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**Detection of \textit{bro} \(\beta\)-lactamase gene of \textit{Moraxella catarrhalis} isolated from Sudanese Patients presenting with Respiratory and Otitis Media Infections**

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**Abstract**

**Background:** \textit{Moraxella catarrhalis} (\textit{M. catarrhalis}) 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. \(\beta\)-lactamase production is associated with chromosomal carriage of two similar genes: \textit{bro-1} and \textit{bro-2}). They may lead to 2-3 fold decrease in expression of lactamase enzyme.

**Objective:** To detect \textit{bro} \(\beta\)-lactamase gene of \textit{Moraxella catarrhalis} isolated from Sudanese patients presenting with respiratory and otitis media infections.

**Materials and methods:** 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 \textit{Moraxella catarrhalis} strains were isolated by conventional procedures. Positive \textit{M. catarrhalis} isolates were confirmed by polymerase chain reaction (PCR) technique. \(\beta\)-lactamase production was inspected for each isolate using nitrocefin disks. \(\beta\)-lactamase positive \textit{M. catarrhalis} isolates were examined for the presence of \textit{bro} \(\beta\)-lactamase gene using restriction fragment length polymorphism (RFLP) technique.

**Results:** 19 samples (4.7\%) of those collected (400) were found positive for \textit{Moraxella catarrhalis}. Of these, 15 isolates (78.9\%) showed typical bands of \textit{M. catarrhalis} while 4 isolates (21.0\%) were found negative. All of \textit{M. catarrhalis} isolates were confirmed as \(\beta\)-lactamase producer. From these 11 isolates (73.3\%) were found positive for a \textit{bro-1} gene by RFLP technique whereas 4 isolates (23.7\%) were found \textit{bro} gene negative.

**Conclusion:** All \textit{Moraxella catarrhalis} strains of this study produce \(\beta\)-lactamase enzyme, and they may spread their \(\beta\)-lactamase property to other organisms and lead to multiple bacterial drug resistance

**Key words:** Genotyping, \textit{Moraxella catarrhalis}, Respiratory and otitis media infections.

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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\(^1\).

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\(^2\).

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\(^3\).

There has been rapid acquisition and spread of β–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\(^4\).

In general, approximately 95 to 99% of clinical *M. catarrhalis* isolates produce β-lactamase, a startling and unpredicted rate of increase in β-lactamase positivity since the identification of the first β-lactamase producing isolates in 1977\(^5\).

Recent reports in Turkey indicated that β-lactamase production was more than 93%\(^6\).

β-lactamase production itself is associated with chromosomal carriage of two similar genes called (bro-1, bro-2); the difference being a single amino acid change in the bro-2 protein, and a 21 base pair deletion in the bro-2 promotor region that leads to 2-3 fold decrease in expression of bro-2 beta lactamase. The sequence and genetic context of bro genes suggest that bro-2 was acquired by interspecies gene transfer, possibly from a Gram positive organism; and that bro-1 involved from bro-2 and spread by horizontal transfer via subsequent transformational events. Isolates carrying bro-1 are usually more resistant to ampicillin than those carrying bro-2\(^7\).

Because the overwhelming majority of clinical isolates possess a bro gene, clinical isolates were almost invariable resistance 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)\(^8\).

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This study, therefore, was conducted to detect bro β-lactamase gene of *Moraxella catarrhalis* and to highlight the importance and significance of *Moraxella catarrhalis* pathogenicity 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 2010 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, immunocompetency, 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:

\[ n = \left(1.96\right)^2 \frac{pq}{L^2}; \]

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 acetzolamide). Plates were incubated at 37°C in a CO₂ candle jar for 24-48 hours. Then isolates in these primary

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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 37°C 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*.

The following criteria were considered for determining the pathogenic significance of *Moraxella catarrhalis* isolate:

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). 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).

DNA extraction was performed by employing GF-1 bacterial DNA extraction method (Vivantis). 1-3 ml of an overnight *M. catarrhalis* culture was pelleted by centrifugation at 6000x g for 2 min. at room temperature. The supernatant was decanted completely. Then 100 ul of buffer reagent 1 (R1) was added to the pellet and the cells were re-suspended by pipetting. 10 ul of lusozume (50 mg/ml) were then added to the cell suspension, mixed thoroughly, and incubated at 37°C for 20 min. The digested cells were pelleted by centrifugation at 10,000xg for 3 min. The supernatant was decanted, the pellet was re-suspended in 180 ul of buffer reagent 2 (R2), and 20 ul of proteinase K were added. This pellet was mixed, and incubated at 65°C for 20 min. with interrupted mixing every 5 min. Two volumes of buffer BG were added and tubes were mixed by inverting several times until a homogeneous solution was obtained. Then tubes were incubated for 10 min. at 65°C, 200 ul of absolute ethanol were added, and immediately mixed to prevent
uneven precipitation of nucleic acids.
The samples were then transferred into a column that was assembled in a clean collection tube provided by the supplier. These columns were centrifuged at 10,000 x g for 1 min., the flow was discarded, the columns were washed with 750 ul of washing buffer that was previously treated with absolute ethanol, and centrifuged at 10,000 x g for 1 min. The columns were re-centrifuged at 10,000 x g for 1 min. to remove residual ethanol that might affect the quality of DNA and inhibit the enzymatic reactions. The columns were then placed into clean micro-centrifuge tubes, 100 ul of preheated buffer were added, left to stand for 2 min. and then centrifuged at 10,000 x g for 1 min. to elute DNA. The eluted DNA was stored at – 20°C. The amplification was conducted using CONVERGYS pettier thermal cycle (Germany).
Measurement of DNA concentration was performed by a Bio Eppendorf spectrophotometer. Also an aliquot of the DNA was electrophoresed on 1% agarose gel to ensure the purity of the extraction. Confirmation of *Moraxella catarrhalis* isolates was carried out by PCR using GenePack DNA PCR tests (GeneON-Germany). A master mix reagent was prepared. 10µl specific dilution buffer (Gene On), 2 µl bovine serum albumin (BSA), 7µl sterile water, and 1µl template DNA were added separately to each lyophilized DNA-PCR test tube to give a final volume of 20 ul.
To differentiate between *bro-l* and *bro-2* genes of *Moraxella catarrhalis* isolates, the PCR was employed using primer pairs. Primers used in this study (Invitrogen, USA) were forward and reverse primers. Their sequences were:
Forward Primer (5’ to 3’) – TRGTGAAGTGATTTRRMTTG
Reverse Primer (5’ to 3’) – GCAATTATTTAAACTGATGTA
100 µm primer stock was prepared in the tube containing the pellet for both forward and reverse primers. This was done by adding 386 ul and 580 µl to the pellet respectively. Then 10 µm stock primer was prepared using RV/O formula. Both primers of 100 um and 10 um were stored at -20°C. A master mix reagent was prepared. 25µl 2X tag master mix, 2 µl up-stream primers (10 µm stock), 2 µl down-stream primers (10µm stock), 18.2 µl molecular grade water, 0.8 µl MgCl, and 2µl template DNA with concentration of 30ng/µl were added separately to each PCR test tube to give a final volume of 50 ul.
The agarose gel was prepared by placing 2 gm. agarose (GeneOn, Germany) into a 250 ml conical flask together with 100 ml of 1x TBE and the flask was covered by an aluminum foil to prevent water evaporation. The mixture was heated on a hot plate until agarose boiling. The mixture was left to cool down for 5 min. to about 60°C. Then 1-3 ul of ethidium bromide was added, and the gel was poured slowly into the gel tank which was inserted by combs in its correct position. Bubbles arising were pushed sideways by a disposable tip. The tank was left to set for at least 30 minutes and 1x TBE running buffer was poured into the tank to submerge the gel to 2-5 mm depth.
The 100 bp DNA ladder (GeneOn, Germany) used was composed of 10 individual DNA fragments with a concentration of 0.2 µg/ul. The markers were prepared by adding 1 volume of ladder into 4 volumes of loading dye. 2ul of loading buffer were added to 7ul of each sample.

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The first well was loaded with markers and the rest were loaded with controls & test samples respectively. The gel tank was closed and the process was started. It was stopped when the bromophenol blue dye had run 3/4 the length of the gel. Then the gel was carried to the dark room to visualize over the ultra violet transilluminater and to photograph using gel documentation system.

These pairs yield amplicons differing in size by 21 bp (bro-1 165 bp and bro-2 144 bp). To confirm bro-1 or bro-2 a restriction fragment length polymorphism (RFLP) was performed. To determine restriction fragment length polymorphism, a master mix reagent was prepared. 2µl restriction buffer (New England biolabs, USA), 0.5 µl restriction enzyme Tsp509i (New England biolabs, USA), 0.8 µl PCR product, and 4.5 µl molecular grade water were added separately to each PCR test tube to give a final volume of 15 ul. After that the tubes were incubated at 65°C for 30 minutes.

Electrophoresis was performed on the enzyme digests using 3.5% agarose. The 50 bp DNA ladder (GeneOn, Germany) used was composed of 10 individual DNA fragments with a concentration of 0.2 ug /ul. 2ul of loading buffer were added to PCR product. Tsp509i cleaved the bro-1 region of interest into two visible fragments of 55 and 91 bp, while bro-2 was left with a visible fragment of 91 bp (Fig. 1).

![Fig. 1: Moraxella catarrhalis PCR amplicon on 2% agarose gel](image)

Lane 1= Molecular weight marker   Lane 2 and Lane 3 = Positive control
Lane 4= Negative control     Lane 5 – Lane 8 = Positive *M. catarrhalis* bands (550 bp).

**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. Patients investigated were 253 (63.2%) males and 147 (36.8%) females. 105 children

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(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 \textit{M. catarrhalis} culture results and age groups (p = 0.027), while there is no significant correlation were observed between \textit{M. catarrhalis} culture results and gender groups (p = 0.603)

\textit{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 \textit{Moraxella catarrhalis} was not isolated from sputum samples collected from them. All isolates showed positive β-lactamase test as per nitrocefin disk assays. On the other hand, the MIC\textsubscript{50} value (where 50% of the isolate were inhibited) and MIC\textsubscript{90} value (where 90% of the isolate were inhibited) obtained for positive \textit{bro} gene strains of \textit{Moraxella catarrhalis} showed that these strains were more inhibited by ciprofloxacin & amoxicillin.

Using GenePack DNA PCR, 14 isolates (78.9%) out of the total 19 \textit{Moraxella catarrhalis} isolates were found to show typical band of 550 bp size as indicated by the standard 100 bp molecular weight DNA marker (Fig. 1). The remaining five \textit{Moraxella catarrhalis} isolates were PCR negative. No significant correlations were observed between \textit{M. catarrhalis} culture results and PCR (p= 0.42).

Table (I) exhibited results of PCR- restriction fragment length polymorphism (RFLP) screening and β-lactamase testing that were performed for all 14 \textit{Moraxella catarrhalis} positive PCR isolates to differentiate between the two genes: \textit{bro}1 and \textit{bro} 2. This technique showed that 11 (73.3%) of these isolates were associated with \textit{bro} 1 β-lactamase genes; and three isolates (26.7%) were negative. This association was more noticed among adults 63.6 % (7/11) than among children 36.4% (4/11). All isolates found to be \textit{bro} gene positive were also found to produce β-lactamase enzyme when tested by the nitrocefin disc test.

The frequency rate of \textit{bro} 1 gene among the gender groups was not significant (p= 0.537). Also the frequency rate of \textit{bro}1 gene among the age group was not significant (p= 0.923).

<table>
<thead>
<tr>
<th>Patients</th>
<th>\textit{bro}-1 gene</th>
<th>\textit{bro}-2 gene</th>
<th>\textit{bro} gene negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Children</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>0</td>
<td>3</td>
<td>14</td>
</tr>
</tbody>
</table>

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Discussion

The difficulty in distinguishing *M. catarrhalis* from *Neisseria* species explains why *M. catarrhalis* had been overlooked as a respiratory tract pathogen. The present study showed that the frequency rate of *Moraxella catarrhalis* was (19/4.75%). A slightly higher frequency rate (6.9%) was reported by Tamang and his co-workers 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 nearly similar result was reported by Broides and his colleagues who found an isolation rate of 4.8% in children less than 5 years.

Kilpie and his co-authors reported a frequency rate from 10-23% in otitis media patients less than 15 years. 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 had reported that lower respiratory infection due to *M. catarrhalis* was diagnosed among 77.7% males and among 22.25% females.

In the present study all *Moraxella catarrhalis* isolates were β-lactamase producers. Anita and his colleagues 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. 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. The MIC<sub>90</sub> of ampicillin and amoxicillin-clavulanic acid for *bro-1* gene isolates reported in the present study were 8 and 1 mg/l; while it was 16 and 0.25 mg/l in the study of Mushtaq and his colleagues. Furthermore, MIC of ciprofloxacin for *bro-1* gene *Moraxella catarrhalis* isolates was 15 mg/l as compared to 0.06 mg/l reported by Hus and his co-workers.

In the present context 11 *Moraxella catarrhalis* isolates (78.6%) were found *bro-1* gene positive, and three were *bro* gene negative. Cossins and his co-authors reported an isolation rate of 95.2% *Moraxella catarrhalis* *bro* gene positive; while Rong and his colleagues were able to find an isolation rate of 97.5%. Different figures were reported by Levy and his colleagues who found an isolation rate of 4.8% *Moraxella catarrhalis* *bro* gene positive.

In the present study, three *Moraxella catarrhalis* strains (21.4%) were found negative for *bro-1* and *bro-2* genes. This result was similar to that reported by Cossins and his co-workers and Khan and his co-authors who found a negative isolation rate of 4.8% and 5% respectively. This may suggest the presence of a third *bro* gene as postulated by Rong and his colleagues.

Conclusion: All *Moraxella catarrhalis* strains of this study produce β- lactamase enzyme, and they may spread their β- lactamase property to other organisms and lead to multiple bacterial drug resistance.

References


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