Journal of Science and Technology Vol. 13
ISSN -1605 – 427X
Natural and Medical Sciences (NMS No. 2)
sust-journal@hotmail.com

December/2012

Phenotypic and Genotypic Detection of *Moraxella Catarrhalis* Among Patients With Respiratory Tract and Otitis Media Infections

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ABSTRACT

Respiratory tract infections are considerably prevalent worldwide and identifying their aetiological agents is of great medical and therapeutic value. The aims of the present study are to determine the prevalence of *Moraxella catarrhalis* among Sudanese patients infected with respiratory tract infections (upper and lower) and to determine the antibiotic sensitivity pattern of *Moraxella catarrhalis* isolates as well as the risk factors. Samples, which were collected from four hundred patients with upper and lower respiratory tract infections, were cultured. Then suspected *Moraxella catarrhalis* colonies were biochemically tested. Next, positive isolates were confirmed using polymerase chain reaction technique. Finally, antibiotic sensitivity tests were carried out and beta-lactmase production was inspected for each isolate using nitrocefin disks. After tests, 19 (4.7%) from the collected samples were positive for *Moraxella catarrhalis*. Of these, 15 (78.9%) isolates showed typical bands of *M. catarrhalis* while 4 (21.0%) isolates were negative. This study shows that *Moraxella catarrhalis* is an important respiratory tract pathogen in Sudan. The emergence of antibiotic resistance in *Moraxella catarrhalis* suggests that the incidence of these infections may continue to rise.

المستخلص

تعتبر امراض الجهاز التنفسي من الامراض الشائعة والمنتشرة علي مستوي العالم , وتحديد المسبب يعتبر ذو فائدة تشخصية وعلاجية يساهم في تعجيل شفاء المريض ويقلل من نسبة المضاعفات.اجريت هذه الدراسة لتحديد وجود باكتيريا موراكسيلا النزلية في شريحة مرضي الجهاز التنفسي العلوي والسفلي وايضا هدفت الدراسة لتحديد حساسية المضادات الحيوية لهذه البكتريا وايضا العوامل التي يمكن ان تودي الي الاصابة بالبكتريا.جمعت العينات من 400 مريض وشملت البلغم ومسحه من الاذن وتم زراعة العينات في اوساط غذائية تساعد البكتريا علي النمو وتم عمل الاختبارات الكيميائية لتأكيد نوع البكتريا المستهدفة .ثم تم اجراء اختبار البلمرة التسلسلي للبكتريا المعزولة التي تم التعرف علي انها باكتريا موراكسيلا النزلية وبعد ذلك تم عمل اختبار الحساسية للمضادات الحيوية واختبار انزيم البيتا لاكتميز. تم عزل 19 ميكروب باكتيريا موراكسيلا النزلية بنسبة 4,7% وتم تحديد نسبة 78,9% من البكتريا المعزولة موجبة لتفاعل البلمرة التسلسلي ونسبة21,1 % سالبة لهذا الاختبار. وخلصت الدراسة الي وجوب التعاطي مع هذه البكتريا علي الوجه السليم خاصة وان كل البكتريا المعزولة افرزت انزيم البيتا لاكتميز.

KEYWORDS: Beta-lactamase, tributyrin, polymerase chain reaction, nitrocefin disks.

INTRODUCTION

Moraxella catarrhalis, formerly called Neisseria catarrhalis and Branhamella catarrhalis, is a Gram negative aerobic diplococcus frequently found as a commensal of upper respiratory track [1, 2]. Over the last 20 to 30 years, the bacterium has emerged as a genuine pathogen and now is considered as an important cause of upper respiratory tract infections in otherwise healthy children and elderly people [3-5]. Moreover, Moraxella catarrhalis is an important cause of lower respiratory tract infections, particularly in adults with chronic obstructive pulmonary disease (COPD) [6].

Additionally, it has been reported as one of the main pathogens of community-acquired pneumonia (CAP) [7-11]. In immunocompromised hosts, the bacterium can cause a variety of severe infections including pneumonia, endocarditis, septicemia and meningitis [3,12]. *M. catarrhalis* is now accepted as the third most respiratory tract pathogen after *Streptococcus pneumonia* and *Hemophilus influenza* [3, 8].

Reports of hospital outbreaks of respiratory diseases caused by *M. catarrhalis* have established the bacterium as a nosocomial pathogen [13, 14].

A recent study recognized *M. catarrhalis* as a pathogen in cleft palate repairs [15].

M. catarrhalis population may be subdivided into two distinct genetic lineages phenotypically characterized by their ability to resist the destructive effect of human serum (i.e., complement resistant versus complement sensitive) and difference in their ability to adhere to human epithelial cells [16]. Recent report indicates that a population expansion (including the acquisition of virulent genes) probably occurred within seroresistant lineage of *Moraxella catarrhalis* around the time of hominid expansion 5 million years ago [17].

There has been rapid acquisition and spread of Beta–lactmase resistance of *Moraxella catarrhalis* in the last 20 to 30 years to the extent that approximately 95% to 99% of clinical isolates now appear to resist one or more beta lactamase [18-20].

MATERIALS and METHODS

Control strains of *Moraxella catarrhalis* (American type culture collection) were used as the reference strains. These included: ATCC2324, ATCC 25238, ATCC 25240, and ATCC 49143.

Clinical Samples

A total of 110 specimens were collected from children suffering from middle ear infections with ear discharge from Khartoum center of ENT, head and neck surgery and a total of 290 sputum samples were collected from patients attending Alshaab Teaching Hospital and Soba university hospital with signs and symptoms of lower respiratory tract infections. Regarding otitis media, the diagnosis was made by a pediatrician, a family physician and an otolaryngologist. Concerning sputum, samples were collected in clean, wide mouth, and leak proof specimen containers. Data were collected using standardized questionnaire eliciting information such as date, name, gender, smoking, underlying disease, and history of antibiotic treatment.

Bacterial Culture

The samples were inoculated on sheep blood agar chocolate blood agar , Columbia blood agar supplemented with5% sheep blood, vancomycin, amphotricin B, and acetozolamide [21], and incubated over night aerobically at 37^{o}C with 5% CO_2 as well as at room

temperature. *Moraxella catarrhalis* bacteria were identified according to colonial appearance, Gram stain, catalase reaction, oxidase reaction, reduction of nitrate, ability to grow on nutrient agar at room temperature, DNAs production and tributyrin test.

All the isolates were tested for beta – lactmase production using nitrocefin disks (Sigma- Aldrich, Germany).

DNA Extraction

Isolates were incubated over night at 37°C on blood agar plates using GF-1 bacterial DNA extraction kit from (Vivantis Company, Germany). This kit uses a specially treated glass filter membrane fixed into a column to efficiently bind DNA in the presence of high salt. This kit applies the principle of mini column spin technology and the use of optimized buffers ensures only DNA is isolated while cellular proteins, metabolites, salts and other low molecular weight impurities are subsequently removed during the washing steps

Polymerase Chain Reaction

Moraxella catarrhalis isolates were confirmed using GenePack DNA PCR tests provided by (GeneON, Germany). The latter utilize a technique based on polymerase chain reaction. Thus, the specific DNA was detected in the suspected colonies.

All the reagents required for PCR (Tag DNA polymerase, dNTPs, specific primers, salt and stabilizers) were lyophilized in PCR tubes (0.2 ml).

The PCR thermocycler parameters used were initial denaturation step at 95°C for 3 minutes, followed by repeated 37 cycles composed of three stages; 95°C for 30 seconds, 56°C for 60 second and 72°C for 2 minutes, and then a final extension step at 72°C for 10 minutes. After thermocycling, the samples were loaded on agarose gel stained by ethidium bromide. Typical band positive for *Moraxella catarrhalis* was 550 bp.

Susceptibility of Moraxella *catarrhalis* to antimicrobial agents was determined by Kirby Beauer disc diffusion method on Muller Hinton agar. Antibiotics tested included amoxyclav, ampicillin, azithromycin, ceftazidime, ceftriaxon, cephalexin, cephotaxime, chloramphenicol, ciprofloxacin, co- trimoxazole, gentamycin, and erythromycin.

RESULTS

Moraxella catarrhalis was isolated from 19 patients; 12 males (63.2%) and 7 females (36.8%). 14 of the patients were adult (73.7%) with the mean of age being 48.5 (range: 22-72) and 5 were children (26.3%) with the mean of age being 23month (range: 5-40 month).

14 (4.8%) out of 290 sputum samples, showed *Moraxella catarrhalis* isolates. 5 (4.5%) out of 110 specimens collected from children with ear discharge, confirmed *Moraxella catarrhalis* isolates (table 1).

Table 1: Clinical data of patients with Moraxella catarrhalis isolates

Clinical datum	Children	Adult	
		Present (%)	Absent (%)
Otitis media	5	-	-

Liver disease	-	1 (5.3)	18 (94.7)
Diabetes mellitus	-	2 (10.5)	17 (89.5)
Chronic bronchitis	-	1 (5.3)	18 (94.7)
Pneumonia	-	2(10.5)	17 (89.5)
Chronic cough	-	8 (42.1)	11 (57.9)
Smoking	-	3 (15.8)	16 (84.2)
Age more than 50 years	-	8 (42.1)	11(57.9)
History of pulmonary TB		2 (10.5)	17 (89.5)

All the isolates of *Moraxella catarrhalis* were Gram-negative diplococci, hockey puck sign positive, catalase positive, oxidase positive, DNAse producing, nitrate reducing, tributyrin positive and able to grow on nutrient agar at room temperature. All isolates showed beta lactmase production. Characteristics of Moraxella catarrhalis used in its identification are listed in table 2.

Table 2: Characteristics of Moraxella catarrhalis used in its identification

Colonial morphology on blood agar	round, opaque colonies
Colonial morphology on chocolate blood agar	pinkish-Brown hockey puck
Colonial morphology on semi selective media	round, opaque colonies
Gram stain	Gram-negative diplococcic
Oxidase test	Positive
Catalase test	Positive
Deoxyribonuclease (DANas) test	Positive
Reduction of nitrate	Positive
Growth on nutrient agar at room temperature	Positive
Tributyrin test	Positive

When PCR was performed to confirmed *Moraxella catarrhalis* isolates 15 (78.9%) isolates showed typical band of 550 bp size as

indicated by the standard DNA marker while 5 samples were PCR negative (see figure 1).

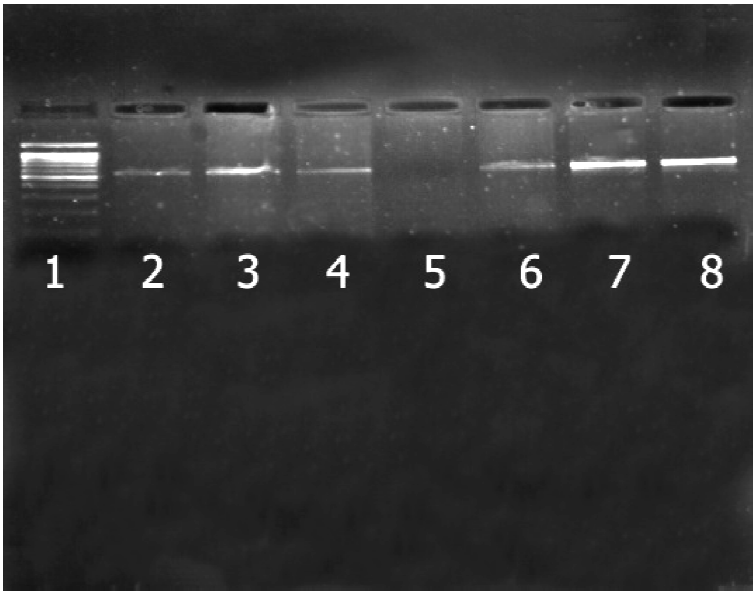

Figure1: The amplicon of Moraxella catarrhalis after PCR on 2% agarose gel: lane1= marker; lane 2= positive control; lane 5= negative control; lanes 3-4 and lanes 6-8 isolates positive for Moraxella catarrhalis (550 bp).

All isolates were noted to be sensitive to amoxyclav, azithromycin, ceftazidime, ceftriaxon, cephalexin, cephotaxime, chloramphenicol, ciprofloxacin and cotrimoxazole and 90% were sensitive to erythromycin, while they were wholly resistant to ampicillin.

DISCUSSION

Moraxella catarrhalis is a Gram negative aerobic diplococcus which is frequently found as a commensal of upper respiratory tract and has been recovered exclusively from human over the last few decades [22].

It has emerged as a genuine pathogen, it is now considered as an important cause of upper respiratory tract infections in otherwise healthy children and elderly adult [22,23]

The *Moraxella catarrhalis* rate in the present study is 4.7%. This is semi similar to that observed by Nishioka *et al.* who had carried out a study in Japan and found the isolation rate of *Moraxella catarrhalis* to be 4.26% [24]. A higher rate (6.9%) was detected among elderly population by Tamang and his fellows [25].

Pollard and colleagues in 1986 found the isolation rate of *Moraxella catarrhalis* was 5.3% which is more or less similar to that of present study [26].

Unlike the aforementioned findings, Ahmed *et al.* in 1994 and Sarubbi *et al.* in 1990 found the rate of isolation of *Moraxella catarrhalis* to be much lower (0.89 and 2.5%, respectively) [27,28]. On the other hand, Hager et al in 1987 found the rate of isolation of *Moraxella catarrhalis* in pure culture was 61% [6], and this figure is much higher from that of current study.

In the present study, 5 (4.5%) *M. catarrhalis* was isolated from children aged from 1 month to 5 years with a percentage of (4.7%). The bacteria were not isolated from those whose ages ranged between 6 and 15 years old**.** This result disagrees with the result obtained by Constantinescu et al who isolated *M. catarrhalis* in culture in 9% of children younger than 5 years and 33% of children aged 6-10 years in his study [29]. Nearly similar to the results obtained in this research, a study by Broides *et al.* in 2009 had reported that *M. catarrhalis* occur in 4.8% of children less than 5 years [30]. Another result reported by Vergison in 2008 shows that the *M. catarrhalis* proportion in children with otitis media was (3–20%) [31].

In some reports, an increasing rate of *M. catarrhalis* isolation from the middle ear fluid (MEF) in AOM has been shown. Kilpi et al have reported an increase from 10% to 23% within 15 years, and a similar pattern has also been reported in the United States [32]. In Costa Rica, the prevalence of *M. catarrhalis* isolated from the MEF of children with AOM aged 3–144 months increased from 2.5% of all pathogens during 1992–1997 to 7% during 1999–2004 and was most commonly found in children aged <24 months during the dry season [33]. However, in the present study, *M. catarrhalis* has been consistently found as a single pathogen in only~1% of MEF isolates. The reasons for the higher relative importance of *M. catarrhalis* as a pathogen in AOM in certain geographical areas and the different rates of *M catarrhalis* AOM in other areas are unknown.

The underlying factors which are associated with *Moraxella catarrhalis* were studied previously. Age was a critical determinant of the pathogenic significance of the isolates of *Moraxella catarrhalis*: the more the advanced age is, the greater the pathological significance of the isolates [25, 34].

In the present study, being of advanced age (42.1), having chronic cough (productive cough) (42.1) and smoking (15.8) are the common predisposing factors. This agrees with the study done by Tamang *et al.* which found the most important predisposing factors were old age and history of smoking [25].

Studies have shown that the elderly are at greater risk of respiratory tract infections which

are caused by *Moraxella catarrhalis* when compared to young adults. Most of these patients had underlying lung disease and other conditions like diabetes mellitus, corticosteroid therapy and malignancy [8, 26, 35, 36].

In the present study rate of *M. catarrhalis* isolated from sputum of elderly is (42.1%). The significance of this finding is strengthened by the fact that after the age of 50 years there may be a reduction of immunoglobulin G and M titers along with damage of the respiratory tract by viral infections that may promote invasion by *M. catarrhalis* [3].

This study noted that there has been no strong correlation between chronic obstructive pulmonary disease (COPD) and *Moraxella catarrhalis* in Sudan. This observation disagrees with that of Timothy and his fellows who concluded that *M.* catarrhalis likely causes approximately 10% of COPD [37].

Antibiotic sensitivity pattern showed high level resistance to penicillin, this is due to rapid increase in prevalence of beta lactamase producing strains of *Moraxella catarrhalis*. In the present study, 100% of isolates are B-lactamase producers. This is semi similar to the observation of Anita et al in 2011 and Hanan in 2000 who found that the proportion of beta lactamase producing isolates was 84 % [36, 38]. Proportions higher than 90% were found in several studies. In 2007, Esel and his fellow found that the percentage of b-lactamase producing strain of *Moraxella catarrhalis* was 94% [39]. Another report in turkey found that 96.9% of clinical isolates and 90.6% of carrier strains produced B-lactamase [40]. A recent study conducted in Taiwan showed the rate of beta lactamase production was 97.8% [41]. The dramatic increase in frequency of beta lactamase producing *M. catarrhalis* could be regarded as the fastest dissemination of any known beta-lactamase producing bacteria within all bacterial species [42].

The beta lactamase activity of *M. catarrhalis* is inhibited by beta- lactam inhibitors such as clavulanic acid and sulbactam[8]. This fact can be reflected by the finding that all isolates in this study were sensitive to amoxycilin- clavulanic acid combination. However, this sensitivity pattern is in discrepancy with that reported by Tamang et al which found that only 4% of *Moraxella catarrhalis* isolates were resistant to both amoxicillin-clavulanate and ceftriaxone and 8% were resistant to ciprofloxacin [25].

The present study shows that all isolates were sensitive to amoxicillin-clavulanate and ceftriaxone. This observation is similar to that of Tamang *et al.* [25].

It has been demonstrated in vitro that *Moraxella catarrhalis BRO* enzyme can confer protection against B-lactam to other co-existing respiratory pathogens residing in the host. This phenomenon, referred to as indirect pathogenicity, of *Moraxella catarrhalis* may lead to antibiotic failure when treating a mixed infection containing both susceptible bacteria and resistant *Moraxella catarrhalis* strains [8, 23].

This, along with the great increase in the percentage of strains producing B-lactamase, makes it necessary to report the isolation of *Moraxella catarrhalis* when seen both in isolation and when co-existing with other pathogens. This will help in the selection of appropriate antibiotics and also in combating infections by other pathogens which may be other- wise protected by B- lactamase produced by *Moraxella catarrhalis.*

CONCLUSIONS

This study shows that *Moraxella catarrhalis* is an important respiratortract pathogen in adults and children in Sudan. The emergence of antibiotic resistance in *Moraxella catarrhalis* suggests that the incidence of these infections may continue to rise.

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