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COMPLEMENT RESISTANCE, ADHERANCE, B-LACTAMASE PRODUCTION AND GENOTYPING OF *Moraxella catarrhalis*

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Abstract- A total of 96 (69.1%) isolates of *M. catarrhalis* were isolated from 139 out patients of both sex with RTI (either Tonsilities, Otitis media, Sinusitis, or Pneumonia) admitted to or presenting at two hospitals in Al-Najaf city. Out of the bacterial isolates of RTI samples, there were 72 (75%) isolates of *M. catarrhalis* appeared to adhere with the epithelial cells and all isolates show resistance to complement. Phenotypic assay was performed to determine the presence of β-lactamase enzyme by the 40 *M. catarrhalis* isolate using nitrocefin disk. while in genotypic β-lactamase assay, the*bro*-1 gene found in 25 (62.1%) isolates, while*bro*-2 gene was presented only in 3 (7.5%) isolates. Randomly amplified polymorphic DNA (RAPD) analysis was performed for nine isolates by using four primers (P1,P2,P3,P4) and PCR technique. These primers show a large number of types with differnent bands, which probably reflects the high degree of genetic diversity present within this species, a diversity which appears to be a feature of *M. catarrhalis* infection and colonization and which may present problems for vaccine design.

Keywords- *M. catarrhalis*, complement, adherance, β-lactamase, RAPD

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Introduction

Moraxella catarrhalis is a human-restricted, encapsulated, gram-negative mucosal pathogen. Further, though previously thought to be a commensal of the upper respiratory tract, the bacterium is now increasingly recognized as a true pathogen of both the upper respiratory tract and the lower respiratory tract of humans. It is the third most common bacterial cause of childhood otitis media (OM) after *Haemophilus influenza* and *Streptococcus pneumoniae* and it is responsible for up to 20% of cases [1,2]. Next to *H. influenzae*, *M. catarrhalis*is the second most common cause of exacerbations of chronic obstructive pulmonary disease (COPD) [3]. In immunocompromized hosts, the bacterium can cause a variety of severe infections including pneumonia, endocarditis, septicemia, and meningitis [4].

Adhesion to mucosal surfaces is mediated by binding of several *M. catarrhalis* macromolecules to surface receptors on eukaryotic target cells or to components of the ECM. various studies, show that *M. catarrhalis* adhesion is a multifactorial event mediated by many adhesion macromolecules. For example, pili may initiate adhesion at long range, while OMPs may be involved during more intimate contact [5].

In particular, several macromolecules that contribute to *M. catarrhalis* adhesion, including OMPs [3]. The general mechanism of cellular adherence of *M. catarrhalis* to host cell surfaces has been studied by Rikitomi, et al [6]. The presence or absence of fimbriae did not influence the capacity of the bacterium to adhere or to cause hemagglutination Indeed, the mechanisms of binding appeared different for adherence and hemagglutination.

Several studies have shown the importance of complement resistance to *M. catarrhalis* pathogenicity [7], with isolates from the upper respiratory tract of children or healthy adults tending to be complement sensitive, whilst isolates from children and adults with lower respiratory tract infections tend to be complement resistant [8].

Research into *M. catarrhalis* ß-lactamase production has shown that 3 different isotype groups may be identified, BRO-1, BRO-2 and BRO-3 [9]. However, by far the most common types are BRO-1 and BRO-2, being found in approximately 94% and 5% of ß-lactamase producers respectively [10]. Research into *M. catarrhalis* ß-lactamase production has shown that 3 different isotype groups may be identified, BRO-1, BRO-2 and BRO-3 [9]. However, by far the most common types are BRO-1 and BRO-2, being found in approximately 94% and 5% of ß-lactamase producers respectively [10]. Randomly amplified polymorphic DNA (RAPD) analysis, which is less time-consuming, has not been applied to the genotyping of *M. catarrhalis* [11]. Therefore, there is an increase demand to investigate the role of *M. catarrhalis* in respiratory tract infection (RTI). Molecular studies on *M. catarrhalis* have received a little attention in

Iraq. The present study is carried out to achieve the Adherence, Complement resistance, and ß-lactamase genes (*BRO*-1and *BRO*-2) in clinical isolates using PCR technique. In addition genotyping of *M. catarrhalis* by using (RAPD) analysis method.

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Materials and Methods

Patient and Sample Collection

This study was carried out in two hospital in in Najaf governorate (Al -Hakeem and Al-Zahra Maternity and Children). during the period between November 2011 to January 2012.A total of 139 sample (67 throat swabs, 32ear swab, 30 sputum, 10 nose swabs) were collected from out patients suffering from respiratory tract infection (pharyngitis, otitis media, pneumonia, sinusitis respectively), they included both sex with different age groups. All samples were cultured on blood agar, chocolate agar, nutrient agar, brain heart agar media. The media were incubated in CO_2 at 37°C for 24-48 hrs. depending on morphological features of the colonies and microscopically examination with Gram's stain, pure culture on chocolate agar plates were made from each single group of colonies. The pure cultures were prepared for biochemical tests to differentiate *M. catarrhalis* from other bacteria.

Adherence to Epithelial Cells

Oropharyngeal epithelial cells were collected from a healthy adult male by scraping the oropharyngeal with a cotton swab for detect the adherence of *M. catarrhalis* according to Ahmed, et al [12].

Complement Resistance Assay

The culture-and spot test [13]. was used to detection complement resistance of *M. catarrhalis* isolates, in which the bacteria was grown overnight in shaking incubator at 37°C in brain heart broth media containing 0.5% BSA to prevent clumping of the bacteria during growth. the bacteria were suspended in PBS to concentration of approximately 1.5×10^8 bacterial cell per ml. samples (100 ml each were spread over blood agar plates). After broth had been absorbed into the agar, duplicate 50-µl sample of 5% human blood serum dropped onto the plate. 50% heated inactivated (56°C;30 min) serum was used as control on every plates. To allow optimal complement activation, the plates were pleased in 37°C incubator immediately after serum application (overnight incubation).

Detection of β- Lactamase

The production of β-lactamase was assessed with nitrocefin disks (Fluka, Switzerland). To detectβ-lactamase activity, a commercially available chromogenic cephalosporin disk and β-lactamase assay were used, containing nitrocefin as substrate Each disk impregnated with nitrocefin was moistened with sterile D.W., and a loop full of the bacteria cells on Muller-Hinton agar was inoculated on the disk surface. The disks were examined for yellow-to red or pink change as evidence of appositive reaction [14].

Genomic DNA Extraction

The wizard genomic DNA purification kit (Promega/USA) is designed for isolation of DNA from Gram negative bacteria.

Plasmid DNA Purification

The Pure Yield™ Plasmid DNA purification kit (Promega/USA) is designed for isolation DNA plasmid.

PCR Assay and Primers Selection

The PCR assay was performed to detect the genotyping of isolates by RAPD method and presence of *BRO-1* and *BRO-2* gene. According to the Vu-Thien, et al [15] for RAPD analysis gene and Levy and Walker [16] for *BRO* gene as shown in [Table-1]. These primers synthesized by Alpha DNA company, Canada. The Alpha DNA primers were prepared depending on manufacturer instruction by dissolving the lyophilized product with TE buffer molecular grad after spinning down briefly. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

Table 1- PCR Amplification Primers: (Alpha DNA)

All PCR programs consisted of 35 cycles			
Primer Name		**DNA Sequences (5'-3')**	**Product size bp**
β-lactamase Bro1	F	CACCCYGTRGGACAAGC	235 bp
Bro2	R	AATGACGGCGTTGCATC	214 bp
RAPD primers			
P1		TCACGATGCA	
P2		GCCCCCAGGGGCACAGT	
P3		GTAATACGACTCACTATAG	
P4		GGAAACAGCTATGACCATG	

PCR Cycling Profiles

Polymerase chain reaction assays were carried out in a 25 µl reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure, as in [Table-2]

Table 2- PCR Cycling Profiles

Gene Name	Initial denaturation Time	Denaturation Time	Annealing Time	Extension Time	Elongation time
bro1& bro2	94/10 min	94 /30 s	55 / 1min	72/ 40 s	72 / 7 min
P1	95 / 3 min	95 / 1min	35 / 1min	72 / 1min	72 / 5 min
P2	95 / 3 min	95 / 1min	55 / 1min	72 / 1min	72 / 5 min
P3	95 / 3 min	95 / 1min	55 / 1min	72 / 1min	72 / 5 min
P4	95 / 3 min	95 / 1min	55 / 1min	72 / 1min	72 / 5 min

Results and Discussion

During the period from November (2011) to February (2012), 96 (69.1%) isolated of *M. catarrhalis* were isolated from a total of 139 out patients of both sex (85 male and 54 female) with URTI admitted to or presenting at two hospitals in Al-Najaf governorate. All these culture sterile isolates were identified on the basis of Microscopic examination, colonied morphology and comparison of the biochemical characteristics with standard description in MaccFadin [17] and Mims, et al [18].

Moreover, the biochemical tests with APINH miniaturized diagnostic test were confirmed that all these isolate as *M. catarrhalis* figure.

Adherance to Epithelial Cell

In this study, there were 72(75%) of *M. catarrhalis* isolate show it ability to adhered with epithelial cell. Attachment to the epithelium respiratory tract is likely to be an essential step in the pathogenesis of *M. catarrhalis* infection. The general mechanism of cellular ad-

herence of *M. catarrhalis* to host cell surfaces has been studied previously by Rikitomi, et al [6].

In vitro adherence study with HEp-2 cell cultures demonstrated that strains derived from infections adhere more efficiently than do mere colonizers [19].

For a number of pathogens, high-molecular weight surface-exposed proteinaceous structures have been shown to be involved in adherence [20]. A similar protein, UspA1, is expressed by *M. catarrhalis.* Because the OMP UspA1 has been implicated in attachment of *M. catarrhalis* to epithelial cells. Hays [1] referred that most *M. catarrhalis* strains able to adhere appeared to possess *uspA1,* whereas most of the nonadherent strains did not. Indeed, the vast majority of strains clustered in the lower branch were found to be *uspA1*-positive, whereas most of the strains from the upper branch appeared to lack the*uspA1*gen.

Complement Resistance

The complement resistant or sensitive phenotype of the 40 *M. catarrhalis* isolates used in this study had been previously determined using the "culture-and-spot" test by Verduin, et al [13]. This is a rapid and simple test for determining the complement resistance phenotype of *M. catarrhalis*, which exhibits a statistically significant concordance with the serum bactericidal assay and is base on the survival of bacteria on a blood agar plate after the application of a drop of 50% serum.

The pathogenesis of *M. catarrhalis* relies on its capacity to resist the human host defense, including complement. The complement system is very harmful for Gram-negative pathogens, including *M. catarrhalis*, and bacterial complement resistance is one of the most important virulence mechanisms [21]. *M. catarrhalis* has thus developed several efficient strategies to circumvent complement. It has been demonstrated that UspA1 and UspA2 interact and inhibit the alternative pathway of complement by noncovalently binding C3 [22]. In the present study we show.

Research by Verduin, et al [13]. showed that a least one resistance mechanism involves inhibition of formation of the membrane attack complex of complement, involving the binding of vitronectin (a natural inhibitor of complement found in serum) to the UspA2 protein present on the surface of *M. catarrhalis*. An *M. catarrhalis* mutant lacking the UspA2 gene was found to be sensitive to complement-mediated killing, whilst the parent isolate was resistant.

In this study, all isolates 100% (96/96) were found to be resistant to the effect of complement in human serum. The percentage of complement resistant *M. catarrhalis* isolated appears to be relatively high when compared to some studies [23] involving healthy children (100% versus 30-60%, Other studies have yielded similar results [24]. The percentage complement resistant isolates could have been influenced by the fact that the children enrolled on the study had previously experienced episodes of AOM disease, possibly resulting in an enhanced immune response (including complement mediated responses) against potential bacterial pathogens.

Complement resistance in *M. catarrhalis* has been previously associated with disease causing isolates. In relation to other studies, the percentage of complement resistant isolates from this set of USA isolates (98%) was high. This could be attributable to the young age of the patients from which the isolates were cultured. During the first two years of life, infants have relatively native immune systems and are likely to come into contact with many novel infectious agents possibly leading to morefrequent activation of the complement system in this age group. In this scenario it is likely that complement resistant *M. catarrhalis* isolates would have a distinct advantage in establishing infection.

Hays, et al [25] indicated that *M. catarrhalis* is only a weak activator of the MBL arm of the complement system. In theory, this lack of MBL activation could be advantageous to the organism in allowing it to remain "hidden" from the MBL arm of the complement system (i.e. protected from lectin pathway-induced complement activation). Verhaegh, et al [2] performed both phenotypic and genotypic analyses on a large cohort of global clinical *M. catarrhalis* isolates cultured from children and adults with respiratory disease.

Both UspA1 and UspA2 were able to inhibit activation of the alternative and classical pathways and C3a generation in activated human serum and thereby they most likely contribute to survival of *M. catarrhalis* in the human host.

Determining the β -lactamase Enzyme

Phenotypic Assay (Nitrocefin Disk)

Phenotypic assay was performed to determine the presence of β-lactamase enzyme by the 40 *M. catarrhalis* isolate using nitrocefin disk. The β-lactamase positive isolate change the color of the disk from yellow to pink within 15 minutes, while the β- lactamase negative isolate did not change the color of the disk. In the present study 36 isolate (90%) were found to produce the ß-lactamase enzyme phenotypically [Table-3].

ß-lactamase Gene Assay

BRO-1 and *BRO*-2 genes were detected by PCR methods by using the primers and the interpretation described by Levy and Walker [16]. The PCR product were tested for the presence of the *bla* gene encompassing 235 bp for BRO-1 or 214 bp for BRO-2 as shown in [Fig-1].

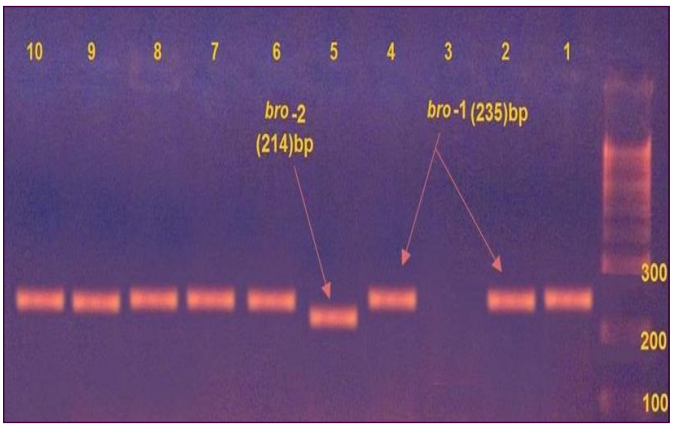

Fig. 1- Agarose Gel Electrophoresis (2%) of PCR Amplified of *BRO* gene (235) bp for BRO-1, (214) bp for BRO-2 of *M. catarrhalis* Isolates

In this study theβ-lactamase assay, the *BRO*-1 gene found in 25 (62.1%) isolates,while *BRO*-2 gene was presented only in 3 (7.5%) isolate as shown in [Table-3].

Our results agreement with Bootsma, et al [26] and Johnson, et al [27], who revealed that although *M. catarrhalis* is susceptible to a number of antimicrobial agents, greater than 90% of *M. catarrhalis* isolates are resistant to penicillin by means of BRO-1 or BRO-2 β-lactamase production. The sequence and the genetic context of *BRO* genes suggest that BRO-2 was acquired by interspecies gene transfer, possibly from a gram-positive organism, and that BRO-1

evolved from BRO-2 and spread by horizontal transfer via subsequent transformational events. Isolates carrying BRO-1 are usually more resistant to ampicillin than those carrying BRO-2 and have become more widespread and predominant. Levy and Walker [16] reported that BRO-2 of *M. catarrhalis* present at rates less than 15% in the1980s, 4.8% from 1984 to 1994, 2.1% from1994 to 1995, and 3.1% in 1997 and 1998.

Table 3- β-lactamase production by M. catarrhalis isolate

Isolate code number	β-lactamase Production		
	Phenotaypic A	Genotypic BRO-1	Genotypic BRO-2
1	+	+	-
2	+	+	-
3	+	+	-
4	+	+	-
5	+	+	-
6	+	+	-
7	+	+	-
8	+	+	-
9	+	+	-
10	+	+	-
11	+	-	+
12	-	+	-
13	+	-	-
14	-	-	-
15	+	+	-
16	+	+	-
17	+	-	-
18	+	-	-
19	+	-	+
20	+	+	-
21	+	+	-
22	+	+	-
23	+	-	-
24	+	+	-
25	+	-	-
26	-	-	-
27	+	+	-
28	+	+	-
29	+	+	-
30	+	+	-
31	+	-	-
32	+	-	-
33	+	-	-
34	+	+	-
35	-	+	-
36	+	-	+
37	+	+	-
38	+	-	-
39	+	+	-
40	+	-	-

Although initial reports suggested the presence of plasmid encoded BRO-1 [8], it is currently believed that the genes encoding BRO-1 and BRO-2 are located on the chromosome. At present, it is unclear whether BRO-1 and BRO-2 are the product of one gene or are encoded by separate genes. BRO-2 is relatively rare, invariable occurring in less than 15% from isolate and on the basis of sequence similarity between BRO-2 and BRO-negative isolate, Bootsma, et al [28] hypothesized that BRO-2 like allele was originally transferred into *M. catarrhalis* and that BRO-I was generated by a duplication in the promoter with subsequent spread enhanced by selection for more active enzymic activity via greater enzyme production. The nucleotide sequence of the BRO-2 allele differs from BRO-1 by five nucleotides within the coding region, one of which causes an amino acid replacement of unknown significance.

Levy and Walker [16] found BRO-2 gene comprised 2-10% of the population per year with the evidence of a decline over time. These report are compatible with present study in which this BRO-2 is presented in 7.5%.

The nitrocefin disc assay for β-lactamase rapid detection and the BRO gene PCR assay showed correspondence in 77.8% of the isolate while Levy and Walker [16] showed correspondence in 98% of the isolate.

There appears to be no relationship between beta-lactamase production (another virulence factor) and complement resistance in *M. catarrhalis* [29], possibly reflecting the plasmid borne nature of ß-lactamase production or the multifactorial nature of complement resistance. An *M. catarrhalis* mutant lacking the UspA2 gene was found to be sensitive to complement-mediated killing, whilst the parent isolate was resistant [30].

Genotyping of *M. catarrhalis* by RAPD Analysis

Randomly amplified polymorphic DNA (RAPD) analysis was performed as previously described by Vu-Thien, et al [15] by using four primers (P1,P2,P3,P4) and PCR technique. In this study all DNA extracted from *M. catarrhalis* isolated by kit. (Wizard Minipreps DNA kit (Promega) without any plasmid. The RAPD products were resolved by electrophoresis in a 2% agarose gel and detected by staining with ethidium bromide. strains were considered different from one another if their patterns. differed by one prominent band in three repeated experiments. small differences in the intesities of major bands or loss of some weak bands was ignored.

The primer P1 yielded eight patterns (different types) composed of 2-6 fragments, of which one permanent bands (383bp) were obtained for every isolated [Fig-2]. The primer P2 yielded nine patterns from the 9 clinical isolates. these patterns were composed of 1-6 fragments, ranging from (129to up to 1389) bp in size [Fig-2]. Withe primers P3 andP4 patterns with one to five fragments were obtained, establishing nine patterns as shown in [Fig-2].

Moreover, the RAPD procedure with these primer was unsuccessful for some isolates. Our results partially compared with Vu-Thien, et al [15] results, Who compared the use of RABD and PFGE (by using six primers for RABD and five restriction endonuclease for PFGE) for the analysis of 13 *M. catarrhalis*isolates,11successive strians isolated from sputa of five children and two isolate obtained the same day from twins, were compared. RAPD (P2) in Vu-Thien, et al [15] yielded nine types from the 13 isolates and correlate perfectly with nine PFGE types. Our results show no correlation perfectly with any RABD types and PFGE types of Vu-Thien, et al [15] although used the same primers (P1,P2,P3,P4).

Walker, et al [31] studied the genetic diversity of *M. catarrhalis* with 2 molecular typing methods, multiple-locus Southern blotting with random probes and a single-locus polymerase chain reaction RFLP method. Although not enirely concordant, both methods showed high genetic diversity between isolates, supporting the hypothesis of frequent recombination relative to spread of clones. Other studies used either ribotyping [33] or pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA analysis [14] for typing of *M. catarrhalis* strains.

Both β-lactamase-positive and β-lactamase-negative strains were found in all main branches, suggesting horizontal transfer of the β-lactamase gene. In contrast, 2 virulence traits, complement resistance and adherence to epithelial cells, were strongly associated with 1 of the 2 subspecies. The branch depth suggested that com-

plement-resistant adherent strains diverged from a common ancestor more recently than did complement-sensitive non adherent strains.

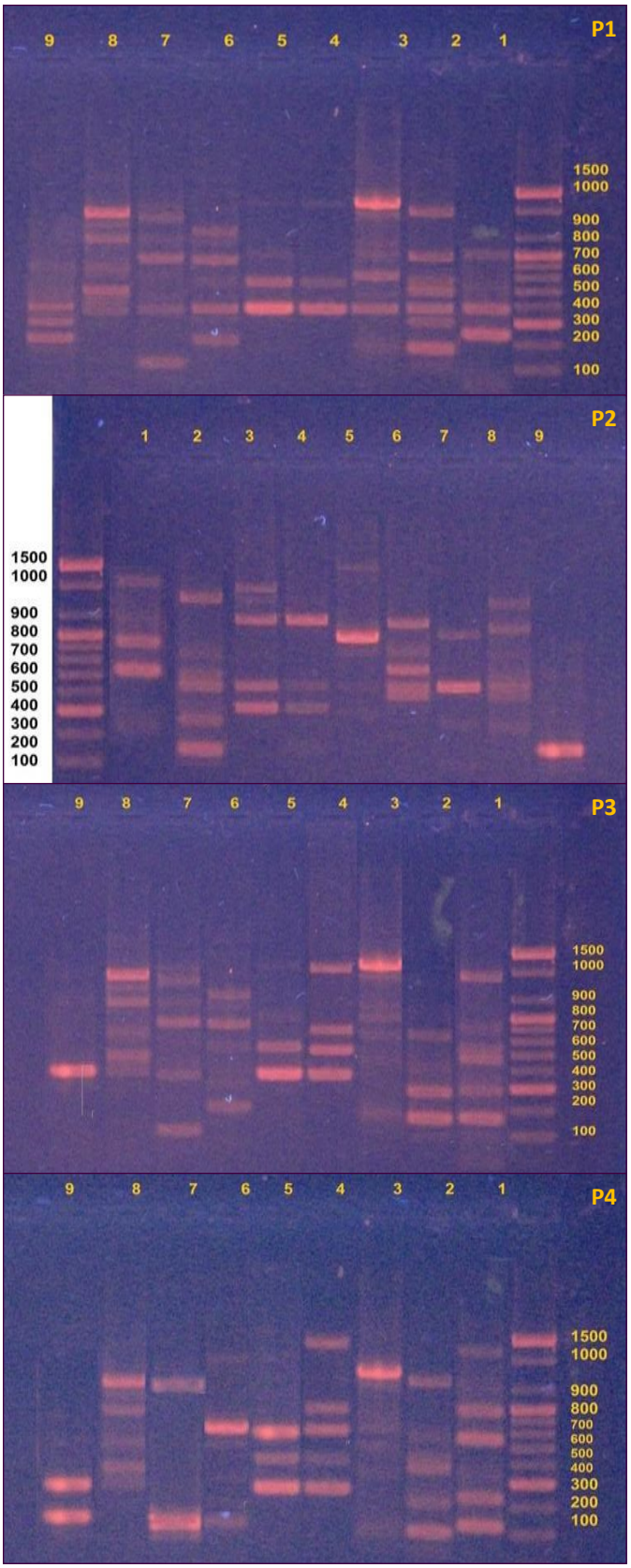

Fig. 2- RAPD amplified products of the *M. catarrhalis* using four random primers P1,P2,P3,P4

While PFGE is a time-consuming procedure, the recently introduced RAPD method has the advantages of simplicity and rapidity. The reproducibility of RAPD analysis was acceptable, though variation in the intensities of certain bands was sometimes noted, probably resulting from the DNA preparation [32]. We evaluated the effectiveness of the RAPD assay with four different primers to distinguish between *M. catarrhalis* strains.

Conclusion

M. catarrhalis have the large number of types probably reflects the high degree of genetic diversity present within this species, a diversity which appears to be a feature of *M. catarrhalis* infection and colonization and which may present problems for vaccine design.

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