**Introduction**

*Moraxella catarrhalis* (*M. catarrhalis*) is a gram-negative diplococcal commensal of respiratory tract of human that is also frequently implicate as a cause of other human diseases.[1,2] The species is frequently associated with respiratory tract infections, including acute and chronic otitis media, sinusitis, acute bronchitis, and pneumonia.[3]

As a pathogen, *M. catarrhalis* tends to be generally associated with upper respiratory tract infections in children and lower respiratory tract infections in adults especially adults with preexisting pulmonary disease.

Several virulence-associated genes have been identified in *M. catarrhalis* including outer membrane protein UspA1, UspA2, Hag, OMPCD CopB, and lipooligosaccharide.[4-8]

In general, approximately 95%-99% of clinical *M. catarrhalis* isolates produce β-lactamase, a startling and unprecedented rate of increase in β-lactamase positivity since the identification of the first β-lactamase producing clinical isolates in 1977.[9-11] Recent reports in Turkey indicated that β-lactamase production is more than 93%.[12,13] β-lactamase production itself is associated with chromosomal carriage of two similar genes called BRO-1 and BRO-2, the difference between which is attributed to 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 β-lactamase.[14]

The sequence and genetic context of BRO gene suggest that BRO-2 was acquired by interspecies gene transfer, possibly from a gram-positive organism[15,16] and that BRO-1 evolved 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.[15]
Because the overwhelming majority of clinical isolates possess a BRO gene, 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, for example, ciprofloxacin, tetracycline, for example, doxycycline; and macrolides, for example erythromycin.[17]

Materials and Methods

Ethical clearance
The study was approved by the National Ethical Committee, Ministry of Health, Sudan. Written consent was obtained from every participant before he/she was enrolled in the study.

Study design
The study was a cross-sectional laboratory-based study conducted in Khartoum State.

Collection of the samples
Four hundred sputum samples and ear swabs were collected from eligible patients attending Al-Shaab teaching hospital, Khartoum Center of ENT and Soba University Hospital in Sudan during the period from June 2010 to August 2011.

Phenotypic characterization
The samples were inoculated onto sheep blood agar, chocolate blood agar, and Columbia blood agar supplemented with either vancomycin, amphotericin B, or acetozolamide.[18] They were incubated aerobically at 37 with 5% CO₂ as well as at room temperature for overnight. *M. catarrhalis* isolates were identified according to colonial appearance, Gram staining, catalase reaction, oxidase reaction, reduction of nitrate, ability to grow on nutrient agar at room temperature, DNase production and tributyrin test.

All the isolates were tested for β-lactamase production using nitrocefin disks (Sigma Aldrich, Germany).

DNA extraction
DNA extraction was performed for *M. catarrhalis* isolates using GF-1 bacterial DNA extraction kits (Vivantis Company, Germany). The extraction procedure was carried out according to manufacturer instructions.

Genotypic characterizations
Fifteen *M. catarrhalis* isolates were confirmed using GenePack DNA polymerase chain reaction (PCR) kit (GeneOn, Germany). The PCR thermocycler parameters used were as follows: 95°C for 3 min followed by 95°C for 30 s, 56°C for 60 s, 72°C for 2 min for 37 cycles, and then 72°C for 10 min. After thermocycling, the samples were loaded on 1% agarose gel which was stained by ethidium bromide. A typical band positive for *M. catarrhalis* was 550 bp.

Antibiotic sensitivity testing
Minimum inhibitory concentration values were determined using HiComb (Himedia, India). This system uses dry chemistry technology and consists of two comb-shaped strips made of an inert material with antibiotics disc at the end. Mueller-Hinton agar plates (Oxoid) were used for susceptibility testing, they were inoculated with a suspension of organisms having the opacity of 0.5 McFarland turbidity standard. Inoculated plates were allowed to dry before strips containing antibiotics were applied to the surface of the agar. After incubation for 22-24 h at 37°C in an atmosphere of 5% CO₂, the MICs were read directly from the intersection of the inhibition ellipse with the test strip MIC scale. MIC results were evaluated according to the National Committee for Clinical Laboratory Standards guidelines.

Control strains
The control strains included for both phenotypic and genotypic characterization were ATCC2324, ATCC25238, ATCC25240, ATCC49143 (American type culture collection), and CCUG58268 (culture collection, University of Gothenburg).

**BRO gene PCR-restriction fragment length polymorphism (RFLP)**
To detect BRO β-lactamase types and to distinguish between them, a PCR was firstly performed using one set of primer: BROF 5’-TRGTGAAGTGATTTTKRRMTTG-3’ AND BROOR 5’-GCAATTTATTAACTGGAT-3’, which yielded amplicons differing in size by 21 bp (bro-1 165 bp and bro-2 144 bp) with the following PCR protocol: 94°C for 5 min followed by 37 cycles of 94°C for 30 s, 51°C for 90 s, and 72°C for 30 s with final extension at 72°C for 7 min.[19]

To confirm bro-1 or bro-2, 2.5 uL of PCR product was digested using 2.5 U of the restriction enzyme Tsp509i (New England Biolabs, USA) for 30 min at 65°C.[19] Electrophoresis was performed for enzyme digest using 3.5% agarose at 60V for 25 min. Tsp509i cleaved the bro-1 region into two visible fragments of 55 and 91 bp, while bro-2 was left with a visible fragment of 91 bp.[19] The agarose gel was stained with ethidium bromide and visualized on ultraviolet transilluminator.
Results

Four hundred patients \( (n = 400) \) were enrolled in this study after being given their informed consent. The majority of the candidates were suffering from lower respiratory tract infection \( (290, 72.5\%) \), compared with \( 110 \ (27.5\%) \) with middle ear infection. \( M. \text{ catarrhalis} \) was isolated and identified by conventional methods \( \text{(data not shown)} \) from \( 15 \ (3.75\%) \) patients among which \( 5 \ (33.3\%) \) were found to be children with middle ear infection \( \text{(age range between 5 and 40 months)}, \) whereas \( 10 \ (66.7\%) \) were adults suffering from lower respiratory tract infection \( \text{(age range between 26 and 70 years)} \).

GenePack DNA PCR tests was adopted to confirm \( M. \text{ catarrhalis} \) isolates, all isolates showed typical bands of 550 bp as indicated by the standard DNA marker \( \text{[Figure 1]} \).

Regarding the result of antimicrobial sensitivity, all isolates \( (n = 15) \) showed positive \( \beta\)-lactamase test according to nitrocefin disk assay. The MIC\(_{50}\), MIC\(_{90}\), and MIC range \( \text{[ug/mL]} \) values obtained for \( M. \text{ catarrhalis} \) isolates are listed in Table 1.

Using RFLP, 11 \( \text{Moraxella} \) isolates of the 15 \( \beta\)-lactamase producers \( (73.3\%) \) were BRO-1 positive with a band size of 146 bp after the first PCR run \( \text{[Figure 2]} \). The digest of the first run product which elicited two fragments of 55 and 91 bp was presented in Figure 3. The remaining four isolates \( (23.7\%) \) were BRO negative.

Discussion

The production of new \( \beta\)-lactamase enzyme, designated BRO \( \text{[from Brahamella and Moraxella]} \) by this bacterium was an event that occurred virtually simultaneously around the world.\[20\]

Two distinct BRO \( \beta\)-lactamase enzymes namely BRO-1 and BRO-2 have since been found in strains of \( M. \text{ catarrhalis} \) which were identical in substrate and inhibition profile but differed by single amino acid. The enzymes are encoded by chromosomal genes, and these genes can be relatively transformed from cell to cell by conjugation.\[3,20\]

There has been a rapid rise in the prevalence of worldwide \( \beta\)-lactam-resistant \( M. \text{ catarrhalis} \) since the first reports documented in the late 1970s. This is considered to be the fastest dissemination of any known bacterial \( \beta\)-lactamase.\[21\]

Our findings suggested that all of \( \text{Moraxella} \) isolates \( (100\%) \) were \( \beta\)-lactamase producers, similar results

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Range (mcg)</th>
<th>MIC(_{50})</th>
<th>MIC(_{90})</th>
<th>MIC Range ug\mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>A:240-0.01</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1-1</td>
</tr>
<tr>
<td></td>
<td>B:8-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac</td>
<td>A:240-0.01</td>
<td>0.1</td>
<td>1</td>
<td>0.1-1</td>
</tr>
<tr>
<td></td>
<td>B:4-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ce</td>
<td>A:240-0.01</td>
<td>3</td>
<td>1</td>
<td>1-3</td>
</tr>
<tr>
<td></td>
<td>B:30-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl</td>
<td>A:240-0.01</td>
<td>1</td>
<td>5</td>
<td>1-10</td>
</tr>
<tr>
<td></td>
<td>B:30-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>A:240-0.01</td>
<td>4</td>
<td>8</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>B:4-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>A:240-0.01</td>
<td>0.25</td>
<td>10</td>
<td>0.25-10</td>
</tr>
<tr>
<td></td>
<td>B:5-0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cp</td>
<td>A:240-0.01</td>
<td>5</td>
<td>15</td>
<td>3-15</td>
</tr>
<tr>
<td></td>
<td>B:30-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>A:240-0.01</td>
<td>3</td>
<td>3</td>
<td>3-7.5</td>
</tr>
<tr>
<td></td>
<td>B:30-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cf</td>
<td>A:240-0.01</td>
<td>0.3</td>
<td>0.3</td>
<td>0.004-0.01</td>
</tr>
<tr>
<td></td>
<td>B:2-0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C = chloramphenicol, Ac = amoxyclav, Ce = cephotaxime, Cl = ceftriaxone, Am = amoxycillin, G = centamicin, Ca = ceftazidime, T = tetracycline, Cp = cephalexin, Ca = ceftazidime, Cf = ciprofloxacain

Figure 1: The amplicon of \( \text{Moraxella catarrhalis} \) after polymerase chain reaction on 2% agarose gel: Lane 1 = marker; 2 = Positive control; 5 = negative control; 3-4, and 6-8 positive for \( \text{Moraxella catarrhalis} \) (550 bp)

Figure 2: The bro gene of \( \text{Moraxella catarrhalis} \) after first run of polymerase chain reaction on 2% agarose gel: Lane 1 = marker 100 bp; 2 = positive control (146 bp); 8 = negative control; 3-7 positive BRO of \( \text{Moraxella catarrhalis} \) (between 100 and 200 bp)

Figure 3: The bro gene of \( \text{Moraxella catarrhalis} \) after restriction fragment length polymorphism on 3.5% agarose gel: Lane 1 = marker 50 bp; 2 = positive control; 8 = negative control; 3-7 positive bro-1 for \( \text{Moraxella catarrhalis} \) (two fragment of 55 and 91 bp)
were reported in central, east, and Baltic European countries where it was mentioned that β-lactamase producer may exceeds 99%. Similar studies reported 95%-99% of β-lactamase producers.

In the current analysis, 73% of the isolates were confirmed as BRO positive. All of them were belonging to BRO-1 gene. This result is in line with that of Mushtag et al., (2009) who found that 90% of Moraxella isolates were BRO positive. No BRO-2 gene was detected in this study. These results disagree with most of the studies that confirmed the existence of BRO-2 among β-lactamase producing strains with ratio of more than 10%,[12,13,19,23] This difference may be attributed to the geographical variation which will reflect on microbial genetics. Moreover, the host genetics as well as abuse of antibiotic in developing countries like Sudan may also participate in this difference. However, since this is the first report from Sudan, there are no previous studies from the region to rely on.

In this study, four penicillin-resistant isolates failed to yield PCR product but were still nitrocefin-positive. This may suggest the presence of a third BRO enzyme as postulated by Christensen et al., 1991.[26] However, Levy and Walker (2004),[27] concluded that PCR failure is probably caused by mismatching between the primer and the target sequences rather than the absence of functional gene.

In this study, all the 11 BRO-positive Moraxella isolates were susceptible to amoxyclav, azithromycin, cefazidime, ceftriaxone, cephalaxin, cephotaxime, chloramphenicol, ciprofloxacin, and cotrimoxazole and 10/11 (90.9%) were sensitive to erythromycin but were resistant to ampicillin while one isolate (9.1%) was resistant to erythromycin. Although M. catarrhalis are known to be highly susceptible to macrolides, tetracycline, fluoroquinolones, second- and third-generation cephalosporins and β-lactam/β-lactamase inhibitors combinations, resistance to erythromycin, tetracycline, and ciprofloxacin has been reported occasionally.[28-30]

**Conclusion**

The major β-lactamase gene of M. catarrhalis in Sudan is bro-1 gene. No bro-2 gene was detected in this study. The hypothesis of the presence of a third bro-enzyme needs more focusing because in this study about four (26.7%) of the isolates failed to yield BRO-PCR product. However, M. catarrhalis in Sudan is susceptible to most of the widely used antibiotics.

**References**


Source of support: Nil. Conflicts of Interest: None declared.