Ministry of Higher Education
and Scientific Research
University of Babylon
College of Medicine

Genomic diversity of *Moraxella catarrhalis* isolated from clinical specimens in Babylon province

A Thesis

Submitted to the Council of College of Medicine ,University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctor philosophy in Science / Medical Microbiology

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2023 AD.   **1445 AH.**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّن الْعِلْمِ إِلاَّ قَلِيلاً

صدق الله العليّ العظيم

Supervisor Certification

I certify that, this thesis (**Genomic diversity of *Moraxella catarrhalis* isolated from clinical specimens in Babylon province)**
was prepared by Nebras Awda Kadhim under my supervsion at the College of Medicine, University of Babylon as partial fulfillment of the requirements for the Degree of phD philosophy in medical Microbiology .

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In view of the available recommendation, I forward this thesis

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Acknowledgements

Summary :

Moraxella. catarrhalis is an important aetiological agent of otitis media (OM) in infants and children, and acute exacerbations of chronic obstructive pulmonary disease (COPD) in the elderly and heavy smokers .*M. catarrhalis* is the third most prevalent pathogen associated with OM worldwide, behind *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) and the second most frequently isolated pathogen associated with exacerbations of COPD, behind NTHi and equal with *S. pneumoniae* .

A total 300 specimens, only 15 isolates of *M. ctrrhalis* were obtained from two different specimens, includes (ear swab , sputum) from patients suffering with otitis media and COPD by standard bacteriological methods. All specimens were obtained from patients or individuals who admitted to the Imam Al-Sadiq Teaching Hospital, and private medical clinics in Babylon province during the period from(March to July 2022).

All specimens were subjected to aerobic culturing on different media and it was found that out of the total 300 specimens, 60 (20%) specimens showed positive bacterial culture. No growth was seen in other 50(16%)spicemens and Positive culture of other bacteria190(63.3). Among (60) positive culture were culturing 15 positive samples was identified as *M. catarrhalis*

These isolates then subjected to molecular detection method using specific primer based on *copB* gene as a genetic marker for confirmed identification of *M. catarrhalis* by polymerase chain reaction, the results revealed that 15(100%) were positive for *copB* .

Some virulence factors of all *M. catarrhalis* isolates were studied such as *uspA1* and *uspA2* gene , PCR was performed using specific primer on genomic DNA from *M .catarrhalis* isolate . PCR amplification

of uspA1 gene indicated that 14(93.3%) which include 11 isolate from ear swab and 5 isolate from sputum, while *uspA2* gene was observed in 11(73.3%) isolates which include 6 isolate from ear swab and 5 isolate from sputum.

Specific primer was used for detection of *Haemagglutinin* gene .It was found that *Hag* was observed in 6(40%) isolate which include 4 isolate from ear swab and 2 isolate from sputum.

Moraxella.catarrhalis adherence protein (McaP) gene was also detected in *M. catarrhalis* by using specific PCR primer .It was found in only 3(20%) isolate of ear swab.

Also, *mapA* gene was detected in *M. catarrhalis* isolates by using specific primer , it was found in only 4(26.6%) isolate of ear swab .

In this study, multilocus sequence typing method (MLST) were used to investigate the discriminatory ability, reproducibility, and the genetic relationship between 15 *M. catarrhalis* isolates. The sequence data obtained for MLST for determining the population structures analyzing the extent of linkage disequilibrium between alleles for phylogenetic relationships between 15 isolates, depended on the 8 housekeeping genes frequently used for MLST analysis of *M. c*atarrhalis (*abcZ, adk , efp, fumC ,glyBeta , mutY, ppa & trpE)* .

The mean GC content of sequnces of eight gene fragments ranged from 40%(*ppa*) to 49%(*mutY*):Trimmed fragment size of the 8 selected loci ranged from 372 b-p (*trpE*)_ 537 bp (*glyBeta*).The nucleotide diversity ranging from 0.00922 to 0.03165 pergene . Moreover , The number of polymorphic sites per locus varied between 12 (*trpE*) _ 54(*ppa*) and harbored a total of 251 SNP. the dN/dS ratios were determined to be more than 1 for the eight genes which indicated positive selection .

According to allelic profile it was found that the presence of allelic variant (SNP, insertion, or deletion) between isolates. In the case *glyBeta* was more variant or mutant than other 7 housekeeping genes, contrary to the *trpE* which was the least variant.

eleven sequence types (STs) were identified through MLST analysis of the *M. catarrhalis* isolates . All *M. catarrhalis* isolates showed polyphyletic lineage and revealed two distinct clusters, cluster A contain one isolate 13 isolates of this cluster was divided into subclusters, while cluster B divided into two branches & each branch contain 2 isolates.

The split graphs for the seven gene (*abcZ , adk , efb ,fum C,glyBeta, mutY&* PPa) revealed network like with parallelogram structures indicating that intergenic recombination had occurred during the evolutionaty history of these genes . However ,the split graphs of *trpE* are tree like structures suggesting that the descent of these genes was clonal and absence of recombination . The split decomposition analysis of combined eight MLST Loci display network like structure with rays of different length.

MLST of 15 isolates from different clinical specimens revealed 11 different sequence typed that grouped into two major clonal complex (cc1 & cc2) by use of eburst . Among the 2cc,cc1 was the largest and comprised 7 link ST, namely N1,N2,ST494,ST501,ST23,ST26& ST11.

In order to determine the distribution and variability of R-M systems in *M. catarrhalis.* Type I found only in 3 sample (20%) while type III found in 12 (80%) samples.

The presence of sequence related to enzyme of the R-M system was observed in all *M.catarrhalis* isolates . The result of type I was (22.3%) from OM infection and (16.7%) from respiratory infections.

while the result of type III was (77.7%) in OM infection and (83.3%) in COPD.

To investigate the distribution of RM system gene (T1mod, T1res, modM1,modM2,modM3) of *M.catarrhalis* isolates pcr analysis of DNA was performed, overall , *M.catarrhalis* isolates were examined consisting of 9 middle ear effusion isolates and 6 sputum from COPD, T1mod and T1RES genes were present in 3 isolates for both .However, mod1 was present in one isolate , mod3 was present in two isolates .while mod2 was found in nine isolates with mod3 the most common (60%) .Interestingly ,a statistically significant association was found between RM(I, II) and disease. modM2 was most prevalent in respiratory infection (66.6%) compared to (55.5%) of middle ear isolates.

The presence or absence of R-M system in 15 *M . catarrhalis* isolates was generated ,with isolated ordered based on phylogeny .comparison of R-M system between 15 isolates found that two main cluster the first A cluster which contain R-M type III system which include (12 isolates) ,while cluster B which contain R-M system type I which include (3 isolates).Differences in R-M system presence were also observed between isolates which obtained from different site of infection.

On the other hand the gene sequence analysis of RM system (I and III) was also studied , the nucleotide sequence of RM system (I and III) were aligned . in the type I mod the nucleotide identity was (97-100 %), so there are 21 mutation in mod in which 4 mutation in (mc20 isolate) and 17 mutation in (mc26 isolate) while no mutation occur in mc2.

Also , in the type I RES ,the nucleotide identity was (94-100%)there are 9 mutation in RES in which 3 mutation in (mc20 isolate) and 6mutation in (mc 26 isolate) while there is no mutation observed in (mc2 isolates).

However ,in wasType III modM2 the nucleotide identity (99-100%) , there were 7 mutation in modM2 in which three mutation in (mc1 isolate)and one mutation in each of (mc 33 , mc25 , mc28 and mc29) while the other isolate mc3 , mc21 and mc22 there is no mutation .

Finally in the Type III modM3 the nucleotide identity (99-100%) and there is one mutation in mc30 isolate while there is no mutation in mc5 .

Dedication

To the woman who raised me as in life and irrigated me by her kindness ..... to dear and noble .....my mother.

To my sisters, my brother and my nieces with their ever efforts to support and encourage me all the to complete my study.

Special thanks to my friends for their scientific advices and support during the period of the research.

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ABBREVIATIONS

a MAb	A monoclonal antibody
AOM	acute otitis media
adk	Adenylate kinase
abcZ	ATP-binding protein
Bpa A	Bisphenol A
CSOM	chronic suppurative otitis media
COPD	Chronic obstructive pulmonary disease
C4BP	C4b binding protein
CEACAM1	arcinoembryonic antigen-related cell adhesion molecule 1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
efp	elongation factor P
fumC	fumarate hydratase
glyRS	glycyl-tRNA synthetase beta subunit
HMEEC	human middle ear epithelial cells
MapA	*M. catarrhalis* acid phosphatase A
MLEE	multilocus enzyme electrophoresis
McaP	*M. catarrhalis* adherence protein
MLST	multilocus sequence typing
MID/Hag	*Hemagglutinin/Moraxella catarrhalis* Immunoglobulin D-

	Binding Protein
MCP-1	Monocyte Chemotactic protein-1
ME	middle ear
mutY	adenine glycosylase
NTHi	non-typeable Haemophilus influenzae
OMVs	outer membrane vesicles
Oca	oligomeric coiled coil adhesins
OMPs	outer membrane protein
OME	otitis media with effusion
PCR	Polymerase chain reaction
ppa	Pyrophosphate phospho-hydrolase
pH	Power of hydrogen (H+)
PC	phosphatidylcholine
ROS	reactive oxygen species
RSV	respiratory syncytial virus
ST	sequence type
TbpB	tert-Butyl peroxybenzoate
trpE	anthranilate synthase component I
TAA	trimeric autotransporter adhesin
uspA1 and *uspA2*	Ubiquitous surface protein A1 and A2
YadA	Yersinia adhesion

Introduction:-

Moraxella catarrhalis is non-motile unencapsulated aerobic diplococcus. Gram-negative is the third most common cause of otitis media in infants and young children, accounting for 15-20% of cases (*Karalus et al.,* 2000*)*.

Metabolically it can be characterized by the lack of fermentation of glucose, lactose, sucrose and maltose; it is able to carry out the positive oxidase, catalase. Also can reduce nitrate and nitrite; performs hydrolysis of tributyrin (an isomeric glyceryl ester of butyric acid) and does not utilise 5% sucrose to form polysaccharide (Hays *et al* .,2006).

Bacterium was first isolated in 1896, it was considered to be a harmless commensal of the upper respiratory tract for a long period of time. The bacterium rapidly colonizes the nasopharynx soon after birth asymptomatically (Blakeway *et al*., 2017) . The bacterium has now firmly established its position as an etiological cause of human respiratory tract (De Vries *et al*., 2010) However, it is associated with a number of respiratory infections affecting both children and adults, including laryngitis, bronchitis and pneumonia (Bernhard *et al*., 2014) . It is a causative agent of otitis media in children and lower respiratory tract infections in adults suffering from chronic obstructive pulmonary disease (COPD) (Schwingel *et a*l., 2019; Tan *et al*., 2020) . Rarely, *M. catarrhalis* can also cause endocarditis, sepsis and meningitis (Aebi *et al*., 2011). Thus, idea has arisen that organism is not simply a commensal colonizer and important bacterial pathogen.

Acute otitis media is defined as an infection of the middle ear space. It is a spectrum of diseases that includes acute otitis media (AOM), chronic suppurative otitis media (CSOM), and otitis media with effusion

(OME). Acute otitis media is the second most common pediatric diagnosis in the emergency department, following upper respiratory infections(Meherali *et al* ., 2019). Infection of the middle ear can be viral, bacterial, or coinfection. The most common bacterial organisms causing otitis media are *Streptococcus pneumoniae,* followed by non-typeable Haemophilus influenzae (NTHi) and *Moraxella catarrhalis*.(Ubukata *et al* ., 2018). *M. catarrhalis* is associated with 10-15%of acute otitis media cases. This bacteria is frequently found as a commensal of the upper respiratory tract (Yamanaka *et al* ., 2008).

COPD is characterized by irreversible airflow obstruction and airway inflammation (George *et al* ., 2016).Bacteria are commonly identified in stable state and at exacerbations with increased bacterial load and dysbiosis associated with exacerbations (Wang *et al* .,2016).The most common pathogens identified are *Haemophilus influenzae* and *Streptococcus pneumoniae* in both stable disease and exacerbations, but several other pathogens are identified including *Moraxella catarrhalis* (Wilkinson *et al* ., 2017). Using molecular approaches *M. catarrhalis* in sputum samples typically has a prevalence of 10%–20% in exacerbation samples, whereas in stable disease it is more unusual with a prevalence of 5%–10%.(Wang *et al* ., 2018). Even though *M. catarrhalis* is not the most prevalent bacteria in COPD sputum samples at exacerbation the abundance of this bacterium increases consistently in a subgroup with an associated increase in sputum inflammatory mediators.

M. catarrhalis outer membrane protein B, or *CopB,* was originally identified by use of a MAb targeted against the outer membrane of *M. catarrhalis* . It is possibly the same protein labeled OMP B in the study by Bartos and Murphy of conserved outer membrane proteins of *M. catarrhalis*; it has also been called OMP B2 (Kyd *et al* 1998).

The expression of *CopB* is increased under iron starvation conditions. *CopB* is involved in iron acquisition (Aebi *et al.*,1996) and serum resistance .

M. catarrhalis uses several virulence mechanisms, which facilitate colonization of the respiratory tract and opportunistic disease. Adhesion molecules expressed on the bacterial cell surface, including *UspA* proteins, *Hag/MID*, and others, mediate *M.catarrhalis* binding to host surface receptors expressed on epithelial cells lining the respiratory tract.(Vries *et al* .,2009)This organism can also invade and survive within epithelial cells, thus evading immune detection and innate immune defenses (Tan *et al* .,2005).

The *M. catarrhalis* species is a panmictic population of strains that is divided into two main phylogenetic lineages based on molecular typing methods such as multilocus sequence typing (MLST) (Wirth *et al.*, 2007). The multiple locus sequence typing (MLST) have been globally recognized as highly discriminative standard strategies in modern epidemiological studies of salmonellosis (Ghaderi *et al.*, 2015). MLST, which is a typing method based on the sequencing of housekeeping genes and characterizes isolates on the basis of variation in nucleotide sequences of each locus of the selected genes. The different sequence at each locus are assigned with specific allele numbers and each unique combination of alleles, often called as allelic profile is assigned a sequence type (ST), which is the unambiguous descriptor of the strain (Sharma *et al.*, 2016).

R–M systems are classified into three groups (type I, II and III) based on differences in the subunit structures of their enzymes, cofactor requirements, recognition sites and enzymatic mechanisms for reviews of R–M systems). The components of type III R–M systems catalyse two distinct reactions: (1) the modification enzyme/methylase (Mod) is

required for sequence recognition in both modification and restriction reactions, and catalyses the post-replicative addition of a methyl group to an adenine residue in a specific DNA sequence; (2) the cognate restriction endonuclease (Res) recognises the same sequence and catalyses double stranded cleavage of unmethylated foreign DNA in the presence of the *mod* gene product(Hadi *et al* ., 1983). Type I R–M systems similarly require a complex of the *hsd* RMS gene products (the <u>r</u>estriction, <u>m</u>odification and <u>s</u>pecificity subunits) for endonuclease activity. In contrast, the restriction and modification proteins of type II R–M systems act independently of each other.

Aim of study:-

The study is aimed to study of genomic diversity of Moraxella catarrhalis strains isolated from clinical sample*s.*

Objectives:-

1-Isolation and detection of Moraxella catarrhalis from, ear swab &sputum samples

2- Molecular diagnosis of by using specific primer.

3-Study of some virulence genes of *Moraxella catarrhalis* .

4- Identification the phylogenetic diversity in *Moraxella catarrhalis* isolated by using MLST

5- Examine the distribution of R-M systems identified in *M. catarrhalis* genomes.

6- Characterize the variable Restriction- Modification systems(R-M) system loci found in *M. catarrhalis.*

1.2 Literatures review:

1.2.1 characteristics of *Moraxella catarrhalis*:

The genus Moraxella belongs to the family Moraxellaceae, the most significant species is *Moraxella catarrhalis* (*M. catarrhalis),* also known as *Branhamella catarrhalis*, *Micrococcus catarrhalis*, and *Neisseria catarrhalis*. (Shi *et al*., 2018).

Moraxella species are Gram negative rods or cocci, but often with a tendency to resist decolourisation. The rods are often very short and plump, approaching a coccus shape 1.0 - 1.5 x 1.5 - 2.5μm. Cells usually occur in pairs or short chains with one plane of division. Polymorphism is enhanced by lack of oxygen and by incubation at temperatures above the optimum. The cocci are usually smaller (0.6 - 1.0μm in diameter) and occur singly or in pairs with adjacent sides flattened, and sometimes tetrads are formed. The Cells may be capsulated, its non-motile and aerobic or facultatively anaerobic , but some strains may grow weakly under anaerobic conditions. The bacterium is nonspore-forming , catalase and oxidase positive(Bacteriology *et al*., 2015).

On the blood agar, the colonies of *M. catarrhalis* appear round, gray, opaque and convex and they can easily be pushed intact over the surface. This phenomenon is the so-called "hockey puck sign" (kano *et al*.,2021).

Chocolate agar and blood agar were used to culture the specimens, which were incubated for 24 h at 37 °C. Bacterial growth was identified by the examination of colony morphology, gram staining, and microscopic analysis. Additional identification tests were the oxidase test, catalase test (Shi *et al* ., 2018).

M . catarrhalis staining and colonies are identical to *N . meningitidis* so it can be differentiated from the latter via its ability to growing at room temperature on blood or chocolate agar

, while *N. meningitidis* does not. A variety of biochemical tests can distinguish *M. catarrhalis* from *Neisseria .M. catarrhalis* produce oxidase, catalase, and DNAse (detected using DNAse test agar with methyl green); reduce nitrate and nitrite; and hydrolyze tributyrin. In addition, *M . catarrhalis* does not ferment glucose , maltose, lactose , sucrose nor fructose while *N. meningitidis* ferments glucose and maltose (Bernhard *et al* .,2012).

Bacterium was first isolated in 1896, it was considered to be a harmless commensal of the upper respiratory tract for a long period of time. The bacterium rapidly colonizes the nasopharynx soon after birth asymptomatically (Blakeway *et al.*, 2017) . The prevalence of colonization of the upper respiratory tract is high in infants and children but decreases substantially in adulthood. (Forsgren *et al.*,2001).

Colonization of *M. catarrhalis* in healthy individuals is facilitated by various factors such as age, family size, socioeconomic status, vaccination status, and seasonal variation. It has a significant association with age as the colonization seems to be higher in children than in adults*(*Thapa *et al.*,2017). *Moraxella catarrhalis* is bacterium that accounts for many pathologies of humans including otitis media, sinusitis, pneumonia, and conjunctivitis (LaCroce *et al.*, 2019).

Unlike other pathogens, it is generally susceptible to many antibiotics; however, newer drug resistance is emerging (Behzadi *et al.,*2021). The emergence and extensive spread of antimicrobial resistance in the natural microbial community are poorly understood (Dolinsky *et al.,*2022). Recent findings have suggested that penicillin resistance is mediated by the formation of beta-lactamases encoded by the genes bro-1 and bro-2 (Verhaegh *et al.*,2022).

Sensitive methods, such as polymerase chain reaction, to detect *M. catarrhalis* and other bacterial pathogens in respiratory secretions are in

development. Indeed, *M catarrhalis* DNA in middle ear effusions can be detected by polymerase chain reaction in children with otitis media (Hall-Stoodley *et al* ., 2006) The application of such sensitive assays are likely to contribute important observations about the epidemiology and disease patterns of *M. catarrhalis*, but they are not commercially available.

1.2.2Classfication of *Moraxella catarrhalis* :

M. catarrhalis was considered a nonpathogenic member of the resident flora of the nasopharynx. It was one of the species belonging to the so-called nongonococcal, *nonmeningococcal Neisseriae*, considered to be members of the normal flora. The name of the species has caused considerable confusion. The bacterium was first described in 1896 and was called Micrococcus catarrhalis. Later it was renamed *Neisseria catarrhalis*. In1963,Berger showed that the original genus *Micrococcus catarrhalis* actually contained two distinct species, *Neisseria cinerea* and *N.catarrhalis*(Berger *et al* .,1963).

In 1984, Branhamella catarrhalis was reassigned to the genus Moraxella as Moraxella (Branhamella) catarrhalis (Bovre *et al.,* 1984). This genus now contains both coccoid and rod-shaped bacteria, which are genetically related.

M. catarrhalis is the most widely accepted and currently preferred name for this bacterial species. (Skerman et al ., 1980*).*

Subkingdom: Negibacteria

Class, Gammaproteobacteria

Order, Pseudomonadales

Family, Moraxellaceae

Genus, Moraxella

Species : Moraxella catarrhalis

M. catarrhalis (formally known as *Branhamella catarrhalis*) has undergone several name changes in the past 100 years. It was first described at the end of the nineteenth century when it was named *Micrococcus catarrhalis*, and it was later changed to *Neisseria catarrhalis* because of its similarity in phenotype and ecological niche to species of Neisseria In 1970, the bacterium was transferred to a new genus, *Branhamella,* based on differences between *N. catarrhalis* and the type species of the genus Neisseria and six other species of this genus (Catlin *et al*., 1970).

These species could be separated based on the results of nitrate and nitrite reduction and tributyrin conversion testing. Because of the wide phylogenetic separation between *N. catarrhalis* and the so-called "true"Neisseria species, observed by avariety of a methods, the bacterium was moved to the new genus Branhamella in honour of Sara E.Branham (Catlin *et al* ., 1970).

1.2.3Epidemiology of *Moraxella catarrhalis* :

M. catarrhalis colonises the nasopharynx in early childhood(Verhaegh *et al* .,2011*)*. Many factors affect nasopharyngeal carriage of *M. catarrhalis*, such as the presence of siblings, respiratory illnesses and visiting nursery schools (Verhaegh *et al* .,2010). By the age of 6 months the cumulative colonisation rate varies between 22% and 55% (Verhaegh *et al* .,2010).

Otitis Media is particularly prevalent in Indigenous Australian children, with one study reporting that as many as 95% of infants had already suffered, acute otitis media (AOM) or otitis media with effusion (OME) in the first 8weeks of life. OM is the main cause of general practitioner consultations, antibiotic prescribing and surgical procedures in children in the developed world(Luke *et al* ., 2017). *M. catarrhalis* causes 709 million cases of acute OM (AOM) globally each year; 51 %

of which are in those ages four and under. AOM is considered one of the most prevalent childhood conditions, with approximately 80 % of all children suffering at least one episode of AOM by 3 years of age (Murphy *et al* ., 2022) .

Furthermore, several nosocomial outbreaks of *M. catarrhalis* infections in adults and in children have been reported. Winter and spring season as well as multibed wards were found to be significant risk factors for nosocomial transmission (Levy *et al* .,2009*).

In healthy children, a seasonal cyclic variation of colonisation, with a peak in autumn/winter, has been demonstrated . Other studies reported seasonal peaks of *M. catarrhalis* infections in winter and spring. This seasonality is also observed in viral respiratory tract infection such as respiratory syncytial virus (RSV) (Duppenthaler *et al* ., 2003). It has been demonstrated that children with a high nasopharyngeal RSV load have an increased risk for the development of acute otitis media (AOM), which suggests that viral infection often paves the way for subsequent bacterial AOM(Pettigrew *et al* ., 2011). Another potential factor is the physiologic cold shock response of *M. catarrhalis* (Heiniger *et al* .,2005). Cold shock describes the physiologic rapid reduction of temperature in the upper respiratory tract to approximately 26 °C when humans breathe cold air for a prolonged period of time, a phenomenon which occurs mainly during the winter season in temperate and cold climates. This physiologic cold shock has been shown to up-regulate the expression of important virulence factors, such as adherence to epithelial cells, iron acquisition, complement resistance and immune evasion (Spaniol *et al* ., 2011). Furthermore, cold shock increases the release of interleukin-8, a pro-inflammatory cytokine in pharyngeal epithelial cells (Spaniol *et al* .,2009).

These mechanisms in turn may lead to an increased bacterial density during the cold season, which has been shown to increase the risk of the development of AOM. The seasonality of viral respiratory tract infections and the physiologic cold shock response appear to be important contributors to the seasonal peak in *M. catarrhalis* infections. In adults the pharyngeal carriage rate is noticeably lower and varies between 1% and 5%. It increases again in adults older than 60 years of age. Specific mucosal IgA antibody responses against outer membrane proteins have been detected in early childhood, but they do not prevent colonisation (Stutzmann *et al* ., 2003). The presence of bactericidal serum anti-*M. catarrhalis* antibodies have been detected in both children and adults (Bakri *et al* ., 2002). The IgG3 antibody subclass response to *M. catarrhalis* is assumed to play an important role. The development of mature specific IgG antibodies is age-dependent. The subclass IgG1 develops during the first year of life and the subclass IgG3 after the second year of life, respectively. It has been demonstrated that children younger than 4 years of age have very low titers of IgG antibodies against *M. catarrhalis* (Goldblatt *et al* ., 1990*)*. This fact could explain the high colonization rate of >80% and the high rate of AOM in children younger than 2 years of age. After the introduction of the conjugate pneumococcal vaccines, the colonization pattern in children has changed towards an increased prevalence of *M. catarrhalis*, *H. influenzae* and the non-vaccine serotypes of *S. pneumonia*. *M. catarrhalis* was found significantly more often in immunized children with AOM (Revai *et al* .,2006).

1.2.4.Clinical manifestation of *M.catarrhalis*

1.2.4.1 Otitis media(OM):

Otitis media is a spectrum of related diseases that occur predominantly in children and is divided into two primary clinical presentations, acute otitis media (AOM) and otitis media with effusion

(OME). AOM involves purulent inflammation of the middle ear with associated acute-onset local and systemic symptoms (otalgia, otorrhoea and fever) ,while OME involves the accumulation of middle ear fluid in the absence of acute symptoms of infection and often succeeds AOM(Atkinson *et al*.,2015).

Poorly managed OM has severe consequences for afflicted children, including scarring of the tympanic membrane following repeated perforation, which can lead to acute or chronic hearing loss and subsequent difficulty in learning. Worldwide studies have consistently revealed a high incidence of OM, with children under 5 in Oceania among the most affected (Monasta *et al*.,2014).

Otitis media with effusion (OME) is a condition in which there is fluid in the middle ear, but no signs of acute infection. As fluid builds up in the middle ear and Eustachian tube, it places pressure on the tympanic membrane. The pressure prevents the tympanic membrane from vibrating properly, decreases sound conduction, and therefore results in a decrease in patient hearing (Emmett *et al*.,2018).

The most common symptom of OME is hearing loss, caused by an impaired transduction of sound waves due to the presence of ME effusion(MEE). Persistence of OME for 3 months or more is considered a chronic condition named chronic otitis media with effusion (COME). A persistent hearing loss may negatively impact speech development, behavior and progress at school (Schilder *et al*., 2016*)*.

CSOM(Chronic suppurative otitis media) is a chronic inflammation of the middle ear and mastoid mucosa with a non-intact tympanic membrane from which discharge (otorrhea) is present. It is one of the most common chronic infectious diseases worldwide, often occurring in the first 5 years of life and more common in developing countries (Mittal *et al* .,2015).

OM is typically a polymicrobial infection that predominantly involves at least one of three bacterial otopathogens, *S. pneumoniae*, non-typeable *Haemophilus influenza* (NTHI) and/or *M. catarrhalis*, and any of several upper respiratory tract viruses (e.g. *rhinovirus, respiratory syncytial* virus and *influenza virus*) (Luke *et al., 2017). M. catarrhalis* is the second or third most common pathogen to cause acute otitis media together with *Streptococcus pneumoniae and nontypable H. influenzae* (Broides *et al.*,2009).

It should be noted that tympanocentesis and culture of middle ear fluid is required for the correct microbiologic diagnosis of bacterial AOM. Tympanocentesis is not routinely performed and the rate of *M. catarrhalis* AOM may thus be underestimated. Compared to *S. pneumoniae* and *H. influenzae*, *M. catarrhalis* causes a relatively mild course of AOM. S. *pneumoniae* AOM are clinically more severe than those caused by *H. influenzae* and *M. catarrhalis* and are more often associated with high fever, tympanic membrane bulging and redness and severe otalgia (Otsuka *et al.*,2022).

Multiple factors influence the pathogenesis of acute otitis media in children. One of the most important factors is upper respiratory tract viral infection. During infection, pathogen migrate into the middle ear along the eustachian tube and cause inflammation, leading to congestion of the Eustachian tube, which in turn causes a negative pressure in the middle ear .Bacteria migrating or aspirated into the middle ear cavity can proliferate and cause AOM. *M. catarrhalis* possesses different virulence factors, which play an important role in this sequence of events(Bernhard *et al.*,2012*).

Biofilm formation by pathogenic bacteria in the nasopharynx followed by shedding of biofilm cells has also been pointed as an important contributor to OM, particularly AOM. Dispersed cells may

enter into the ME(middle ear) cavity, taking advantage of the negative pressure in the ME due to ET(Eustachian tube) dysfunction. Once in the ME, these bacteria can establish a new biofilm and induce disease. Since bacteria in biofilms can resist to the antimicrobial agents administered more effectively, recurrent episodes of OM (RAOM) are probable to occur (Coticchia *et al*., 2013*)*.

The polymicrobial biofilms associated with AOM are incredibly resistant and difficult to treat using classic antibiotic protocolss (Korona *et al*., 2018) This is a result of conferred β-lactamase protection, quiescent bacteria within biofilms, poor antibiotic penetration and persister cells.

When taken in combination with the continued prevalence of AOM in the post-vaccine era, these challenges demand novel preventative and treatment strategies. Because all of these otopathogens can colonize asymptomatically, the interactions that occur in the nasopharynx that prevent or promote co-colonization play an important role in the steps that eventually lead to pathogenesis(Murphy *et al* ., 2015).

1.2.4.2. Chronic obstructive pulmonary disease (COPD):

Chronic obstructive pulmonary disease (COPD) is a chronic multifactorial inflammatory disease whose main pathophysiological mechanisms include airflow limitation, pulmonary emphysema, and chronic bronchitis (Brown *et al*.,2018*)*.

Among the aspects that characterize COPD pathogenesis, neutrophil-mediated oxidative stress (or reactive oxygen species, ROS) is one of the most important hallmarks (Choudhury and Macnee, 2017). There is significant theoretical support for the hypothesis that ROS contributes to the pathogenesis of COPD (Footitt *et al*., 2016). Lungs perse are particularly vulnerable to oxidative stress due to the relatively high oxygen environment, increased blood supply, and

exposure to environmental pathogens and toxins. Additional factors contributing significantly to this burden are cigarette smoke and, in COPD patients under treatment, extensive antibiotic exposure (Marino *et al.*, 2015).

The course of COPD is marked by recurrent periods of worsening symptoms, called exacerbations (Pavord *et al.*, 2016), responsible for disease progression, increased morbidity and mortality. Several epidemiological studies have reported that nontypeable *Haemophilus influenzae* (NTHi) and *M. catarrhalis* are the most prevalent bacteria found in the sputum of individuals with exacerbations of COPD (D'anna *et al.*, 2020). and their co-infections reach up to 20–30% (Perez *et al.*, 2019).

There are many pulmonary and systemic comorbidities in COPD patients, such as bronchiectasis, asthma, heart failure, cardiovascular diseases, sleep apnea, malnutrition, and frailty(Matsunaga *et al.,* 2020).

The inflammatory process can alter the bronchi, bronchioles, and pulmonary parenchyma, leading to progressive restriction of airflow, resulting in emphysema and chronic bronchitis. The pathogenesis of emphysema includes destruction of alveolar septa, increased air space, and loss of elastic recoil due to hyper inflammation and oxidative stress (Gharib *et al* .,2018). Chronic bronchitis involves the over production and hyper secretion of mucus by goblet cells, thereby reducing air flow(*Kim et al .,2015*).

COPD is a progressive airway disease consisting of persistent airflow limitation and chronic inflammation, and is currently the fourth greatest cause of death worldwide. COPD sufferers experience alternating periods of stability and acute exacerbations of the disease characterized by the sudden worsening of respiratory symptoms (sputum production, purulence and tenacity, cough and dyspnea). Frequent exacerbations have

a detrimental impact on lung function and quality of life, and drastically increase the risk of mortality (Suissa *et al.*,2012).

COPD is characterized by irreversible airflow obstruction and airway inflammation.*(George et al.,2016)* Bacteria are commonly identified in stable state and at exacerbations with increased bacterial load and dysbiosis associated with exacerbations (Wang *et al.,*2016) .The most common pathogens identified are *Haemophilus influenzae* and *Streptococcus pneumoniae* in both stable disease and exacerbations, but several other pathogens are identified including *Moraxella catarrhalis*.(Wilkinson *et al.*,2017).

M. catarrhalis is also the second most common cause of exacerbation in chronic obstructive pulmonary disease (COPD)(Wilkinson *et al.*,2017). which is the third largest cause of global morbidity responsible for 3 million deaths in 2016.(World Health Organization *et al.,*2018).

1.2.5. Pathogenesis of *Moraxella catarrhalis*:

Moraxella catarrhalis pathogenicity, like other microorganisms, depends on the ability for binding to epithelial and mucus layer and escape from the host defense mechanisms (Liu *et al.,* 2016). *M. catarrhalis* can attach to numerous types of cells, involving epithelial cells of bronchial, small airway, and type 2 alveolar cells (Vries *et al.*, 2013) .

M. catarrhalis causes mucosal infections in children and adults. The pathogenesis of infection appears to involve contiguous spread of the bacterium from its colonizing position in the respiratory tract to cause clinical signs of infection. Colonization of the upper respiratory tract with middle ear pathogens, including *M. catarrhalis*, is a necessary first step in

the pathogenesis of otitis media. However, colonization alone is not sufficient to cause disease. An inciting event, such as a viral infection, in a child colonized with a middle ear pathogen is probably necessary for bacteria to move to the middle ear and cause otitis media. In the case of infection in adults with COPD the acquisition of a new strain is critical in the pathogenesis of infection (John *et al.,* 2020).

M. catarrhalis causes mucosal infections in children and adults. The mechanisms of colonization and pathogenesis of *M. catarrhalis* have been extensively studied and many virulence factors have been identified. The most important virulence strategies involve: (1) evasion of complement-mediated killing mainly via interference with regulatory proteins; (2) polyclonal, non-specific B cell activation and redirecting of adaptive immunity; (3) hiding inside lymphoid tissue, which is the main reservoir facilitating the host invasion; (4) formation of biofilm; and (5) participation in protease-antiprotease imbalance(Parameswaran et al ., 2009).

Some of these strategies can be driven in part by the release of outer membrane vesicles (OMVs), which contain several virulence factors facilitating the delivery of periplasmic and outer membrane components to the host *(*Kaparakis-Liaskos *et al*. , 2015*).* Moreover, OMVs can favour pathogen coexistence and colonization after their interaction with the other bacterial species(Schaar *et al.,*2011).

M. catarrhalis also appears to be able to invade host epithelial cells (Jordan *et al*.,2010), the intracellular survival of pathogens being an important aspect of host immune evasion. Moreover, once attached to the host mucosal surfaces, *M. catarrhalis* has the ability to interact and/or compete with the commensal flora and has the apparatus to survive and multiply under challenging nutrient-limiting conditions. Such conditions

may result in the formation of microcolonies and biofilms (Christensen *et al.,*2010).

1.2.6 Virulence factors of *Moraxella catarrhalis* :

Several virulence factors of *M. catarrhalis* have been identified and characterized, and many of these are raised through the plasma membrane and are either generalized to the outer membrane protein (OMPs) or secreted outside the cell. These molecules then mediate processes such as adherence to epithelial cells, complement resistance, biofilm formation, and nutrient acquisition in order to colonize and cause disease in the human host.

Usually, the virulence factors associated with whole bacteria or can be driven in part by the release of outer membrane vesicles (OMVs) (Augustyniak *et al*., 2018) It is not identified if virulence is linked with certain strains or subpopulations of *M. catarrhalis*, or if variances in clinical appearance can be attributed to the heterogeneous expression of specific *M. catarrhalis* virulence factors in the socializing population (Blakeway *et al*., 2017*)* .

Generally, some of these mechanisms which contain several virulence factors facilitating the transfer of periplasmic and outer membrane components to the host (Kaparakis-Liaskos *et al* .,2015) Moreover, OMVs can confer pathogen existence and colonization after their interaction with the other bacterial species (*Schaar et al., 2011*) . *Moraxella catarrhalis* is commonly found in several community of clinical isolates. This pathogen uses several virulence mechanisms to colonize and survive in its host (Perez and Murphy *et al* .,2017*).*

There are a number of virulence factors and virulence-associated genes, have been identified in a number of respiratory pathogens, as *M. catarrhalis* isolates. Pathogenicity of some virulent

strains contributes to possess a highly mutable genes (Blakeway *et al.*, 2017*).* Moreover, many related genes have been identified in the genomes of a wide variety of bacterial species, suggesting the presence of the outer membrane protein genes and the potential for pathogenesis specially Ubiquitous surface protein A1 and A2 (*uspA1* and *uspA2*)antigens (Spaniol *et al.*, 2008).

Addition to *uspA1* and *uspA2*, a number of different gene products of *M. catarrhalis* have been associated with colonization and complement evasion, *including* CopB.(Sethi *et al* .,1997).

1.2.6.1 *outer membrane proteins* (OMPs) *Cop B* **Protein:**

In order for *M. catarrhalis* to survive in the human host , the bacterium must have a way to acquire iron. In mammals, only about (0.1%) of iron is estimated to be in the extracellular space and is bound to either transferrin, which mostly found in serum and lymph , or lactoferrin , that mostly found in phagocytic cells and mucosal secretions (Hays *et al* ., 2006).

CopB was identified as a major outer membrane protein and potential vaccine candidate from *M. catarrhalis* (Helminen *et al.*, 1993). However, subsequent studies demonstrated that the immune response in human convalescent sera was primarily associated with TbpB(Transferrin- binding protein B) (Campagnari *et al.*, 1996; Mathers *et al.*, 1997), which may have reduced the interest in pursuing *CopB* as a vaccine candidate. In parallel, early studies identified as a *CopB* homologue, FrpB, as a major iron regulated outer membrane protein in *Neisseria meningitidis* (Ala'Aldeen et al., 1994).

CopB an important antigen for the human immune response. It plays a role in mediating resistance to the bactericidal activity of normal human serum (Helminen *et al.*, 1993). The predominant antibody

response to chronic lower respiratory tract infection with *M. catarrhalis* in patients with bronchiectasis is to OMP B1, a 81-kDa minor OMP.

Hays (2009) has shown that *M. catarrhalis* is able to utilize iron from the human transferrin and lactoferrin iron binding proteins . A main factor allowing *M. catarrhalis* to survive and cause disease on the human respiratory mucosa is the ability to acquire essential iron. The iron-limited environment of the human host requires pathogens to be diverse in their repertoire of iron acquisition systems . Also the acquisition of iron by *M. catarrhalis* is mediated by several surface-exposed iron- binding proteins, one of these outer membrane protein is *CopB* protein , formerly known as *Omp B2* (Pearson *et al* ., 2004) , which its expression is up-regulated under iron starvation conditions .

This outer membrane protein has been found in all isolates (Bullard *et al* .,2007) , and the patients with *M. catarrhalis* can make antibodies against *CopB* protein , so this protein is able to mount a bactericidal immune response against *M. catarrhalis*. In humans, (IgA) antibodies from healthy adults are directed against *CopB* , and many studies indicate that *CopB* might be an immunogenic antigen with vaccine potential, and antibodies to this protein might be protective (Aebi *et al* ., 1997).

1.2.6.2 Ubiquitous surface protein A1 and A2 (*uspA1* and *uspA2*):

UspA consists of three major proteins including *uspA1, uspA2*, and a closely related protein known as hybrid *uspA2H*, since it has the property of both *uspA1* and *uspA2* and contributes both in adhesion and serum resistance (Brooks *et al*., 2008*).

Ubiquitous surface protein *UspA1* and *UspA2* were listed as top antigens for *M. catarrhalis* vaccine candidates in the past. However, observations indicate that *UspA1* and *UspA2* have diverse sequences with varied structures resulting in different phenotypes and divergent functions in interacting with host targets among strains and clinical isolates.

Therefore, these proteins are no longer high in terms of priority in the list of vaccine targets. *uspA1* and *uspA2* are heat-modifiable proteins with predicted molecular weight of 83 and 60 kDa, respectively, but they appear 130 kDa (UspA1) and above 200 kDa (*UspA2*) after denaturation in SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). UspAs are adhesins and autotransporters with an oligomeric coiled-coil structure. They also play a role in serum resistance (Nordstrom *et al.,*2005)and other virulence mechanisms.

   M. catarrhalis attaches to epithelial cells *via uspA1*, which binds arcinoembryonic antigen-related cell adhesion molecule 1(CEACAM1) (Hill and Virji, 2003*)*, and as a consequence suppresses the human inflammatory response (Slevogt *et al*., 2008). *uspA1* also binds extracellular matrix proteins laminin (Tan *et al*., 2006*)* and fibronectin (Tan *et al.*, 2005;Agnew *et al.*, 2011), whereas UspA2 binds preferentially to laminin (Tan *et al*., 2006*),* Another important function associated with *UspA* proteins is serum resistance. Both *uspA1* and *uspA2/A2H* have been proposed to bind the C3d domain of C3, inhibiting both the classical and alternative pathways of the complement cascade (Hallström *et al*., 2011). Interestingly, among the larg number of bacteria analyzed, only *M. catarrhalis* has this unique ability to interfere with the innate immune system of the complement system by binding C3, Furthermore, *UspA1* and *UspA2* appear to bind to the complement inhibitor C4b binding protein (C4BP) in a dose dependent manner. Finally, UspA proteins block generation of the opsonin C3a, which may result in decreased inflammatory reactions (Hallström *et al*., 2011). This last would be consistent with binding C3d (Lambris *et al .,*2008*).*

   UspA proteins belong to the trimeric autotransporter adhesin (TAA) family. Although no full-length structure of *uspA1* is available, there are structures of three *UspA1* fragments. Two structures (3NTN and

3PR7) (Agnew *et al.*, 2011) together give a fragment comprising *UspA1*, containing the head, the neck, and 33 amino acids of the stalk domainThe head domain consists of 14-to-16 residue repeats placed parallel to each other forming a trimeric left-handed parallel β-roll, first identified in YadA(Yersinia adhesion). The neck (region 276–334) is a positively charged region of the *UspA1* structure forming large loops (Agnew *et al.*, 2011); it belongs to the long neck type (Hartmann *et al.*, 2012) as found in SadA (2YO2, 2YNZ) SadA promotes biofilm formation and host cell adherence in *Salmonella* (Hartmann *et al.*, 2012) or Bpa A (*Bisphenol A)* (3LAA) The structure of part of the stalk of *UspA1* (*UspA1*) has been solved (2QIH) (Conners *et al.*,2008) It is supposed to bind CEACAM1 . It reveals a continuous left-handed trimeric coiled-coil stalk with, as expected, an underwound periodicity of 3.5 residues per turn, characteristic for TAA proteins (Conners *et al.*, 2008).

Both *uspA1* and *uspA2*, consistent with their functional activities, have been localized to the surface of *M. catarrhalis*, where they are accessible to antibodies (McMichael *et al* ., 1998). *UspA1* and *UspA2* were shown to be antigenically conserved among *M. catarrhalis* clinical isolates and contain surface-exposed epitopes. However, there are various 'cassettes' of peptide sequence in the UspA variants from a variety of independent *M. catarrhalis* isolates (Brooks *et al*.,2008*)*. Modular assortment of unrelated 'cassettes' of peptide sequence results in divergence of individual UspA proteins. Exchange of certain variant cassettes accounts for strain-specific differences in UspA protein function and confers differing phenotypes among different *M. catarrhalis* isolates (Brooks *et al*.,2008). Another study demonstrates that *UspA* proteins exchange their functional regions *in vivo* and this genetic change contributes more heterogeneity in the sequence and function of the protein family than previously believed (Hill *et al* .,2012).

The *UspA1* protein is thought to form "lollipop" like structures on the bacterial surface , that are approximately 60 nm in length and found to play an important role in biofilm formation in *M. catarrhalis* (Verhaegh *et al.,* 2011) .

Both *uspA1* and *uspA2* induce naturally acquired antibodies in children and adults (Murphy et al ., 2005). *UspA1-* and *UspA2*-specific antibodies from guinea pig and mice also show bactericidal activities.

Immunization with individual *UspA1* and *UspA2* proteins enhances bacterial clearance from the lungs in mice challenged with *M. catarrhalis* (McMichael *et al* .,1998). In sum, *UspA* proteins have been extensively characterized and tested and their potential as vaccine antigen targets has diminished over time.

1.2.6.3 Hemagglutinin/*Moraxella catarrhalis* Immunoglobulin D-Binding Protein(MID/Hag):

Haemagglutinin (hag), also known as MID (Superantigen Moraxella immunoglobin D binding protein), is an important surface protein that mediates the adherence of *M. catarrhalis* to various host cells, most notably human middle ear epithelial cells (HMEEC) (Bullard *et al* .,2005) The surface protein Hag (also designated MID) has previously been shown to be a key adherence factor for several epithelial cell lines relevant to pathogenesis by *M. catarrhalis*.

The hemagglutinin protein (*hag*) from *M. catarrhalis*, was first described by Sasaki *et al*. , (1998) as a 200-kDa outer-membrane protein . It was responsible for lollipop-like formations on the bacterium's surface ,this protein is also referred to as *M . catarrhalis* Ig D binding protein (*MID*) (Pearson *et al.* , 2002).

This protein belongs to a family of trimeric autotransporter proteins called oligomeric coiled coil adhesins (Oca) (Bullard *et al .,* 2005).

Hag is a major adhesin expressed by *M. catarrhalis* , and has been identified in the most isolates (Mollenkvist *et al*., 2003). Essentially , *MID / Hag* is a multifunctional outermembrane protein that fulfills an important role in pathogenesis (Forsgren *et al*. ,2003) .

The *mid* gene was cloned and expressed in *Escherichia coli*. The complete *mid* nucleotide gene sequence was deter-mined, and the deduced amino acid sequence consists of 2,123or 2,139 residues, depending on two alternative translation starts. The sequence of *MID* has no similarity to other Ig-binding proteins and differs from all previously described outer membrane proteins (OMPs) of *M. catarrhalis*. *MID* was found to exhibit unique Ig-binding properties(**Gjörloff** *et al* ., 2002).

The surface protein *Hag/MID* that acts as an adhesin and hemagglutinin, exhibits unique immunoglobulin (Ig) D-binding properties and binds to both soluble and membrane-bound IgD on B cells. Our previous study demonstrated that exposure of *M. catarrhalis* to 26°C down-regulates *hag* mRNA expression (**Heiniger** *et al* .,2005), indicating a possible involvement of *Hag* in the cold shock response.

The *mid* gene was detected in 98 different strains as revealed by homology of the signal peptide sequence and a conserved area in the 3′ end of the gene (Mo̎llenkvist *et al* .,2003).

The importance of *MID/Hag* is underlined by the consistently strong immune response observed in humans. *mid* also stimulates a prominent mucosal immune response in COPD patients. Most patients develop mucosal IgA against *MID*, *UspA1*, and*UspA2*, whereas fewer patients develop IgA responses against other OMPs like TbpB and *CopB*. Besides stimulating a significant mucosal response, a strong serum IgG response to *MID* can also be found in the majority of COPD patients who cleared the organism (Murphy *et al* .,2005).

Hallstrom *et al* ., (2008) mentioned that *Hag* mediates binding to a number of different human cell types   and also mediates   *M. catarrhalis* cells binding to each other . This protein was shown to participate in *M. catarrhalis* binding to human erythrocytes , lung epithelial cells and directly mediates attachment to human middle ear epithelial cells (HMEE) (Holm *et al.,* 2003). Disruption of *hag/mid* in several *M. catarrhalis* strains substantially decreased adherence to these cell types , while expression of *hag* in *E. coli* increased attachment to middle ear cells at least 17-fold (Bullard *et al.,* 2007).

The *Hag/MID* protein also contributes to evasion of the immune response by inducing non-specific IgM production from B-cells without T-cell help , potentially serving to redirect the adaptive immune response. *Hag* was the only *M* . *catarrhalis* outer-membrane protein that all patients made an IgA immune response to it (Murphy *etal.,*2005) and patients who cleared *M. catarrhalis* from the respiratory tract demonstrated a mucosal immune response   specific   to *Hag* . Furthermore, infants salivary IgA were shown to be directed against this protein. On the other hand , Vidakovics and Riesbeck , (2009) had published that *Hag/MID* had been determined to be involved in biofilm formation by *M. catarrhalis*.

1.2.6.4 *M. catarrhalis* adherence protein (*McaP*):

M. catarrhalis adherence protein (*McaP*), was first identified by Lafontaine and later described by the same group as one of the essential outer membrane protein responsible for adhesion (Lipski *et al* .,2007). Surprisingly, no significance reduction of adherence was found in mutant *M. cat*arrhalis that lacked only the *McaP* gene compared to wild-type *M.catarrhalis* The other adhesion molecules, such as *UspA1, UspA2*, and *Hag*, could compensate the function of *McaP*.

The *M. catarrhalis* adherence protein (*McaP)* is another highly conserved autotransporter. Unlike *UspA1* and *Hag*, *McaP* is a conventional autotransporter. Conventional autotransporters differ from trimeric auto transporters in that the former functions as a monomer and therefore possesses a much longer translocator domain containing mulitple pore-forming beta-barrels. *McaP* is composed of an N-terminal signal sequence and passenger domain, a short linker sequence, and a C-terminal transporter domain (Lipski *et al* .,2007).

In addition to being an adhesin, *McaP* also exhibits esterase and phospholipase B activities, evidenced by the ability of *McaP* to cleave short-chain fatty acids as well as phosphatidylcholine(PC)and lysophosphatidylcholine (Timpe *et al*., 2003). However,the biological relevance of these lipolytic activities has not been determined.

Lipski *et al.*,(2007) showed that the two functions of adhesion and lipolytic activity of this protein are separable. Since adherence and lipolytic activity are frequently associated with virulence in other organisms.

1.2.6.5 *M. catarrhalis acid phosphatase A (MapA):*

The N-terminal portion of the *M. catarrhalis acid phosphatase A* (*MapA)* was most similar (the BLAST probability score was 10^{-10}) to bacterial class A nonspecific acid phosphatases. The central region of the *MapA* protein had similarity to passenger domains of other autotransporter proteins, whereas the C-terminal portion of *MapA* resembled the translocation domain of conventional autotransporters. Cloning and expression of the *M. catarrhalis mapA* gene in *Escherichia coli* confirmed the presence of acid phosphatase activity in the *MapA* protein. The *MapA* protein was shown to be localized to the outer membrane of *M. catarrhalis* and was not detected either in the soluble

cytoplasmic fraction from disrupted *M. catarrhalis* cells or in the spent culture supernatant fluid from *M. catarrhalis* (Todd *et al* ., 2007).

The *mapA* gene was flanked upstream by an ORF, designated *prmA*, encoding a predicted ribosomal protein L11 methyltransferase and downstream by a gene encoding a predicted penicillin-binding protein 1B.

MapA was an autotransporter protein, the predicted *Map*A translocation domain was used to replace the predicted translocation domain of the *McaP* protein (Lipski *et al 2007*) .

1.2.7. Multilocus sequence typing (MLST):

Multilocus sequence typing (MLST) combined a number of technical and conceptual developments of the last two decades of the 20th century to provide a universal, portable, and precise means of typing bacteria (Urwin *et al* ., 2003) . The approach owed much to the pioneering technique of multilocus enzyme electrophoresis (MLEE), from which it acquired its name. A key conceptual development was the recognition that bacteria do not necessarily have a clonal population structure, leading to the realization that patterns of genetic exchange among bacteria, and therefore of descent, could only be resolved by the analysis of nucleotide sequence data from multiple locations of the chromosome (Maynard *et al* .,2000) . Developments in high-throughput nucleotide sequence determination and analysis permitted the generation of definitive genetic data from any locus on the chromosome of multiple isolates. An advantage of nucleotide sequence data is that they can be disseminated via the Internet, particularly the World Wide Web (Jolley *et al* ., 2004) .

MLST has since been applied to a number of different bacteria and eukaryotic organisms as a tool for the epidemiological analysis and surveillance of pathogens as well as to investigate their population

structure and evolution. MLST has also been deployed in studies of the population structure of nonpathogenic bacteria (Maiden *et al* ., 2006).

Multilocus sequence typing (MLST) has been widely employed in epidemiological investigations of various scales(Qin *et al*., 2009), including population, pathogenicity, and evolution studies, of several bacteria.

Morever ,MLST, which is a typing method based on the sequencing of housekeeping genes and characterizes isolates on the basis of variation in nucleotide sequences of each locus of the selected genes. The different sequence at each locus are assigned with specific allele numbers and each unique combination of alleles, often called as allelic profile is assigned a sequence type (ST), which is the unambiguous descriptor of the strain (Sharma *et al*., 2016*)*.

The Multilocus sequence typing (MLST) is a technique for distinguishing accurately between different isolates within a species. MLST is based on the principles of phenotypic multi-locus enzyme electrophoresis (MLEE). MLEE is a typing method that relies on differences in electrophoretic mobility of different enzymes present within a bacterium(Sabat *et al*.,2013).

However , it provides a uniform, expandable typing method that can be used for long-term epidemiology (Maiden   *et al.,*2006). The developed such an MLST scheme for *M. catarrhalis* that is based on the sequences of eight housekeeping gene fragments. This scheme is freely available for interrogation.

The *M. catarrhalis* MLST scheme uses internal fragments of the following 8 house-keeping genes: *glyRS* (glycyl-tRNA synthetase beta subunit), *ppa* (Pyrophosphate phospho-hydrolase), *efp* (elongation factor P), fumC (fumarate hydratase), *trpE* (anthranilate synthase component I),

mutY (adenine glycosylase), *adk* (Adenylate kinase), *abcZ* (ATP-binding protein).

Multilocus sequence typing (MLST) is an unambiguous procedure for characterising isolates of bacterial species using the sequences of internal fragments of 8 house-keeping genes. Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequences. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST) (Maiden *et al*., 2013).

In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The rationale is that a single genetic event resulting in a new allele can occur by a point mutation (altering only a single nucleotide site), or by a recombinational replacement (that will often change multiple sites) - weighting according to the number of nucleotide differences between alleles would erroneously consider the allele to be more different than by treating the nucleotide changes as a single genetic event (Jolley *et al., 2018*).

In a typical MLST approach, recombination is expected to occur with a much higher frequency than point mutations. Therefore, one does not look at the total sequence similarity between strains. Instead, each sequence for a given locus is screened for identity with already known sequences for that locus. If the sequence is different, it is considered to be a new allele and is assigned a unique (arbitrary) allele number.

eBURST is an algorithm that can be used to subdivide MLST data into nonoverlapping groups of STs with a user-defined level of similarity in their allelic profiles (Feil *et al*.,2004). The most stringent definition of

an eBURST group, where all STs assigned to the same group must share alleles at least five of the seven MLST loci with at least one other ST in the group, identifies clusters of closely related genotypes that are considered to be descended from the same founder and that are defined as clonal complexes (Feil *et al*.,2004).

The total number of STs within each clonal complex identified by eBURST was rather low and probably reflects sampling strategy. In general, eBURST may identify few clonal complexes, and few large clonal complexes, in populations where sampling has largely been designed to uncover the genetic diversity within the species (Spratt *et al*.,2001).

MLST provides a number of advantages over other typing approaches. First, it uses sequence data and can therefore detect changes at the DNA level that are not apparent by phenotypic approaches, such as serotyping, and by MLEE that uses the migration rate of proteins in starch gels. Second, it is a generic technique that can be readily reproduced and does not require access to specialized reagents or training. Third, modern methods of direct nucleotide sequencing, based on the polymerase chain reaction (PCR), do not require direct access to live bacterial isolates or high-quality genomic DNA. These techniques can be performed on killed cell suspensions, avoiding all the difficulties associated with the transport and manipulation of pathogens, or on clinical samples, such as the cerebrospinal fluid or blood of a patient undergoing antibiotic therapy, from which a live bacterial isolate might be difficult to obtain. Fourth, the data generated are fully portable among laboratories and can be shared through-out the world via the Internet. Finally, the Initrnet can also be used to disseminate MLST methods, providing standardization of approaches (Maiden *et al*.,2006).

1.2.8 Restriction–Modification systems(R-M):

In many bacterial species, restriction–modification (R-M) systems are among the few genes associated with distinct phylogenetic lineages or virulent versus a virulent subpopulations(Tan *et al.,* 2016).

R-M systems are ubiquitous in bacteria and are particularly abundant in naturally competent species. R-M systems consist of a restriction endonuclease and a DNA (adenine or cytosine) methyltransferase that cleave and methylate DNA at specific DNA sequences, respectively.

R-M systems have traditionally been described as a type of bacterial defense mechanism to protect the host cell from invasive foreign DNA (e.g., bacteriophages). However, R-M systems have been demonstrated to perform several additional roles, including maintaining speciation(Vasu and Nagaraja .,2013), DNA repair and epigenetic regulation of gene expression (Srikhanta *et al.,* 2005 ; Atack *et al* .,2018*)*

Four main types of R-M systems exist (Types I–IV) that differ in their subunit composition, cofactor requirements and mechanism of action (Roberts *et al.,* 2003).Type I R-M systems are a complex of three subunits: A restriction endonuclease (HsdR) that cleaves unmethylated DNA, a DNA methyltransferase (HsdM) that methylates DNA and protects the host genome from cleavage, and a specificity subunit (HsdS) that determines the recognition sequence of the complex.

Type II R-Msystems consist of a restriction endonuclease (Res) and a methyltransferase(Mod) that act independently of each other. Type II are the most abundant, present in 39.2% of bacterial genomes (Tesson *et al.,* 2022) with a mean of ~0.5 systems per genome (Oliveira *et al* .,2014). Type II R-M systems consist of two enzyme activities: a restriction endonuclease which cuts double-stranded DNA (dsDNA) at targets and a methyltransferase which modifies targets to protect them

from cleavage. These enzymes are typically encoded by separate genes located close together in the genome. The targets of restriction are short sequences of 4-8 bases which are usually palindromic i.e. they are equal to their own reverse complement (Pingoud and Jeltsch., 2001) due of the symmetrical subunits of the protein multimers that recognize the target (Arber *et al*., 1969). Any occurrences of the restriction target in the cell's own DNA should be protected from restriction by the methyltransferase. In contrast, DNA originating from a different species or strain should lack this methylation at target sites and will be cleaved by the restriction endonuclease when it enters the cell.

The Type II R-M enzymes usually act as monomers, dimers or even tetramers.

Type III R-M systems consist of an independent methyltransferase (Mod) that contains a DNA target recognition domain (TRD; also known as the DNA recognition domain) and methylates DNA, and a restriction endonuclease (Res) that must form a complex with Mod to recognize and cleave DNA (Blakeway *et al*., 2014). The Type III DNA methyltransferases ModM and ModN are phase variable, and that there are three modM alleles (modM1-3) that vary in their TRDs (Blakeway *et al.*, 2014). the phase variable TypeIII methyltransferase ModM2 controls expression of multiple genes in aphase varion ,including genes involved in colonization, and protection against host defenses.

The restriction endonucleases recognize specific DNA sequences close to or at the recognition site and cleave the DNA. The methylases transfer a methyl group from the donor (AdoMet) directly to double-stranded DNA and form m4C, m5C or m6A. Type III R-M enzymes are composed of two protein subunits that function either in DNA recognition and modification (Mod) or restriction (Res). To cleave DNA, both subunits are necessary, as well as an absolute requirement for ATP

hydrolysis. After recognition of the DNA sequence, an ATP-dependent DNA translocation occurs, as with the Type I restriction enzyme. The Mod subunit can function independently of the Res subunit to methylate DNAto form m6A (reviewed by Bourniquel and Bickle, 2002; Tock and Dryden, 2005).

ModM is a phase-variable type III DNA methyltransferase that acts as an epigenetic regulator. ModM phase variation is mediated by a 5¢-(CAAC)n-3¢ repeat tract in its ORF (Seib *et al* ., 2002), and switching of ModM expression alters the expression of a distinct set of genes, known as a phase-variable regulon or phasevarion, via differential methylation of the genome (Blakeway *et al* ., 2014). Three modM alleles (modM1, modM2 and modM3) have been identified that differ in their central DNA recognition domain and potentially regulate the expression of different phasevarions.

Phase-variable Type III DNA methyltransferases in *M. catarrhalis* (Blakeway *et al.,* 2014), *N. meningitidis* (Jen *et al,.* 2014), *N. gonorrhoeae* (Srikhanta *et al.*, 2009), and *H. influenzae* (Atack *et al.*, 2015) have been shown to act as epigenetic regulators of gene expression and differentially affect the virulence of these bacteria. We previously identified three modM alleles (modM1-3) and it was shown that ModM2 and ModM3 methylate different .

Several Type III DNA methyltransferases exhibiting SSR(Server – Side Rendering) mediated phase-variable expression have also been identified in *M. catarrhalis* (Blakeway *et al*., 2018; Tan *et al*., 2020) Phase variation of Type III DNA methyltransferases causes differential methylation of the genome between clonally derived cells, epigenetically altering the expression of multiple genes (a phasevarion) that are otherwise not associated with readily identifiable genetic markers of phase variation (e.g., SSRs) (Seib *et al*., 2020). Adding further

complications, many of the phase-variable Type III DNA methyltransferases characterized to date have multiple target recognition domain (TRD) alleles, and it is hypothesized that every allelic variant regulates the switching of expression of a distinct phase varion, substantially increasing the number of phase-variable genes in the species (Atack *et al*., 2018).

Type IV R-M systems are composed of a single enzyme that only cleaves methylated DNA. Many host-adapted bacterial pathogens contain R-M systems that are phase-variable. Phase variation is the random and reversible, high frequency on/off or graded switching of gene expression, which is typically mediated by simple DNA repeats in host adapted bacteria. (Ham *et al.*, 1993),

As well as functioning as defense systems, R-M systems can also be viewed as selfish elements that serve to propagate themselves. Because the methyltransferase decays more quickly than the endonuclease, a Type II R-M system can function as an addiction system to ensure its own persistence (Ichige and Kobayashi 2005), similar to toxin antitoxin systems (Mruk and Kobayashi., 2014). This addictive quality may contribute to their occasional occurrence on MGEs such as plasmids: around 10.5% of plasmids carry R-M systems (Oliveira *et al*., 2016) and experiments have shown R-M system carriage can lead to increase plasmid stability in cells (Kusano *et al*., 1995).

However, various mechanisms mediate phase variation, including slipped strand mispairing of simple DNA sequence repeats . site-specific recombination (e.g., DNA inversion) (Zieg *et al*., 1977) or domain shuffling(Manso *et al*., 2014) or epigenetic mechanisms (e.g., differential methylation and competition between regulatory proteins Dam and Lrp affect expression of the *Escherichia coli pap* operon). Phase variation of DNA methyltransferases due to changes in DNA sequence repeats

(Blakeway *et al*., 2014) or domain shuffling (Manso *et al*., 2014), causes differential methylation of the genome, which epigenetically alters the expression of multiple genes in systems known as phase varions (phase-variable regulons) (Srikhanta e*t al.* 2005; Atack *et al*., 2018). In every case identified, switching of expression of phase varions alters the pathobiology of the organism, and controls expression of current and putative vaccine candidates (Atack *et al.*, 2018).

In every case identified, switching of expression of phase varions alters the pathobiology of the organism, and controls expression of current and putative vaccine candidates (Atack *et al.,* 2018).

2 . Materials and methods

2.1 Equipments and Instruments :

The equipments and instruments used in this study were listed in Table (2-1).

Table (2-1) :Laboratory equipments and instruments

No.	No Instrument	Company	Country
1	Incubator	Memmert	Germany
2	Water bath	Memmert	Germany
3	Oven	Memmert	Germany
4	Distillator	GFL	Germany
5	Centrifuge	Hettich	Germany
6	Micropipettes 5-50 µl , 100-1000 µl, 0.5 – 10 µl	Eppendorf	Germany
7	DNA extraction tubes 100 µl	Eppendorf	Germany
8	PCR tubes 50µl.	Eppendorf	Germany
9	Millipore filter (0.45mm)	Satorins membrane Filter Gm, BH, W.	Germany
10	Sterile swab	Lab.Service	S.P.A.
11	Autoclave	Stermite	Japan
12	Light microscope	Olympus	Japan
13	Dualed Blue /White Transilluminator and Documentation system	Bioneer	Korea
14	Sensitive electron balance	A & D	Lebanon

15	Vortex	Clever	USA
16	Thermocycler apparatus	Clever	USA
17	Gel electrophoresis	Clever	USA
18	UV-transilluminator	Clever	USA
19	Refrigerator	Concord	
20	Eppendrof centrifuge	Fisons	England
21	Hood	Labogene	Danemark

2.1.2 Chemical Materials:

The main chemical materials and stains that used in this study were listed in table (2-2).

Table (2-2) : Chemical materials.

No.	Chemicals	Manufacturer (origin)
1	Absolute ethyl alcohol	Fluka / Germany
2	DNA ladder marker	Promega / USA
3	Decontamination nucleases solution	Bio-world
4	Loading dye (orange blue), Agarose, Master mix	Promega / USA
5	Glycerol	Fluka / England
6	Gram Stain Kit	Syrbio / Switzerland
7	Hydrogen peroxide	Fluka / England

8	Oxidase	Himedia / India
9	Nuclease free water (1.25) ml	Promega / USA
10	Tris EDTA (TE)	Bio Basic / Canada
11	Ethidium bromide	Promega/ USA
12	Tris-Borate-EDTA (TBE10X) buffer	Bio Basic / Canada
13	100 bp DNA ladder	Promega/ USA
14	Greenstar	Bioneer / korea

2.1.3 Biological Materials:

The main biological materials used throughout this study were listed in Table (2-3).

Table (2-3) Culture media and their manufacturer

No.	Culture Media	Manufacture (Origin)
1	Brain Heart Infusion Broth	Himedia / India
2	MacConkey Agar	Oxoid / UK
3	Blood agar base	Himedia / India
4	Brain heart infusion agar	Himedia / India
5	Nutrient Broth	Himedia / India

2.1.4 Commercial Kits:

The commercial kits have been used in the present study are listed in the table (2-4).

Table (2-4) Commercial kits used in the present study

No.	Type of Kit	Manufacture (Origin)
1	**DNA extraction kit**	Geneaid / UK
2	**DNA ladder 100- 20000 bp**	Promega / USA
3	**Green master mix**	Promega / USA
4	**primers of** copB,uspA1,uaspA2, Hag,mcaP,mapA, abcZ, adk , efp, funC ,glyBeta , mutY, ppa & trpE, Multi-F, Multi M1, MultiM2, Multi M3,T1modF,T1modR,T1resF,T1resR.	Microgen / Korea
5	**Gel/PCR DNA Fragments Extraction Kit**	Geneaid / UK

Table (2-5) DNA extraction kit components (Geneaid / UK)

No.	Component	Size
1.	Gram + Buffer	30 ml
2.	GT Buffer	30 ml
3.	GT Buffer	40 ml
4.	W1 Buffer	45 ml
5.	Wash Buffer (add Ethanol)	25 ml (100 ml)
6.	Lysozyme	110 mg
7.	Proteinase K (add ddH$_2$O)	11 MG *2 (1.1 ml)

8.	Elution Buffer	30 ml
9.	CD Columns	100
10.	2 ml Collection Tubes	200

Table (2-6) Master Mix Used in PCR (Promega / USA)

No.	Materials
1	DNA polymerase enzyme (Taq)
2	**dNTPs (400 μm dATP, 400 μm dGTP, 400 μm dCTP, 400 μm dTTP)**
3	**MgCl2 (3mM)**
4	**reaction buffer (pH 8.3)**

Table (2-7) Contents of the Reaction Mixture

No.	Contents of reaction mixture	Volume
1.	Go Taq Green master mix	12.5 μl
2.	Upstream primer	1.5 μl
3.	Downstream primer	1.5 μl
4.	DNA template	3 μl
5.	Nuclease free water	6.5 μl
Total volume		25 μl

Table (2-8) DNA ladder

No.	Materials
1.	Ladder consist of 13 double-stranded DNA with size 100-2000 bp.
2.	Loading dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA).

2.2 Methods

2.2.1 Preparation of Reagents and solutions

2.2.1.1 Catalase Reagent

Hydrogen peroxide (3%) was prepared from stock solution in a dark bottle and it has been used for detection of the ability of the isolates to produce catalase enzyme (Cappuccino and Welsh, 2019).

2.2.1.2 Oxidase Reagent

Oxidase reagent has been prepared by dissolving 0.1 gm. of Tetramethyl p-phenyl diamine- dihydrochloride in 10 ml of distilled water and it must prepared freshly in a dark bottle (Cappuccino and Welsh, 2019).

2.2.1.3 Glucose ,Maltose and Lactose Solutions:

The solution was prepared by dissolving 1gm of glucose , maltose and lactose in 100 ml distilled water, and sterilized by filtration (Gadeberg *et al*., 1983).

2.2.2 Preparation of cultural media:

2.2.2.1 MacConkey agar

This medium used to isolate and differentiate between bacteria which ferment and non-ferment lactose, prepared by weight (51.5 gm.) of medium, dissolving in (1 L.) of distilled water, heated to boiling with frequent agitation to completely dissolve the medium and sterilized by autoclave, then cooled to 55ºC and poured into Petri dishes, then incubated in an incubator at 37ºC for 24 hours to remove any contaminated medium (MacFaddin, 2000).

2.2.2.2 Brain heart infusion broth

This medium using to activate, grow and as stock culture for isolates Salmonella typhi; it is prepared by dissolving (37 gm.) of medium in (1L.) of distilled water, then pouring to sterile test tubes and sterilizing by autoclave. The tubes were sterilized in autoclave at 121o C 15 Ibs of pressure for 15 min (MacFaddin, 2000)

2.2.2.3 Blood Agar Medium (pH:7.1):

Blood agar medium was prepared by dissolving 40gm blood agar base in 1000ml of distilled water . This media was autoclaved at 121oC for 15 minute, and then cooled to 50oC.Then , (5%) of fresh human blood was added. This medium was used to cultivate bacterial isolates (Forbes *et al*., 2007).

2.2.2.4 Chocolate Agar Medium:

Chocolate agar medium has been prepared by dissolving 40 gm of blood agar base in 1000 ml D.W. and sterilized by autoclaving. Then 8% of human blood was added to the medium after cooling to 80 Cº. This

medium was especially used for isolation and cultivation of bacteria that need 5-10% CO_2 tension (Baron *et al*., 1994).

2.2.2.5 Brain Heart Infusion (BHI) Broth - Glycerol Medium (Maintenance Medium):

This medium was prepared by mixing 5 ml of glycerol with 95 ml of BHI broth (sterilized by autoclave). It was used for preservation of bacterial isolates as stock for a long time (Collee *et al*., 1996 ; Forbes *et al*., 2007).

2.2.2.6 Nutrient Agar Medium

Nutrient agar medium was prepared according to the manufacturing company (28 gm/1L). It was used for general experiments, cultivation and activation of bacterial isolates when it is necessary (MacFaddin, 2000).

2.2.2.7 Sugar Fermentation Medium :

This medium consists of:

Medium base: 0.0082 gm of phenol red was added to 100 ml of Brain heart infusion broth, the pH was adjusted to 7.4, and then this media had been autoclaved .

Sugar solution: 1 gm of each of the following sugars (glucose ,lactose and maltose), were added to the broth separately and sterilized by filtration by Millipore filter , later poured into sterile plain tubes. (Forbes *et al*., 2007)**.**

2.2.3 Subjects of the Study

This study involved (300) specimens which collected from patients with otitis media and chronic obstructive pulmonary disease(COPD

)were admitted into hospitals of Babylon Province: Imam AL-Sadiq Hospital and and private medical clinics in Babylon province during the period from (March to July 2022).

2.2.3.1 Cross sectional Study

Sample Collection
(300 ear swab and sputum)

↓

culturing of ear swab and sputum sample on (Blood agar ,MacConkey agar andChocolate agar)

↓

Biochemical tests

↓

DNA Extraction

Detection of M.catarrhalis by specific gene *copB*

Detection of five virulance genes by PCR

Molocular dection of R-M

Multilocus sequence typing MLST

usp A1   usp A1   hag   mcap   mapA
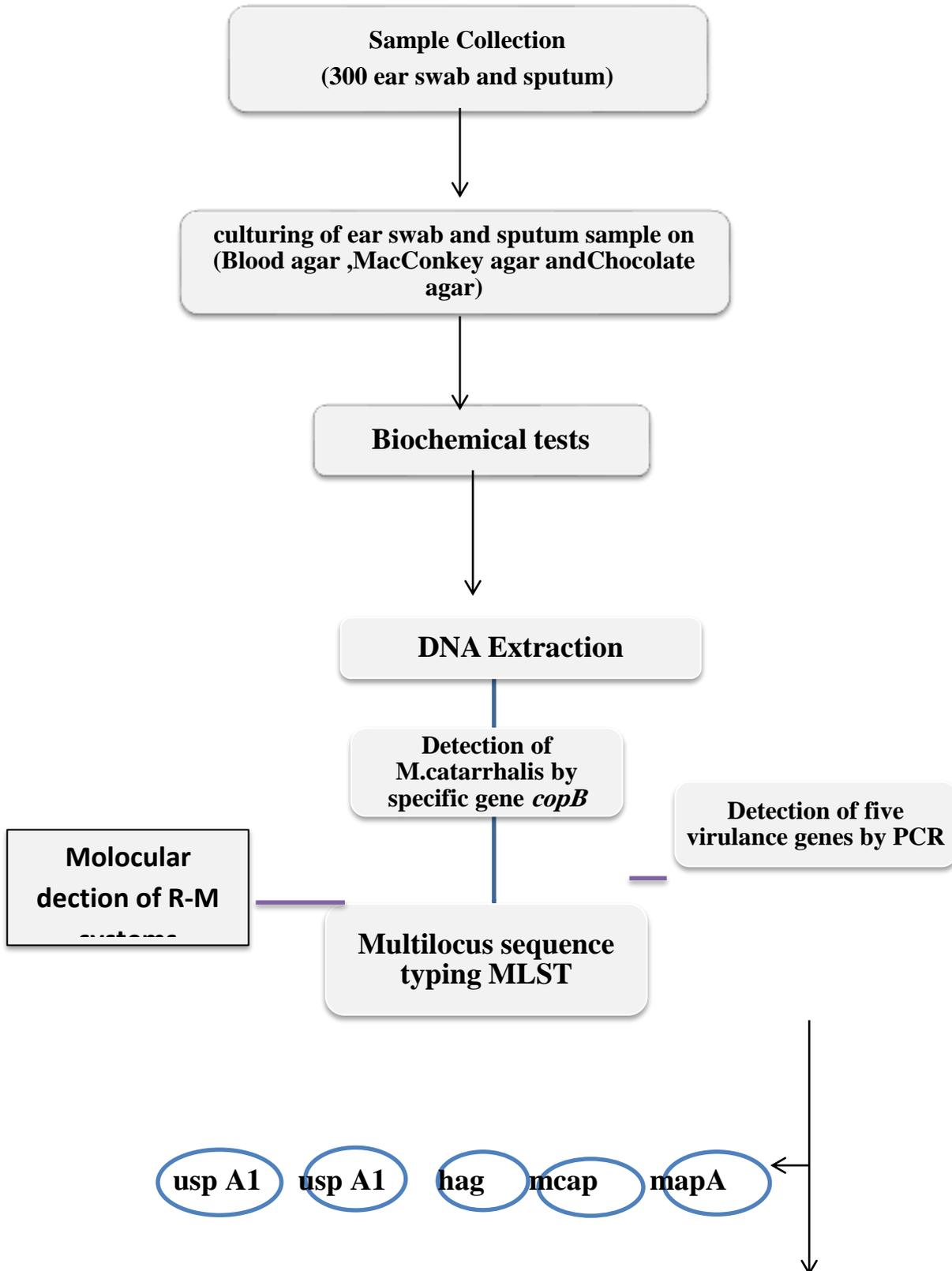
figure (2-1) Scheme of Study Design.

2.2.3.2 Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. Verbal consent was taken from each patient or his parents before sampling. Investigative standards were rigidly preserved, primarily concerning confidentiality. Moreover, this study was undisclosed, participation of patients was optional, and verbal consent was received before data uptake process was started. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee (at College of Medicine University of Babylon).

2.2.4 Isolation and identification of Moraxella catarrhalis

2.2.4.1 Collection of Specimens

The proper specimens collected for bacteriological analysis are described below. Those specimens were collected in proper ways to avoid any possible contamination.

2.2.4.2 Ear swab:

In the case of otitis media the isolates recovered from the middle ear are present in the nasopharynx, indicating that the middle ear isolate came from the nasopharynx via the eustachian tube. *M .Catarrhalis* is recognized as one of the most common causes of respiratory tract infection (RTI) . It considerd the third largest bacterial cause of otitis media (OM)(Ngo *et al.*,2016).

The specimens are generally collected from patients with otitis media by disposable swab , The swabs are taken from the middle ear . Swab for culturing should be placed in tubes containing normal salin to manitain the swab moist until taken to laboratory or placed in its cover

immediately and transformed to the laboratory during half hour of taking. Each specimen was immediately inoculated on the blood agar plates, chocolate agar plates and MacConkey's plates. The plates has been inoculated on culture media and incubated aerobically for 24hrs. at 37°C.

2.2.4.3 Sputum:

The specimens were generally collected by instruction of the COPD patient to give the early morning sputum after rinsing of the mouth thoroughly and before eating any meal, then the sputum put in a clean container and transferred to the laboratory immediately ,and the obtained sputum was examined grossly for amount ,color and consistency (purulent , mucoid) . Sputum being obtained by wooden applicator and cultured on the ordinary media .Those specimens were collected under the help of advisory to avoid any possible contamination. Each specimen was immediately inoculated on the blood agar plates, chocolate agar plates and MacConkey's plates. The plates had incubated aerobically for 24hrs. at 37°C.

2.2.4.4 Exclusion criteria

More than fourteen cases are excluded from the study due to the absence of study criteria when patient use antibiotics after taken the history of patients.

2.2.4.5 Biochemical tests

A. Catalase Test

Catalase is an enzyme that catalase the release of oxygen from hydrogen peroxide. MacConkey agar medium was streaked with the selected bacterial colonies and incubated at 37C for 24 hrs., then the growth was transferred by the wooden stick and it was put on the surface

of a clean slide, a drop of (3% H2O2) was added. Formation of gas bubbles indicates positive result (Cappuccino and Welsh,2019).

B. Oxidase Test

This test depends on the presence of certain bacterial oxidases enzyme that would catalyze that the transport of electrons between electron donors in the bacteria and a redox dye (tetramethyl-p-phenylene-diamine dihydrochloride), the dye was reduced to a deep purple color. A strip of filter paper was soaked with a little freshly made reagent, and the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep purple color which appeared within 5-10 seconds (Cappuccino and Welsh, 2019).

C. Sugar Fermentation:

This test was done to detect the ability of bacteria to ferment different types of carbohydrate including (glucose ,lactose and maltose). This test was performed as follows :

Sugar fermentation medium was inoculated by the suspected bacterial colonies and the tubes were incubated at 35-37ºC for 18-24hrs, incubation as long as 30 hrs may be needed to confirm a negative result. Looking for the results next day.

a) Change the color of broth from red to yellow indicates positive result (acid formation i.e. sugar fermenter).

b) No change in color indicates negative result (no sugar fermentation) (MacFaddin, 2000; Collee *et al,* 1996)**.**

2.2.5 Molecular study

2.2.5.1 Extraction of genomic DNA from bacterial culture:

DNA was extracted from Bacterial isolates, using the classical method by Presto Mini gDNA Bacteria Kit (Geneaid, Uk) protocol for bacterial genome was used: as the following: the supernatant was subjected to the extraction protocol.

1. A volume of 1 ml of an overnight culture was added to a Micro-centrifuge tube (size 1.5ml) and were centrifuged for 1 minute at 14-16,000xg, then the supernatant was discarded.

2. GT buffer (200 µl)was added to the pellet cells and completely re-suspended using a vortex or pipette.

3. Proteinase k(20 µl)was added and incubated at 60 ˚C for at least 10 minutes ;the tubes were inverted every 3 minute during the incubation time.

4. GB Buffer (200 µl)was added to the sample and mixed by vortex for 10 seconds then incubated at 70 ˚C for at least 10 minutes, this step was performed to ensure the sample lysate is clear, the tube were inverted every 3 minute during the incubation time, and in this time pre-heated the required Elution Buffer.

5. Absolute ethanol (200 µl)was added to the sample lysate and mixed immediately by shaking vigorously, a GD column was placed in 2ml collection tube, then the mixture was transferred to the GD column, finally it was centrifuged for 2 minutes at 14-16,000 x g.

6. GD column was placed in a new 2ml collection tube and the collection tube was discarded.

7. W1 Buffer (400 µl) was added to the GD column, centrifuged for 30 seconds at 14-16,000 x g, and discarded flow-through then GD column placed back in the 2ml collection tube.

8. Wash Buffer (600µl) was added to the GD column. Then it was centrifuged at 14-16,000x g for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube. And the tubes were centrifuged again for 3 minutes at 14-16,000x g to dry the column matrix.

9. The dried GD column was transferred to a clean 1.5 ml micro-centrifuge tube and 70µl of pre-heated elution buffer were added to the centre of the column matrix. The tubes were let still for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 14-16,000x g for 30 seconds to elute the purified DNA and stored at-20 ˚C.

2.2.5.2 Measuredment DNA concentration and purity:

The extracted DNA was checked by using Nano drop spectrophotometer, which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nano drop software, chosen the appropriate application (Nucleic acid, DNA).

2. A dry paper-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestals for blank the system.

3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of extracted DNA

carefully pipet onto the surface of the lower measurement pedestals ,then check the concentration and purity of extracted DNA(Krebset *et al*., 2009).

2.2.5.3 Primer of virulence genes:

Table (2-9) Primer used in PCR assays for virulence gene of Moraxella catarrhalis.

Genes	Primer sequence (5´- 3´)	Size bp	PCR conditio	Reference
mapA F	ATAGGATCCGCACCAGCCTCATCAA		94ºC 4min 1x	
	AATGGATCCTTGTGCCAGTGCCATT		94ºC 2min	
mapA R			55ºC1min 30x	
		140	72ºC 1min	Hoopman
			72ºC 8min 1x	*et al*.,2008
mcaP	CGCAATAAAGATCACCATGCTTG		94ºC 4min 1x	
mcaP R	CGGGATCCCGCTGACACATTGCATT AAA		94ºC 1min1x	
			57ºC 1min 30x	
			72ºC 1min	Verhaegh
		220	72ºC 7min 1x	*et al*.,2008
uspA1 F	CGTTATGCACTAAAAGAGCAGGTC		95ºC 1min 1x	
uspA1 R	GCATCTGACCAGCTTAGACCAATC		94ºC 1min	
			53ºC 1min 35x	
			72ºC 1min	Aebi *et al*.,2011
		247		
			72ºC 5min	

Primer	Sequence	Size	PCR Conditions	Reference
hag F	GTCAGCATGTATCATTTTTTAAGG		94ºC 4min 1x	
hag R	TGAGCGGTAAATGGTTTAAGTG		94ºC 2min	
			56ºC 1min 30x	
			72ºC 1min	Verhaegh *et al.*,2008
		175	72ºC 7min 1x	
copB F	AAAAGACGAAAGCACGGCTA		95ºC 5min 1x	
copB R	CATAAAGCGACCTTGGTGGT		94ºC 1min	
			65ºC 1min 35x	
			72ºC 1min	Designed in this study
		187	72ºC 8min 1x	
UspA2 F	CGCTGTAACCAGTGCCATGA		95ºC 1min 1x	
UspA2 R	ACGATAGCCAGCACCGATAG		94ºC 2min	
			48ºC 1min 35x	
		1100	72ºC 40s	Aebi *et al.*,2011
			72ºC 5min	

2.2.5.4. Dissolving and Preparation of Primers

All primer pairs used in this study were dissolved using TE Buffer, 1X (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA-Na2. Firstly, the primer stock tube prepared and then the working solution would prepare from primer stock tube. According to the instruction provided by primer manufacturer (Bioneer / Korea) the TE buffer were added to get 100 Pico mole/ microliter concentration of primer stock solution. The working solution prepared from stock by dilution with TE buffer to get 10 Pico mole/ microliter and kept in -20 ºC.

2.2.6 Detection of Amplified Products by Agarose Gel Electrophoresis .

The PCR amplification of products were analyzed by Agarose gel electrophoresis using 1 % agarose gel prepared by dissolving 1 g of agarose mixed with 100 ml of 10 x Tris Borate EDTA (TBE) buffer (10 ml TBE + 90 ml sterile distilled water) heated to boil on hot plate. The agarose gel was cooled down to 45°C where 5μl of ethidium bromide stain was added (Jegasothy *et al.*, 2000). The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was powered gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covering the surface of the gel, 5μl of DNA sample was transferred into the signed wells in agarose gel, and in one well we put the 5μl DNA ladder mixed with 1μl of loading buffer.

The electric current was allowed at 70 volts for 30 min. UV transilluminator was used for the observation of DNA bands, and gel was photographed using a digital camera.

2.2.7. 1 Multi Locus Sequence Typing (MLST)

In this study, The *M. catarrhalis* MLST scheme uses internal fragments of the following Eight house-keeping genes: *glyRS* (glycyl-tRNA synthetase beta subunit), *ppa* (Pyrophosphate phospho-hydrolase), *efp* (elongation factor P), *fumC* (fumarate hydratase), *trpE* (anthranilate synthase component I), *mutY* (adenine glycosylase), *adk* (Adenylate kinase) and *abcZ* (ATP-binding protein. Primer and PCR conditions were available on MLST database (https:// pubmlst.org/ bigsdb?db= pubmlst_mlst_seqdef&page=schemeInfo&scheme_id=6)and (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-mcatarrhalis.html),

Table (2-10) Primer used in PCR assays for MLST of *Moraxella catarrhalis*.

Gene	PCR Primers	PCR Product		
glyRS F *glyRS* R	F 5'-GCACCGAAGA GTTGCCACCA-3' R 5'-ACGCA ACGGGCAAATCCACC-3'	762**bp**	94ºC 1 min 1x 95ºC 1min 58ºC 1min 30x 76C 1min 75ºC 10min	
Ppa F *Ppa* R	F 5'-AATAAAATTCTA GATGCTGGC-3' R 5'-ACTTATT GCTCTGTCCAGCG-3'	523 **bp**	94ºC 1 min 1x 95ºC 1min 52ºC 1min 30x 76C 1min 75ºC 10min	
Efp F *Efp* R	F 5'-CTCTGATTGA CAACTGGCAGG-3' R 5'-GATATTC GCCAGTACGCG-3'	582 b p	94ºC 1 min 1x 95ºC 1min 52ºC 1min 30x 76C 1min 75ºC 10min	**Zhao** *etal., 2022*
FumC F *FumC* R	F 5'-CTCTGATTGA CAACTGGCAGG-3' R 5'-GATATTC GCCAGTACGCG-3'	675 bp	94ºC 1 min 1x 95ºC 1min 52ºC 1min 30x 76C 1min 75ºC 10min	
Trp E F *Trp E R*	F 5'-TTATCCCGCATCGAAAATGG-3 R 5'-GGTTTC ATCCCATTCAGCC-3	545 bp	94ºC 1 min 1x 95ºC 1min 52ºC 1min 30x 76C 1min	

				75ºC 10min	
MutY F *MutY* R	F 5'-GGCAATACCATCATC AGCCG-3 R 5'-GGTAA CTGACTTTGAACGCC-3'	609 bp		94ºC 1 min 1x	
				95ºC 1min 52ºC 1min 30x 76C 1min	
				75ºC 10min	
Ad k F *Ad k* R	F 5'-GG CATTCCTCAAATCTCAAC-3' R 5'-GATGGGCTTTA TTGTCAAATG-3'	631 bp		94ºC 1 min 1x	
				95ºC 1min 54ºC 1min 30x 76C 1min	
				75ºC 10min	
Abc Z F *Abc Z R*	F 5'-ACATGCTGATGA TGGTGAG-3' R 5'-CA CTGGCAAGTTCAAGCGC-3'	610 bp		94ºC 1 min 1x	
				95ºC 1min 52ºC 1min 30x 76C 1min	
				75ºC 10min	

2.2.7.2 Sequencing of MLST PCR products:

All PCR products obtained above were cleaned and submitted for sequencing as follows. The PCR product was cleaned of amplification primer using the Gel/PCR DNA Fragments extraction kit (Geneaid, U.K) as per manufacturer's instructions. Purified DNA was sequenced at Macrogen company (Korea) with the sequencing primers for each gene as outlined in Table (2-10). Bidirectional Sanger sequencing method was carried out on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

2.2.7.3 Bioinformatic analysis of MLST:

The raw Sequence data was trimmed and aligned to the control sequences. The standard sequences for alignment were taken from MLST database. Multiple alignments were done by using Clustal W v2.0 (Thompson, *et al.* 1994) of Geneious Prime Software V2021.1 (Biomatters, Inc., North America). Identification of ST and allele profile was done by interrogation of gene sequences against the international MLST database at https://pubmlst.org/ and http://www. genomicepidemiology.org/.

Regarding the identification of phylogenetic relationships among *M. catarrhalis* isolates, the merged edited sequences were used to generate phylogenetic tree using the PhyML maximum likelihood by using MEGA X v10.0.5 (Kumar *et al.*, 2018). Regarding recombination tree, Split decomposition analyses were performed with Splits Tree, version 4, by using LogDet distances, equal edge lengths, and 1000 bootstrap replicates. eBURST analysis was done according to Feil et al., 2004 ; Ribeiro-Gonçalves et al., 2016.

2.2.8.1 Restriction -Modification Sequences

Molecular assay in this study includes, 5 genes for RM system The oligonucleotide primers for all genes used in this study were obtained from previous studies and pubmlst.org, each one has specific nucleotide sequences and product size. The primer sequences and PCR conditions that used are listed in tables (2-11).

Table (2-11) Primer used in PCR assays for RM system of *Moraxella catarrhalis*.

Primer	sequence		Description	PCR condition	
Multi-F	5-GATGGCGTGATATTTAT CAGTATTGATG-3		Conserved modm forward primer upstream of variable target recognition domain (TRD)	**94ºC 4min 1 1x** **94ºC 2min** **55ºC 60s 30x** **72ºC 1min** **72ºC 5min** 1x	
MultiM1	5GTACAAAGCTTCTTGAT AATACAGCTC-3	400bp	Reverse primer specific for modM1 allele TRD	**94ºC 4min 1x** **94ºC 2min** **55ºC 60s 30x** **72ºC 1min** **72ºC 5min** 1x	Blakeway *et al.,* 2018
MultiM2	5-CAGCCGAATAACCTTG AGTAGATG-3	300bp	Reverse primer specific for modM2 allele TRD	**94ºC 4min 1x** **94ºC 2min** **55ºC 60s 30x** **72ºC 1min** **72ºC 5min**	

Multi M3	5-CAAGGTTTGGCTACTT TTCCTCG-3	500bp	Reverse primer specific for modM2 allele TRD	**94ºC 4min 1x**	
				94ºC 2min **55ºC 60s 30x** **72ºC 1min**	
				72ºC 5min	
T1 modF	5-GGCAAATCGCCAACG ATGTCAG-3		Type 1 R-M system Methyltransferase Forward primer	**94ºC 4min 1x**	
				94ºC 2min **55ºC 60s 30x** **72ºC 1min**	
				72ºC 5min	
T1mod R	5-CCTGAACCACAAGCA GGGTC-3	637bp	Type 1 R-M system Methyltransferase reverse primer	**94ºC 4min 1x**	
				94ºC 2min **55ºC 60s 30x** **72ºC 1min**	
				72ºC 5min	
T1 res F	5-CCTGATAAAGCAGGCG TGATAG-3		Type 1 R-M system restriction endonuclease forward primer	**94ºC 4min 1x**	
				94ºC 2min **55ºC 60s 30x** **72ºC 1min**	
				72ºC 5min	

T1res R	5-GTTTTCGCTGGCGTA TCCTC-3	158bp	Type 1 R-M system Restriction endonuclease reverse primer	94ºC 4min 1x	
				94ºC 2min 55ºC 60s 30x 72ºC 1min	
				72ºC 5min	

2.2.8.2. Bioinformatic analysis of R-M:

The raw sequence data was trimmed and aligned to the control sequences. The standard sequences for alignment were taken from MLST database. Multiple alignments were done by using Clustal W v2.0 (Thompson, *et al.* 1994) of Geneious Prime Software V2021.1 (Biomatters, Inc., North America). Identification of RM systems (type I and III) was done by Blast search against the NCBI nr database and the REBASE database (Roberts et al. 2010). Alignments and visualization of multiple R-M systems were performed using CLC Genomics Workbench (V 22.0.2, QIAGEN Aarhus A/S). The phylogenetic tree was done by using similarity matrix by unweighted pair group method with arithmetic mean (UPGMA) method using NTSYSpc (v2.02e, Applied biostatistics inc.)

3.1. Isolation and Identification of *Moraxella catarrhalis:*

A total of 300 clinical specimens were collected during this study which obtained from patient Suffering different infection such as otitis media and COPD . All specimens were subjected to aerobic culturing on blood agar and it was out of the total (300 specimens),250(83.3%) specimens showed   positive bacterial culture. No growth was seen in other 50(16%) specimens which indicate the presence of microorganisms that may be cultured with difficulty such as anaeraobic bacteria, virus, fungi and other agent .Among (250) positive culture, only 15   sample show positive was the   method by which culture were considered positive identified as *M. catarrhalis* as shown in the table(3-1) and figure (3-1).

These isolates than subjected to molecular detection method using specific primer based on *copB* gene as a genetic marker for confirmed isolation of *M. Catarrhalis* by PCR . The results revealed that only 15 out of 60 were positive for PCR as shown in table (3-1) .

Table(3-1) prevalence of *Moraxella   catarrhalis*   among other etiological agents detected by culture and PCR .

No of sample	No of Negative bacteria culture	No of Positive culture of other bacteria	Positive culture of *M. catarrhalis*	on moleculer *(copB)* positve
300	50(16%)	190(76%)	60(24%)	15(6%)

3.1.2 Culturing of bacteria:

The identification of *M. Catarrhalis* depends on the cultural and biochemical characteristics and also microscopic patterns. The organisms are grams-negative rods or cocci, but often with a tendency to resist decolourisation. The cocci are usually smaller (0.6 - 1.0µm in diameter) and occur singly or in pairs with adjacent sides flattened, and sometimes tetrads are formed. There is one medically important species, *M. catarrhalis*. They are non-motile and aerobic, but some isolates may grow weakly under anaerobic conditions.

The bacterium is nonspore-forming , catalase positive , nonmotile and diplococci shaped with flat adjacent sides . Aerobic or facultatively anaerobic , also they are oxidase positive .

M. catarrhalis is cultured on enriched media at 37 °C under an atmosphere of (95 %) O2 and (5%) CO2 and observed to be grown well on blood agar and chocolate agar . On blood agar plates , colonies tend to be large , grey, smooth , opaque and convex in nature and may be readily pushed intact over the surface of agar using a sterile loop, and also appeared to be non-pigmented and non-haemolytic.

M. catarrhalis is catalase and oxidase positive, and is unable to produce acid from glucose, lactose, maltose fructose and sucrose.(Bernhard *et al.* ,2012).

However, some characteristic of *M.catarrhalis* should be considered to confirm the identification of this bacteria through using specific markers via PCR techniques. The diagnostic features of bacteria were summarized in Table(3-2).

Table (3-2): The most important traditional test used in the present study.

Tests	Results
Growth on blood agar	Gray color,convex, opaque colonies
Hemolysis	Non- haemolytic
Colonies morphology	Diploocci (mostly in pairs)
Gram Stain reaction	Gram negative
Catalase	Positive
Oxidase	positive
Fermentation Maltose	negative
glucose fermentation	negative
Motility	Non motile

3.1.2.1 Confirmed diagnosis of *M. catarrhalis* by PCR using specific primer (*copB*).

The one purpose of this study was to develop molecular diagnostic test to identify bacterial isolates based on the specific primer (*copB* gene)profiling, comparison with of traditional criteria and that may be good condition to be used as genotyping (marker) for confirmatory identification of *Moraxella catarrhalis* from patients.

The DNA was extracted from 60 specimens was collected from ear swab and sputum which used in conventional PCR.it was carried out using the DNA samples for amplification of specific *copB* primer; according to the sequences and program listed in Table (2-9). After that gel electrophoresis showed that , only 15 isolates gave specific 187bp DNA fragment when compared with allelic ladder; as shown in figure (3-1)
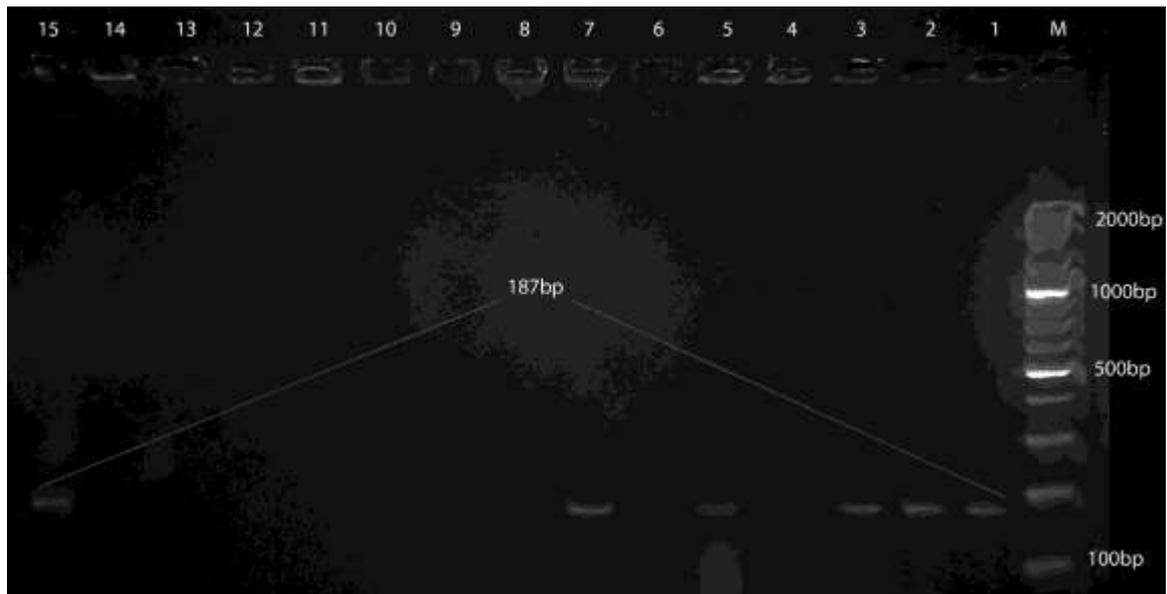
Fig. (3-1) Agarose gel electrophoresis of Uniplex-PCR products obtained with bacterial strains using *Moraxella catarrhalis*-specific primers.. lanes 1, 2, 3, 5, 7and 15represent the identified *Moraxella catarrhalis*, Lane M represent 100bp universal DNA ladder.

According of the result above the *Moraxella catarrhalis* depend on *CopB* gene in PCR it was observed that only (15) isolates of *M. catarrhalis* out of (60) were decomented as *M. catarrhalis* by biochemical test .

CopB is present only in *M . catarrhalis* and this gene is specific for *M. catarrhalis*. These it can facilitated downstream analyses such as molecular detection. *M. catarrhalis* is an opportunistic bacterium considered as pathogen that causes Otitis media and COPD . The importance of *M.catarrhalis* with studies on metabolic pathways and analysis on gene.

The result obtained by (Nawa *et al.,* 2022) who were found the percentage of *copB* (100%) in *M. catarrhalis* isolates. While ,other result obtained by Eghbali et al ., (2019) who found the percentage of *copB* (90.7%) in *M. catarrhalis* isolates. Also Ramadan *et al* ., (2017) found *M. catarrhalis* is responsible for (11.5%) of all cases of lower respiratory tract.

Most studies indicate that all isolates possess the *copB* gene (Bullard *et al* .,2007 ;Verhaegh *et al* ., 2008). although one study showed that *copB* is more frequently present in *M. catarrhalis* isolated from children and adults with RTI (50 %) than in carriage isolates from children (0 %) (Mitov *et al* ., 2010). The reason for this difference in distribution is not clear, but may reflect localized geographical variance of *M. catarrhalis*.

The *copB* gene sequences are housekeeping gene and highly conserved and specific for this organism .Therefore, it is a useful marker for the molecular detection of *M. catarrhalis* by PCR. *CopB* is largely conserved among strains of *M. catarrhalis* and contains discrete regions which show moderate heterogeneity among strains(Sethi *et al* .,1997).

CopB, known as *Omp B2*, is one of the proteins whose expression is induced under iron limiting conditions. The *copB* is an iron repressible protein that enables these bacteria to obtain iron bound to transferrin, lactoferrin, and hemoglobin, including complement resistance.(Chochua *et al* .,2016).

These proteins were up regulated in response to iron limitation , and the *copB* mutant was severely impaired in its Cop B protein appears to have an important role of *Moraxella catarrhalis* serum resistance , so the *copB* mutants improved higher sensitivity to the human serum as mentioned under various levels of Iron in the environment . *CopB*, an 80-kDa major OMP has been consistently present in all strains studied with minor variations in apparent molecular weight(Aebi *et al* ., 1996). Is an important antigenic target for IgG antibodies to *M. catarrhalis* in children. Whether this OMP is OMP B1 or *CopB* is not known. Further studies of *CopB* should address several important issues. The role of this OMP as an antigen in diverse populations after acute infections with *M.*

catarrhalis, i.e., children with otitis media and adults with chronic obstructive pulmonary disease.

Genes encoding the third OMP (OMP B2) were found in nearly half of the clinical *M. catarrhalis* isolates. OMP B2 determines resistance to normal serum bactericidal activities; however, it participates alone or jointly with OMP B1 receptors in lactoferrin and transferrin binding and in competing with the plasma and mucosa proteins for iron acquisition (Myers *et al* .,1998).

Culture based methods are slow-it can take days between when patient first is seen by a doctor and time the result back from the clinical laboratory., for this reason, molecular method are much faster than the culture based method ,so the *copB* gene are a gold stander for identify *Moraxella catarrhalis* beyond phenotypic method.

However, this approach will enhance the accuracy, sensitivity specificity ,special and cost effectiveness in the detection of *M. catarrhalis* than culture technique and the PCR is the best choice for diagnosis of infection with *M. catarrhalis* . However ,molecular technique has over convective methods ,it can provide results in 24 hr .where as routine culture followed by biochemical test need 36-48hr .

3.1.3 Distribution of *M. catarrhalis* isolated from different clinical specimen.

In this study , a total of 300 specimens which isolated 200 Ear swabs obtained from patients suffering from otitis media and100 sputum specimens from COPD who were attended to Al-Iamam AL-Sadiq General Teaching Hospital in Babylon in a period ranging from (March to July 2022).

Table (3-3): Distribution of *M. catarrhalis* isolated from Two different clinical specimens.

Sources of isolates	No. of samples	No. of *M.catarrhalis* isolates	
Ear swab	200	9	60%
sputum	100	6	40%
Total number	300	15	100%

Hussein *et al* .,(2015) found the prevalence of *Moraxella catarrhalis* is approximately (8%) isolate from otitis media. While Timothy *et al* .,(2022) found that the rate of *M.catarrhalis* was(20%) in acute otitis media, and Ibrahim (2012). who were found the prevalence of *Moraxella catarrhalis* is 7 (6.4%) isolates from patient with otitis media.

on the other hand George *et al.,* (2018) found 22(62.8%) isolates of *Moraxella catarrhalis* from patient with COPD. While Shaikh *et al* ., (2015) who found the prevalence of *Moraxella catarrhalis* is 22(20%) isolates from patient with COPD.

Variations in *Moraxella catarrhalis* isolation between studies can be attributed to a variety of factors, including sanitary practices in hospitals and staff ,their geographical regions, environmental conditions, isolation and identification techniques, social and cultural level of patients, and use of antibiotics that may lead to bacterial resistance development, or differences in sample size; all of these factors may combine and play an important role in inhibiting or stimulating bacterial resistance development.

Chronic obstructive pulmonary disease (COPD) is a major cause of global morbidity and mortality, resulting in increased economic and social burden (Gold *et al.*,2021*)*. Variance among countries and between different groups in the prevalence of this disease is often directly related to smoking prevalence, although environmental pollution is also a significant risk factor in many countries. The prevalence and burden of

COPD will increase in the coming decades due to continued exposure to risk factors and aging of the world population(Halpin *et al*., 2019).

Moraxella catarrhalis is a human-restricted, respiratory tract pathogen that is a major cause of middle ear infections, or otitis media (OM), in young children and exacerbations in adults with chronic obstructive pulmonary disease (COPD). OM and COPD exacerbations cause substantial morbidity in both patient populations, and can lead to fatality in COPD patients.(Murphy and Parameswaran.,2009). There is also significant financial burden associated with infections in these 2 clinical settings, with doctor visits, emergency room visits, lost wages due to missed days at work, and cost of treatment(Mustafa *et al*., 2017).

There are more than 700 million cases of acute otitis media (AOM) diagnosed globally each year, with 50% of affected children under 5 years of age (Monasta *et al*., 2014). *Moraxella catarrhalis*, non-typeable Haemophilus influenzae (NTHi) and *Streptococcus pneumoniae* cause approximately 95% of AOM cases creating an incredible economic burden on healthcare systems (Broides *et al*., 2009).In addition to being the most common reason for doctor's office visits among children, AOM is also the most common reason for antibiotic use in the pediatric population. studies have shown antibiotic resistance and decreased sensitivity developing among the major otopathogens. Further, the polymicrobial biofilms associated with AOM are incredibly resistant and difficult to treat using classic antibiotic protocols (Korona-Glowniak *et al*., 2018).

Otitis media (OM) is a particularly important respiratory illness during early childhood. The most common bacterial species cultured from the nasopharynx of children during OM episodes are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *M. catarrhalis*, either as single pathogens or as co-cultures (Constantinescu *et al* .,2016).

Chronic obstructive pulmonary disease (COPD) is a chronic multifactorial inflammatory disease whose main pathophysiological mechanisms include airflow limitation, pulmonary emphysema, and chronic bronchitis (Brown *et al* ., 2018). The course of COPD is marked by recurrent periods of worsening symptoms, called exacerbations, responsible for disease progression, increased morbidity and mortality.

Several epidemiological studies have reported that non typeable Haemophilus influenzae (NTHi) and *M. catarrhalis* are the most prevalent bacteria found in the sputum of individuals with exacerbations of COPD (D'anna *et al*., 2020) and their co-infections reach up to 20–30% (Perez and Murphy, 2019) .

Considering the increasing clinical relevance of *M. catarrhalis* and NTHi in COPD, we decided to shed light on the mechanisms underlying the interactions between these bacteria and neutrophils, focusing on the pathways related to the oxidative stress response.

M. catarrhalis is a major cause of exacerbations in chronic obstructive pulmonary disease (COPD), after *Streptococcus pneumoniae* and *Haemophilus influenza* (Verduin *et al*., 2002).
M. catarrhalis is the most common bacterium isolated from sputum, middle ear, sinus, oral, and throat swabs. Since *M. catarrhalis* has been considered a harmless commensal bacterium for a long time, there is relatively little cognition about pathogenicity characteristics and its virulence factors. Generally, the pathogenicity of this bacterium, like other microorganisms, depends on the ability to escape from the host defense mechanisms and binding to cellular and mucus layer, as well (Eltaib *et al* .,2015*)*.

3.2. Molecular Detection of some virulence genes in *M.catrrhalis*.

3.2.1 Molecular detection of Ubiquitous surface protein A1 and A2 *uspA1& uspA2*

Ubiquitous surface protein UspA1 was investigated by PCR techequie using specific primers for this gene. It was found that *uspA1* marker was observed in 14(93.3%) isolates which included 9 isolates from ear & 5 isolates from sputum . while uspA2 was present in 11 (73.3%) isolates which included 6 isolates from ear &5 isolates from sputum as shown in figure (3-2) ,(3-3).

Ibrahim (2012) found *uspA1*at rate (99%) isolates from ear. on other hand Denise *et al* .,(2022) that found *uspA1*at rate(98%).while, Nawa *et al.,* (2022) that found the *uspA1* percentage at (92.3%) and *uspA2* (69.2%).respectivelyrd on the other hand Blakeway *et al.,* (2017) that found the *uspA1* percentage at (98%) and *uspA2* (77%).

However , the absence *uspA* in some isolates either means the gene is only partially present or could mean that the gene is present but is altered.
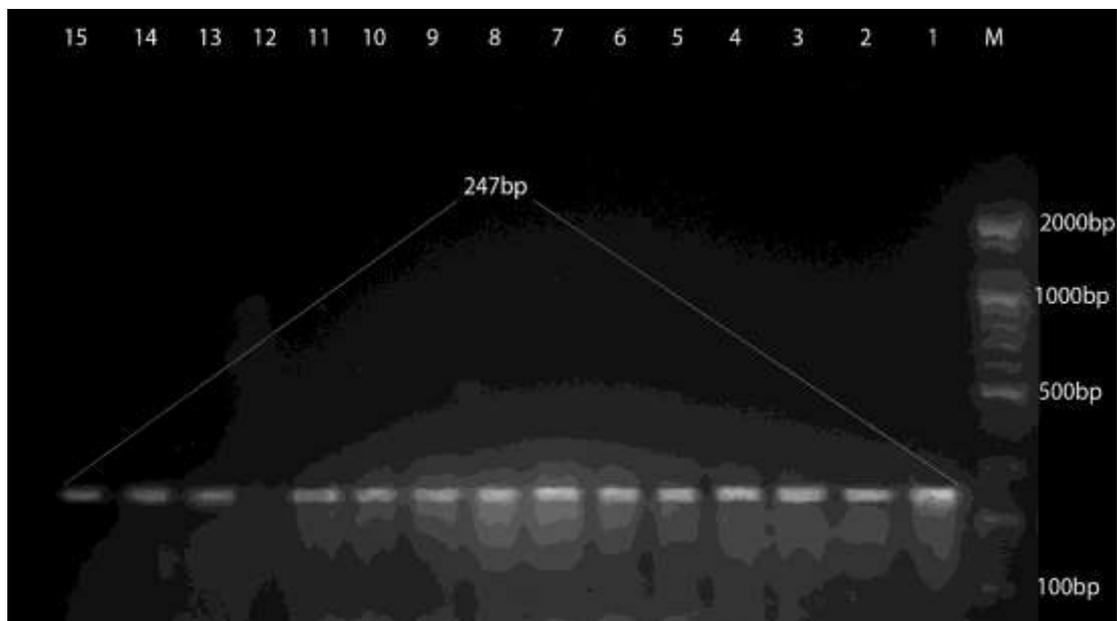

Fig. (3-2): Agarose gel electrophoresis of Uniplex- PCR products obtained using *Moraxella catarrhalis*-uspA1 primer. lanes 1, 2, 3, ,4,5,6,7,8,9, from era swab and10,11,13,14,15 from sputum at 247bp.

Fig. (3-3): Agarose gel electrophoresis of Uniplex- PCR products obtained using *Moraxella catarrhalis*-**uspA2 primer. lanes 1, 3, 4, 5, 7,8, from ear swab and 10,12,13,14, 15 from sputum represent the identified** *Moraxella catarrhalis*, **at 1100bp.**

However , positive result of this marker will render the bacteria to attach to the host mucosal surfaces , which is an important step in colonization. The function of *M*. catarrhalis *uspA* proteins is demonstrated by their roles in adhesion, invasion, and protection against the human complement system. If this marker is positive, the bacteria will attach to the host mucosal surfaces, a critical step in colonization. *In vitro* attachment of *M. catarrhalis* to certain cell lines, including Chang conjunctival epithelial cells, laryngeal epithelial cells, and alveolar epithelial cells, is required by the *UspA1* protein (Hallstrom *et al* .,2011).

UspA1 belongs to the major adhesins of *M. catarrhalis*. Interestingly, the *UspA1* gene is present in both phylogenetic lineages, but only the the seroresistant type 1 expresses the corresponding protein on its surface . *UspA1* and *UspA2* were also found to bind to components of the extracellular matrix proteins such as fibronectin and laminin (Tan *et al* .,2005). *UspA1* expression varies in accordance to phase variation, which in turns mediates the binding to host cells. (Heiniger *et al* .,2005).

M. catarrhalis possess the virulence genes *uspA1* and *uspA2*, which encode proteins that enable the bacterium to adhere to multiple epithelial cell types leading to biofilm formation and suppression of inflammatory response(Bernhard *et al* .,2012) Additionally, these virulence factors can neutralize C3 and C3d and inhibit the human complement system by binding to the complement inhibitor C4b binding protein, thereby protecting *M. catarrhalis* from the bactericidal action of human serum.(Hallström *et al* .,2011).

uspA1 also can be determine invasion of the epithelial cells , and also this phenomenon seems to be based mostly on *UspA1* interaction with fibronectin and α-5-β 1 integrin(Manolov *et al* .,2009).

3.2.2 Molecular detection of Haemagglutinin (*hag*):

Molecular detection of hemagglutinin protein (designated as *Hag*) was done by using specific marker. .It was found that only 6(40 %) isolates gave positive result with this primer which included 4 isolates from ear swab& 2 isolate from sputum as shown in Figure (3-4) **.**
Ibrahim (2012) found *hag* gene in 3(42.8%) isolates from ear. on the other hand Eghbali *et al* .,(2019) found *hag* gene in 84.4% isolate . While Denise *et al* .,(2022) that found *hag* gene in 80% isolate. The differences between our and other results may be due the difference in the sequence of this marker and number of isolates.
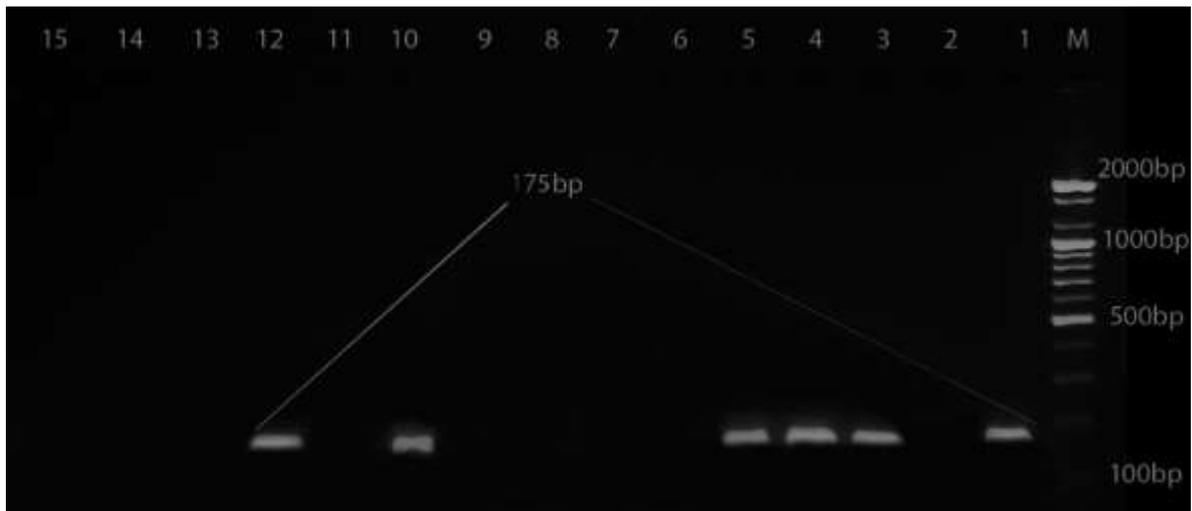
Figure (3-4): Agarose gel electrophoresis of Uniplex- PCR products obtained using *Moraxella catarrhalis-hag* primer. lanes 1, 3, 4, 5 from ear swab and 10,12, from sputum represent the identified *Moraxella catarrhalis*, at 175bp.

For *Moraxella catarrhalis* , however , there is no clear correlation between isolates that cause specific disease and the presence or expression of a particular virulence factor.

hag was found in (80 %) of child-carriage isolates (Verhaegh *et al* ., 2011), (90 %) of child RTI and (91 %)of adult RTI isolates. Another study showed that *mid/ hag* was present in(100 %) of isolates from various clinical presentations, indicating that there was no association between gene presence and carriage or disease.

Bullard *et al.,* (2005) were demonstrate that *hag* is expressed during M. *catarrhalis* infection / colonization and that *hag* may make a viable vaccine candidate .

Moraxella catarrhalis hag protein , which is also called the *Moraxella catarrhalis* IgD binding protein (MID), is present on the *M. catarrhalis* cell surface, as well as that of the *UspA1* and *UspA2* proteins. *mid/hag* is a multifunctional protein that fulfills an important role in the pathogenesis *M. catarrhalis* .The most extensively studied property of *Hag* is its ability to the bind Immunoglobin D (IgD) . This adhesin also mediates the binding of *M . catarrhalis*to human erythrocytes This

property is called hemagglutination (Forsgren *et al*.,2003). Bullard *et al.*, (2007) indicated that *Hag* directly mediates adherence to lung cells and collagen, but is not sufficient to confer binding to conjunctival monolayers.

Lipski *et al*; (2007) reported the(Hag/MID) protein is more variable at the amino acid level among isolates of various origins , and this is identical with the (Forsgren *et al* .,2001*)* who mentioned that *MID* has a different composition as shown by amino acid and DNA sequence analysis. It appears that *hag* gene may not be equally important in influencing autoagglutination ability in all isolates of *M. catarrhalis*(Verhaegh *et al., 2008)Hag* deletions caused reduction in adherence , which suggests that this factor is very important in attachement of *Moraxella catarrhalis*.

mid/hag is located in the outer membrane and mediates haemagglutination and non-immune binding of IgD by *M. catarrhalis*, while it also functions as an adhesin for cells derived from the human lung, middle ear and ciliated bronchial epithelium. However, also like the *UspA* proteins, *MID/Hag* is subject to phase variation, mediated by a polyG tract in its ORF, resulting in the presence or absence of functional *MID/Hag* and the respective adherence phenotype. (Verhaegh *et al* ., 2008)In isolates that have a mid/hag gene, *mid/hag* is more frequently expressed by child RTI isolates than adult RTI isolates (92 vs 73 %). It is unknown whether the association of *MID/Hag* expression with child versus adult disease isolates is due to phase variation, and further investigation is required. This may suggest that while *MID/Hag* expression bestows a selective advantage during infection of the child host, selection against *mid/hag* expressing isolates may occur in adults following an adaptive immune response, with the phase variation of *MID/Hag* contributing to immune evasion. The presence of the *mid/hag*

gene was also associated with the RB1 lineage in isolates from child carriage (Verhaegh *et al* ., 2011), and isolates from children and adults presenting with respiratory disease (Verhaegh *et al* ., 2008).

3.2.3 Molecular detection of protein CD, *M. catarrhalis* adherence protein (*McaP*)gene:

Moraxella catarrhalis adherence protein (McaP) was also detection in *M.catarrhalis* isolates. .It was shown that only three (20%) isolates from ear swab gave positive result with this primer as shown in Figure (3-5) .

Eghbali *et al* ., (2019) found *that mcaP* gene was present in (100%) isolate. on other hand, Ajeel *et al* .,(2021) found *mcaP* gene was present at rate at (28.5%)of isolates.

Figure (3-5): Agarose gel electrophoresis of Uniplex- PCR products obtained using *Moraxella catarrhalis-mcaP* **primer. lanes 1, 3, 4 from ear swab represent the identified** *Moraxella catarrhalis***, at 220bp. Lane M represent 100bp universal DNA ladder.**

However , the absence of this marker for 11 isolates may be attributed to the variation in gene sequence because of this variation in the amino acid sequences .

M.catarrhalis is population exhibited diverse pathogenicity *in vivo* .Adhesion and invasion are two consecutive steps in *M.catarrhalis* conlonization and infection .

McaP is a 62 kDa protein that confers adhesive properties towards Chang, A549 and polarised human bronchial cells. (Timpe *et al* .,2003). *McaP* was shown to be an adhesin expressed via the *Moraxella catarrhalis* which also displays esterase and phospholipase B activities (Timpe *et al*., 2003).Although *mcaP* gene is highly conserved in *Moraxella* as mentioned by (Verhaegh *et al* .,2008).

Moraxella catarrhalis adherence protein (*McaP*) belongs to the auto transporter family of the protein which expressed on the bacterial cell surface(Akimana *et al* .,2007). Moreover, This gene is considered autotransporter because of this protein has a phospholipase activity and also an esterase activity that was demonstrated by (Akimana *et al* .,2007).

3.2.4 Molecular detection of *mapA* gene:

Molecular detection of *mapA* was done by using specific marker. .It was found that 4(26.6%) isolates gave positive result with this primer as shown in Figure (3-6) .

Ajeel *et al*;(2021) who were found *mapA* was present only in 3(42.8%) isolates of *M. catarrhalis*.

Expression of bacterial virulence factors may depended on some suitable environmental factors.it expected that some specific factors may be present in the infected hosts, but not or only insufficiently available under the conventional *in vitro* condition of cultivation .
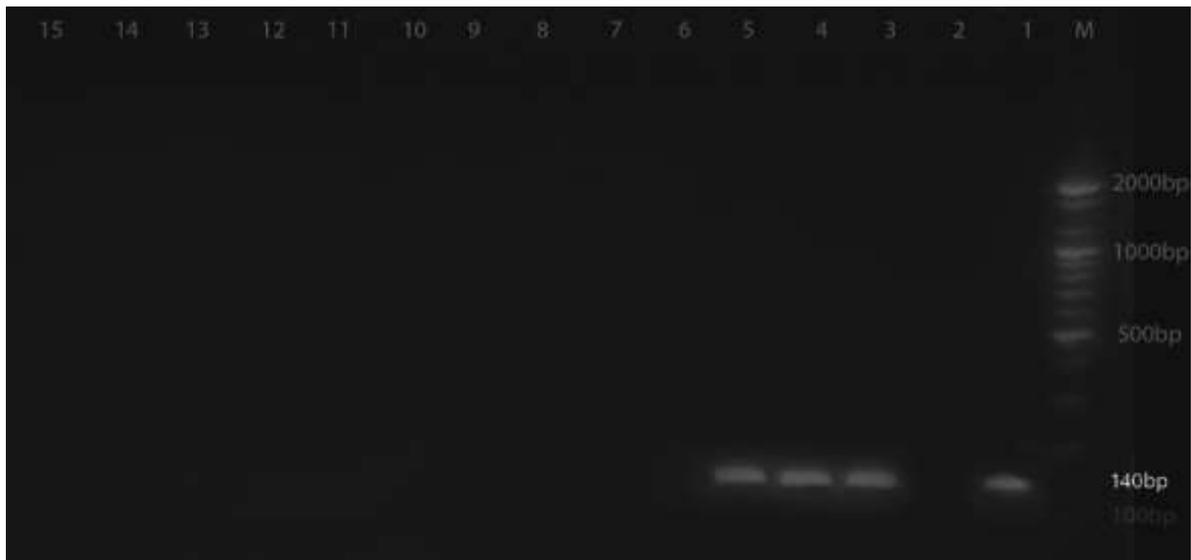
Figure (3-6)): Agarose gel electrophoresis of Uniplex- PCR products obtained using *Moraxella catarrhalis-mapA* primer. lanes 1, 3, 4,5 from ear swab represent the identified *Moraxella catarrhalis*, at 140bp. Lane M represent 100bp universal DNA ladder.

The *mapA* gene is one of the essential genetic factors encoding the production of acid phosphatase. The ability of *M . catarrhalis* to produce this enzyme regardless of presence or absence of *map A* gene , may also give an impression that this enzyme may be encoded by another genes available in *Moraxella* genome .

Bull *et al.* , (2002) referred to that despite having a common functional identity, the isoenzymes of acid phosphatases differ widely regarding the chromosomal origin, molecular weight , amino acid homology and sequence length .

A previous study have been done by Hoopman *et al* . ,(2008) indicated that the *mapA* gene plays importance role in acid phosphatase enzyme production in *M . catarrhalis* strains. But other studies stated that this enzyme can be produced by different types of bacteria like Salmonella enterica and Escherichia spp. (Grose *et al*., 2005; Ishikawa *et al*., 2000).

The fact that the *MapA* is expressed at a relatively low level in comparison to other *M . catarrhalis* autotransporters (*UspA2*) may be related to the fact that its enzymatic function is catalytic and does not require abundant protein expression (Hoopman *et al*., 2008)**.**

The cleavage and release of phosphate from organic sources by this acid phosphatase may be necessary for the uptake of an essential nutrient. (Todd *et al*., 2007).

3.3 Multilocus sequence typing of *Moraxella catarrhalis***:**

To identify each locus accurately ,it was used sequence method. For all isolate ,the eight gene were successfully sequenced and analyzed by MLST ,polymorphic site ,GC content ,K (rate of nonsynonymous (dN) ,synonymous (dS) substitutions and the nucleotide diversity for each locus (*abcZ, adk , efp, funC ,glyBeta , mutY, ppa & trpE*) were determined as shown in table (3-4).

Table (3-4). Nucleotide and allelic diversity of the 8 housekeeping genes evaluated

Locus	Size (bp)	Alleles	Polymorphic sites	GC content (%)	Nucleotide Diversity	*k*
abcZ	429	6	32	44	0.02779	11.92381
adk	471	8	24	44	0.02054	9.67619
efp	414	7	24	45	0.02356	9.75238
fumC	465	6	32	43	0.02329	10.82857
glyBeta	537	10	45	43	0.03165	16.93333
mutY	426	9	28	49	0.02732	11.63810

ppa	393	9	54	40	0.03058	12.01905
trpE	372	4	12	47	0.00922	3.42857

k **: Average number of nucleotide differences.**

The mean GC content of sequnces of eight gene fragments ranged from 40%(*ppa*) to 49%(*mutY*):Trimmed fragment size of the 8 selected loci ranged from 372 b-p (*trpE*)_ 537 bp (*glyBeta*).The nucleotide diversity ranging from 0.00922 to 0.03165 pergene . Moreover , The number of polymorphic sites per locus varied between 12 (*trpE*) _ 54(*ppa*) and harbored a total of 251 SNP.

In present study, MLST was used to explore the population structure and evolution of 15 *M. catarrhalis* isolates from different clinical specimens which may provide better information concerning their biological properties. To initiate analysis ,the sequence diversity of the 8 housekeeping genes was calculate . This step was carried out to measure whether these selected loci had sufficient typing discrimination. the number of alleles in these gene loci ranged from 4 to 10. compared with nucleotide sequence diversities reported in *M. catarrhalis* **table (3-4).**

The proportion of nucleotide substitution that changed the amino acid sequence (nonsynonymous base substitution [dn] and the proportion that did not synonymous base substitution [ds]) were calculated the ratio (dn/ds) measure the level of selection in a protein coding gene. The ratio of dn/ds indicates purifying selection if dn/ds <1, positive selection if dn/ds >1. However ,the high ratios of nonsynonymous to synonymous substitution indicat a role for diversitying selection these loci ,and the d_N/d_S ratios which were determined to be more than 1 for the *eight* genes which indication positive selection .

The MLST protocol was used to examine the sequence diversity of eight housekeeping genes from *M. catarrhalis* isolates, and it had enough discriminatory power to classify isolates within a single species. One

locus (*trpE*) had low polymorphism, indicating that the sequences were similar across species. The *ppa* had 54 sites suggesting recombination was evident &representing asignificant source of genetic diversity of *M. Catarrhalis,* fifteen *M. catarrhalis* isolates were typed using MLST protocol.

According to allelic profile, it was found that the presence of allelic variant (SNP, insertion, or deletion) between isolates. In the case *glyBeta* was more variant or mutant than other 7 housekeeping genes, contrary to the *trpE* which was the least variant. The genes chosen for the present MLST scheme seem to be representative of the general polymorphism seen in housekeeping genes of *M. catarrhalis*.

The polymorphic sites found in the present sequence data might therefore be useful in the development of a molecular bacterial typing scheme based on detection of single nucleotide differences.

In MLST protocol different sequence at each locus are assigned with specific allelic profile and assigned as a sequence type which is the unambiguous descriptor of the strain. knowledge, this is the first study report that describes the development and application of MLST in Iraq to characterize this important human pathogen. However, the result of this study present sequence type (ST8,ST23,ST26,ST1051,ST11,New ST,ST494,ST501 ,ST197&ST1498).

Isolates could be divided into 11 ST using combined data from 8 loci as shown in table (3-5).Among which two novels STs based on new combination of the known alleles. The most abundant ST was ST26 containing 3 isolates (mc3,mc25,mc33) followed by ST23and ST8 each containing two isolates (mc2,mc20) , (mc1,mc21) receptively , the remaining STs only contained one isolates. The ST26 was found in ear and sputum. the isolates in ST23 and ST8 were all from ear swabs.

Table (3-5): Allelic profiles based on 8 housekeeping genes from all isolates of *Moraxella catarrhalis* evaluated.

Sample name	ST	abcZ	adk	efp	fumC	glyBeta	mutY	ppa	trpE
mc1	8	2	6	2	2	3	6	6	2
mc2	23	16	15	1	15	12	17	16	9
mc3	26	3	8	2	3	6	18	3	2
mc5	1051	2	17	11	2	36	31	34	2
mc7	11	3	8	2	9	8	8	8	5
mc20	23	16	15	1	15	12	17	16	9
mc21	8	2	6	2	2	3	6	6	2
mc22	New	34	6	3	3	30	26	21	2
mc23	New	34	6	3	3	30	26	21	2
mc25	26	3	8	2	3	6	18	3	2
mc26	494	84	72	9	64	89	92	82	36
mc28	501	84	59	9	44	111	92	87	36
mc29	197	8	3	6	2	17	9	17	2
mc30	498	3	18	55	2	15	15	8	2
mc33	26	3	8	2	3	6	18	3	2

In the study by Zhao *et al* ., (2022) were found in the analysis of the MLST patterns of the 210 isolates, 105 STs were identified including 70 new STs ,with ST449 (n=13),ST64(n=11),ST215(n=10),ST462(n=9),and ST394(n=8) predominating.

However the variation between this study and other studies due to the limited number of isolates, or may be due to undergoing natural accumulation of sequence variation in housekeeping genes. Moreover, isolates obtained from diverse geographical locations, and during extended periods of time may give more genetic variability.

The maximum likehood method was used to analyze the DNA sequences of each of the eight genes, which were found to be well suited for determining phylogenetic relationships among *M. catarrhalis* isolates. The housekeeping genes are assumed to be appropriate for population genetic reserch. The phylogenetic relationship among *M. catarrhalis* were shown in figure (3-7). the DNA sequence were aligned and analyzed for each gene fragments. The phylogeny of these ST, an MLST phylogenetic tree of all the *M. catarrhalis* strains was inferred maximum like hood approach from concatenated sequence. All *M. catarrhalis* isolates showed polyphyletic lineage and revealed two distinct clusters, cluster A contain 13 isolates of this cluster was divided into subcluster ,while cluster B divided into two branches & each branch contain 2 isolates .
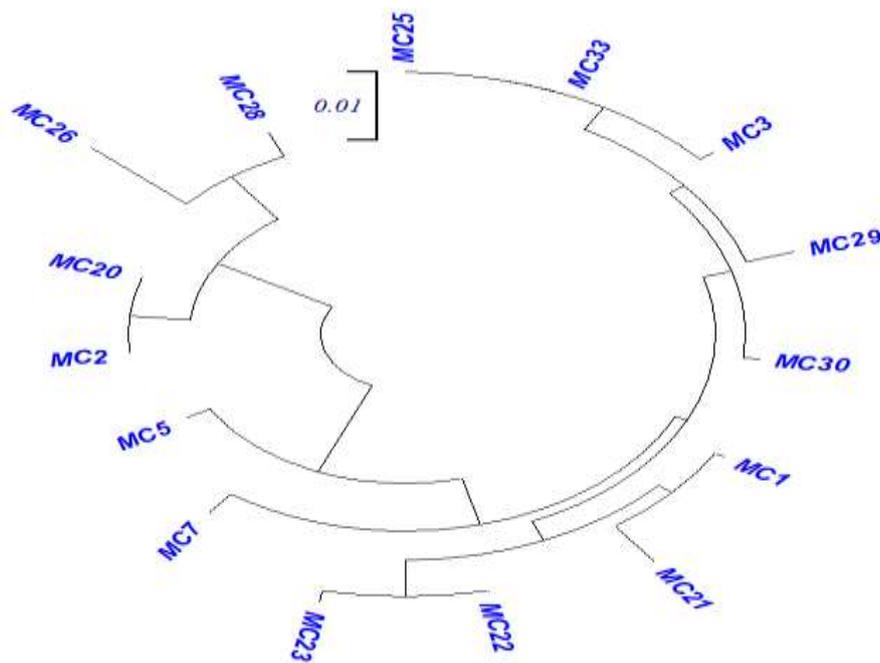

Fig. (3-7): Circular phylogenetic analysis based on concatenated sequences of 8 housekeeping genes from 15 *Moraxella catarrhalis* isolates by maximum likelihood method.

The phylogenic relationship between bacterial organism, potential problems associated with its ability to resolve the relationship between closely related isolates. due to an extremely low rate of natural mutation.

The present finding provided strong evidence that *M. catarrhalis* strains possess a high level of temporal stability and phylogeographical structuring ,supported largely by the phylogeographical signals observed in the phylogenic tree. However, the phylogeny tree for each gene was shown in appendix (1).

Splite decomposition analysis was performed on each locus separately and on the concatenated sequences of all ST ,as shown in the split graphs figure (3-8).
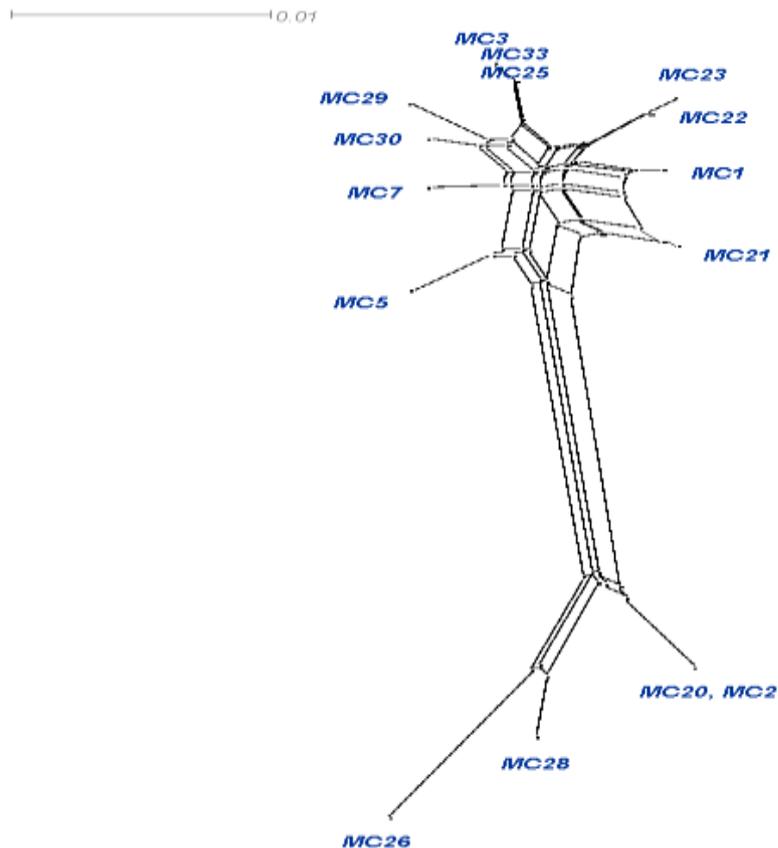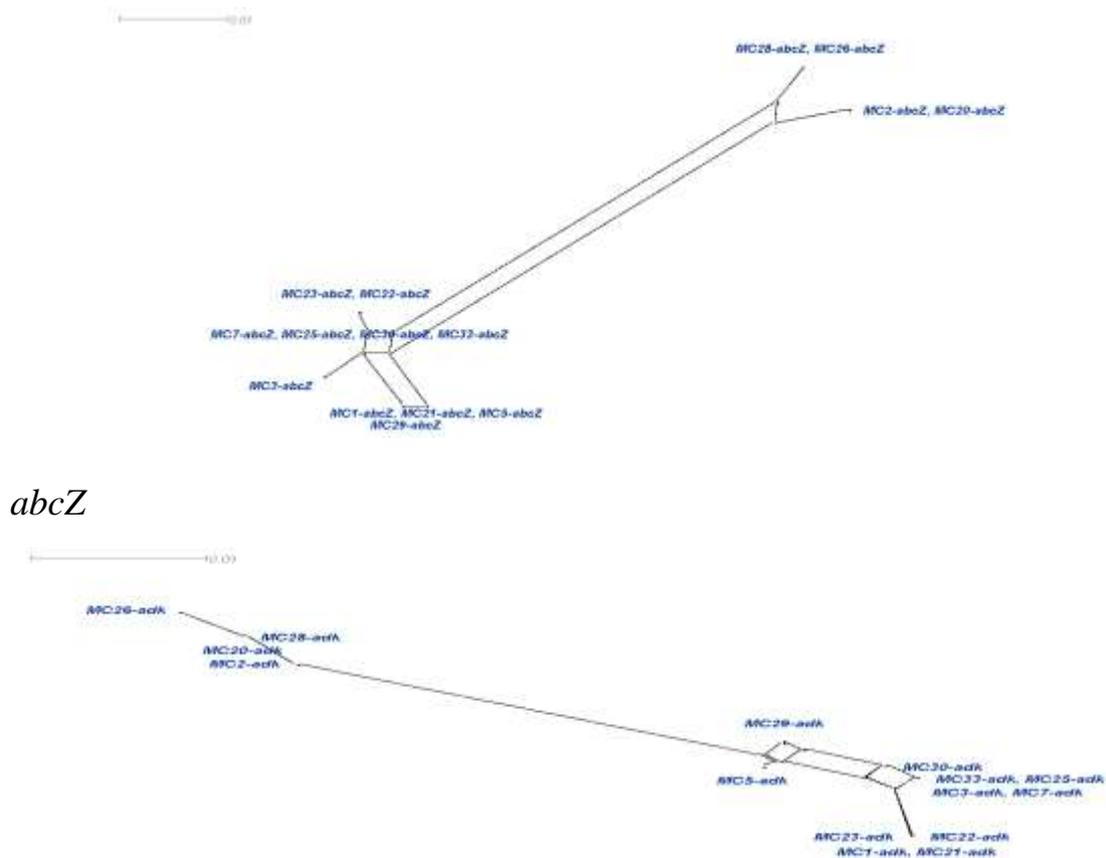
Fig. (3-8): Split-decomposition analysis based on concatenated sequences of 8 housekeeping genes from 15 *Moraxella catarrhalis* **isolates. Note: multiparallelogram formations indicate recombination events.**

The split graphs for the seven gene (*abcZ , adk , efb ,fum C,glyBeta, mutY& PPa*) revealed network like with parallelogram structures indicating that intergenic recombination had occurred during the evolutionaty history of these genes . However ,the split graphs of *trpE* are tree like structures suggesting that the descent of these genes was clonal and absence of recombination . The split decomposition analysis of combined eight MLST Loci display network like structure with rays of different length as shown in figures (3-9).

abcZ

efp

Fig. (3-9): Split-decomposition analysis *abcZ , adk , efb ,fum C,glyBeta, mutY& PPa, trpE* gene sequences of 8 housekeeping genes from 15 *Moraxella catarrhalis* isolates. Note: multiparallelogram formations indicate recombination events.
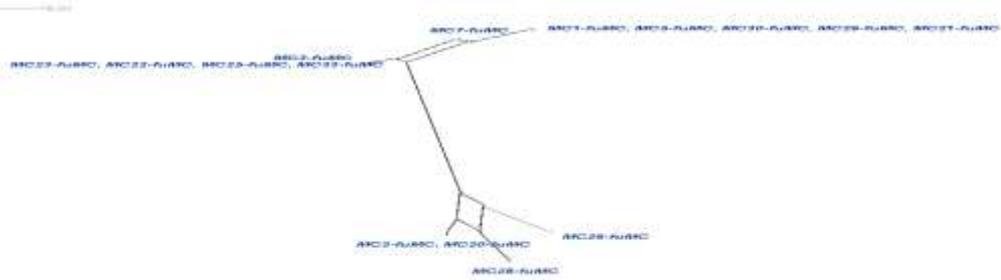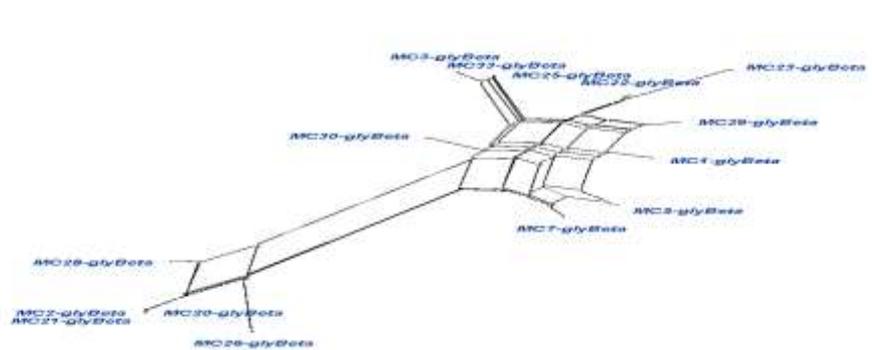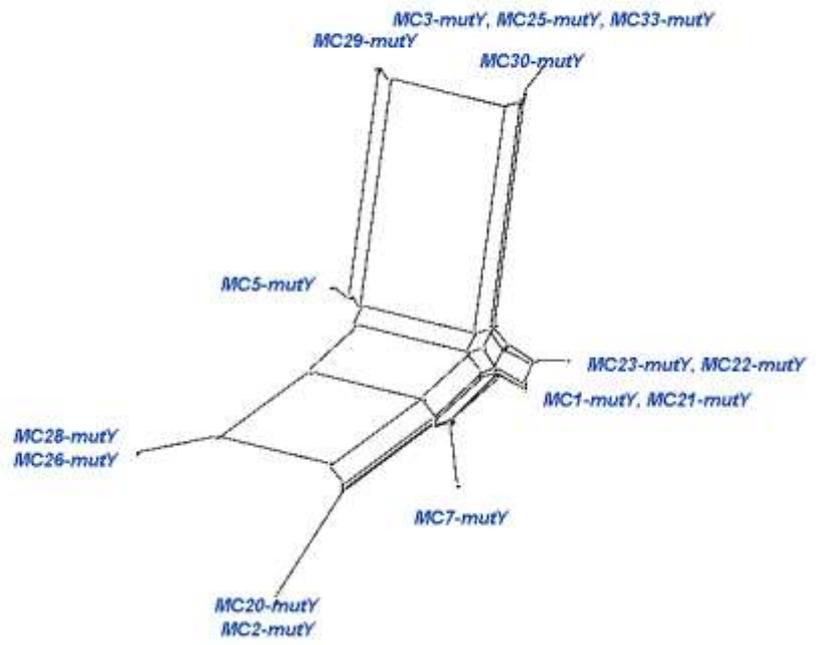
continuous

fumC

glyBeta
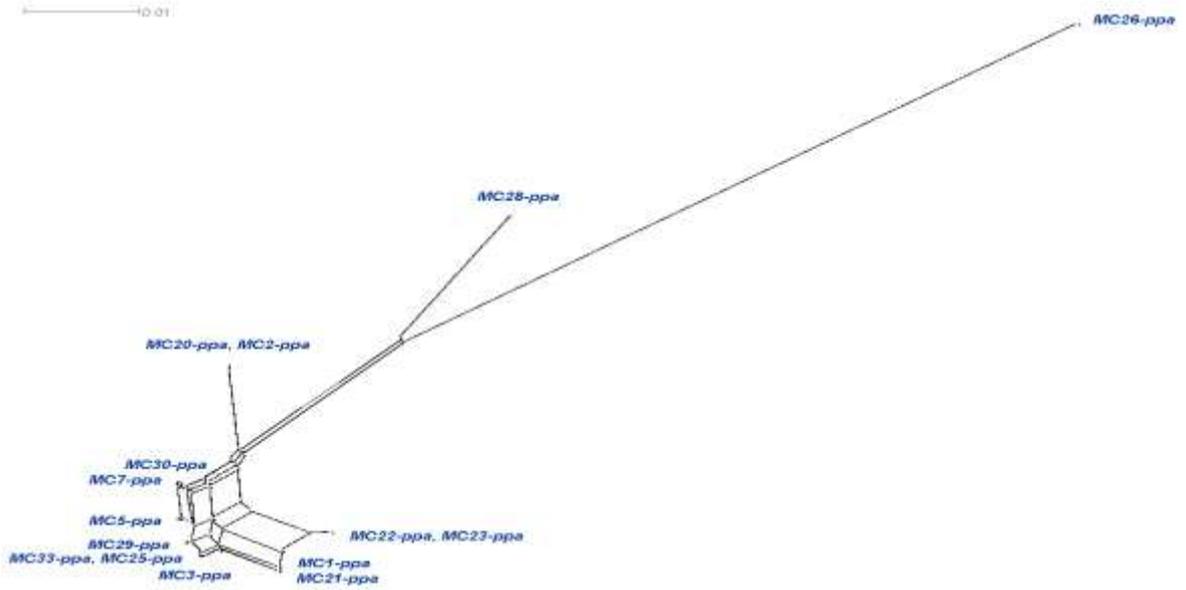
mutY

Ppa
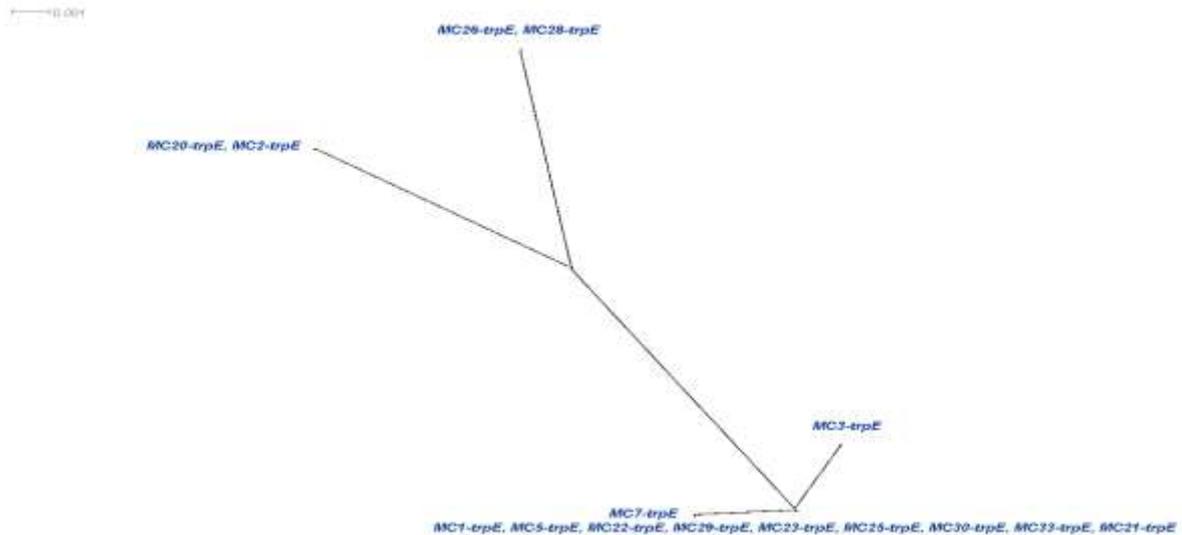
trpE

Fig. (3-9): Split-decomposition analysis *abcZ , adk , efb ,fum C,glyBeta, mutY& PPa, trpE* **gene sequences of 8 housekeeping genes from 15** *Moraxella catarrhalis* **isolates. Note: multiparallelogram formations indicate recombination events.**

The 11 STs representing all isolates divided into subpopulation was completely disconnected. Split decomposition analysis based on the allelic profils of isolates have provided evidence of recombination that play a role in generating genotyping diversity among isolates. In this study, tree like structures or parallelogram. Shaped structures were commonly found in the split graphs for all the night housekeeping genes evaluated, illustrating that recombination had occurred in these MLST loci.

eBURST is an algorithm that uses MLST data to identify groups of closely related sequence types. In the current study, It was used to investigate the possible similarity, variability, and evolutionary relationships between different *M. Catarrhalis* ST types. The genetic backgrounds of the STs identified in this study were found to be diverse. As shown in figure (3-10)2CC(CC1&CC2) were observed for the 11 ST.

Among the 2cc,cc1 was the largest and comprised 7 link ST, namely N1,N2,ST494, ST501,ST23,ST26& ST11.These included six isolate from ear swab,& four isolates from sputum.

Clonal complex 2,representing 4ST, which includes three isolates from ear swab & two isolates from sputum Therefore ,it is unsurprising that the relationship between the isolates from clinical different source was closely related. Often, only some isolates of the same source or location were clustered togather, whereas the rest were dispersed across other clusters.
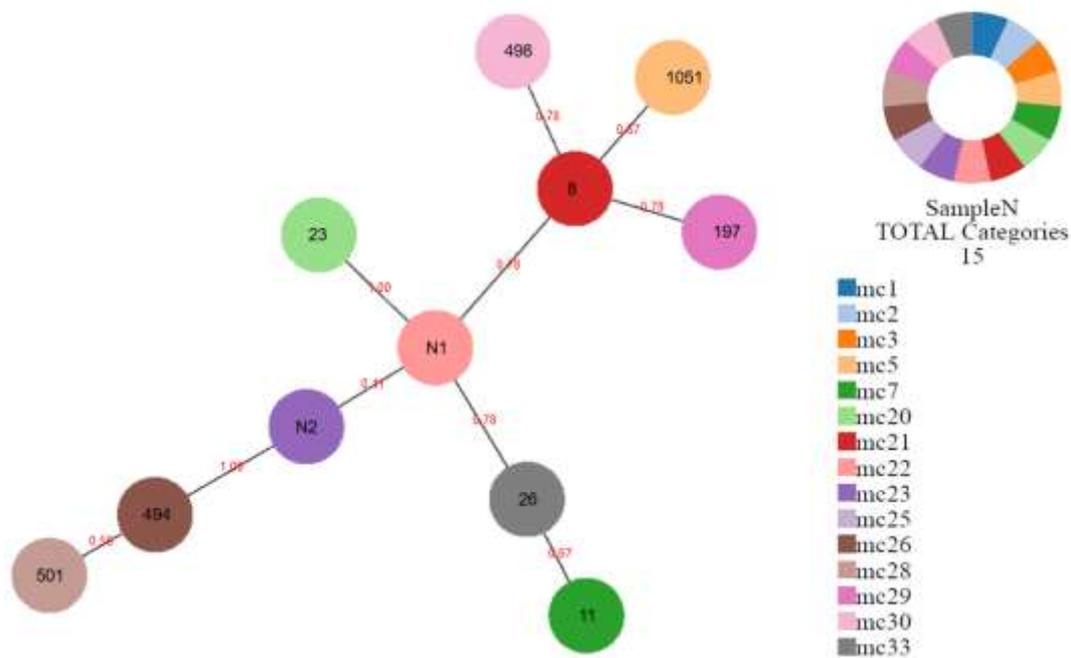
Fig (3-10): Comparative eBURST analysis showing the clonal assignment and the relative distances of the identified STs .

The sequence of fragments of eight housekeeping genes from 15 of *M. catarrhalis* isolates provide data that can be used to address aspects of the population & evolutionary biology of the species.

M. catarrhalis frequent horizontal DNA exchange has eliminated the phylogenetic signal in each housekeeping gene. determined the degree

of allelic variations in eight housekeeping genes of *M. catarrhalis* by using a sample of 15 isolates originated from different clinical specimens. The degree of isolates differentiation by MLST appear adequate for use in epidemiological investigation, as the number of different types obtained by MLST. Single locus phylogenetic tree were non congrunent ,suggesting that recombination plays a role in the generation of diversity of *M, catarrhalis* population .MLST was performed for only 15 isolates to determine the STs due to its high cost &labor intensive. The high genetic variability amongst Moraxella isolates in this study provides some information on the local dissemination and genetic relatedness.

The MLST was developed as a scalable typing system to determine the diversity and phylogenetic relationships of the isolates based on eight housekeeping genes, and it provide reproducibility, comparability, and transferability between laboratories.

Most previous studies showed that MLST was useful to accurately identify bacterial lineages, but few studies have considered the relationships between isolates &their source. In this study, we used MLST to type *M. catarrhalis* isolated from different clinical specimens and look for relatedness to isolates that were pathogens. These representative isolates were unique in their diversity of sources and provide some necessary information required to understand genetic diversity persistence & movement in this species .

The genetic diversity among these strains may be related to gene deletion, insertion, duplication, or high rate of horizontal gene transfer mechanism.

Results of this study indicates that the majority of *M. catarrhalis* studied may have descended from ancestor that exist many years ago. However, genetic variation in pathogen population is a major barrier to disease control.

MLST as a tool for epidemiological studies to investigate the evolutionary pathogen and clonal lineages of bacteria. MLST differentiated strains into sequence of 8 housekeeping genes with appropriate level discrimination using allelic differences.

The study was showed the genetic relationships between the *M. catarrhalis* clones. The presence of dominant clone in the specimens of hospital showed the presence of shared infection source among the patients.

Different allelic profile in our isolates showed a decrease of possiblity of specimens to be from a clonal lineage. So, elaborating the relationship among the *M. catarrhalis* isolates is becoming increasingly important. The ability for *M. catarrhalis* to jump into host is a major threat to public health.

Although the number of isolates studied was not large, this work has shown that the specimens collected are highly divers. This is due either to a dynamic evolution of the local strain of the organism, or to the continuous introduction of new isolates from abroad. This study provide valuable information that is important for the understanding of the poor adaptation of *M. catarrhalis* with uncommon STs, which may otherwise be capable of disseminating globally.

The data of this study have public-health implication, where the high diversity and emergence of a new clone of *M. catarrhalis* in Babylon province calls attention for the epidemic and the recognition of the strains with new clones that could protrude in the future, that in turn very important for understanding the evolution.

MLST is based on allelic variation in housekeeping genes, and while it monitors change over just a small portion of the genome, its highly discrimination and provides insight into genetic structure. Also, it reveals highly detailed information on genetic changes in specific

housekeeping genes, and thus provides direct insight into evolutionary changes of the core genome. In addition, MLST proved useful for detection of novel and previously known strain and for inferring relatedness among isolates.

Generally, molecular typing methods are intended to tackle two different levels of epidemiological problems, which reflect different insights toward solving a local or global epidemiology in different timeframes. In one hand localized outbreak of disease in a short period of time should be assessed and on the other, relation between strains causing a disease in one geographic area with those observed around the world during a longer period would be investigated. These two different conceptual views demand different appropriate scheme of molecular typing, so that isolates recorded in same molecular type are likely to be descended from a younger ancestor and those belonging to more distant ancestors are expected to differ in type unless a relative higher clonal population would be under study.

eBURST analysis of MLST datasets of highly recombinogenic species results in a single large straggly eBURST group, which results from the incorrect linking of unrelated groups of strains (Turner *et al*.,2007).

In particular, the Multilocus Sequence Typing (MLST) is an ordinary typing method that is based on the characterizing bacterial species via sequencing of internal fragments of multiple housekeeping genes. Usually, seven housekeeping gene evaluate by the MLST method via the internet (Kalia *et al*.,2001;Willems *et al*.,2001). In this process, each sequence of internal fragments compares with the other alleles that they were already characterized. Then, each sequence classified at one of those seven housekeeping genes category. Ultimately, by the combination

of obtained data, the allelic profile will be construct and each distinct profile consider as absolute sequence type.

findings expand previous research on *M. catarrhalis* strain change to include detailed phylogenetic typing. We found that the diversity of M. catarrhalis strains is very high; however, we were unable to determine whether acquisition of a new strain was important for the onset of an exacerbation because new acquisition was common in most samples, regardless of whether it was a new stable or new exacerbation visit. Although we cannot rule out the possibility that specific *M. catarrhalis* strains are linked to an exacerbation, the number of different MLST types identified in such a small sample size makes this unlikely (Sethi *et al* .,2002*)*.

Wang *et al* .,(2018)were found number of limitations. Despite the fact that the sample size for genomic sequencing of *M. catarrhalis* cultures from a large observational study was the largest to date, the number of samples and subjects remained modest, and larger longitudinal studies are needed to determine whether acquisition of a new strain is associated with exacerbations, other clinical features, and inflammatory profiles. It would also be necessary to test multiple isolated colonies from all samples to determine the presence of more than one strain at the same time. However, simultaneous carriage was uncommon in samples where more than one colony was sequenced. The study relied on sputum sampling, which presented challenges such as consistent sampling in large populations and oral contamination. We are confident that these data reflect lower airway samples because the success rate for sputum samples was very high in this group and salivary contamination was low.

MLST is widely used in epidemiological investigations of large-scale outbreaks as well as sporadic cases. This technique is highly

reproducible and allows pathogenic clones to be compared across laboratories, regions, and countries(*Zhao et al .,2022*).

3.4 Restriction –modification systems of *Moraxella catarrhalis*:

Fifteen *Moraxella catarrhalis* isolates were obtained from different clinical specimens include (ear swab and sputum) used to study R-M system(MultiM1, MultiM2, MultiM3,T1mod,T1res)it was screened by multiplex PCR technique as shown in figure (3-11).

Fig (3-11): Restriction-modification (R-M) system screening by Multiplex PCR. A representative multiplex PCR reaction is shown. Different sized amplicons are generated depending on which of the mutually exclusive locus 1 restriction-modification (R-M) systems (Type I & Type III). Type I R-M system: 637 bp for MOD(2,6,11) and 158 for RES (2,6.11), modM1(5): 400 bp, modM2(1,3,7,8,9,10,12,13,15): 300 bp, modM3(4,14): 500 bp. Lanes 1-15 represent the 15 study isolates.

3.4.1 Distribution of *M. catarrhalis* R-M Systems:

In order to determine the distribution and variability of R-M systems in *M. catarrhalis*. Type I showed only in 3 isolates (20%) while type III that found in 12 (80%) isolates as show in the table (3-6).

Table (3-6): Distribution of RM systems (type I and III) .

Result	Type I RM-system N (%)	Type III RM-system N (%)	P value
Positive	3 (20)	12 (80)	<0.001*
Negative	12 (80)	3 (20)	
Total	15 (100)	15 (100)	

* represent a significant difference at p<0.05.

However the Distribution of RM systems (type I and III) according to infection type was also studied, the presence of sequence related to enzyme of the R-M system was observed in all *M.catarrhalis* isolates . The result of type I was (22.3%) from OM infection and (16.7%) from Respiratory infections. while the result of type III was (77.7%) from OM infection and (83.3%) from COPD.as shown in the table.(3-7).

Table (3-7): Distribution of RM systems (type I and III) according to infection type.

Result	*Type I RM-system* N (%)	*Type III RM-system* N (%)	*P value a*
OM Infection	2/9 (22.3)	7/9 (77.7)	<0.001*
COPD	1/6 (16.7)	5/6 (83.3)	<0.001*
Total	3/15 (20)	12/15 (80)	

Note: a statistical analysis between RM-systems (Type I and Type III), * represent a significant difference at p<0.05.

To investigated the distribution of RM system gene (T1MOD, T1RES, modM1,modM2,modM3) of *M.catarrhalis* isolates, PCR analysis of DNA was performed, overall , *M.catarrhalis* isolates were examined consisting of 9 middle ear effusion isolates and 6 sputum from COPD, T1modM and T1RES gene was present in 3 isolates for both gene(table 3-8) .However, modM1 was present in one isolate , modM3 was present in two isolates .while modM2 was found in nine isolates with modM2 the most common (60%) .Interestingly ,a statistically significant association was found between RM(I, III) and disease. modM2 was most prevalent in respiratory infection (66.6%) compared to (55.5%) of middle ear isolates , the complete list of isolates their site of isolation and their RM system gene were shown in table (3-8) and appendix(2)

Table (3-8): Distribution of RM system genes (*T1MOD, T1RES, modM1, modM2 and modM3*) according to infection type.

Source	Type I RM-system		Type III RM-system			P value
	T1MOD	T1RES	modM1	modM2	modM3	
OM Infection n9	2/9 (22.3)	2/9 (22.3)	1/9 (11.1)	5/9 (55.5)	1/9 (11.1)	<0.001*
Respiratory infections N6	1/6 (16.7)	1/6 (16.7)	0/6 (0)	4/6 (66.6)	1/6 (16.7)	<0.001*

* represent a significant difference at p<0.05.

It was suggested that modM isolates are more frequently associated with OM and COPD than the Type I R-M containing isolates in present surveyed collections. . It was shown that ModM2 regulated genes that were previously identified as being involved in colonization and disease.

Differential regulation of ModM phasevarions may provide a selective advantage to ModM ON versus ModM OFF variants, or vice versa, in carriage or disease (either OM or COPD), however further investigation of ModM ON/OFF switching in these populations is required.

In type III RM system three modM allele identified in *M. catarrhalis* each allele differs most extensively within the putative DNA recognition domain .The present different allele suggested that multiple phase variation exist within *M.catarrhalis* each modM allele regulation different of genes.

significant difference was observed in the distribution of R-M systems or modM alleles in child isolates regardless of the patient's OM status. Similarly, difference was observed between strains isolated from COPD patient sputum samples during an exacerbation versus periods of stable colonization.

Blakeway *et al*., (2014). previously investigated the distribution of the modM2 and modM3 alleles in a collection of 81 nasopharyngeal carriage and OM-associated middle ear isolates from children and found a statistically. that modM gene was present in all isolates investigated ,

with modM2 being the most common allele (83% of isolates) ,followed by allele 3 (16% of isolates), with allele 1 present in only one additional isolate (1%). While (Blakeway *et al* ., 2018) were found that all OM associated middle ear isolates contained a Type III R-M system modM gene with the Type I system never occurring in these isolates. This suggests that strains containing a nonphase-variable Type I R-M system may be attenuated in their ability to infect or persist in the middle ear when compared with strains containing a phase variable Type III modM gene. Analysis of the distribution of modM alleles in these 549 isolates revealed that modM2 was the most common allele, present in 82% of isolates, while modM3 was found in the remaining 18% of isolates. When isolates were split by clinical manifestation, modM3 was found in child OM-associated middle ear isolates at a significantly higher frequency than child nasopharyngeal carriage isolates.

This discrepancy may be due to potential geographical differences in circulating strains or differences in presentation or severity of OM among study populations. The variation of this result with other result due to some factors such as geography ,origin of specimens , the time of conduction of studies ,epidemiological and regional factors. in addition to the results affected by sampling biased.

The prevalence of phase variable and R-M system in bacteria suggests that important , ther to unrecognised functions are being fulfilled .There are possible implication for several cellular processes such as inter –and intra-species transformation and genetic regulation .

The domince of one R-M system in *M.catarrhalis* isolates helps these isolates to better to invade both micro and macro environments and make their host more resistant and fittest subpopulation .

The two types of R-M system genes were reported to be distributed in the studied isolates , while the functional analysis showed that the mod

gene is the main player in colonization in the regulation of different gene expression.

Type III R-M system might allow *M.catarrhalis* isolates to better colonize different environmental condition and different sites of infection ,as well as to smartly evade host immune responses. moreover, the investigated phase variable R-M genes might be instrumental in generating genetic diversity by changing their phases from on to off or vice versa, thereby increasing colonization and providing overall survival and fitness advantages to *M.catarrhalis* isolates in Babylon population .

The wide spread distribution of R-M system in host adapted pathogenic bacteria suggest that this novel mechanism of coordinated random switching of multiple genes may be a commonly used strategy for generation of distinct ,differentiated ,cell type with distinct niche specialization in host adapted bacterial pathogen .

It was shown that ModM2 regulated genes that were previously identified as being involved in colonization and disease, however genes regulated within the ModM3 phasevarion have not been identified and are under investigation. In other significant respiratory colonizers, including Neisseria meningitidis (Seib *et al* ., 2011) and NTHi (Brockman *et al* .,2016), phase variation of DNA methyltransferases causes the differential regulation of virulence factors and vaccine candidates that are important in the progression of disease.

The modulation of the expression of ModM, a phase-variable type III DNA methyltransferase, affects the expression of various genes, known as a phase-variable regulon (Seib *et al* ., 2002). The three alleles of modM (modM1, modM2, and modM3) potentially regulate the expression of multiple genes associated with colonization, infection, and protection against host defences (Blakeway *et al* ., 2014).

3.4.2 *Moraxella catarrhalis* R-M system are associated with phylogenetic

The presence or absence of R-M system in 15 *M . catarrhalis* isolates was generated ,with isolated ordered based on phylogeny as shown in figure (3-12) comparison of R-M system between 15 isolates found that two main cluster the first A cluster which contain R-M type III system which include (12 isolates) ,while cluster B which contain R-M system type I which include (3 isolates).Differences in R-M system presence were also observed between isolates which obtained from different site of infection.

In the current study, we have found that the genetic diversity in *M.catarrhalis* depended on source of isolation and occurrence of mutation .It is interesting to note that ,there was no evident correlation between the observed isolates variability and the specimen from which isolates originated.
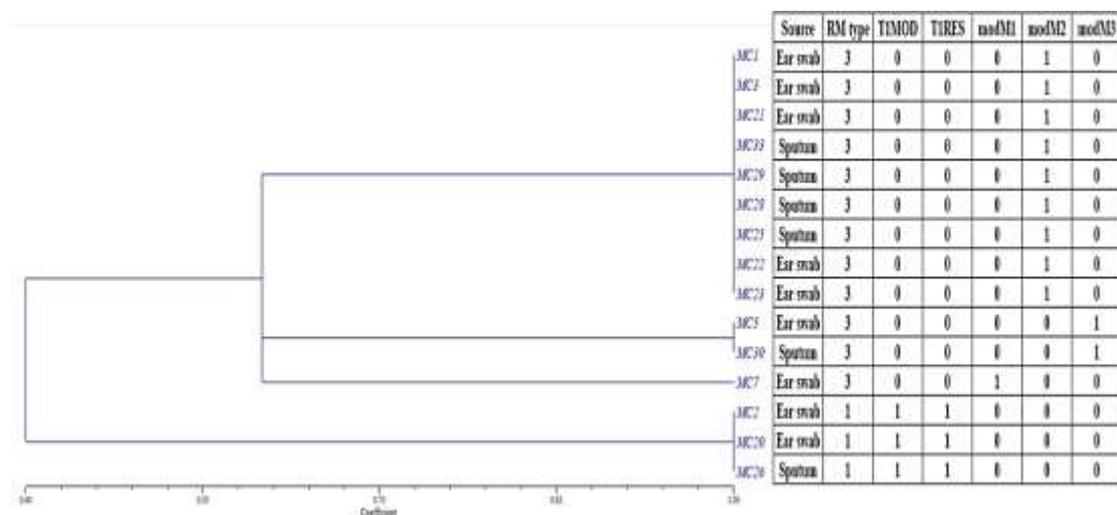
Source	RM type	TIIMOD	TIRES	modM1	modM2	modM3
MC1	Ear swab	3	0	0	0	1	0
MC3	Ear swab	3	0	0	0	1	0
MC21	Ear swab	3	0	0	0	1	0
MC33	Sputum	3	0	0	0	1	0
MC29	Sputum	3	0	0	0	1	0
MC28	Sputum	3	0	0	0	1	0
MC23	Sputum	3	0	0	0	1	0
MC22	Ear swab	3	0	0	0	1	0
MC24	Ear swab	3	0	0	0	1	0
MC5	Ear swab	3	0	0	0	0	1
MC30	Sputum	3	0	0	0	0	1
MC7	Ear swab	3	0	0	1	0	0
MC2	Ear swab	1	1	1	0	0	0
MC20	Ear swab	1	1	1	0	0	0
MC26	Sputum	1	1	1	0	0	0

Fig (3-12): UPMG phylogeny of *Moraxella catarrhalis* isolates depending on RM system profile. Note: 1=Positive, 0=Negative.

The data presented in this study show that a diverse range of systems exist in *M. catarrhalis*, and that many of these show associations with the phylogeny of the species. From these data, it is tempting to speculate that the acquisition of lineage associated R-M systems has

shaped the *M. catarrhalis* population structure, potentially contributing to the differing genomic content, and by extension, the pathogenic potential of the *M. catarrhalis* phylogenetic lineages. In addition, a number of highly variable R-M systems that exhibit allelic variation, and are potentially phase variable were identified in *M. catarrhalis*. Our data demonstrate that there are a sizeable number of R-M systems present in *M. catarrhalis* that may contribute to clade specific evolution.

The distribution of R-M system in *M. catarrhalis* along phylogenetic line phylogenetic analysis demonstrates that many *M.catarrhalis* isolates contain potentially phase variable (type III) . This could imply that there is less selective pressure to generate phenotypic diversity in these organisms as they exist in a more predicatable environment and use the conventional "sense and respond" gene regulation paradigm of adaptability, that is, these organisms contain many more two-component sensor-regulator pairs than small genome pathogens that contain multiphase-variable methyl transferases (Shang *et al* ., 2018).

R-M systems are ubiquitous in bacteria, where they have fundamentally been associated with defense of the cell from incoming DNA, such as from bacteriophages. The presence or absence of particular R-M systems has been associated with phylogenetically distinct clades in bacteria such as N. meningitidis (Budroni *et al*., 2011), *Staphylococcus aureus* (Roberts *et al*., 2013), and Burkholderia pseudomallei (Nandi *et al*., 2015). Furthermore, phase variable Type I and Type III R-M systems have been associated with epigenetic regulation in systems called phasevarions (Atack *et al*., 2018). All characterized phase varions contain genes important for human infection, and genes that encode potential vaccine candidates (Atack *et al*., 2018).

It is unknown whether differences in R-M system possession are responsible for the population structure of *M. catarrhalis*, or are a consequence of the independent evolution of the phylogenetic lineages. R-M systems have been associated with the maintenance of speciation and intra species population structure by limiting horizontal gene transfer and homologous recombination between genomes with non cognate R-M systems, while permitting the genetic flux between bacteria containing cognate R-M systems (Oliveira *et al*., 2016).

On the other hand the gene sequence analysis of RM system (I and III) was also studied , the nucleotide sequence of RM system (I and III) were aligned . In the type I MOD the nucleotide identity was (97-100 %), so there are 21 mutation in MOD in which 4 mutation in (mc20 isolate) and 17 mutation in (mc26 isolate) while there no mutation occur in mc2 as shown in figure (3-13).
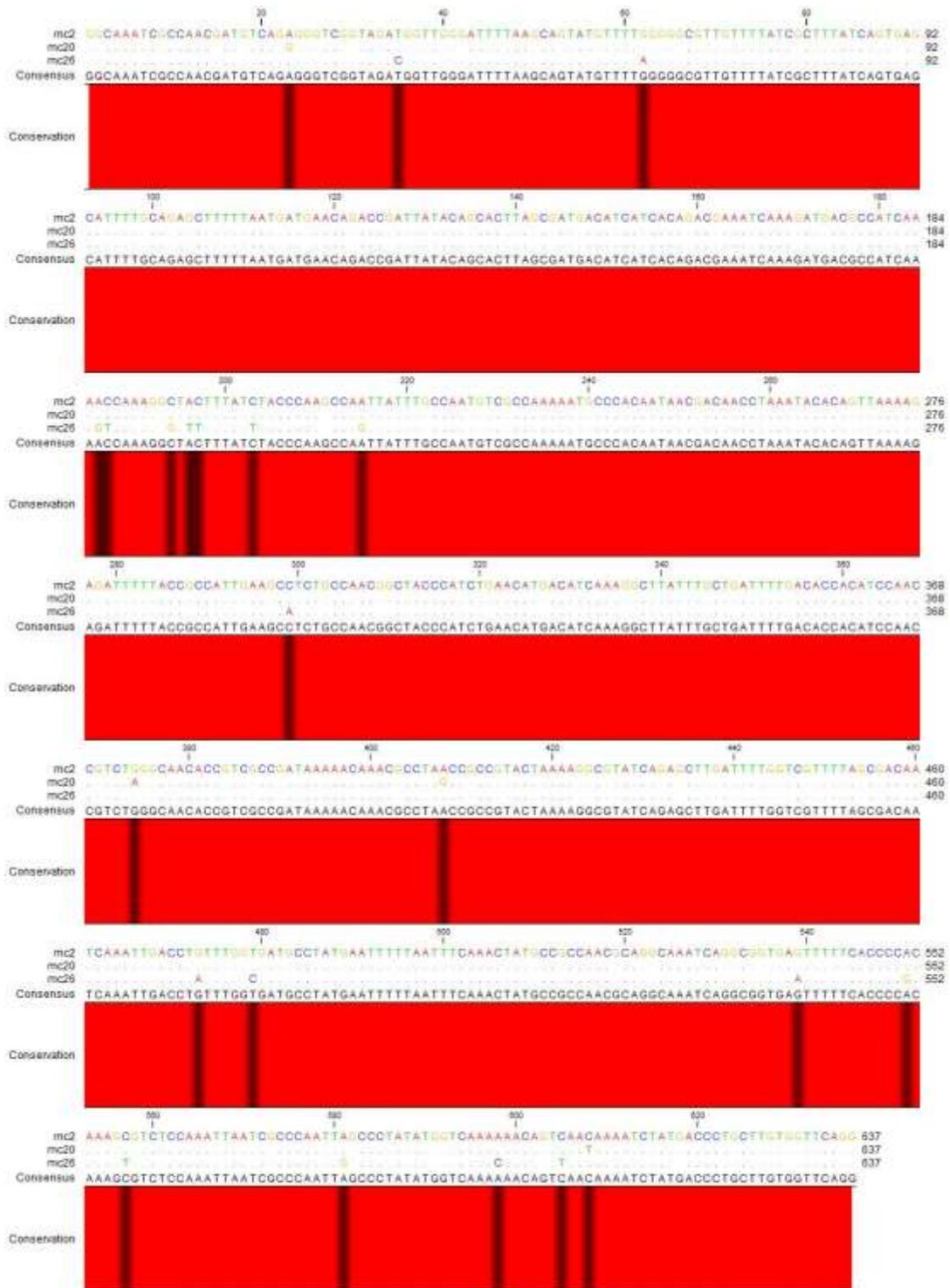
Fig (3-13): Type1 MOD alignment with illustration of mutation positions.

Also , in the type I RES ,the nucleotide identity was (94-100%)there were 9 mutation in RES in which 3 mutation in (mc20 isolate) and 6mutation in (mc 26 isolate) while there is no mutation observed in (mc2 isolates) as shown in figure (3-14).
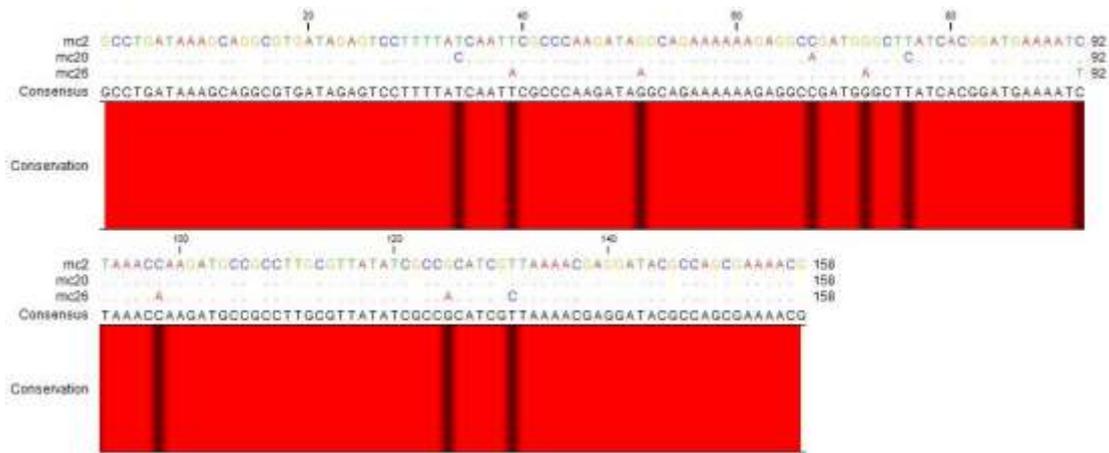
Fig (3-14): Type1 RES alignment with illustration of mutation positions.

However ,in the Type III modM2 the nucleotide identity (99-100%) , there are 7 mutation in modM2 in which three mutation in (mc1 isolate)and one mutation in each of (mc 33 , mc25 , mc28 and mc29) while the other isolate mc3 , mc21 and mc22 there is no mutation as shown in figure (3-15).
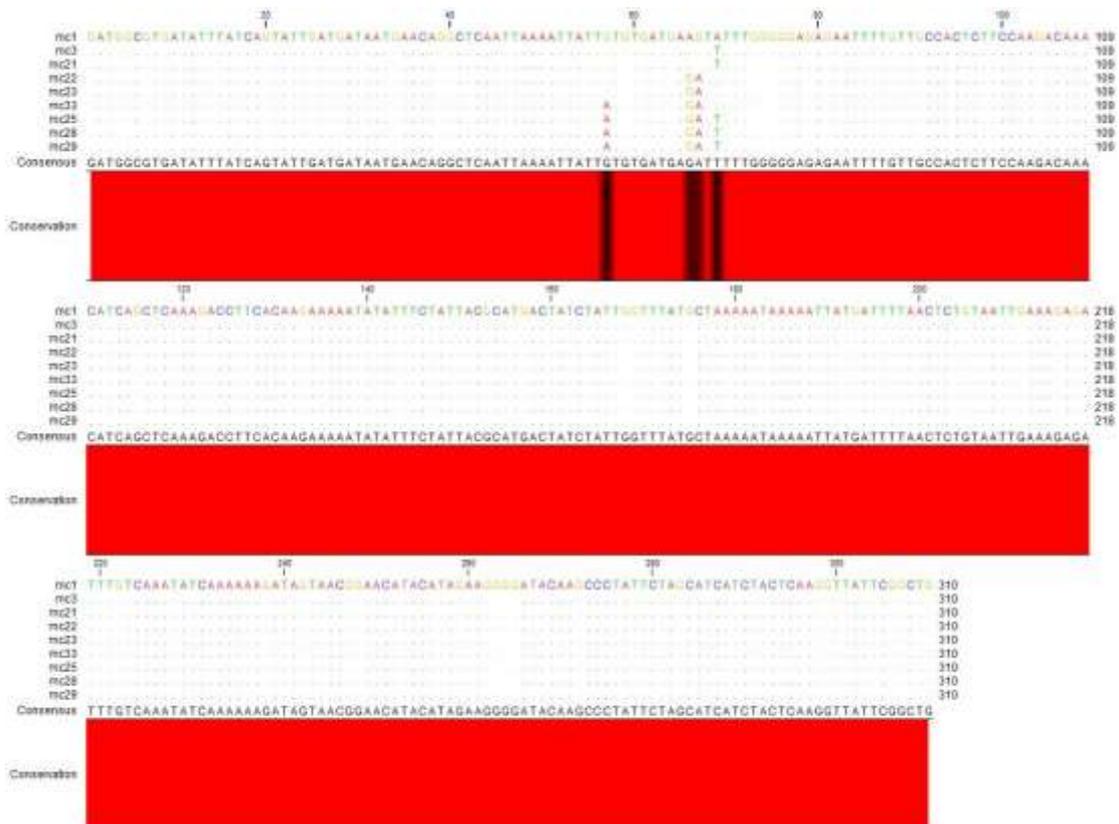

Fig (3-15): Type III modM2 alignment with illustration of mutation positions.

Finally in the Type III MODM3 the nucleotide identity (99-100%) and there is one mutation in mc30 isolate while there is no mutation in mc5 as shown in figure (3-16).
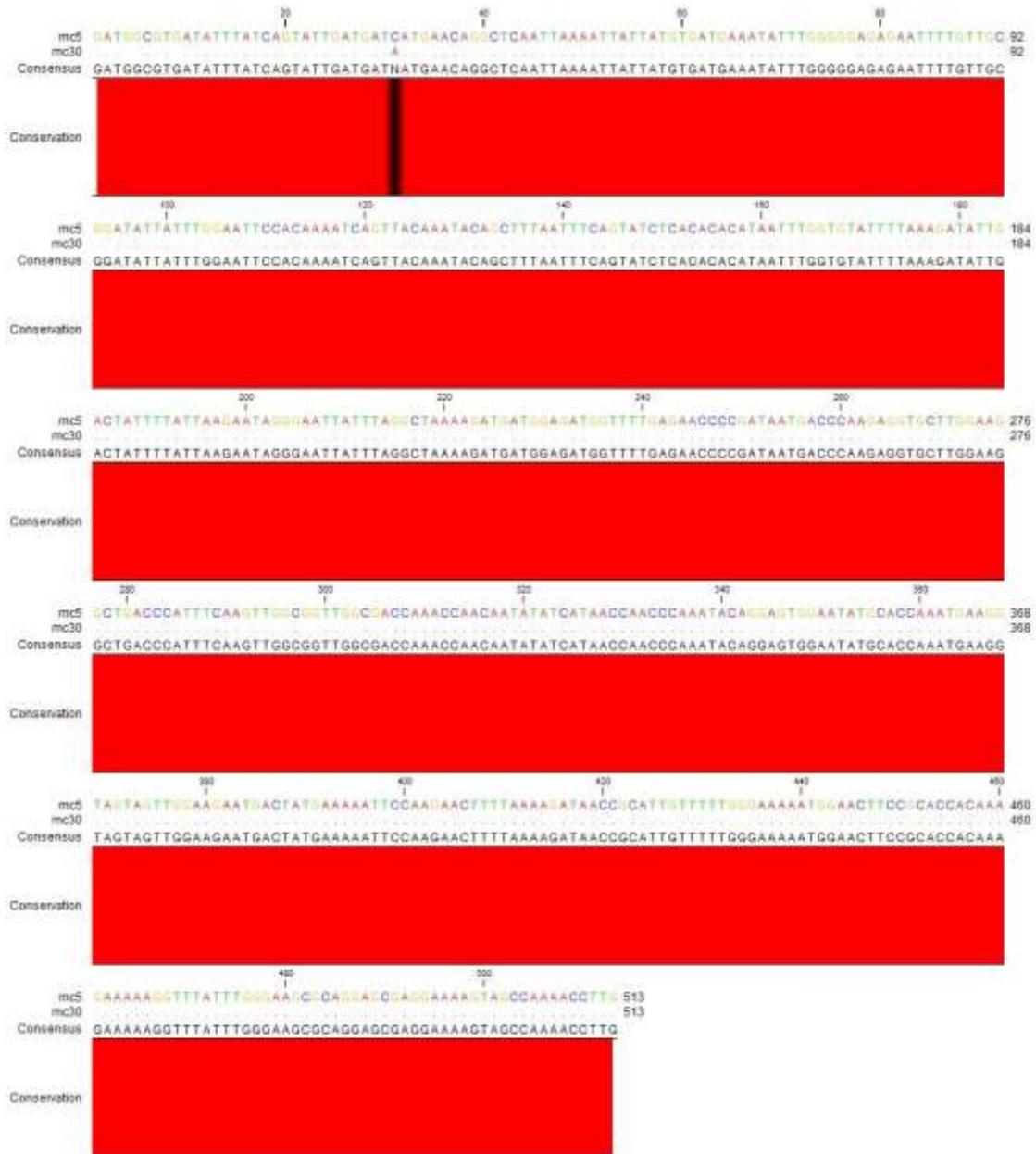

Fig (3-16): Type III modM3 alignment with illustration of mutation positions.

There   are more than on mutation in some isolates and this display that the type and location of mutation that were found could lead to difference in effect of these mutation and this mutation is point mutation .

High through put sequencing offer opportunities for understanding bacterial evolution within the host and promise to shed light on the in vivo dynamic of bacterial carriage and infection .the role of chance ,circumstance and genetic in invasive bacterial disease is let to be determined , but the exhavstive characterization of bacterial genetic version within the host is an important step.

In the molecular experiments , it can be useful for the introduction of point mutation in the genes that are important to create or remove restriction enzymes sites.

Phase variation is known to mediate bacterial adaptability and virulence (Moxon *et al* ., 2006).Phase-variable Mods, which control the coordinated switching of expression of multiple gene ,further enhance this adaptability. Phase varions are increasingly being recognized as playing an important role in host-adapted pathogen.

Switching of phase varion expression has also been shown to modu-late diverse phenotypes associated with virulence Phase variation is the high frequency, reversible, random ON/OFF or graded switching of gene expression .

Phase-variable gene expression is an important aspect of bacterial pathogenesis that aids in adaptation to changing host microenvironments, and which can aid immune evasion which has implications for vaccine development . (Tan *et al* ., 2016).

The presence of phasevarions in bacterial pathogens results in increased phenotypic diversity of these organisms. Where multiple allelic variants of phase-variable methyltransferases are present, each unique Mod allelic variant has been shown to methylate a unique target sequence, and regulate a different phasevarion (Seib *et al* ., 2015).

Bacteria have developed many biological strategies to live in different environments. The presence of Restriction-Modification systems

(R-M) is considered one of these strategies (Murray, 2002). The genome of many species of bacteria sequenced so far is homologous to R-M genes, which are associated with important biological functions (Bujnicki, 2001; Kobayashi, 2001).

Some authors have considered R-M gene as selfish DNA units. Once transferred to and established in a cell clone , the R-M gene complex could be difficult to eliminate because its losslesds to cell death by the action of restrication enzyme (kobayashi et al ., 2001).

There is also considerable evidence that R-M complexes are involved in bacterial genome rearrangements. These rearrangements are a consequence of the generation of DNA fragments originated by the action of restriction enzymes in heterologous non-methylated DNA. Once in the cell, these DNA fragments could be the target of host enzymes that recombine them in the recipient genome (Chinen *et al.*, 2000; Nobusato *et al.*, 2000; Kobayashi, 2001).

R-M systems are a common characteristic of *Moraxella catarrhalis* species, and that they probably have important roles in the genome organization and architecture, as well as in the adaptability of these bacteria to different niches found in their respective hosts.Phase variation of Type III methyltransferases has been shown to regulate the expression of genes important for infection of the human host in organisms studied to date (Seib *et al*., 2020).

aConclusions:

The present study was concluded the following criteria:

1- Detection of *M. catarrhalis* using specific primer (*copB*) is more accuracy and specific than biochemical test.

2- The bacterial isolates were found to possess more than one virulence factor such as *uspA1,uspA2,hag ,mcaP,* and *mapA* which detected by molecular procedure.

3- The *uspA1* gene exhibited the highest prevalence in the present study , while the *mcap* gene showed the lowest prevalence among *M. catarrhalis* isolates.

4. MLST has emerged as an important tool to study the long-term epidemiology and the population structure and patterns of evolutionary descent. The high genetic variability amongst Moraxella catarrhalis isolates in this study provides some information on the local dissemination and genetic relatedness.

5. Notwithstanding high discriminatory power, nucleotide changes accumulate in housekeeping genes in long period of time. That is why the allelic profile of isolates persists unchanged over a longer timeframe, which make MLST a desirable tool for global epidemiology.

6. Significant association between R-M system and disease.

7-R-M system are a remarkable characteristic of M. catarrhalis and are probably involved in the adaptation of these bacteria to different environmental conditions.

8.Genetic diversity of M.catarrhalis depended on source of infection and occurence of mutation .

9.phase variation Mod M isolates more frequent associated with OM and COPD than the type I R-M system. `

Recommendations :

Depending on the finding of this study the recommended   objective include:

1. Direct and rapid identification of *M. catarrhalis* in clinical samples through using molecular technique which minimize the mixed growth.

2. Using Real-time PCR to detect pathogen as the main canstive agent depending on the copy number.

3. Detection of mutation rate in *M.catarrhalis* through using DNA microarray .

4. Detection of whole genome sequencing of *Moraxella catarrhalis* .

5. Further studies can determine the exact role of CRISPR-*cas* with virulence factor of *M. catarrhalis* .

6. Re-evaluation of antibiotics currently used to treat *M. catarrhalis* especially after the bacteria have developed resistance to most of them.

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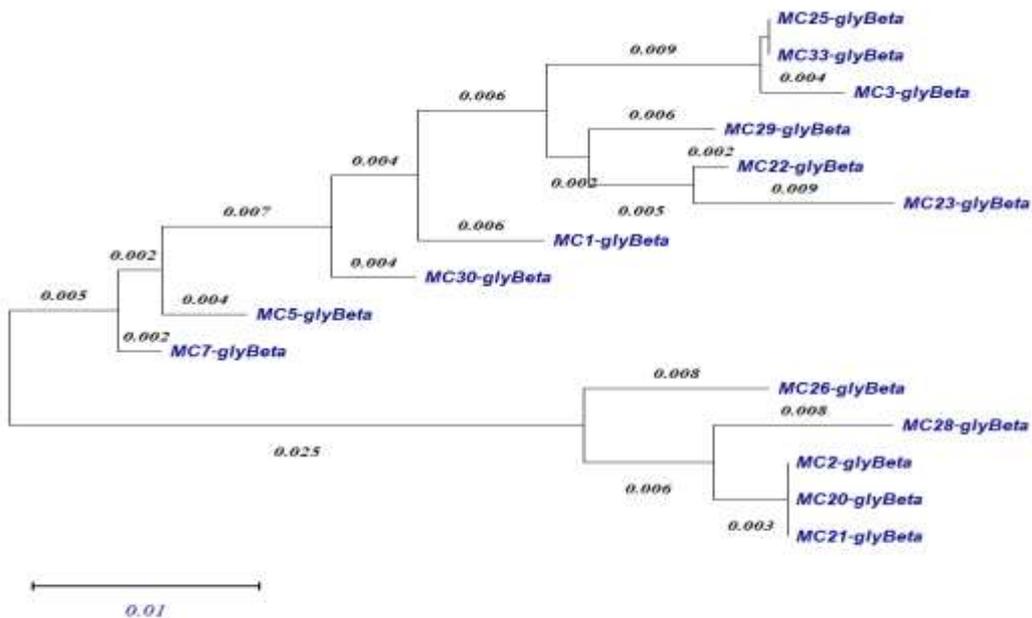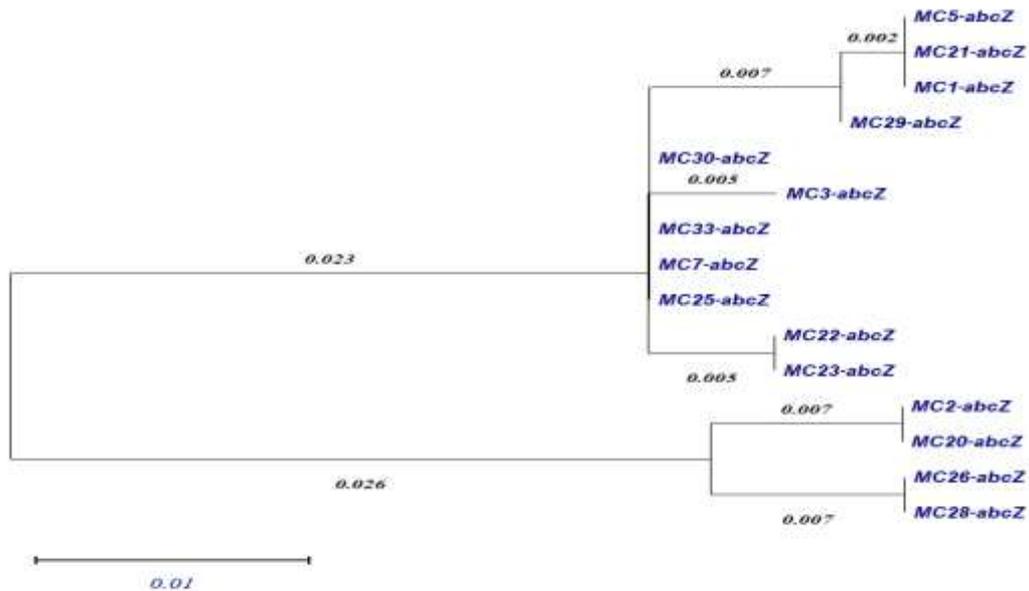
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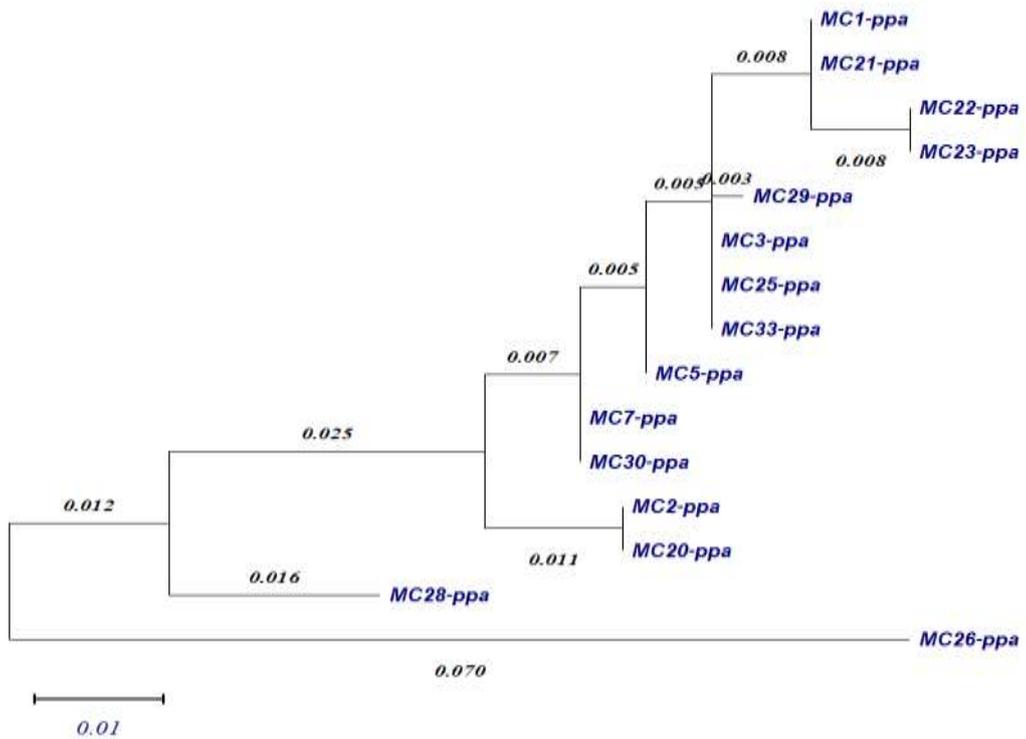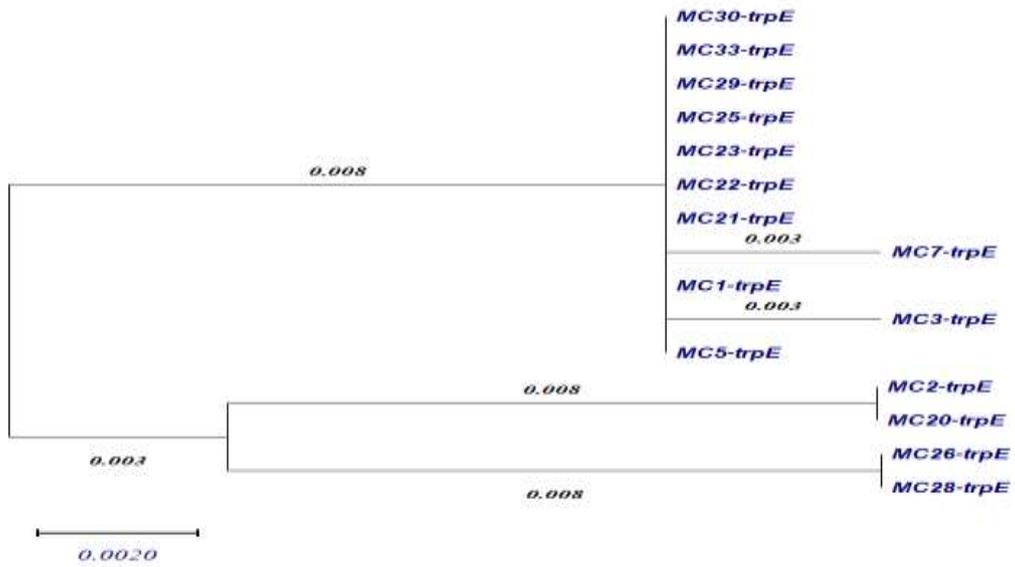
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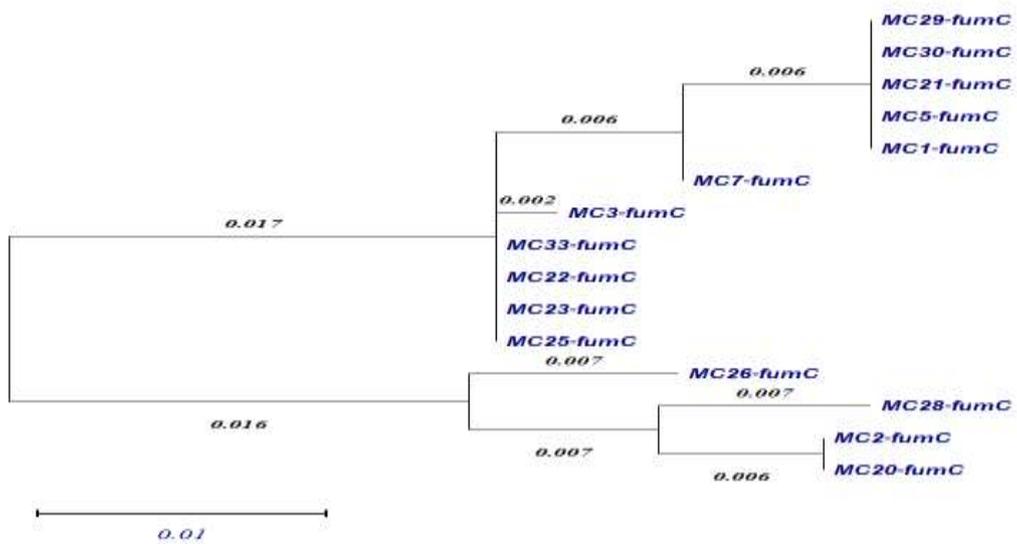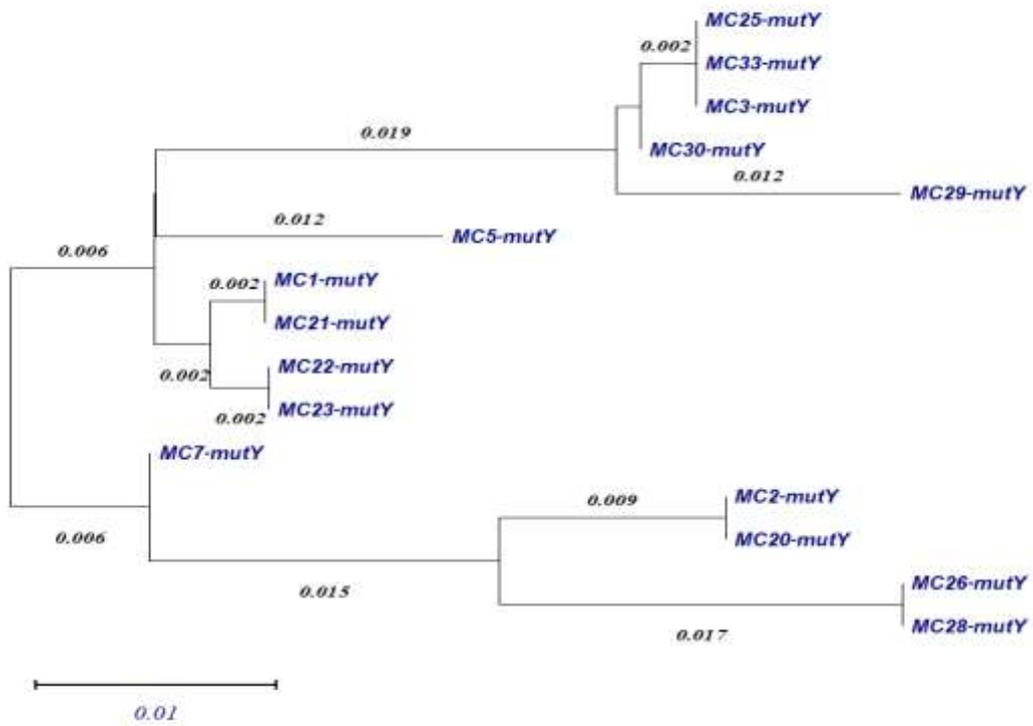
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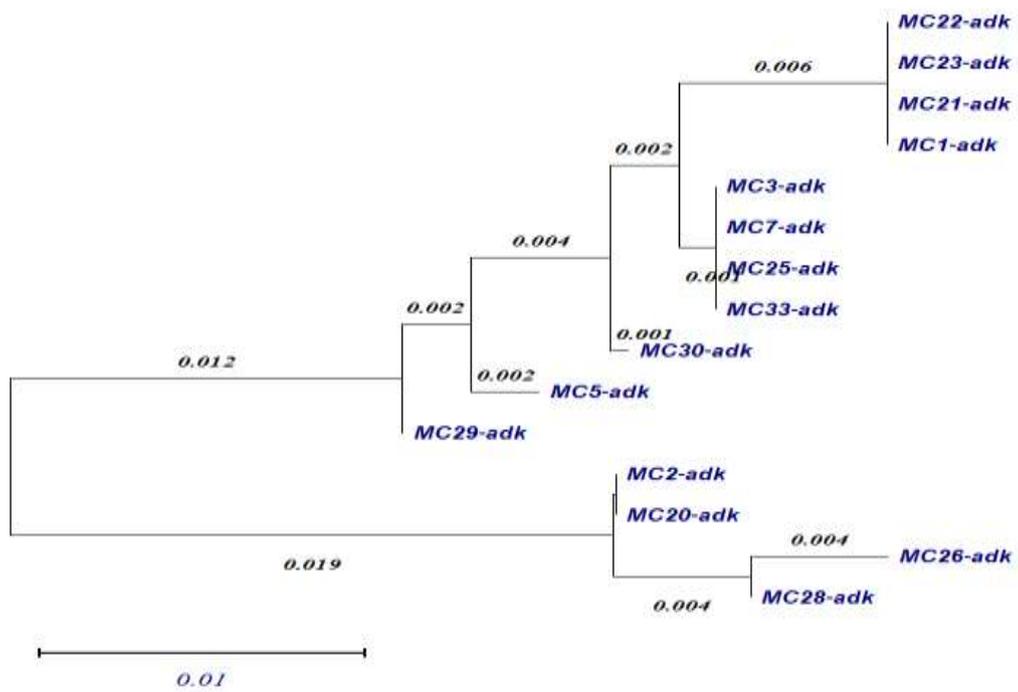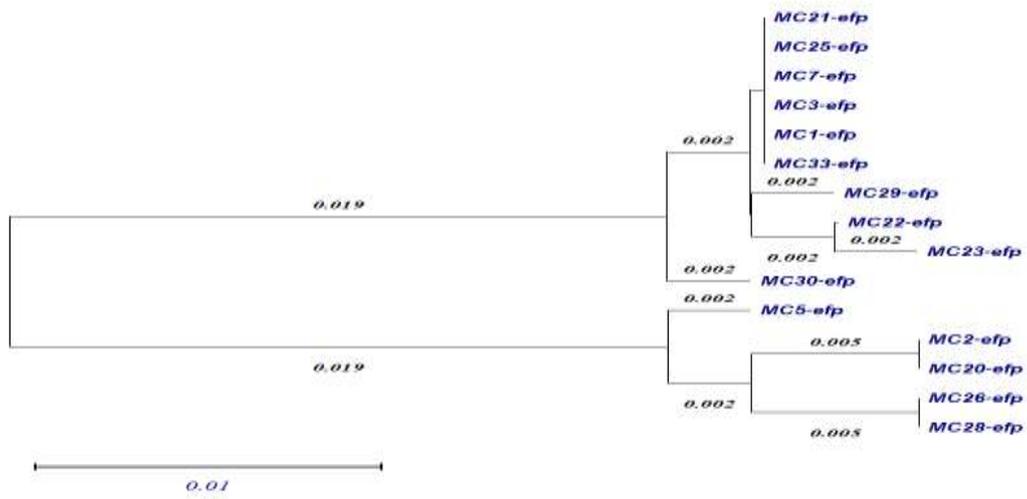
Appendix (1): Phylogram analysis based on (*abcZ, glyBeta, trpE, ppa, mutY, fumC, efp, adk*) sequences from 15 *M oraxella catarrhalis* isolates by maximum likelihood method.

MC30-trpE

MC33-trpE

MC29-trpE

MC25-trpE

MC23-trpE

0.008 MC22-trpE

MC21-trpE

0.003 MC7-trpE

MC1-trpE

0.003 MC3-trpE

MC5-trpE

0.008 MC2-trpE

MC20-trpE

MC26-trpE

0.003 MC28-trpE

0.008

0.0020

MC1-ppa

0.008 MC21-ppa

MC22-ppa

MC23-ppa

0.008

0.005 0.003 MC29-ppa

MC3-ppa

0.005 MC25-ppa

MC33-ppa

0.007 MC5-ppa

MC7-ppa

0.025 MC30-ppa

MC2-ppa

MC20-ppa

0.012 0.011

0.016 MC28-ppa

MC26-ppa

0.070

0.01

Appendix (2): RM system profile among studied stains and the different sources.

Sample name	Source	RM-system	Type I RM-system		Type III RM-system		
			T1MOD	T1RES	modM1	modM2	modM3
1	Ear swab	3	-	-	-	+	-
2	Ear swab	1	+	+	-	-	-
3	Ear swab	3	-	-	-	+	-
4	Ear swab	3	-	-	-	-	+
5	Ear swab	3	-	-	+	-	-
6	Ear swab	1	+	+	-	-	-
7	Ear swab	3	-	-	-	+	-
8	Ear swab	3	-	-	-	+	-
9	Ear swab	3	-	-	-	+	-
10	Sputum	3	-	-		+	
11	Sputum	1	+	+	-	-	-
12	Sputum	3	-	-	-	+	-
13	Sputum	3	-	-	-	+	-
14	Sputum	3	-	-	-	-	+
15	Sputum	3	-	-	-	+	-

Note: +, Positive; -, Negative.

التنوع الوراثي

لبكتريا *Moraxella catarrhalis* المعزولة من عينات

سريرية في محافظة بابل

اطروحة

مقدمة إلى مجلس كلية الطب / جامعة بابل وهي جزء من متطلبات نيل درجة

دكتوراه فلسفة في العلوم / الأحياء المجهرية الطبية

من قبل

نبراس عودة كاظم عبيس

بكلوريوس كلية العلوم / جامعة بابل (2010)

ماجستير / أحياء مجهرية طبية / كلية الطب / جامعة بابل (2018)

بإشراف

الأستاذة الدكتورة

لميس عبد الرزاق عبد اللطيف

1445هـ   **2023**