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Detection of fimv *gene* in *Stenotrophomonas maltophilia* isolated from clinical samples

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Abstract
Stenotrophomonas maltophilia is a low-virulence opportunistic pathogen that has been identified as an emerging hospital pathogen. Therefore this study was conducted to characterize and investigate some virulence factors particularly the detection of biofilm formation to determine the role of this bacteria in causing infection in Mosul city. 375 clinical samples were collected from patients suffering from various infections who frequently visited Mosul hospitals for both genders. The samples were cultured on MacConkey medium and then incubated at 37 C°. The results showed that 11 of the isolates were Stenotrophomonas maltophilia were obtained from different infections, (5)45% of S. maltophilia was isolated from UTI, (2)18% from burns, (2)18%from sputum, in addition to (1)9% from each of wound and medical devices. The bacteria were diagnosed by traditional methods with the Vitek-2 system in addition to molecular methods based on 16S rRNA. A slime layer of eleven strains were detected using Congo agar medium. The results showed that (6)55% of strains gives positive results. while (5)45% gaves negative results. Biofilm formation by microtiter plate, (8)73% of the strains were strong and (3)27% were moderate. While the tube method results were heterogeneous as it showed (5)45% are highly productive, (4)36% are medium, (1)9% are weak and (1)9% showing no productivity compared to the control. The adhesion ability was also molecularly diagnosed by designing primers *fimv* gene in current study. The results showed that 73%of the strains produced a positive result after amplifying by PCR at 736 bp.

Keywords: *Stenotrophomonas maltophilia*, biofilm, *fimv gene*

Introduction

S. maltophilia was first isolated in 1943 by J. L. Edward and named as '*Bacterium bookeri*.' In 1958, Hugh and Ryschenkow isolated a strain from an oral carcinoma patient and named it as *Pseudomonas maltophilia*; they also reclassified '*B. bookeri*' as *Pseudomonas maltophilia* [1]. In 1963 the bacterium was isolated from Japanese rice paddies named as *Pseudomonas melanogena* [2].

Using DNA-rRNA hybridization techniques, it was discovered that the genus *Pseudomonas* has five rRNA homology groups, this information was used by Swings in 1981 to propose that *P. maltophilia* be reclassified in the genus *Xanthomonas* as *X. maltophilia*, until 1993,in a large study of *Xanthomonas* strains, an analysis of 295 phenotypic characteristics, DNA-rRNA hybridization and sequencing of PCR-amplified 16S rRNA genes have resulted in naming of *X. maltophilia* as *S. maltophilia* by Palleroni and Bradbury [3].

Stenotrophomonas species are non-fermenting gram-negative rods. Currently sixteen species are recognized within the genus: *S. acidaminiphila*, *S. bentonitica*, *S. chelatiphaga*, *S. daejeonensis*, *S. ginsengisoli*, *S. humi*, *S. indicatrix*, *S. koreensis*, *S. lactitubi*, *S. maltophilia*, *S. nitrireducens*, *S. pavanii*, *S. pictorum*, *S. rhizophila*, S. *terrae*, *S. tumulicola*. To note that *S. africana* is no longer recognized as a species and has been added to *S. maltophilia*. However, the taxonomy of the genus is not completely resolved. *S. maltophilia* forms a complex of which the isolates show considerable heterogeneity in genetic and phenotypic characteristics [4].

S. maltophilia can be difficult to identify because it frequently co-isolates with other microorganisms in samples taken from patients, including *P. aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Acinetobacter baumanii*, *Escherichia coli*, *Enterobacter*, and *Candida albicans* [5].

Major risk factors regarding the infection of *S. maltophilia* include malignancies, persistent respiratory conditions, immunological deficiencies, and prolonged hospitalization. Therefore, those who are suffering from underlying illnesses, cancer, chemotherapy, radiation, and organ recipients are the most susceptible [6]. These risk factors highlight particular characteristics of *S. maltophilia*, such as its capacity to live on almost any moist surface, its ability to colonize moist surfaces through the formation of biofilms, and its use of a number of pathways that confer resistance to a variety of antimicrobial drugs [7] [8] [9]. The biofilm production by *Stenotrophomonas maltophila* is a prominent feature of its virulence. The formed biofilm is composed of bacterial cells adherent to the surface and stick to each other via an extracellular matrix, consisting of polysaccharides, proteins, lipids, nucleic acids. and minimally active bacteria, which can become mature very rapidly, colonizing new surfaces within less than 24 hours. It ensures higher resistance to antibiotics and antiseptic solutions, counteracting the immune defense mechanisms of the host, and contributes to the progression of CF lung disease and other chronic respiratory diseases [10] [11].The process starts with adherence to the surface, followed by irreversible attachment, then final maturation, and is mediated through its motile flagella, fimbriae, pili, adhesins, and the outer membrane lipopolysaccharide positively charged surface, quorum sensing by diffuse single molecules, and extracellular polymeric substances. This biofilm enables *Stenotrophomonas maltophilia* to firmly adhere to both animate surfaces, such as respiratory epithelial tissue, and inanimate surfaces, such as ventilation tubes and circuits. This allows it to avoid the natural human phagocytosis and

complement-mediated immune killing defenses, resist most antibiotic activity up to 1,000 times more, and spread throughout the surface [12]. Infections caused by biofilm-producing bacteria are difficult to treat and eradicate because they rarely respond to conventional antibiotic treatments. Therefore, peritoneal catheters should be removed early in cases of failure to respond to treatment [13].

Material and methods

Sample collection and identification

From July to December (2022), 375 specimens including (urin, sputum, burns, wound, wound, medical devices, Throat swabs, blood and CSF samples) were collected from patients suffering from different clinical infections from hospitals in Nineveh Governorate. After collecting the specimens, they were cultured on the commonly used media (MacConkey) which are initially based on isolation and initial diagnosis. And then adopting the biochemical tests from them IMVIC test,catalase test, coagulase and oxidase [14],In addition to using VITEK-2 Compact System to confirm the diagnosis Identification.

Slime layer detection

This method worked by using Congo red agar, the colonies were examined to determine whether they were positive, with the appearance of a bright black color due to the secretion of gelatinous polysaccharides by the isolates, or negative with the appearance of a pink to red color due to the absence of gelatinous polysaccharides [15, 16].

Biofilm detection

Biofilm detection for 11 isolates from *Stenotrophomonas maltophilia* were done by microtiter plate method and tube method [15].

Detection of biofilm by microtiter plate method

This method worked according to [17].

The isolates were classified into four categories, according to the mean optical densities (ODi) in relation to the ODc results.

If ODi ≤ ODc; considered non-adherent, ODc≤ODi≤2*ODc; considered moderately adherent and if 2*ODc≤ODi which considered strongly adherent [18].

Detection of biofilm by using Tube method

This method worked according to [15] [19].

Molecular detection of *Stenotrophomonas maltophilia*

Whole genomic DNA was extracted from suspected isolates of *S.maltophilia* using the DNA extraction kit supplied by(Geneaid, USA). DNA was extracted according to the steps recommended by the company. The universal primers 27F AGAGTTTGATCMTGGCTCAG and 1522R AAGGAGGTGATCCARCCGCA were used to amplify the full region of the 16S rRNA gene [20]. The PCR program for the 16S rRNA gene was set as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles of amplification including a denaturation step at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. A final extension step was set at 72 °C. The concentration of primers (1 µM each) and the total amount of template DNA (100 ng) were added as recommended by the manufacturer, PCR products were separated on 1% agarose gel.A 100 bp DNA marker (New England Biolabs, UK) was used as a molecular weight marker.The PCR amplification products were forwarded to Korea's Micro laboratory. Using the Basic Local Alignment Search Tool (BLAST) aprogram, the 16s rRNA sequences were determined and compared to the sequences in NCBI. The relationship between the diagnosed strains under study was determined by comparing sequences within Clustal W Using the Mega 10 program and the Unweighted Pair- Group Average Method.

Molecular detection of adhesion gene *fimv* for *S.maltophilia*

Adhesion genes *fimv* were detected using primers designed during the current study that targets a 736 bp (F, 5’ CCATCGCTGAGCTTCCTGAT 3’ and R, 5’ CCTGTCGCAGTTGTTCGTTG 3’). The PCR program for the *fimv* gene was set as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles of amplification including a denaturation step at 95 °C for 30 sec, annealing at 57 °C for 1 min and extension at 72 °C for 1 min. A final extension step was set at 72 °C.

Results and discussion

Sample collection and identification

MacConkey medium was used, which distinguishes lactose fermenting bacteria from nonlactose fermenting bacteria. Biochemical assays were performed on lactose non-fermented isolates to estimate results for the diagnosis of *Stenotrophomonas maltophilia*. These tests included catalase, oxidase, motility, and fermentation of sugars on TSI agar. *S.maltophilia* were negative for the Indol test, methyl red, Voges - Proskauer, TSI, and urease test, only the citrate test was variable among the isolates. Diagnostic results showed that *S. maltophilia* was identified in eleven (11) strains among all bacterial isolates. The highest (5) of *S. maltophilia* wasfrom UTI infection compared to other sites of infections. Two (2) were recovered from burn infections and sputum from patients suffering from cystic fibrosis, in addition to (1) isolate from each of wound and medical devices. While no isolate was recovered from throat, blood and CSF samples as in figure (1).

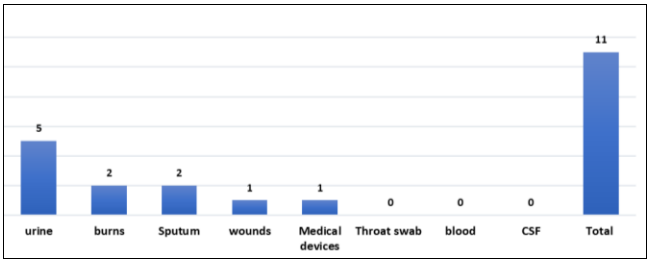

Fig 1: Distribution of *Stenotrophomonas maltophilia* obtained from different clinical specimen according to the specimen source.

The *S. maltophilia* strains isolated by [2] from the blood, throat swab, sputum, and urine from the institute for Mother and Child Health Care of Serbia were verified according to NCBI blast.

Slime layer detection

Results obtained from testing the ability of isolates of *Stenotrophomonas maltophilia* to the production o fslime layer by using congo red agar medium which showed that 6(55%) of isolates from 11 isolates were able to produce slime layer by producing black color on medium as in figure

(2) and (3). The negative result (45%) for other isolates appears as a red colony on the media.

Results of the study by [21] showed the ability of *Stenotrophomonas maltophilia* to produce slime layer by CRA were High at (66%), moderate (17%), and Non producer (1%).

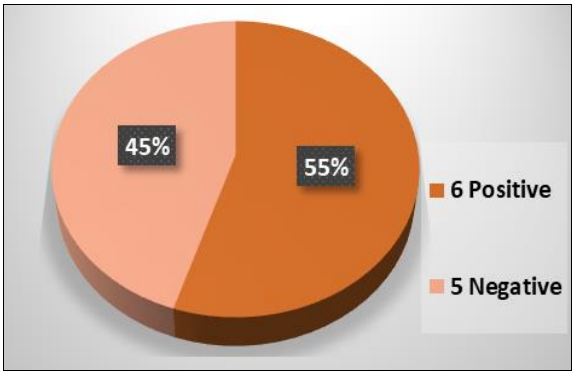

Fig 2: Production of slime layer by *S.maltophilia*

Fig 3: Colonies of *S.maltophilia* on Congo red agar(postive result)

Biofilm formation

A biofilm is an aggregate of microorganisms that adhere to each other on a living or non-living surface, and are embedded within a self-produced matrix of extracellular polymeric substances (EPSs) (including exopolysaccharides, proteins, metabolites, and extracellular DNA [22].

Micro titer plate method

The quantification result of biofilm formation by micro titer plate method revealed that all *Stenotrophomons maltophilia* isolates produced biofilm in microtiter plates at different ranges. (73%) of isolates were characterized as strong biofilm producers, and (27%) of isolates were moderate biofilm production figures (4) and (5).

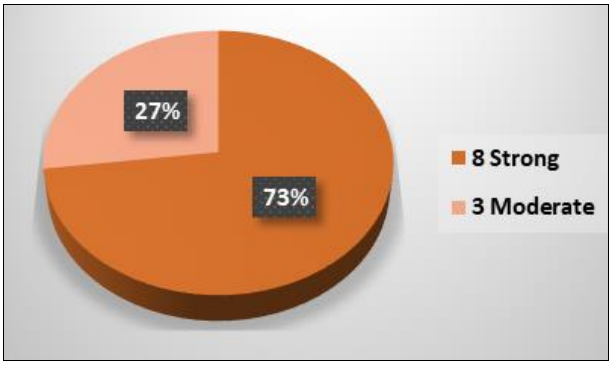

Fig 4: Percentage of biofilm production by Micro-titer plat

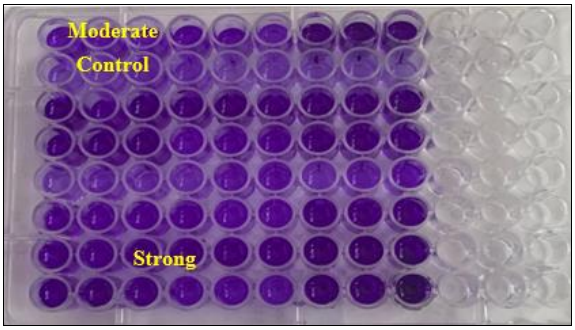

Fig 5: Micro-titer plate for biofilm detection of *S.maltophilia*

The result is consistent with several studies as in study of [23], who demonstrated that all isolates (100%) form biofilm. In the study of [6], the results showed that 51% and 33% of *S. maltophilia* produced strong- and moderate-level biofilms, respectively. while [24] found that all isolates have the ability to produce biofilms with a percentage of 29% strong and 71% moderate. Also results of [21] showed the ability of *Stenotrophomonas maltophilia* by MTP were High (14%), moderate (6%), and nonproducer (0%). [25] demonstrated that the majority of strains (91.7%) were capable of producing biofilm, but only blood-borne strains and those classified as "definite" pathogens produced biofilm levels that were significantly higher than those caused by hospital- rather than community- acquired illnesses.

Tube method (TM)

The tube method (TM), This method is considered a Qualitative assay for the detection the biofilm formation ability based on the thickness and intensity of the biofilm associated with the inner wall of the test tube. The results showed that (5) isolates were strongly adherent and as shown in, Fig (6) and (7). (4) isolates, had moderate productivity, while were (1) weakly adherent and (1) isolates were non-adherent compared to control.

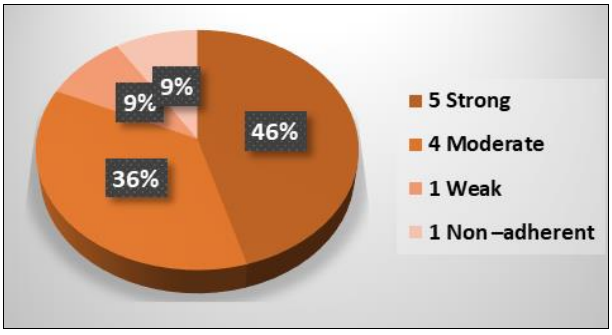

Fig 6: production of biofilm by using tube method

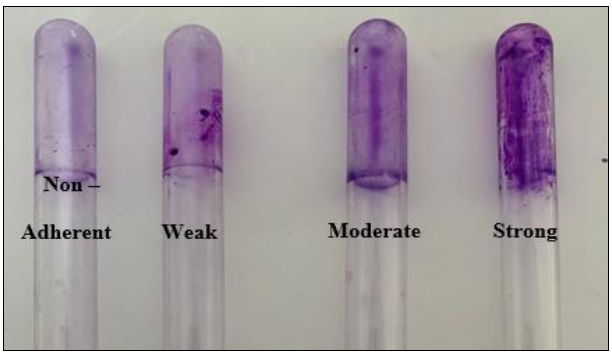

Fig 7: Tube method for biofilm production of *S.maltophilia*

A prominent feature of *S. maltophilia* is capability to adhere to abiotic surfaces, host tissues and bioflm formation, *S. maltophilia* has been identifed on the surfaces of biomaterials used in prosthetic devices, intravenous cannula, dental unit waterlines and nebulizers, The ability of *S. maltophilia* to generate bioflms is now recognized as a significant pathogenicity feature and is believed to be a significant contributor to the persistence of *S. maltophilia* infections in hospital settings [23].Numerous investigations have shown that *S. maltophilia* bacteria contribute to the biofilm development inside the urine catheter in patients lying in the critical care unit. Also,, the increasing use of indwelling medical devices has resulted in a corresponding rise in the incidence of device-related infections. Biofilms are formed by bacteria that colonize medical equipment [26] The results of study were similar with results of [27] indicated that *S. maltophilia* strains may firmly adhere and form biofilm on the surfaces of equipment in hospitals, which helps spread diseases. while the study of [28] indicated that *S. maltophilia* bacteria can form a multi layer of bacterial growth on the surface of medical devices and spread infections, and it proved all previous studies are the extent of resistance of these bacteria to antibiotics, as the biofilm is one of the reasons for this resistance, as it works to protect bacteria from external conditions and protects them from the effect of antibiotics and disinfectants.

Detection *Stenotrophomonas maltophilia* by using universal *16S r RNA* gene

The universal primers 27F and 1522R were used to amplify the whole gene for the 16S subunit. The size of the fragment was 1500 bp as in Fig (8).

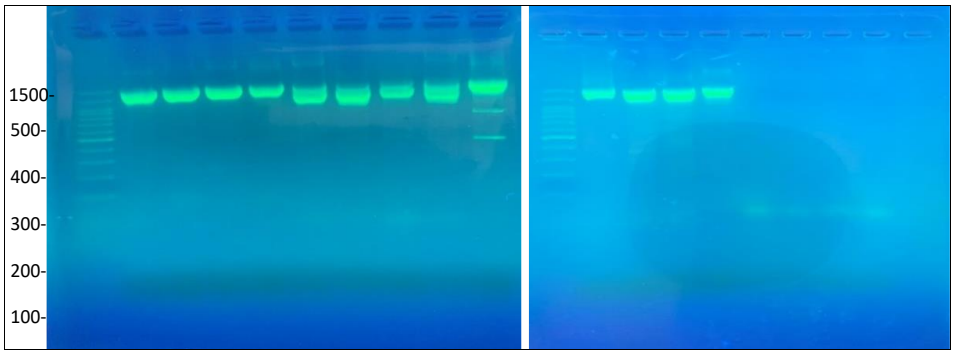

Fig 8: The universal 16S rRNA genetic segment at 1500bp in 2% agarose gel electrophoresis at 70 volt for 50 min, the bands visualized under U.V light. using 1500 bp ladder.

The results of the sequences of the nitrogenous bases of the forward and reverse primer of the 16S rRNA gene of the under study isolates were compared with the strains within NCBI using a program BLAST. Results showed that 11 of isolates belong to *Stenotrophomonas maltophilia* and 3 isolates belong to *Enterobacter hormaechei.*

All (14) sequences were submitted to the gene bank (NCBI) and were given the accession numbers. Within a Molecular Evolutionary Genetics Analysis Mega 10 by using the Unweighted pair group method with arithmetic mean UPGMA based on the method of [28]. Phylogenetic tree for under study strains revealed presence three main clusters A, B, C as in Fig (9).

Stenotrophomonas maltophilia clustered on A, and B while *Enterobacter hormaechei* on C. The first cluster contained 9 strains (BE 16, BE 12, BE 14, BE 15, BE19, BE 21, BE 22, BE 20, BE45) with the bootstrap value (99.86%), the second cluster placed (BE 11, BE 13) together with the bootstrap value of (99.88%) and this strains appeared to contrast from the strains on the first cluster in citrate utilization (negative for citrate test) and also on having some virulence enzyme (didn't have lecithinase and Gelatinase enzymes). The third cluster placed (BE17, BE 18, BE 23) together with the bootstrap value of (99.88%).

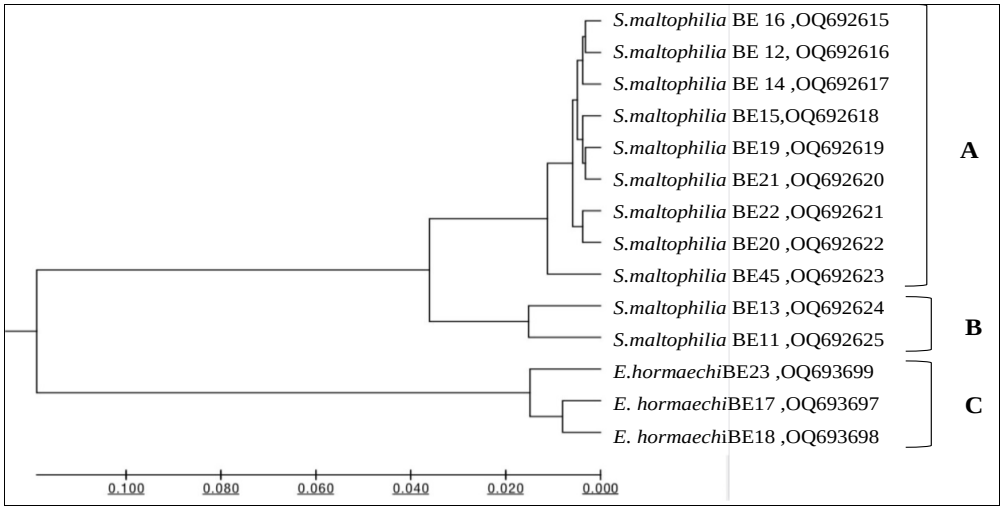

Fig 9: Phylogentic tree analysis of the *S.maltophilia* based on universal 16S rRNA gene.

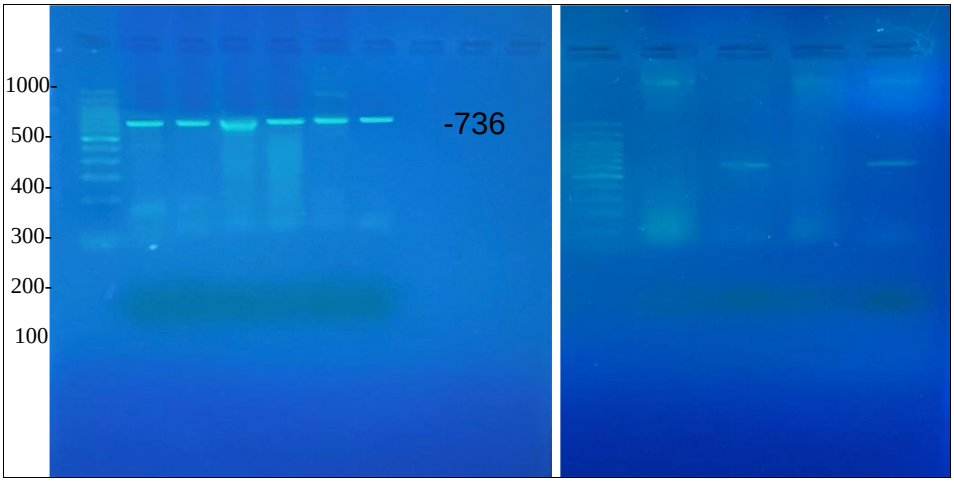

Fig 10: The *fimv* genetic segment at 736 bp in 2% agarose gel electrophoresis at 70 volt for 50 min, the bands visualized under U.V light. Using 1500 bp ladder

[30] reported that one of the most important uses of 16S rRNA gene sequence analysis is to provide genus and species identification for isolates [31] performed sequence analysis by using the NationalCenter for Biotechnology Information (NCBI) blast tool and searched in *Stenotrophomonas maltophilia* isolates obtained from environmental and clinical samples.

Detection of *fimv* gene

The results of PCR amplification of the *fimv* gene that is designed during the current study among *S. maltophilia* isolates appear that 8 isolates give positive results as in Fig (10).

The results showed that the *S. maltophilia* strains that possessed the adhesion gene had the ability to produce a biofilm with high strength, while the strains that were missing this gene were moderate by MTP method as in table (1).

Table1: The relationship between the presence of the *fimv* gene and biofilm production by MTP method

N	Strains	*Fimv*	Biofilm production by MTP	
			Strong	Moderate
1	*S. maltophilia* BE11	+	+	-
2	*S. maltophilia* BE 12	+	+	-
3	*S. maltophilia* BE13	+	+	-
4	*S. maltophilia* BE 14	-	-	+
5	*S. maltophilia* BE15	-	-	+
6	*S. maltophilia* BE16	+	+	-
7	*S. maltophilia* BE19	+	+	-
8	*S. maltophilia* BE20	+	+	-
9	*S. maltophilia* BE21	+	+	-
10	*S. maltophilia* BE22	-	-	+
11	*S. maltophilia* BE 45	+	+	-

Fimv is involved in a number of virulence mechanisms, including twitching motility, type II secretion (T2S) of lipases and proteases, and regulation of cAMP production, and thus Vfr function [32].The *S. maltophilia* fimbriae 1 (*SMF-1*) are composed of a 17 kDa fimbrin subunit which shares significant similarities with the N-terminal amino acid sequences of several fimbrial adhesins (G, F17, K99, and 20K) were found in pathogenic *Escherichia coli* and the CupA fimbriae of *Pseudomonas aeruginosa* [33].High-resolution electron microscopy provided evidence of the presence of fimbriae acting as bridges between bacteria adhering to inert surfaces or cultured epithelial cells, this is the first characterization of fimbriae in this genus. the SMF-1 fimbriae are involved in [31].The study in Kufa city reported by [34] shows that all of the clinical *S. maltophilia* (100%)isolates were carrying(*smf-1*). The presence or absence of fimbriae did not influence the capacity of the bacterium to adhere or to cause hemagglutination. The mechanisms of binding appeared different for adherence and hemagglutination [35].Attachment of bacterium to the epithelium tissue is likely to be an essential step in the pathogenesis of *S. maltophilia* infection, the general mechanism of cellular adherence of *S. maltophilia* to host cell surfaces has been studied previously by [36].

Conclusions and suggestions

The results of the study demonstrated the presence of *S.maltophilia* in Mosul city, which are highly capable of forming biofilms and the presence of the *fimv* gene increase this ability.

Acknowledgements

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