



## MOLECULAR DETECTION OF *MORAXELLA CATARRHALIS* ISOLATED FROM CHILDREN INFECTED WITH OTITIS MEDIA

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### ABSTRACT

*Moraxella* is a fastidious organism that does not ferment carbohydrates, nonmotile, Gram-negative, aerobic and oxidase positive diplococcus. The most important pathogen in the genus is *Moraxella catarrhalis*. It belongs to the family *moraxellaceae* and the bacterium can cause a variety of severe motile that can cause infections of the respiratory system, middle ear (otitis media), eye, central nervous system, and joints of humans. Aims of the current study were to review the impact of *Moraxella catarrhalis* in the otitis media infection, and use the singleplex PCR technique as a rapid identification method for *Moraxella catarrhalis* isolated from children clinically diagnosed with otitis media. In this study ear swabs from 50 children clinically diagnosed with otitis media were collected by sterile cotton swabs and used for conventional methods include culture, Gram staining, biochemical tests and singleplex PCR technique as a molecular method. Singleplex PCR was performed using primer pair targeted to the 16S rRNA gene of *Moraxella catarrhalis*. The conventional methods include culture, Gram staining and biochemical tests showed positive results in 12(24%) out of 50 (100%) children clinically diagnosed with otitis media, whereas the singleplex PCR method detected positive results in 10 (83.3%) out of 12 (100%) samples that were positive for these conventional methods. The singleplex PCR positive samples were identified by presence of ~235 bp amplicon of 16S rRNA gene of the *Moraxella catarrhalis*. The singleplex PCR method using species-specific primers for *Moraxella catarrhalis* represented a rapid, specific and sensitive method to detect these bacteria in the otitis media infections of human.

**KEYWORDS:** *Moraxella catarrhalis*, otitis media, 16S rRNA gene.

### INTRODUCTION

*Moraxella* species are parasites of the mucous membranes of humans and other warm-blooded animals. The genus *Moraxella*, a member of the *Moraxellaceae* family, Order *Pseudomonadales*, Class *Gammaproteobacteria*, Phylum *Proteobacteria*<sup>[1]</sup>. This family includes large group of a Gram-negative non-motile capsule-less aerobic diplococcus. The *Moraxella* genus contains both rod-shaped (genus *Moraxella*, subgenus *Moraxella*) and coccoid (genus *Moraxella*, subgenus *Branhamella*) bacteria exhibiting similar genetic relatedness, though taxonomic revision of the genus is a continuing process<sup>[2]</sup>. *Micrococcus catarrhalis* it was first described in 1896<sup>[3]</sup>. Pfeiffer gave the organism its first name<sup>[4]</sup>. For a long time *M. catarrhalis* was considered a relatively harmless commensal in the respiratory tract<sup>[5,6]</sup>. At present, it is considered as a third most frequent cause of otitis media (OM)<sup>[7]</sup>, chronic obstructive pulmonary disease (COPD), conjunctivitis, laryngitis, pneumonia, bacteraemia, endocarditis, meningitis, and arthritis<sup>[8]</sup>. Otitis media is defined as the presence of fluid in the middle ear (ME) with absence of clinical signs or symptoms that related to acute ear infection<sup>[9]</sup>. It constitutes the most common respiratory tract infection of infancy and early childhood<sup>[10]</sup>. Otitis media may be acute or chronic supportive type. Children are mostly affected with acute type, and the prevalence of it is approximately 20% also the peak incidence of it occurs at the first year of age with 70% experiencing at least one episode by age 3 years<sup>[11]</sup>, while

adults are mostly affected with chronic supportive types<sup>[9]</sup>.

### MATERIALS & METHODS

#### Samples collection

During the period of study, 50 ear swabs were collected from patients clinically diagnosed with otitis media include 36 females and 14 males in ENT unit of Baghdad Teaching Hospital from the beginning of October to the end of December, 2015. The patient's ages ranged from 6 months to 3 years. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq. Ear swabs were taken from the patients under sterile conditions and immediately transferred to the laboratory to inoculate into brain heart infusion broth for 4-6 hours, then inoculated on blood base agar and chocolate agar (Merck, Germany) at 37°C for 48 hours under aerobic condition as deferential and enrichment media for *M. catarrhalis*<sup>[12]</sup>, then the Gram stains and biochemical tests include urease, oxidase, catalase, DNase, indol, H<sub>2</sub>S production, citrate and motility tests were performed<sup>[13]</sup>.

#### DNA extraction

Genomic DNA was extracted from the *M. catarrhalis* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of an overnight *M. catarrhalis* culture grown at 37°C in brain

heart infusion broth (Sigma, USA) was transferred to a 1.5 ml micro centrifuge tube. The microcentrifuge tube was centrifuged at 14,000 rpm for 3 minutes to pellet the cells and the supernatant was removed. 600 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added and gently pipet until the cells is resuspended. The microcentrifuge tube was incubated in water bath at 80°C for 5 minutes to lyse the cells; then cool to room temperature. 3 µl of RNase solution (wizard genomic DNA purification kit) was added to the cell lysate and the microcentrifuge tube was inverted for 5 times to mix. The microcentrifuge tube was incubated at 37°C for 60 minutes and cool to room temperature. 200 µl of protein precipitation solution (wizard genomic DNA purification kit) was added to the RNase-treated cell lysate and vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate. The microcentrifuge tube was incubated on ice for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The microcentrifuge tube was gently mixed by inversion until the thread-like strands of DNA form a visible mass and centrifuged at 14,000 rpm for 5 minutes. The supernatant was carefully pour off and the microcentrifuge tube was drained on clean absorbent paper. 600 µl of room temperature 70% ethanol was added

and then the microcentrifuge tube was gently inverted several times to wash the DNA pellet. The microcentrifuge tube was centrifuged at 14,000 rpm for 2 minutes and the ethanol was carefully aspirated. The microcentrifuge tube was drained on clean absorbent paper and the pellet was allowed to air-dry for 15 minutes. 100 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added to the microcentrifuge tube and the DNA was rehydrated by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the microcentrifuge tube and the DNA sample was stored at -20°C until use.

#### DNA quantification

The extracted DNA from the *M. catarrhalis* isolates was quantified spectrophotometrically at O.D. 260/ 280 nm with ratios 1.4-1.5. The sensitivity of the M.Cata-F and M.Cata-R primers was evaluated by PCR amplification for serial diluted concentrations (10-100 ng) of purified genomic DNA isolated from *M. catarrhalis*.

#### Primers selection

The primers for 16S rRNA gene of *M. catarrhalis* as the target gene for this study were selected according to (14). This set of primers was designed based on the conserved region in *M. catarrhalis*, primers were synthesized by Alpha DNA, Kanda. The primers sequence of 16S rRNA gene and their size of product are shown in (Table 1).

**TABLE 1:** The primers sequences of 16S rRNA gene of *Moraxella catarrhalis* and their product size

Name of primer	Sequence of primer (5'-3')	Size of product
M.Cata-F	CCCATAAGC CCTGACGTTACG	~235 bp
M.Cata-R	GACGCATTTACCGCTACA	

#### Singleplex PCR master mix

The singleplex PCR reaction of 16S rRNA gene detection of *M. catarrhalis* was performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl<sub>2</sub>, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol M.Cata-F primer and 2.5 µl of 20 pmol M.Cata-R primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil.

#### Singleplex PCR program

Singleplex PCR was carried out in a thermal cycler (Applied Biosystem, 9902, Singapore) according to the PCR program described by (14), with some modification. Briefly, the amplification of 16S rRNA gene of *M. catarrhalis* was carried out with initial denaturation at 95°C for 6 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for M. Cata-F and M. Cata-R primers for 90 seconds, and extension at 72°C for 2 minutes. The thermal cycles were terminated by a final extension for 10 minutes at 72°C.

#### Singleplex PCR products analysis

The analysis of singleplex PCR products of 16S rRNA gene of *M. catarrhalis* were performed on 1% agarose gel.

The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The singleplex PCR products were stained with ethidium bromide and visualized by an image analyzer (ChemImager 5500, Alpha Innotech, USA).

## RESULTS

#### Conventional methods

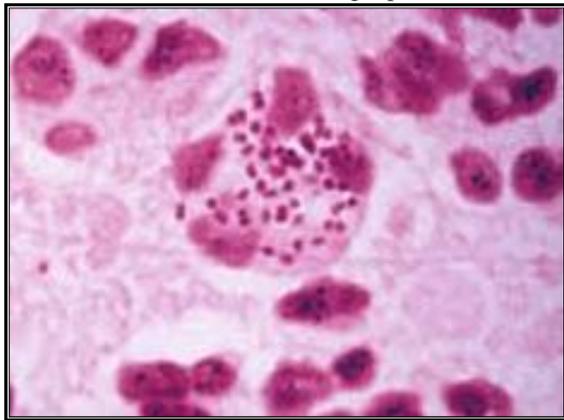
The conventional methods include culture, Gram staining and biochemical tests showed positive results in 12(24%) out of 50(100%) children clinically diagnosed with otitis media, the results of ear swabs culture showed growth of colorless non-hemolytic colonies of *Moraxella catarrhalis* on blood base agar (Figure 1) and grey colonies of this bacteria on chocolate agar (Figure 2). The Gram staining of *Moraxella catarrhalis* was showed a diplococcus Gram-negative with flattened sides (Figure 3). The results of biochemical tests include urease, oxidase, catalase, DNase, indol, H<sub>2</sub>S production, citrate and motility tests showed that these tests were identified presence of *Moraxella catarrhalis* in 12(24%) out of 50(100%) children clinically diagnosed with otitis media, through observation of the positive results of *Moraxella catarrhalis* isolates for urease, oxidase, catalase, DNase, indol and H<sub>2</sub>S production tests, as well as the negative results of these isolates for both of citrate and motility tests.



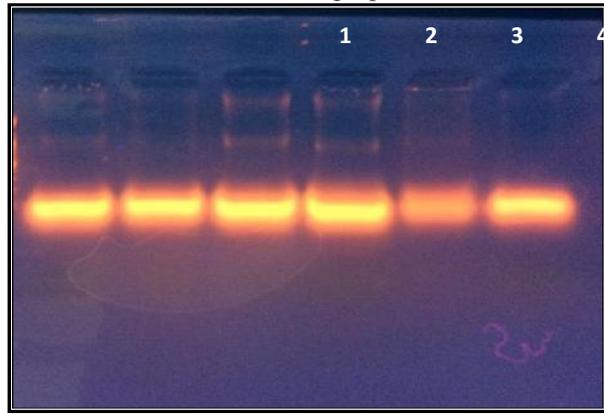
**FIGURE 1:** Colorless colonies of *Moraxella catarrhalis* on blood agar plate.



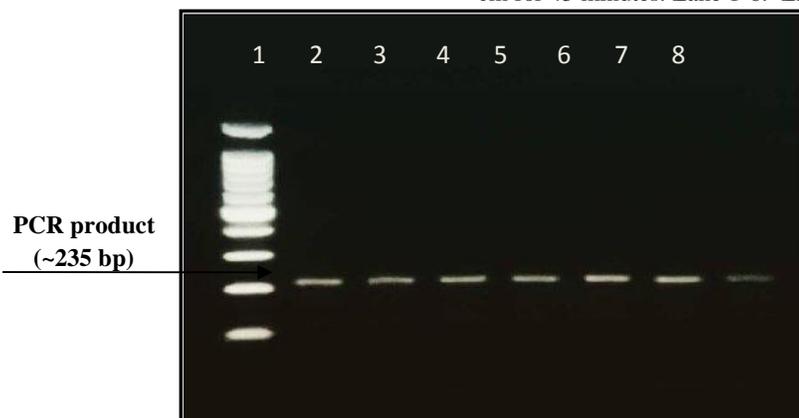
**FIGURE 2:** Grey colonies of *Moraxella catarrhalis* on chocolate agar plate



**FIGURE 3:** Gram-negative diplococcus with flattened sides of *Moraxella catarrhalis*.



**FIGURE 4:** Gel electrophoresis of extracted DNA of *Moraxella catarrhalis* isolates using 1 % agarose gel at 7 volt/ cm for 45 minutes. Lane 1-6: Extracted DNA.



**FIGURE 5:** Gel electrophoresis of singleplex PCR products of 16S rRNA gene for *M. catarrhalis* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-8: 16S rRNA PCR products of *M. catarrhalis* isolates.

#### **Analysis of extracted DNA of *Moraxella catarrhalis* isolates**

After performing of the DNA extraction from *M. catarrhalis* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1 % agarose gel at 7 volt/ cm for 45 minutes (Figure 4).

#### **Analysis of singleplex PCR products of 16S rRNA gene for *Moraxella catarrhalis***

On the basis of the 16s rRNA gene sequence, a product of ~235 bp was amplified by singleplex PCR with *M. Cata-F* and *M. Cata-R* primers. In 50 children clinically diagnosed with otitis media, the singleplex PCR method detected positive results in 10(83.3%) out of 12(100%) samples that were positive by the conventional methods include culture Gram staining and biochemical tests. The singleplex PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5µl of the singleplex PCR product were loaded on 1.5% agarose gel and run at 100 volt/ cm for 60

minutes. The gel was stained with ethidium bromide solution (0.5 µg/ ml) for 15-30 minutes; finally, bands were visualized on UV transilluminator at 350 wave length and then photographed by using photo documentation system. The singleplex PCR result was considered positive for *M. catarrhalis* when there was presence of ~235 bp singleplex PCR product band of 16S rRNA gene for the *Moraxella catarrhalis* on the agarose gel electrophoresis, no amplification was observed with negative control (Figure 5).

## DISCUSSION

*Moraxella catarrhalis*, formerly called *Neisseria catarrhalis* and *Branhsmella catarrhalis* is a Gram-negative bacterium that belongs to the family *Moraxellaceae* where they can be either pathogens or commensals<sup>[6]</sup>. The present study was described the otitis media caused by *Moraxella catarrhalis* in children and identification of this bacteria by using the conventional methods include culture, Gram staining and biochemical tests which were go together with study conducted by<sup>[1]</sup> showed that the bacterial cultures were positive in 39(78%) patients versus 11(32%) patients revealed negative bacterial culture, the most common type of bacterial isolated were *Moraxella catarrhalis* 19(32%) patients was detected by the conventional methods of blood culture, Gram staining, culture; blood base agar, Muller Hinton agar, chocolate agar and VITEC-2 Compact. Also the result of *Moraxella catarrhalis* culture on blood agar agrees with results of the same study showed that the *Moraxella catarrhalis* colonies on blood agar appear tend to be large, grey or non-pigmented, that are generally found single or in pairs, smooth, opaque and convex in nature and may be readily pushed intact over the surface of agar using a sterile loop, this phenomenon is the so-called "hockey puck sign. In addition, the result of Gram staining of *Moraxella catarrhalis* goes together with result of exhibited that in a typical Gram film, the organism appears as a Gram-negative diplococcus with flattened sides, though physical appearance by itself is not enough to separate the species from related contaminating *Neisseria* spp<sup>[8]</sup>. The results of biochemical tests of current study agree with study conducted by<sup>[15]</sup> showed that the biochemical testing with the production of oxidase, catalase expression of a DNase were positive, lack of acid production from glucose, sucrose, lactose, maltose and fructose, also can reduce of nitrate and nitrite, and the hydrolysis of tributyrin being important. The biochemical tests are largely used for bacterial identification in clinical laboratories, the advantages of conventional methods were non costly but the disadvantages of those methods were consuming time, contamination present, false positive result and require a large amount of sample<sup>[15]</sup>. In current study, the singleplex PCR method was used for detection of *M. catarrhalis* by using pair primers targeted the 16s rRNA gene (~235 bp) showed a positive result in 10(83.3%) out of 12(100%) samples that were positive by the conventional methods. The benefits of molecular methods are more sensitive, more qualitative for results, materials available, but the drawback of molecular methods is costly. These explanations made molecular

methods relatively more accurate than conventional methods<sup>[9]</sup>. At a comparison between the conventional and molecular methods, we think that the incubation period is uncertain and inappropriate growth media, in addition to the contamination of culture in identification methods could be the reason for false positive results, this may explain the false positive results in 2(4%) out of 50(100%) children clinically diagnosed with otitis media by using these conventional methodes, whereas the singleplex PCR method detected only 10(20%) of 50(100%) patients who were also gave positive results by these conventional methods. Conventional studies have highlighted the difficulties in identifying *M. catarrhalis* strains based on commercial phenotypic identification systems. Identification of *M. catarrhalis* has long been based on phenotypic and biochemical test, these physiological and biochemical tests were performed on selected isolates using Vitek 2 system and PCR method<sup>[9]</sup>. This explains that the molecular diagnosis of *M. catarrhalis* by the singleplex PCR method was more sensitive and efficiency than the diagnosis of these bacteria by conventional methods. This data agrees with the study by<sup>[16]</sup> who confirms the efficacy of the PCR assay compared to conventional methods of diagnosis in the clinical setting.

## CONCLUSION

Many evidence indicate that *M. catarrhalis* are based upon inaccurate isolate identification, resulting from inadequate identification conventional methods include culture, Gram staining and biochemical tests that lack the resolution needed to discriminate *M. catarrhalis* isolates, on the other hand, 16S rRNA gene appeared to be useful genetic marker for determination of *M. catarrhalis* and singleplex PCR using species-specific primers could be represented rapid, sensitive and specific molecular method for detection of this bacteria in different human infections.

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