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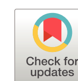
BRIEF REPORT

Study of the genetic diversity of *Moraxella* spp. isolates obtained from corneal abscesses



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Abstract This is the first study of the genetic diversity of *Moraxella* spp. Isolates were detected in an Eye Hospital in the City of Buenos Aires. Due to the high frequency of *Moraxella* spp. observed in corneal abscesses, we decided to validate their identification at the species level, determine their drug susceptibility and perform molecular subtyping. Seventeen (17) isolates obtained from corneal abscesses were evaluated. The identification was carried out using a combination of biochemical tests and MALDI-TOF mass spectrometry. Of these isolates, 88.2% were identified as *Moraxella lacunata*, and 11.8% as *Moraxella nonliquefaciens*. Molecular subtyping was performed using the pulsed-field gel electrophoresis (PFGE) technique. All isolates were typable and thirteen digestion patterns were identified. Based on the obtained results, the PFGE technique using the *SmaI* enzyme can be used for epidemiological studies of strains of these species.
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PALABRAS CLAVE
Moraxella spp.;
PFGE;
Absceso corneal

Estudio de la diversidad genética de aislamientos de *Moraxella* spp. a partir de abscesos corneales

Resumen En este trabajo se presenta el primer estudio de diversidad genética de aislamientos de *Moraxella* spp. detectados en un hospital de oftalmología de la Ciudad Autónoma de Buenos Aires. Debido a la observación de una elevada frecuencia de *Moraxella* spp. en abscesos

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corneales, se decidió confirmar su identificación a nivel de especie, conocer su sensibilidad y realizar la subtipificación molecular. Se analizaron 17 aislamientos provenientes de abscesos corneales. La identificación se realizó mediante una combinación de pruebas bioquímicas y espectrometría de masas, MALDI-TOF MS. El 88,2% fueron identificados como *Moraxella lacunata* y el 11,8% como *Moraxella nonliquefaciens*. La subtipificación molecular se realizó por la técnica de electroforesis en gel de campo pulsado (PFGE). Todos los aislamientos fueron tipificables y se identificaron 13 patrones de digestión. Nuestros resultados muestran que la técnica de PFGE con la enzima *SmaI* es útil para hacer estudios epidemiológicos en cepas de estas especies.
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The genus *Moraxella* comprises catalase and oxidase-positive, nonmotile, non-encapsulated and asaccharolytic Gram-negative coccobacilli or diplobacilli[5], which show slow growth on blood agar and poor growth on chocolate agar. After 24 h, colonies appear circular, small, gray and translucent, turning opaque with increased incubation time[2]. These coccoid or coccobacillary organisms (plump rods), occurring predominantly in pairs and sometimes in short chains, tend to resist decolorization in the Gram stain. Most *Moraxella* species are susceptible to penicillin and its derivatives, cephalosporins, tetracyclines, quinolones, and aminoglycosides. β-Lactamase production has been rarely reported for *Moraxella* species other than *Moraxella catarrhalis*, of which most isolates produce an inducible, cell-associated β-lactamase[8].

These are low-virulence microorganisms, which are commonly found as commensals of the upper respiratory tract[4]. Occasionally, they can cause infections in vulnerable patients. *M. catarrhalis* is a leading cause of otitis media in children and acute exacerbations of chronic obstructive pulmonary disease[3]. *Moraxella* species other than *M. catarrhalis*, such as *Moraxella lacunata*, *Moraxella nonliquefaciens* and *Moraxella osloensis*, are mainly associated with ocular infections such as endophthalmitis, keratitis and blepharoconjunctivitis, sometimes with stromal involvement and corneal perforation; although they have also been isolated from invasive infections in humans, including endocarditis, septic arthritis, meningitis, bacteremia, and pericarditis[7]. *M. nonliquefaciens* and *M. osloensis* are the two species most frequently isolated, approximately in equal numbers, from nonrespiratory clinical material, especially blood cultures from patients at risk. *Moraxella lincolnii* is not frequently isolated from clinical samples. *Moraxella canis* has been isolated from dog bite wounds and from debilitated patients[8]. *M. lacunata* is the causative agent of 2% of bacterial ocular infections .[7] Local predisposing factors include wearing contact lenses, previous eye surgery, ocular trauma, and a previous history of *Herpes simplex* keratitis. Immunocompromised patients, alcoholism, malnutrition, old age, diabetes mellitus and thyroid disease are systemic risk factors[4].

Due to the very frequent finding of *Moraxella* spp. in corneal abscesses, various isolates were referred to the Reference Laboratory (RL) to assess the heterogeneity of species within the genus and also the molecular diversity within the species level, to confirm or rule out whether they are part of a clonal complex.

Of a total of 40 *Moraxella* isolates obtained in the Eye Hospital between January 2018 and November 2019, 17 were randomly selected for an initial study of their identification and clonal diversity. The PFGE technique is widely used for the subtyping of various bacterial species; therefore, we decided to evaluate its functionality in the molecular typing of *Moraxella* spp. To the best of our knowledge, there are no previous reports that evaluate this technique, nor is it established which restriction enzyme shows a discriminatory power that makes it possible to compare and establish the genetic relationship between isolates of this genus, other than *M. catarrhalis*[3].

The characteristics of the population affected by these types of microorganisms were analyzed in accordance with the available epidemiological information. The antibiotic susceptibility of all isolates was tested as well.

Seventeen (17) *Moraxella* spp. isolates obtained from corneal scrapings, which were referred to the RL between January 2018 and November 2019, were analyzed. The median age of patients was 47 years. Thirteen (13) isolates (76.5%) were obtained from male patients. Local risk factors, such as glaucoma (4 patients) and cataracts (1 patient), and systemic risk factors, such as HIV (1 patient), diabetes (3 patients) and arthritis (1 patient), were identified in this study. One or more of these factors were present in nine of the seventeen patients.

The strains were subcultured on blood agar in an atmosphere of 5% CO_2 at 37 °C for 24 h. Isolates were observed under a microscope using the Gram staining technique, and both pigment and hemolysis were recorded. The biochemical characterization was carried out using manual phenotypic tests (oxidase, catalase, growth on MacConkey agar, TSI, gelatin hydrolysis, urease, nitrate reduction, DNase and alkalinization with acetate) and by using commercial miniature-version API® 20NE galleries (bioMérieux, France) according to the manufacturer's instructions. Incubation temperature was 37 °C and the results of the galleries and biochemical tests were read after 24, 48 and 72 h.

Isolates were also identified by mass spectrometry (MALDI-TOF MS Bruker Daltonics, Bruker Taxonomy Database 3.3.1 version + CDC-Microbenet database) using the direct

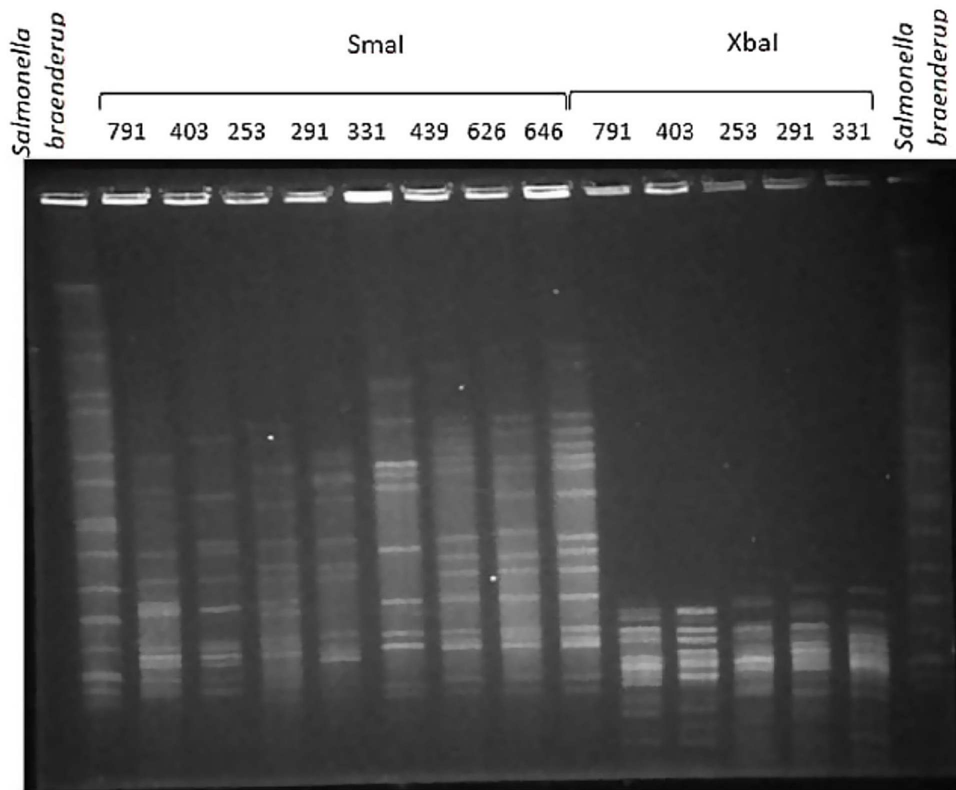

Figure 1 Band patterns obtained by PFGE of *M. nonliquefaciens* (lanes 2 and 3) and *M. lacunata* (lanes 4–9) using SmaI. Patterns observed for XbaI-PFGE can be observed in lanes 10–14 (lanes 10 and 11 *M. nonliquefaciens*; 12–14 *M. lacunata*). Salmonella Braenderup H9812 was used as a molecular weight marker, digested with XbaI.

transfer-formic acid method: MALDI-TOF target plates were inoculated into the spots by picking a freshly grown overnight colony and overlaid with 1 μl of 70% formic acid (Sigma–Aldrich). Each spot was allowed to dry and subsequently overlaid with 1 μl of matrix solution (a cyano-4hydroxycinnamic acid in 50% acetonitrile, 2.5% TFA). The score cut-offs recommended by the manufacturer were used, a score value ≥2,0 indicates species-level identification, a score value between 1,7 and 1,99 indicates genus-level identification, and scores <1,7 indicate no reliable identification. Additionally, the ''10% rule'' was applied, which states that any species scoring >10% below the top-scoring match may be excluded.

Antibiotic susceptibility testing was performed using the disk-diffusion method (the Kirby–Bauer method). The interpretation criterion used is the one established in the CLSI guideline M45-A2 for *M. catarrhalis*. The culture conditions used were an incubation temperature of 35 °C, an atmosphere of 5% CO_2 and Mueller Hinton as the culture medium. The ''susceptible'' category was derived from the extrapolation of the standardized cut-off values for the *M. catarrhalis* species, which is the only species of the genus with standardized cut-off values[1]. The following antimicrobials were tested: amoxicillin-clavulanate, erythromycin, azithromycin, tetracycline, and trimethoprim-sulfamethoxazole.

Patient isolates were analyzed by PFGE to assess genetic relatedness. In brief, chromosomal DNA from the *Moraxella* isolates was digested with restriction endonucleases *SmaI* and *XbaI* to determine their typeability and discriminatory power. Digestion was performed with 30 units of *XbaI* (10 U/μl, Fermentas) for 3 h at 37 °C and with 50 units of *SmaI* (10 U/μl, Invitrogen) for 3 h at 30 °C. Salmonella Braenderup H9812 was used as a molecular weight marker, which was digested using *XbaI* enzyme under the same conditions as the ones previously described. With regard to the running conditions, an initial time of 5 s was used and then a final time of 35 s, the total run-time was 19.5 h. Banding patterns were visually analyzed and interpreted following the criteria described by Tenover et al. (1995) for the typing of bacterial species[6].

Gram staining showed gram-negative coccobacilli or diplobacilli. Isolates were neither hemolytic nor pigmented. Of the 17 isolates analyzed, 15 turned to be *M. lacunata* and 2 were classified as *M. nonliquefaciens* by biochemical tests.

Table 1 shows the traditional biochemical tests that allow to identify different species of *Moraxella*.

Using API® 20NE, the biocode obtained for 15 isolates was 1–0–1–0–0–0–0, 100% concordant with *M. lacunata* species. For the remaining two isolates, the obtained biocode was 1–0–0–0–0–0–0, 100% concordant with *Moraxella* spp. Identification by MALDI-TOF MS showed the following results: 4 isolates were identified as *Moraxella* sp., with score values between 1860 and 1944, 11 as *M. lacunata* with score values between 2012 and 2443; and 2 isolates were identified as *M. nonliquefaciens* with score values between 2146 and 2187. Taking into account the 4 isolates identified to the genus level through MALDI-TOF hydrolyzed gelatin,

Table 1 Traditional biochemical tests that allow the identification of different species of *Moraxella*.

	M. lacunata	*M. nonliquefaciens*	*M. atlantae*	*M. osloensis*	*M. lincolnii*	*P. phenylpiruvicus*	*M. catarrhalis*
Nutritional requirement	–	–	+	–	–	+ (with Tween80)	–
Colony size	Small	Small	Small	Small	Small	Small	Regular
Pyrrolidonyl aminopeptidase	–	–	+	–	–	–	–
Susceptibility to desferrioxamine	R	R	V	S	S	R	R
Acidification of Ethylene glycol	v	–	–	+	–	+	–
Urease	–	–	–	–	–	+	–
Nitrate reduction	+	+	–	V	–	V	+
Nitrite reduction	–	–	–	–	–	–	+
Gelatin hydrolysis	+	–	–	–	–	–	–
DNase	–	–	–	–	–	–	+
Growth on MacConkey agar	–	–	+	v	–	v	–
Acetate	V	–	–	+	–	v	–

Adapted from Vaneechoutte et al.[8]

it is worth mentioning that combining basic biochemical tests and MALDI-TOF MS enabled a correct identification at the species level of the *Moraxella* spp. isolates that were referred to the RL. It should be noted that retrieving *M. catarrhalis* from corneal abscesses in the Eye Hospital is about 1 out of every 100 *Moraxella* isolates, which shows a clear predominance of the *M. lacunata* species in this type of infection.

With regard to antibiotic susceptibility, only 8 of 17 isolates grew on Mueller Hinton. In 8 strains, antibiotic susceptibility testing was carried out in Mueller Hinton medium with 5% horse blood. One of the isolates did not grow on any media tested, therefore, its susceptibility profile could not be determined. All 16 isolates were susceptible to all the antibiotics tested.

Of the two enzymes that were tested, it was only with *SmaI* that interpretable restriction patterns could be obtained. Therefore, *SmaI* was selected to test all the strains. Fig. 1 shows the resolution of the PFGE patterns produced by *SmaI* and *XbaI* in a subgroup of the total isolates tested. Of the 15 *M. lacunata* isolates analyzed, 13 different digestion patterns were obtained. As shown in Fig. 2, the isolates were distributed into eight clonal types (designated with letters A–H) depending on whether there was a difference of four or more bands among the profiles. Clonal type A was divided into subtypes (A1–A6) since there was just one difference of 1 or 3 bands among these isolates. Subtypes A1 and A2 are comprised of two isolates each, which showed the same banding pattern. The two *M. nonliquefaciens* isolates, identified as 791 and 403, showed a difference of more than 4 bands from one another.

With regard to antibiotic susceptibility, all isolates were susceptible to the five antimicrobials tested. As for the antibiotic treatment administered to the infected patients, after the ocular sample was collected, an intensive medical treatment was recommended, which included fortified antibiotic eye drops of vancomycin (50 mg/ml) and ceftazidime (50 mg/ml), and cycloplegic eye drops for pain management. Once the identification and susceptibility results were obtained, vancomycin was removed and the treatment continued with ceftazidime eye drops and, in some cases, with moxifloxacin eye drops as well.

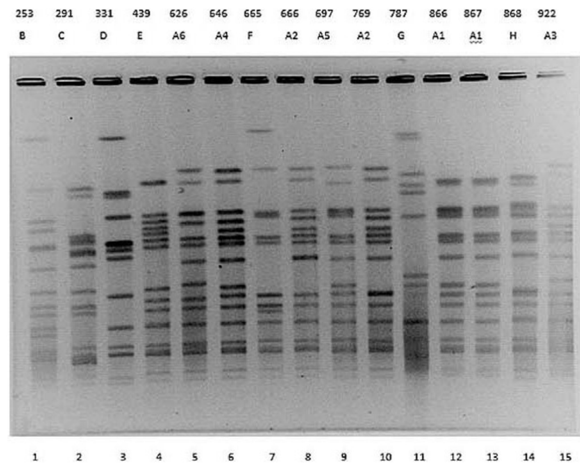

Figure 2 SmaI-PFGE of the clonal types (A–H) and subtypes (A1–A6) obtained from the 15 *M. lacunata* isolates that were analyzed.

Comparing the results obtained by PFGE with restriction enzymes *XbaI* and *SmaI*, it could be observed that the digestion with *XbaI* produced low molecular weight fragments that could not be adequately resolved in the gel; therefore, this enzyme was considered inappropriate for the analysis of these species by PFGE. The same result was documented for *M. catarrhalis* when digestion with *XbaI* was analyzed[9]. Thereby, *SmaI*, another restriction enzyme, was tested. In most of the literature reviewed, *SpeI* is used for PFGE of *M. catarrhalis*, being its drawback that it is a very expensive restriction enzyme, which is not available in our laboratory.

SmaI produced restriction patterns ranging from 11 to 15 bands, which enabled sample comparison and typification.

Thirteen (13) different restriction profiles can be observed among the 15 *M. lacunata* isolates. Subtypes A1 and A2 appear to be genetically indistinguishable, since their PFGE patterns have the same number of bands with the same apparent size. The remaining clonal subtypes represent a single isolate. Due to the genetic diversity obtained, an outbreak could be ruled out and they were, evidently, different isolates. Based on the analysis following Tenover's criterion[6], those isolates that belong to the same clonal type were considered closely related isolates.

This study demonstrated that digestion with a single enzyme, *SmaI*, can confirm or rule out the clonal relationship of *M. lacunata* and *M. nonliquefaciens* isolates by PFGE. Moreover, it was concluded that the use of MALDI-TOF MS and biochemical tests, alone or in combination, allows the identification of *M. lacunata* and *M. nonliquefaciens*. It is important to mention that 16S rRNA gene sequencing is another useful tool to differentiate *Moraxella* species such as *M. catarrhalis*, *M. nonliquefaciens*, *M. lincolnii and M. osloensis*. With reference to antibiotic susceptibility, it should be noted that the report should clarify that the interpretation of the categories ''susceptible'', ''resistant'' or ''intermediate'' is based on the extrapolation of *M. catarrhalis* cut-off points, and that in the future it should be evaluated whether this extrapolation is valid for all *Moraxella* species. This article highlights the importance of surveillance in the distribution of *Moraxella* species in corneal abscesses to recognize changes over time, to detect the emergence of clones and, if they do emerge, to reveal if they are associated with some antibiotic resistance in particular.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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