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Molecular detection of fimH gene from *Klebsiella pneumoniae* isolated from oral cavity patients in AL-Najaf province

Hala R. A. Al-Fahham[1], Hadeel A. Hussin[2], Khalid R. Kareem[3], Zahraa Y. Motaweq[4]

[1]Department of Medical Microbiology, Jabir Ibn Hayyan University for Medical and Pharmaceutical Sciences, Al-Najaf, Iraq.
[2]Department of Medical Microbiology, Faculty of Science, University of Kufa, Al-Najaf, Iraq.
[3]Department of Biology, Faculty of Science, University of Kufa, Najaf, Iraq.

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ABSTRACT

We took 150 clinical specimens from patients ranging in age from 7 to 65 years old who were suffering from gingivitis, dental caries, and dental plaque. Of these, 70 were male and 80 were female. Among the specimens tested, 124/150 (82.7%) showed positive culture on both blood agar and MacConkey agar, while 26/124 (17.3%) showed no growth on MacConkey agar. The results of the culture showed that 72/124 (58.1%) isolates belonged to Enterobacteriaceae, and 44/124 (35.5%) specimens belonged to other Gram-negative bacteria. The results of the culture on blood agar showed 6/124 (4.8%) isolates belong to coagulase-negative staphylococci, and 2/124 (1.6%) isolates belong to coagulase-positive staphylococci. K. pneumoniae and E. cloacae isolates were diagnosed according to cultural and biochemical tests as well as the final among bacterial isolates, *K. pneumoniae* 26 (32.5), which was detected phenotypically resistant to some antibacterial agents after being isolated and identified from clinical samples of the mouth. From clinical samples of the mouth, 26 (32.5%) of the bacteria tested positive for K. pneumoniae, and phenotypic testing revealed that these bacteria exhibited resistance to at least one antibiotic. A large percentage of *K. pneumoniae* isolates showed positive responses to piperacillin-tazobactam (88.8%), colistin (66.6%), and ticarcillin (88.8%). The rate of resistance to trimethoprim-sulfamethoxazole was moderate, at 44.4%. Most of the people who were resistant to Cefepime (33.3%), Aztreonam (22.2%), Gentamicin (22.2%), Ceftazidime (11.1%), Meropenem (0%), and Imipenem (0%), were also resistant to Levofloxacin (0%), Ciprofloxacin (0%), Amikacin (0%), and Tobramycin (0%). The fimH gene, which codes for fimbria type 1, helps K. pneumoniae isolates make biofilms. In 11/14 isolates (78.8%), the gene was found to be increasing.

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Introduction

The mouth is a prime site for microbial proliferation due to its unique anatomical and physiological characteristics, making it a crucial region for investigation. Rocha etal. 2006 mentioned that germs could be transmitted through saliva droplets, or

***Corresponding author Email address**: hala.ridha@jmu.edu.iq (Hala R. A. Al-Fahham)

aspiration through oropharyngeal secretions, or by speaking, coughing, sneezing, or inhaling in congested air. In addition, Fernandes etal. 2000 said thatoral cavity is a diverse ecosystem with a multitude of microorganisms containing over 800 distinct species of bacteria Aas etal. 2005; Jr & Rôças etal. 2010. Most species can only be maintained by adhering to the hard or soft tissues where a biofilm is formed in oral cavity (Socransky & Haffajee 2005). Oral microbiota research also aimed to find out whether there was a connection between good dental hygiene, periodontal health, and COPD flare-ups. Przybyłowska etal. 2016, Liu etal. 2012 , and others. According to Przybyłowska etal. 2016, a prior study found that denture plaque from COPD patients included a substantially greater quantity of potentially harmful germs than that of healthy individuals. Additionally, it was stressed that better periodontal health and dental hygiene practices could be a way to avoid COPD flare-ups. Liu etal. 2012 In addition to the substantial economic burden and adverse effects on quality of life of these diseases Durham et al. 2013; Batcheloretal. 2014, oral bacteria and periodontal infections have been identified as possible risk factors for many systemic diseases Cullinan etal. 2013 Natural human gastrointestinal system flora includes the essential human body pathogens Enterobacteriaceae, which are also present in the mouth for a short period of time. The main causes of oral infections caused by enterobacteria are poor personal hygiene, cross-contamination between the mouth and the rest of the body, self-inoculation of toothbrushes, and the use of antibiotics (Lafayette etal. 2012 ; Majeed & Motaweq, 2024).

Oral cavity is an ideal environment (e.g., suitable temperature and nutrients) for the production, survival and persistence of microbial cells and the subsequent establishment of biofilm dental plaques. The exact number of species from the oral plaque is not known, as some of them are notcultivable, but it is estimated to be between 700 and 1000 species, reaching densities of 108 bacterial cells/mg, many of them being uncultivable (Singh etal. 2015). However, bacteria, followed by a diverse array of archaea fungi, protozoa, and viruses, are the most numerous communities in the oral microbiota. Oral microorganisms are typically commensal species maintaining mutually beneficial relationships with the host. They do not cause disease but rather hinder the attachment of pathogenic organisms to mucosal surfaces (Avila etal. 2009 ; Lazar etal. 2017). Moreover, either the use or misuse of antimicrobial drugs associated with poor oral hygiene will promote the colonization of the oral cavity by these microorganisms, as well as the dissemination of their resistance genes among the members of oral microbiota (Gonçalves etal. 2007; Gaetti-Jardim etal. 2008). In addition, the loss of the equilibrium between the immune response of the host and the virulence of the microbiota has led to many oral infections, such as denture stomatitis and endodontic, periodontal or periapical infections. However, the clinician may suspect the presence of facultative anaerobes and aerobes in the infectious process in the case of a history of prior use of antimicrobials or immune suppression (Brook, 2007). In addition, in spite of the role that facultative anaerobes and aerobes would play in head and neck infections, most of dentists have been instructed to prescribe antimicrobial drugs only directed against strict anaerobes (Brook 2007). Epidemiological studies have indicated that oral infection, especially periodontitis, may be a risk factor for systemic diseases like respiratory diseases, cardiovascular diseases and stroke (Scannapieco 1998; Li et al.2000).

Material and Methods

Sampling and bacterial identification

Fifty clinical specimens were obtained from patients with oral cavity diseases at the Al-Kafeel clinic and a private clinic in Al-Najaf province between the months of September 2023 and April 2024. Patients with oral cavity infections ranging in age from seven years old to sixty-five years old were included in the sample pool. After removing the teeth and from the site of infection, a swab was collected and sent to the laboratory using special transport media. Patients' sex, age, and infection type were documented on the swab. Patients who had abstained from antibiotic use for at least seven days prior to sample collection had their samples taken. All information, like date, sex, and age were recorded in questionnaire, sheet paper. Diagnosis of *K. pneumoniae* using VITEK2 system. These were the outcomes grows successfully on MacConkey agar, producing colorful colonies. There are 26 isolates of *K. pneumoniae* and 20 isolates of other Gram-negative bacteria. In contrast to most other members of the genus, *K. pneumoniae* is catalase-positive and oxidase negative; in addition to the IMVICs tests, they were all negative; only the citrate test differed among isolates, which is consistent with Denton and Kerr etal. 1998.

Preparation of solutions

Gram Stain Solutions

The solutions of Gram sinning (Crystal violate, Safranin, Iodine Alcohol) have been prepared according to manufacturer instructions Sigma/USA). It was used to distinguish between Gram positive and Gram negative

bacteria and observed the bacterial shape (Collee etal. 1996).

McFarland standard solution

The microbial inoculum was standardized at 0.5 McFarland. In microbiology, McFarland standards were used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range (Jain & Varshney, etal. 2011).

Antibiotic susceptibility test of K. pneumoniae

Bauer Kirby's disc diffusion method was used to assess the antibiotic susceptibility of *K. pneumaniae* isolates, as per CLSI etal. 2019. A 0.5 McFarland standard suspension of *K. pneumoniae* organisms was used to inoculate Mueller-Hinton plates, and then disks were placed on top. After incubation for one night, the growth inhibition zones were measured in millimeters. Pedal fern etal. 2003 and following antibiotic Ticarcillic, Piperacillin-tazobactam, Colistin, Trimethoprim-sulfamethoxazole, Cefepime, Aztreonam, Gentamicin Ceftazidime, Meropenem, Impenem, Levofloxacin, Ciprofloxacin, Amikacin and Tobramycin.

Kits

The following kits were used in this study: Gram stain (BDH, UK), FavorprepTM plasmid extraction Mini Kit (Favorgen, Taiwan), FavorprepTM DNA extraction Mini Kit (Favorgen, Taiwan), and VITEK-2 GN-ID + Gp _ID (BioMérieux, France).

Biochemical identification of Klebsiella pneumoniae suspected isolates

Biochemical tests were conducted to identify *Klebsiella pneumoniae* suspected isolates. The oxidase test detects cytochrome c oxidase activity by applying a bacterial isolate to an oxidase reagent, with a positive result indicated by a color change (MacFaddin 2000). The motility test assesses bacterial movement in semi-solid agar, aiding in distinguishing non-motile Klebsiella species (Collee et al. 1996). The indole test uses Kovac's reagent to detect tryptophanase activity, with a red color indicating indole production. The Voges-Proskauer (VP) test identifies acetoin production from glucose fermentation, yielding a red color upon addition of α-naphthol and potassium hydroxide (McFadden 2000). The Methyl Red (MR) test detects strong acid production from glucose fermentation, with a red color confirming positivity. The citrate utilization test evaluates bacterial use of citrate as a carbon source, indicated by a medium color change (Forbes et al. 2007). The urease test detects urea hydrolysis, with a color change signaling positivity (McFadden 2000). The Triple Sugar Iron (TSI) test examines sugar fermentation and gas production, with Klebsiella typically showing an acid/acid (A/A) reaction (Forbes et al. 2007).

Molecular identification

By identifying the fimH gene, we used the PCR assay to identify *K. pneumoniae*, as indicated in tables 1 and 2, which demonstrates that Canadian Alpha DNA created these primers. We used 1% agarose gel electrophoresis to magnify results and determine the PCR size. The gel was stained for roughly 1.5 hours at 80 volts using 4 mL of ethidium bromide. The amplified products were measured using a 100 bp ladder (Bionaer, Korea).

Table 1 Primers utilized in this study according to Fertas et al. (2013).

Primer	Sequence	Amplicon size
fimH	F:ATGAACGCCTGGTCCTTTGC R:GCTGAACGCCTATCCCCTGC	688

Table 2 PCR program of *fimH* primer that applied in the thermocycler

Primary denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
94˚C / 5min	30	94˚C / 30sec	55\40 sec.	72\60 sec.	72\10min

Statistical analysis

The results of each analysis were carried out in triplicate and are shown as means standard deviation (SD). The Statistical Package for the Social Sciences (SPSS) software program, version 20, was used to conduct statistical analysis using Student's t-tests. A P value less than 0.05 was considered significantly.

Results and Discussion

From September 2023 to April 2024, 150 specimens were obtained from male and female patients ranging in age from seven to 65 years old, who were diagnosed with dental caries, dental plaque, and gingival infections. The study included 150 samples of each sex from patients ranging in age from 7 to 65 years old who were suffering from gingivitis, dental caries, and dental plaque. Among the specimens tested, 124/150

(82.7%) showed positive culture on both blood agar and MacConky agar, while 26/150 (17.3%) results of culture showed that 72/124 (58.1%) isolates had belonged to Enterobacteriaceae and 44/124 (35.5%) isolates belong to other Gram negative bacteria. The results of culture on blood agar showed 6/124 (4.8%) isolates belong to coagulase negative staphylococci and 2/124 (1.6%) isolates has been belong to coagulase positive staphylococci.

Bacterial isolates were isolated and identified using the VITEK-2 compact system after 45 Gram-Negative bacteria showed growth. These were the outcomes: 20 additional bacterial isolates and 14 *K. pneumoniae* isolates. Cultures of Gram-negative bacteria, as determined by morphological and biochemical tests, have an exceptionally high rate. A high rate of G-ve bacteria was found in 116/150 cases (77.3%) and 14/116 cases (12.1%) of *K. pneumoniae*, according to the data. 9/116 (7.8%) belonged to *E. coli* followed by *P. aeruginosa* 10/116 (12.5%) then *Proteus mirabillis* 12/116 (10.3%) and *Proteus vulgaris* 2/116(1.7%), only 2/8 (25%) isolates belong to *S. aureus* followed by *S. epidermidis* 6/8 (75%). *K. pneumoniae* 26(32.5) was one of the bacterial isolates identified from diabetic foot ulcer clinical samples; it showed phenotypic resistance to several antibacterial agents. Saranya etal. 2015 & Basnet etal. 2017 found similar results (2017). But Al-Mosawi 2018 found that (9%) of isolates were *Klebsiella pneumoniae*. *Klebsiella* spp., produces a mucoid lactose fermented colony on MacConkey agar and give indole negative, citrate positive non motile and A/A on TSI as (Table 3).

Table 3 Biochemical tests results of *Klebsiella pneumoniae* suspected isolates

Test							
Oxidase	Motility	Indole	VP	MR	Citrate	Urease	TSI
-	-	-	+	-	+	+	A/A

Antimicrobial susceptibility test was conducted for 14 *K. pneumoniae* isolates using disc diffusion way accord. The resistance show in *K. pneumoniae* isolates to Ticarcillin (88.8%) and Piperacillin-tazobactam (88.8%) were all high. The moderate resistance rate was observed for Trimethoprim-sulfamethoxazole (44.4%). The lowest resistance rate was observed for Cefepime (33.3%), Aztreonam (22.2%), Gentamicin (22.2%), Ceftazidime (11.1%), Meropenem (0%) showed no growth on any culture media. Of these, 70 were male and 80 were female. The and Impenem (0%), Levofloxacin (0%), Ciprofloxacin (0%), Amikacin (0%) and Tobramycin (0%). This result agreement with (Ghogare etal. 2018; Jawade etal. 2024) who found that (0%) resistance to Amikacin. and agreement with Phiri etal. 2016 who found that (0%) resistance to Meropenem. Also, this result is near to Sahu & Swain etal. 2019) who found that (83%, 62%, 61%, 28%, 57%, 89% and 56%) resistance to Gentamicin, Ciprofloxacin, Aztreonam, Piperacillin-tazobactam, Cefepime, Ceftazidime, and Impenem. Also, this result disagrees with Al-Hilli etal. 2015 who found that (40%) resistance to Tobramycin.

Identification of Enterobacteriaca

The result of microscopic examination and biochemical test of bacterial isolates that obtained from culturing of 150 specimens collected from gingivitis, dental caries and dental plaque patients showed that 116 isolates were belong to Gram negative bacteria. Our results showed that Gram negative bacteria divided to 72 (62%) isolates belong to Enterobacteriaceae and 44/116 (38%) non-Enterobacteriaceae. The results in table (3) showed that 72/116 (62%) isolates were belong to Enterobacteriaceae that divided to 28/72 (38.8%) isolates *Klebsiella* spp. 14 (19.4%) *K. pneumoniae*, 8 (11.1%) *K. oxytoca*, 6 (8.3%) *K. aerogenes* and 15/72 (20.8%) isolates of *Enterobacter* spp. 12 (16.6%) *E. cloacae* and 3 (4.1%) *E. freundii*, while *E. coli*, *Proteus* spp., *Citrobacter* spp. and *Shigella* spp. reached the number of isolates to 13%, 11.1%, 8.3%, 6.9%, respectively (Table 4).

The conventional identification of bacterial isolates depended on some criteria which included Gram stain, cultural morphology and biochemical tests. The result appears that 8 of isolates were Gram positive bacteria, all isolated grown on blood agar, while positive catalase 8 were grown on mannitol salt agar, which is primary identified as *Staphylococcus* spp isolate. According to coagulase +ve, 2 isolates of Gram positive bacteria gave coagulase positive which was identified as *S. aureus*, because only *S. aureus* produce coagulase enzyme, while 6 isolates gave negative test, Coagulase test is used to differentiate *S. aureus* (positive) from Coagulase Negative *Staphylococcus* (CONS), *S. haemolyticus* yielded small, white, convex colonies. Yellow colonies similar to those of *S.*

aureus were observed on MRSA (Shittu et al. 2004).

Table 4 Distribution of pathogenic bacteria on the type of infection

Type of bacterial isolates	Type of infection (%)			
	Gingivitis	Dental caries	Dental plaque	Total
Klebsiella pneumoniae	5	4	5	14
Enterobacter cloacae	4	5	3	12
Escherichia coli	3	3	3	9
Proteus spp.	3	2	3	8
Klebsiella oxytoca	4	0	4	8
Klebsiella aerogenes	3	1	0	4
Citrobacter freundi	0	2	4	6
Enterobacter freundii	1	0	2	3
Serratia marcescens	1	0	0	1
Serratia liquefaciens	1	0	1	2
Shigella spp.	3	1	1	5
Pseudomonas fluorescens	2	2	1	5
Pseudomonas aeruginosa	7	0	3	10
Acinetobacter lwoffii	3	1	2	6
Acinetobacter calcoaceticus	1	1	1	3
Aeromonas spp.	4	0	3	7
Burkholderia cepacia	5	0	0	5
Pantoea spp.	2	2	4	8
Coagulase positive Staphylococci	1	1	0	2
Coagulase negative Staphylococci	2	3	1	6

Detection of some virulence factors for *K. pneumoniae*

Phenotypic detection of biofilm production

Tissue culture plate (TCP) was utilized to detect the biofilm manufacture of bacterial isolates, the quantification result of biofilm formation by microtiter plate method revealed that the results confirmed that 12(85.7%) of isolates were strong to biofilm formation, 2(14.3%) moderate and 0 weak by microtitier plate as shown in table (4). *K. pneumaniae* can create biofilms, which are clumps of cells that adhere to one another and/or surfaces by the use of a self-produced matrix of extracellular polymeric material. The most crucial clinically significant *K. pneumaniae* biofilms are those that develop on the inside of indwelling devices, such as catheters. Colonization of the pulmonary, gastrointestinal, and urinary tracts as well as the progression of invasive infections, particularly in patients with impaired immune systems, may be facilitated by *K. pneumaniae* biofilms. Type 3 fimbriae and the CP are the most important surface structures that are part of the formation process. Finally, CPs impact cell-to-cell communication and biofilm architecture, while fimbriae mediate persistent adherence (Hassan and Motaweq, 2024).

Table 5 Classification of bacterial adherence and biofilm formation via tissue culture plate technique (TCP)

Mean of O.D value at 630nm	Biofilm formation
< 0.120	None
0.120-0.240	Moderate
>0.240	High

Capsule production detection

Indian ink stain was used to detection of

encapsulated isolates of *K. pneumonia*. The results showed that 57.1% (8/14) of isolates were encapsulated *K. pneumoniae.*

Molecular detection of fimH gene

The molecular detection of *fimH* gene by using a specific primer that encodes fimbria type 1 participates in biofilm formation in *K. pneumoniae*isolates revealed positive amplification in 11/14(78.8%) isolates.

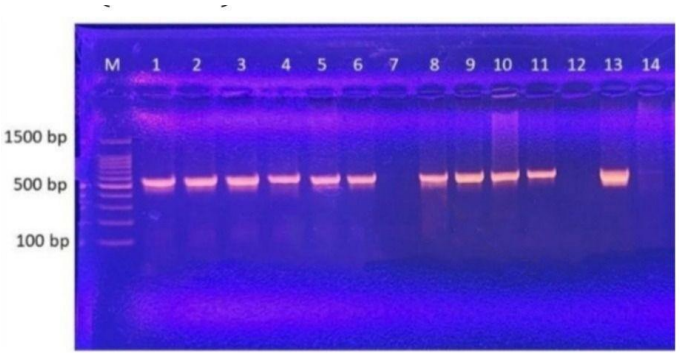

Fig 2. PCR products of *K. pneumoniae*isolates amplified with *fimH* gene primers, having a product size of 232 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1,2,3,4,5,6,8,9,10,11,13) indicate *fimH* gene positive findings

The attachment of fimbrillae to the surfaces of epithelial cells is thought to be crucial. To facilitate bacterial colonization and host cell signaling, fimbriae bind to particular receptors on the host. The pathogen's bacterial destiny and the development of the disease are controlled by fibrial adhesins. A further key factor in determining the organism's pathogenicity is type-1 fimbriae. Jaroni etal. 2014. A key virulence gene of *Klebsiella pneumoniae*, *fimH*, has a strong correlation with biofilm development. Multiple investigations have linked the genes for fimbriae, capsule, and lipopolysaccharides to the process by which *Klebsiella pneumoniae* forms biofilms, beginning with adherence by fimbriae and lipopolysaccharide and progressing through substratum coverage and the development of mature biofilm architecture by capsule. Johnson etal. 2010; Nepal etal. 2011 sample and detected the *fimH* gene in 40% of the isolates. Additionally, the results showed that *fimH* detected 76% of isolates, which is in partial agreement with Hamam etal. 2019. In addition, research by Abdul-Razzaq etal. 2013 demonstrated that the presence of the *fimH* gene was positive in 75% of the *Enterobacter* spp. isolates. Type 1 fimbriae are present in around 66% of *E. aerogenes* isolates and 23% of *E. cloacae* isolates, as noted by Hornick etal. 1991. Consistent with the findings of Hamam etal. 2019, our investigation found that 76% of *K. pneumoniae* isolates had the *fimH* gene. In a study carried by Melo etal. 2014 the *fimH* gene was recorded in all isolated of *K. pneumoniae* recovered in their study. While in 2017 and 2023 carried by Lev etal. 2018 & Kuş etal. 2023 found the percentages to be 64.2% and 91% and, respectively.

Conclusions

The outcomes of this study revealed a great spread of *K. pneumoniae* isolates in AL -Najaf hospitals that produce fimH enzymes and are resistant to many antibiotics, Molecular study for the detection of fimH genetic factor of *K. pneumoniae* microbes, was as 14 fimH with percentages 11/14 (78.8%) with some Virulence factor detection with it.

Ethics approval

The study had been done following recommendations and guidelines obtained from Jabir ibn Hayyan and Pharmaceutical Science University/Iraq. The present study was done without involving any biological materials or genetically modified organisms.

Conflict of interest

The authors declare that they have no conflict of interest.

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