis</i> /7169	–	–

^aE, epistaxis; OM, otitis media.

Table 3b

Colonization^a and disease^b in animals inoculated with a nonhuman primate *Moraxella* isolate.

Animal	Week 1	Week 2	Week 3	Week 4
HB78	(+) P	(+) P	(+) E	(+)
HG81	(+) P, E	(+) P, E	(+) P	(+) P
HC79	(+) P, E	(+) P, E, OM	(+) P, E	(+) P
HD29	(+) P, E	(+) P, E	(+) P, E	(+)

^a (+), CI24 was re-isolated from nasal swab; P, CI24 was predominant.

^b E, epistaxis; OM, suspect otitis media.

Table 4

In vitro growth inhibition of *M. catarrhalis* with rhesus normal flora.

	Chocolate		SBAp (1 µg/mL)		SBAp (4 µg/mL)	
	+CO ₂	-CO ₂	+CO ₂	-CO ₂	+CO ₂	-CO ₂
Inoculum source						
HB57 flora +7169	BE (-)	++ BE (-)	++, hemolysis BE (-)	++, hemolysis BE (-)	No growth	No growth
7169	++ BE (weak +)	++ BE (weak +)	++ BE (strong +)	++ BE (strong +)	++ BE (strong +)	++ BE (strong +)
HB57 flora	++	++	++, hemolysis	++, hemolysis	No growth	No growth

++, ample growth; BE, butyrate esterase.