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Phenotypic Differences in *Moraxella catarrhalis* isolated from Sudanese Patients presenting with Respiratory and Otitis Media Infections

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Abstract

Background: Over the last 20 to 30 years, *Moraxella catarrhalis* (*M. catarrhalis*) had emerged as a genuine pathogen and now it is considered an important cause of upper respiratory tract infection in healthy children and elderly people. It is an important cause of chronic obstructive pulmonary disease, acute and chronic otitis media, sinusitis, acute bronchitis and pneumonia.
Objective: To study the phenotypic differences in *Moraxella catarrhalis* isolated from Sudanese patients presenting with respiratory and otitis media infections.
Materials and methods: This was a descriptive, cross sectional, hospital-based study conducted during the period from June 20l0 to August 2011 at Al Shaab Teaching Hospital and Soba University Hospital, Khartoum (Sudan). 110 swabs were collected from children suffering from middle ear discharge, and 290 sputum samples were collected from patients presenting with lower respiratory tract infection. 19 *Moraxella catarrhalis* strains were isolated by conventional procedures. Phenotypic characteristics for all isolates were studied. All isolates were investigated for β-lactamase activity using nitrocefin disk assays. The susceptibility pattern was also conducted for all strains identified.
Results: 19 *Moraxella catarrhalis* strains were isolated. 14 *isolates* (73.7%) were recovered from sputum samples; and five isolates (26.3%) were recovered from ear discharges. All isolates were Gram-negative diplococci, gray to white in colour, opaque, and smooth. They were catalase positive, oxidase positive, DNase test positive, reduce nitrate to nitrite, tributyrin test positive, and were able to grow on nutrient agar at room temperature. All isolates showed positive β-lactamase test. Other organisms isolated in association with *Moraxella catarrhalis* were *Pseudomonas, Klebsiella, Streptococcus pneumoniae,* and *Haemophilus influenzae.* All *Moraxella catarrhalis* isolates were found sensitive to amoxyclav, azithromycin, ceftazidime, ceftriaxone, cephalexin, cephotaxime, chloramphenicol, ciprofloxacin, and co-trimoxazole. 90 %

of them were sensitive to erythromycin and resistant to ampicillin.

Conclusion: All *Moraxella catarrhalis* strains isolated in this study produce β- lactamase enzyme. *Moraxella catarrhalis* may spread its β- lactamase property to other organisms and lead to bacterial drug resistance.

Key words: Phenotyping, *Moraxella catarrhalis,* Respiratory and otitis media infections.

Introduction

Moraxella catarrhalis is a Gram-negative aerobic diplococci usually a commensal of the respiratory tract of humans. It frequently implicates in human disease. It was considered as one of the main pathogens of community-acquired pneumonia. In immunocompromised hosts, the bacterium can cause a variety of severe infections including pneumonia, endocarditis, septicemia, and meningitis[1].
M. catarrhalis is now accepted as the third most respiratory tract pathogen after *Streptococcus pneumoniae* and *Haemophilus influenzae*. Reports of hospital outbreaks of respiratory disease caused by *M. catarrhalis* had established the bacterium as nosocomial pathogen[2].
Recent studies had considered *M. catarrhalis* a pathogen in cleft palate repairs. Phenotypically *M. catarrhalis* population may be subdivided into two distinct genetic lineages as per their ability to resist the destructive effect of human serum (i.e. complement resistance versus complement sensitivity) and the difference in their ability to adhere to human epithelial cells. Recent reports indicate that a population expansion (including the acquisition of virulent genes) had probably occurred within sero-resistant lineage of *M. catarrhalis* around the time of hominid expansion some of 5 million years ago[2].

There has been rapid acquisition and spread of Beta–lactamase resistance of *M. catarrhalis* during the last 20 to 30 years, and approximately 95 clinical isolates now appear to be resistant to one or more beta lactams. In general, approximately 95 to 99% of clinical *M. catarrhalis* isolates produce beta-lactamase, a startling and unpredicted rate of increase in beta-lactamase positivity since the identification of the first beta-lactamase producing isolates in 1977. Recent reports in Turkey indicated that beta lactamase production was more than 93%[3].
Clinical isolates were almost invariably resistant to penicillin, amoxicillin, and ampicillin. However, the majority of clinical isolates appear to be sensitive to other widely used antibiotics, including fluoroquinolones (ciprofloxacin), tetracycline (doxycycline), and macrolides (erythromycin) [4].
Over the last 20-30 years, *M. catarrhalis* has emerged as a genuine pathogen and is now considered as an important cause of respiratory tract infection in healthy children and elderly people. Recently *M. catarrhalis* is considered to be the third most common and most important isolate after *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* as the causative agent of bronchopulmonary infection, otitis media, which is the leading cause of

conductive hearing loss in children, and of exacerbation of chronic obstructive pulmonary disease in adult, which is the fourth leading cause of death worldwide. Many reports of *M. catarrhalis* infection were published in western countries; however, no such reports were made in Sudan and other developing countries, where cases may not be reported or isolation of *Moraxella catarrhalis* is overlooked and considered as normal commensal of the respiratory tract. Also this may be due to the lack of awareness of *Moraxella catarrhalis* role as a pathogen of the lower respiratory tract.
This study, therefore, was to highlight the importance and significance of *Moraxella catarrhalis* as a pathogen among Sudanese patients.

Materials and methods

This is a descriptive, cross-sectional, laboratory and hospital-based study conducted to determine the frequency of *M. catarrhalis* in children with otitis media as well as adult patients suffering from respiratory tract infection; during the period from June 20l0 to August 2011.The specimens were collected from patients attending Al Shaab Teaching Hospital and Soba University Hospital, Khartoum (Sudan). Inclusion criteria were all patients presenting with chronic respiratory tract infection, immunocomptency, and acute otitis media; as well as patients above 20 years old and children less than 10 years old. Complete information regarding the study risk factors was handed to all participants without concealment what so ever. Confidentiality of information collected from the study participants was maintained. Valid verbal consent of all patients under the study was obtained. Results of specimens collected were handed to all patients included in the study and some results were dispatched to physicians for treatment of patients. Permission to collect the specimens was obtained from the Ear, Nose, and Throat Teaching Hospital, Al Shaab Teaching Hospital, and Soba University Hospital (Khartoum, Sudan). Approval to conduct the study was granted by Sudan University of Science and Technology (Khartoum). The software used for the analysis of data was the Statistical Package for Social Sciences (SPSS) program (version 14). For categorical variables, proportions were compared by the chi-square test as appropriate. The means and medians of the continuous variables were compared by Student's *t* test program depending on the sample distribution. Frequencies, percentages, tables and graphs were used for presentation of the data.
110 swabs were collected from children suffering from middle ear discharge, and 290 sputum specimens were collected from patients presenting with signs and symptoms of lower respiratory tract infection. The sample size (400 samples) was calculated according to the following equation: $n = (1.96)^2 pq / L^2$; where: n = the designed sample size; p = the estimated prevalence (percentage); q = 1-p; L = allowable error. p = 0.04; q = 1-0.04; and L = 0.02.
A structural questionnaire was designed to collect demographical and clinical data. The diagnosis of otitis media was made by a pediatrician, a family physician, or an otolaryngologist. Early morning sputum following a deep cough was collected in clean, wide-mouth, and leak-proof container. Ear discharges were collected using sterile cotton wool swabs. Specimens were labeled and transported same day in Amie's transport media to the laboratory.

Macroscopical inspection of sputum was made to note its appearance, colour, consistency; and if it was purulent, mucoid, salivary, or bloody. Salivary sputa were discarded to insure validity of the specimen. Direct Gram stain was performed for sputum and ear swab specimens. All the specimens were inoculated on Mac Conkey agar, sheep blood agar, and chocolate agar (supplemented with 10µg vancomycin, 2µg amphotericin B, and 10µg sodium acetazolamide). Plates were incubated at 37°C in a Co_2 candle jar for 24-48 hours. Then isolates in these primary plates were identified by their colonial morphology, indirect Gram's stain, positive oxidase test, positive catalase test, nitrate reduction, positive DNAse test, and ability to grow on nutrient agar. Also a positive tributyrin test (Sigma Aldrich-Germany) was performed to differentiate between *M. catarrhalis* and *Neisseria* species, using the ATCC 25240 American type culture collection as positive control. Enough colonies of *M. catarrhalis* were suspended in a tube containing 1ml of normal saline, then tributyrin strips were dropped into the suspension, and test sample was incubated at 37ºC for 18-20 hours, together with positive and negative controls. A positive reaction changes the red color of the test to yellow, indicating positive *M. catarrhalis* (Fig.1).
The following criteria were considered for determining the pathogenic significance of *Moraxella catarrhalis* isolate[5]:
1. Clinical evidence of infection with *Moraxella* consistent with the disease spectrum associated with *M. catarrhalis* (cough with sputum production and otitis media).
2. *Moraxella catarrhalis* as a predominant potential pathogen isolated from an appropriate and adequate specimen.
3. Clinical response on treatment with antibiotic to which isolate was susceptible.
Taking the above criteria into consideration, the interpretation was as follows:
* Significant isolate: if criteria 1, 2 and 3 were present.
* Probably significant isolate: if criteria 1 and 2 were present and 3 could not be assessed.
* Indeterminate significant isolate: if only criterion 1 was present.
* Not significant: if none of the criteria were present.
Control strains used were: ATCC 23246, ATCC 25238, ATCC 25240, ATCC 49143 (American type culture collection) and CCUG 58268 (University of Goteborg type culture collection).

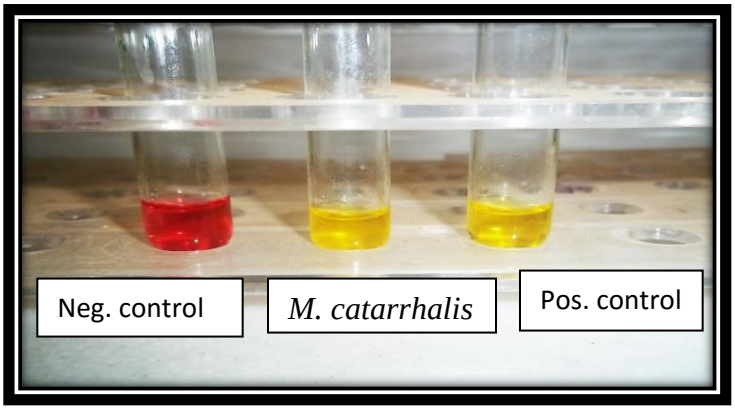

Fig. 1: Positive tributyrin test

Antibiotic susceptibility testing was performed for all isolates of *M. catarrhalis* using Kirby-Bauer disk diffusion method. Antibiotic discs used were amoxyclav, ampicillin, azithromycin, ceftazidime, ceftriaxone, cephalexin, cephotaxime, chloramphenicol, ciprofloxacin, co-trimoxazole, and erythromycin. Mac Farland standard was used for preparing the standard for test and control inocula, with a turbidity suspension of approximately 1.5×10^8 bacterial cells per ml. Mueller Hinton agar plates were inoculated with *M. catarrhalis* isolates, incubated overnight, and susceptibility was interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines.

Nitrocefin disks (Sigma Aldrich-Germany) were used for rapid detection of β-lactamase enzyme produced by *M. catarrhalis*. Nitrocefin disks were first moistened with one drop of deionized water, placed into clean empty petri dish, and several pure colonies were applied onto the surface of disks. ATCC 25240 strain (American type culture collection) was used as negative control and CCUG 58268 strain (University of Goteborg type culture collection) was used as a positive control. Formation of a red spot on the disk indicates β- lactamase producing colonies.

Minimum inhibitory concentration (MIC) was determined by the HiComb method (Himedia, India). 200 mm Mueller Hinton agar (Oxoid) plates were streaked three times with a suspension of *M. catarrhalis* having opacity of 0.5 Mac Farland turbidity using sterile cotton swabs. The plates were allowed to dry up, and HiComb strips were placed on the medium. Other strips were placed on the opposite side of the plates with their higher concentration side towards the edge of plate, and their lower concentration side towards the centre of the plate. The plates were then incubated at 37^{O}C and were examined after 24 hours. MIC was read according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (Fig.2).

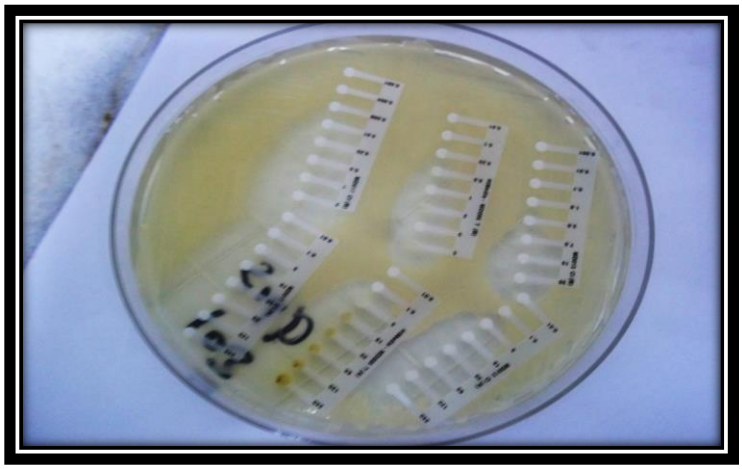

Fig. 2: MIC determination by HiComb method

Results

A total of 290 good quality sputa and 110 ear swabs were screened for *Moraxella catarrhalis.*

19 *Moraxella catarrhalis* strains were isolated by conventional procedures. 14 *isolates* (73.7%) were recovered from sputum samples; and five isolates (26.3%) were recovered from ear discharges. All isolates were Gram-negative diplococci, gray to white in colour, opaque, smooth, and slide across the surface of the agar when nudged with the end of bacteriological loop (hockey puck on ice sign). They were catalase positive, oxidase positive, DNAse test positive, reduce nitrate to nitrite, tributyrin test positive, and were able to grow on nutrient agar at room temperature. All isolates showed positive β-lactamase test as per nitrocefin disk assays.
Other organisms isolated in association with *Moraxella catarrhalis* from sputum samples were *Pseudomonas* aeruginosa and *Klebsiella* pneumoniae. From ear swabs *Streptococcus pneumoniae* and *Haemophilus influenzae* were isolated in association with *Moraxella catarrhalis.*

Patients investigated were 253 (63.2%) males and 147 (36.8%) females. 105 children (26.3 %) and 295 (73.7%) adults were included in this study. The adults investigated were in the age range 22-72 years; while five children were in the age range 5-40 months. Marginally significant correlations were observed between *M. catarrhalis* culture results and age groups ($p = 0.027$), while there are no significant correlation were observed between *M. catarrhalis* culture results and gender groups ($p = 0.603$)
Moraxella catarrhalis was also isolated from patients suffering from liver disease (5.2%), diabetes mellitus (10.5%), chronic bronchitis (5.2%), and pneumonia (10.5%). Some patients (15.8%) were chronic smokers and some patients (42.1%) were more than 50 years old. Two patients were diagnosed as cases of chronic obstructive pulmonary disease but *Moraxella catarrhalis* was not isolated from sputum samples collected from them.
Regarding susceptibility testing, all *Moraxella catarrhalis* isolates were found sensitive to Amoxyclav, azithromycin, ceftazidime, ceftriaxone, cephalexin, cephotaxime, chloramphenicol, ciprofloxacin, and co-trimoxazole. 90 % of them were sensitive to erythromycin and resistant to ampicillin.

Discussion

Over the last few decades, *M. catarrhalis* had emerged as genuine pathogen, and now it is considered an important cause of upper respiratory tract infections in otherwise healthy children and elderly adults[4].
The clinical interest in *M. catarrhalis* is relatively recent, and many laboratories are not reporting *M. catarrhalis* as a pathogen, especially when a well-recognized pathogen (*Streptococcus pneumoniae* or *Haemophilus influenzae*) is isolated as well in the clinical sample[1].
The difficulty in distinguishing *M. catarrhalis* from *Neisseria* species explains why *M. catarrhalis* had been overlooked as a respiratory tract pathogen[5].
The present study showed that the frequency rate of *Moraxella catarrhalis* was 4.75% (19/400). A slightly higher frequency rate (6.9%) was reported by Tamang and his co-workers[6] among elderly population.

In this study five *M. catarrhalis* strains (4.5%) were isolated from children aged 1 month to 5 years. No *M. catarrhalis* strains were isolated from patients aged 6-15 years old. This result was not similar to that of Constantinescu[7] who reported an isolation rate of 9% in children less than 5 years old, and 33% rate in children aged 6-10 years. Nearly similar result was reported by Broides and his colleagues[8] who found an isolation rate of 4.8% in children less than 5 years. Version[9] reported an isolation rate of 3-20% in children with otitis media infection. Kilpie and his co-authors[10] reported a frequency rate from 10-23% in otitis media patients less than 15 years. In Costa Rica, the prevalence of *M. catarrhalis* isolated from middle ear fluid (MEF) among children with acute otitis media (AOM) aged 3-14 months was 2.5%; and it was found more prevalent during the dry season in children aged less than 24 months[11].
Out of the 14 lower respiratory tract infection patients from whom *M. catarrhalis* was isolated in this study, nine patients (64.3%) were males, and five patients (35.7%) were females. Gupta and his colleagues[12] had reported that lower respiratory infection due to *M. catarrhalis* was diagnosed among 77.7% males and among 22.25% females.

In the current study, *M. catarrhalis* otitis media infection was found associated with *Streptococcus pneumoniae and Haemophilus influenzae* in two samples (40%) out of five *M. catarrhalis* isolates. A similar study performed by Ahmed and his co-workers[13]who showed six cases (35.3%) of co-infection out of 17 *M. catarrhalis* otitis media cases.
Age of the patient is a critical determinant of the pathogenicity of isolates of *Moraxella catarrhalis.* With advancing age, the pathological significance of these isolates becomes greater[14].
In the present study, the common predisposing factors were old age (42.1%), chronic productive cough (42.1%), and smoking (15.8%). This agrees with the findings of Tamang and his co-authors[6] who found the most important predisposing factors to be old age and history of smoking.
Recent studies had shown that *M. catarrhalis* binds to the major glycoprotein basement membrane which becomes thickened and interferes with airways among smokers[12].
Other studies had shown that elderly patients are liable to have respiratory tract infections that are caused by *Moraxella catarrhalis.* Most of these patients may have an underlying lung disease as well as diabetes mellitus, corticosteroid therapy, and malignancy[6].
This study found no good correlation between chronic obstructive pulmonary disease (COPD) and *Moraxella catarrhalis* infection; while other workers[13] reported a correlation of approximately 10% with COPD.
In the present study all *Moraxella catarrhalis* isolates were β-lactamase producers. Anita and his colleagues[15] reported an isolation rate of 84% *Moraxella catarrhalis* β- lactamase producers. Another study conducted in Taiwan found a rate of 97.8% β- lactamase production[16].
The dramatic increase in the frequency rate of β- lactamase producing *Moraxella catarrhalis* could be regarded as the fastest dissemination of any known β- lactamase producing bacterial species[17]. In this study *Moraxella catarrhalis* was found sensitive to amoxicillin- clavulanic acid, ciprofloxacin, chloramphenicol, erythromycin, and ceftriaxone.

These findings disagreed with the report of Tamang and his co-workers[6] who found 4% of *Moraxella catarrhalis* isolates were resistance to amoxicillin-clavulanic acid and ceftriaxone as well as 8% of *Moraxella catarrhalis* isolates were resistance to ciprofloxacin.
However Hsu and his co-authors[16] found that all *Moraxella catarrhalis* isolates were susceptible to amoxicillin- clavulanic acid.
On the other hand, Gupta and his colleagues[12] found that *Moraxella catarrhalis* isolates had shown a maximum resistance to co-trimoxazole (82.2%), and ampicillin (71.4%); and a maximum susceptibility to cefotaxime (87.3%), followed by tetracycline (85.7%), ciprofloxacin (84.1%), and gentamycin (77.7%).
From this study it may be recommended that all microbiology laboratories should master the methods needed for the isolation, identification, and antibiotic sensitivity testing of *Moraxella catarrhalis.* Clinicians should select antibiotics needed for treatment of *Moraxella catarrhalis* infection based on a sensitivity report from the laboratory. Development of a vaccine against *M. catarrhalis* infections is highly indicated.
Conclusion: All *Moraxella catarrhalis* strains isolated in this study produce β- lactamase enzyme, and they may spread their β- lactamase property to other organisms and lead to multiple bacterial drug resistance

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