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ORIGINAL ARTICLE

SPECIFIC 23S rRNA GENE FOR DETECTION OF *STENOTROPHOMONAS MALTOPHILIA* ISOLATED FROM CLINICAL SOURCES

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Summary

Background: *Stenotrophomonas* infections are becoming more widespread around the world and can be counted as a “newly emerging pathogen of concern". The present study aimed to detect a variety of *Stenotrophomonas* species (*S. maltophilia*) using specific 23S rRNA gene primers and investigate their multi-drug resistance potential.

Methods: This study includes 375 clinical samples from different clinical sources 175 from males and 200 from females collected from Mosul City Hospital. Identification of *Stenotrophomonas* was conducted through multiple steps including culturing methods, molecular methods in addition to some biochemical tests 11(3%) of isolates belonged to *Stenotrophomonas maltophilia*. The isolates understudy were tested for their ability to resist 10 different antibiotics using the Kirby-Bauer disk diffusion method.

Results: The resistance rate to amoxicillin, gentamicin, and amikacin (100%), cefixime (91%), imipenem (64%), meropenem(55%), Azithromycin (36%), nalidixic acid and trimethoprim (18%), ciprofloxacin(0%). The virulence factors of *S. maltophilia* siderophores were found in all (11) isolates belonging to *S. maltophilia* at a percentage (100%). The result of PCR assay using specific primers designed for detecting 23S rRNA genes of *S. maltophilia* gives amplification for 11 isolates from 14 suspected isolates. Nucleic acid sequencing for the 23S rRNA gene shows that all isolates belong to *S. maltophilia* with a similarity rate (91-99) in NCBI.

Because the 23S rRNA gene sequence in *Stenotrophomonas* species shows more variety in this location this study used specific 23S rRNA gene primers to identify *S. maltophilia*.

Conclusion: The study used phenotypic and molecular diagnostic techniques to isolate the bacteria, including the S rRNA23 gene. The results emphasize the need for increased vigilance in hospitals to prevent the spread of antibiotic-resistant bacteria and the development of new treatment strategies.

Key words: Stenotrophomonas maltophilia; 23S rRNA gene; Siderophores; critically ill patients

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Introduction

Stenotrophomonas maltophilia is a Gram-negative aerobic bacilli that does not ferment lactose, motile non-spore-forming, and non-capsulated bacteria that is widely distributed in the hospital environment (1). Examination of *S. macrophilia* cells under the microscope shows that the cells could be either straight or slightly curved, that is 0.5 to 1.5 mm long. The cells were found to occur singly or in pairs and no accumulation of poly–hydroxybutyrate as intracellular granules was detected. Movement in the media is conducted using several polar flagella. When grown on nutrient media, they generate white to pale yellow smooth colonies and have a glistening appearance, with entire margins. This bacterium is not generally considered a Beta-hemolytic organism (2).

S. maltophilia is a nosocomial pathogen that can spread mainly by ingestion, consumption of contaminated water, and direct contact mainly by the hands of healthcare workers, altogether leading to healthcare-associated illnesses (HCAIs) (3). *S. maltophilia* is widely dispersed among plants, animals, and water sources in aqueous and humid ecosystems (4). *S. maltophilia* is the cause of several diseases, including meningitis, pneumonia, bacteremia, eye infection, enteritis, urinary tract infection, skin, and soft tissue infection (5, 6). A recent evaluation of the bacterium's ability to enter clinical settings led to its recognition as a significant multi-drug-resistant worldwide opportunistic nosocomial pathogen (7, 8).

Virulence factors of *S. maltophilia* are divided into cellular-associated virulence factors and extracellular-associated virulence factors. The cellular-associated virulence factors include lipopolysaccharide, pili or fimbriae, and non-pilus adhesins and flagella (9). The extracellular virulence factors include both DNA and RNA nucleases, protein degrading enzymes, gelatinases, elastases, lipases, siderophores, esterases, hyaluronidases, heparinases, hemolysins, cytotoxins and fibrinolysin (8).

S. maltophilia exhibits resistance to several classes of antibiotics with different structures including β-lactam antibiotics, macrolides, cephalosporins, polymyxins, tetracyclines, aminoglycosides, chloramphenicol, and carbapenem. Resistance of *S. maltophilia* is a result of several factors, some of them are intrinsic resistance while others are acquired, including fluctuation in membrane permeability, acquisition of several chromosomally encoded genes that function as efflux pumps, aminoglycoside-modifying enzymes and β-lactamases (10).

Iron is a major essential compound required to perform various metabolic activities in all living cells. It is involved in the regulation of oxidative stress response, formation of biofilms, and several other pathogenic processes in S. *maltophilia*. Iron is an insoluble element due to its oxidation by environmental oxygen. Iron Acquisition Systems such as siderophores have evolved in bacteria to capture iron in its insoluble form (11). This is a crucial virulence factor for microbes to thrive in iron-limiting environments (12).

The total genome size of *S. maltophilia* is 4,851,126 bp with a G+C content of 66.7% G+C that builds up a single chromosome. Analysis of the genome identified six types of transposable elements. More than 200 genes were found to be unique among the *S. maltophilia* strains with completely sequenced genomes. The majority of these encoded putative proteins and transposases indicate that *S. maltophilia's* core genome is large (13). The use of PCR technique to distinguish *S. maltophilia* depending on the 16S rRNA gene has a limitation when used to detect *S. maltophilia* as a target. This is due to considerable genetic similarity between *S. maltophilia* and other non-fermentative gram-negative bacilli. This method shows low specificity, but the use of PCR to identify 23S rRNA is more efficient. The reason is that the 23S rRNA gene sequence shows more variety in this area between *Stenotrophomonas* species (14). Because the 23S rRNA gene sequence in *Stenotrophomonas* species shows more variety in this location this study used specific 23S rRNA gene primers to identify *S. maltophilia*.

Material and Methods

Specimens collection: Specimens were collected from Mosul hospitals including (Alsalam Teaching Hospital, Mosul General Hospital, Aljumhurii Teaching Hospital, Chest Diseases Hospital, Ibi Sina Teaching Hospital, and Specialized Burn Centre) for the period between July to December 2022. A total of 375 samples were taken including 130) of urine from UTI, (92) sputum from RTI, (18) swabs from the wound, (66) swabs from the burn (18) swabs from a throat infection, (8) CSF), (17) blood, in addition to (26) samples from medical tools.

Specimens culture and biochemical test: All the specimens were transferred immediately via transport media, and streaked on MacConkey agar and blood agar, plates were incubated for 18-24 h at 37°C, The primary identification of bacterial isolates depended on cultural, morphological and biochemical character (15) (16). API and VITEK-2 Compact System were used to confirm the diagnosis.

Antibiotic susceptibility test: Isolates understudy were tested for their ability to resist 10 different antibiotics supplied by Himedia-India. The test was performed using the Kirby-Bauer disk diffusion method. Antibiotic-containing discs were placed on the surface of Muller Hinton media previously inoculated with the tested isolate. Plates were incubated for 18-24 h at 37°C and the diameter of the inhibition zone was measured and compared to (CLSI 2022a).

Siderophores production: This medium was prepared by dissolving 28gm of nutrient agar with 990 mL distal water. The pH was adjusted to 7, sterilized in an autoclave for 15 min at 121°C, left to cool to 50°C, and then 10 mL of 2.2'-dipyridyl at a concentration of 0.2 mg /mL was added (17). Bacterial isolates understudy were cultured on agar plates, incubated at 37°C for 24 h and tested for growth. A positive result for siderophores production was recorded when an isolate produced colonies on this medium.

DNA extraction and PCR protocol: Genomic DNA was isolated from all isolates understudy using a specialized kit supplement by Geneaid company. DNA was stored at -20°C for further applications and processing for molecular identification of *S. maltophilia* using specific primers for detecting 23 S rRNA genes were designed using NCBI primer design (Table 1) and amplification done by conventional PCR (Table 2).

Table 1. Primers and product size of 23S rRNA gene.

Primers	Sequence (5' to 3')	Gene length (bp)	Sources
23S rRNA- F	CCTGCGAAAAGTATCGGGGA	816	Designed using pick primer program within NCBI
23S rRNA-R	TAAGTCGCGATGACCCCCTA		

Table 2. Program and conditions for 23S rRNA gene.

Sequencing	Stage	Number of cycles	Temperature (C°)	Time (minutes)
1	DNA Initial Denaturation	1	95	3:00
2	Denaturation	35	95	00:30
3	Annealing		54	00:30
4	Extension		72	1:30
5	Final extension	1	72	3:00

Electrophoresis was conducted at 70 volts for 45 min, and then PCR amplification was confirmed by the production of a specific band shown on an agarose gel. UV trans-illuminator was used for the observation of the PCR product, the gel was photographed using an iPhone X Max.

Sequence analysis: PCR products of the 23S rRNA gene were sequenced using Sanger sequencing at Macrogen company (Korea). The identity of sequences was confirmed using BLAST 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 (18).

Results

Isolation and identification of S. *maltophilia*: *S. maltophilia* was diagnosed depending on its colony morphology on both MacConkey agar and blood agar, its reaction towards Grams stain, and towards results obtained from specific

biochemical tests as shown in table (3). Final identification was confirmed by the Vitek-2 system. Results showed that only 11(3%) of the isolates belonged to *S. maltophilia*. UTI infection showed the highest percentage (5) isolates of *S. maltophilia* isolates as compared to other sources of specimens.

On the other hand, (2) isolates were recovered from burn infections and (2) isolates from sputum from patients suffering from cystic fibrosis, in addition to (1) isolates from each of the wound and medical devices. This study failed to detect *S. maltophilia* from the throat, blood, and CSF samples (Figure 1).

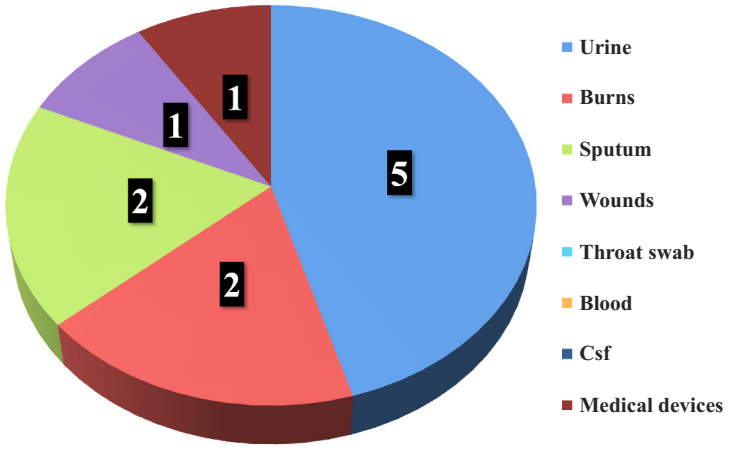

Figure 1. Distribution of *S. maltophilia* obtained from different clinical specimens according to the specimen sources.

As expected *S. maltophilia* was motile due to the acquisition of polar flagella. This bacteria grew well on MacConkey agar plates and colonies appeared medium to small in size, round, and pale yellow colonies due to not-fermented lactose about 0.7-1.8 mm in diameter. As well as is also found in contrast forms (appeared too transparent mucoid colonies with pink colour. While on blood agar appeared grey convex smooth glistening colonies and it is growth was faster in (24) h unlike the MacConkey agar (from 24 to 72 h) (Figure 2).

S. maltophilia are motile, positive towards catalase, and negative towards oxidase which was considered a key to distinguish this species from other species in the genera. In addition, *S. maltophilia* was negative for all IMVIC tests, except citrate utilization which was variable among isolates (Table 3).

Table 3. Colony morphology and reaction towards biochemical tests in *S. maltophilia* isolates.

Test	Result	%
Gram staining	Negative	100% (11)
Aerobic growth	Positive	100% (11)
Anaerobic growth	Negative	100% (11)
Oxidase	Negative	100% (11)
Catalase	Positive	100% (11)
Indole	Negative	100% (11)
Voges –Proskauer	Negative	100% (11)
Methyl red	Negative	100% (11)
Citrate utilization	Variable	82% (9),positive 18% (2),negative
Triple sugar iron test	k/k	100% (11)
H_2S	Negative	100% (11)
Motility	Positive	100% (11)
Urease	Negative	100% (11)

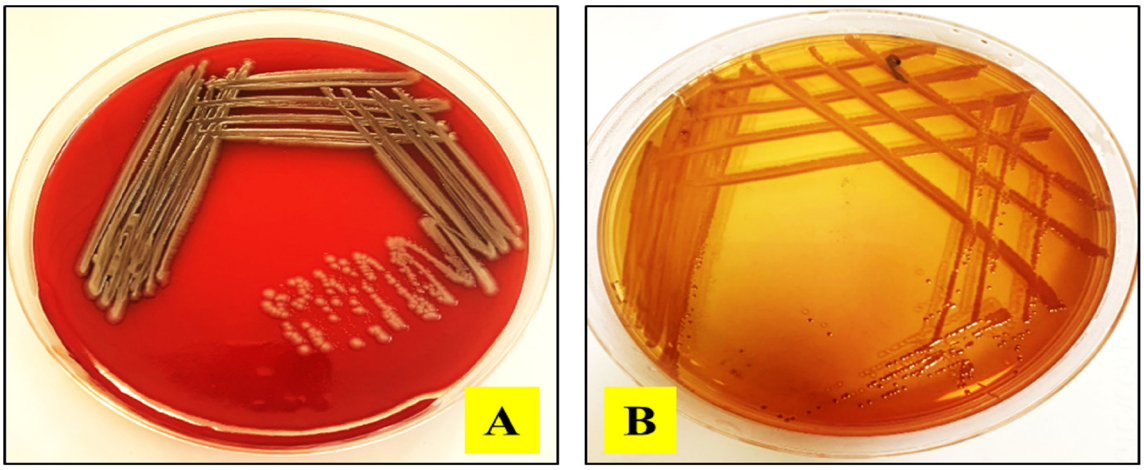

Figure 2. Colonies of *S. maltophilia* on blood and MacConkey agar, A-blood agar, B-MacConkey agar.

Antibiotic susceptibility test of *S. maltophilia*: The antibiotic resistance pattern of *S. maltophilia* isolates was conducted using the common antibiotics used in the treatment of this pathogen by using the Kirby-Bauer disk diffusion method. Results were recorded following the guidelines of CLSI (2022a). Results of the current study indicated that isolates of *S. maltophilia* were given high resistance to most antibiotics represented by all isolates (100%) to appear resistant to Amoxicillin, Gentamicin, and Amikacin, and (91%) of isolates were resistant to cefixime, while (64%) of the isolates were resistant to imipenem and (55%) to meropenem. Resistance to Nalidixic acid appeared in (18%) of the isolates, while resistance to Azithromycin was detected in (36%) and trimethoprim (18%). This study did not detect resistance towards ciprofloxacin (Figure 3).

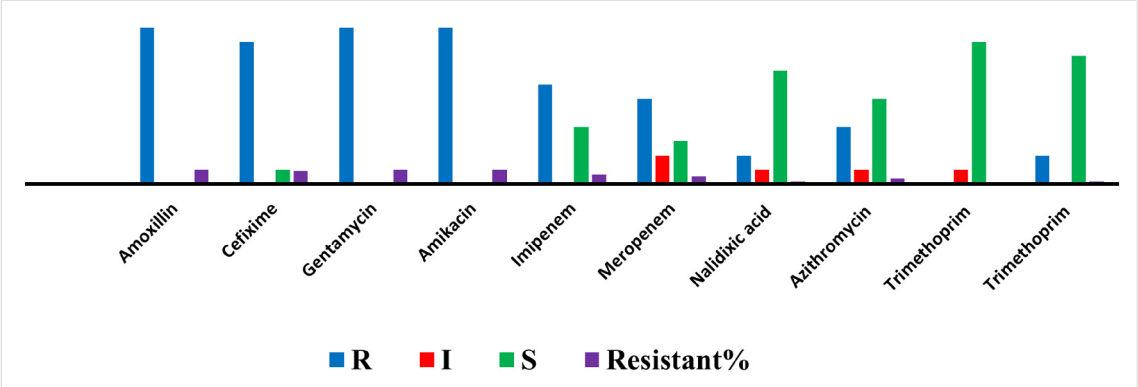

Figure 3. Antimicrobial susceptibility rates among *S. maltophilia* isolates.
(R: Resistance, I: Intermediate, S: Sensitive).

Results of the current study indicated that isolates of *S. maltophilia* were highly resistant to most antibiotics (100%), including resistant to Amoxicillin, Gentamicin, and Amikacin, and (91%) of isolates were resistant to cefixime, while (64%) were resistant to imipenem and (55%) to meropenem. Resistance to Nalidixic acid resistance was (18%) of the isolates, while Azithromycin resistance was recorded in (36%) and (18%) to trimethoprim. In contrast, all isolates were sensitive to ciprofloxacin (100%).

Siderophores production: To detect the ability of *S. maltophilia* for siderophores production. 24-hour- colonies grown on heart and brain infusion agar media were transferred to nutrient agar that contains 2,2 dipyridyl, results showed that the siderophore production rate in *S. maltophilia* isolates was 100%, all (11) isolates were growing on medium (Figure 4).

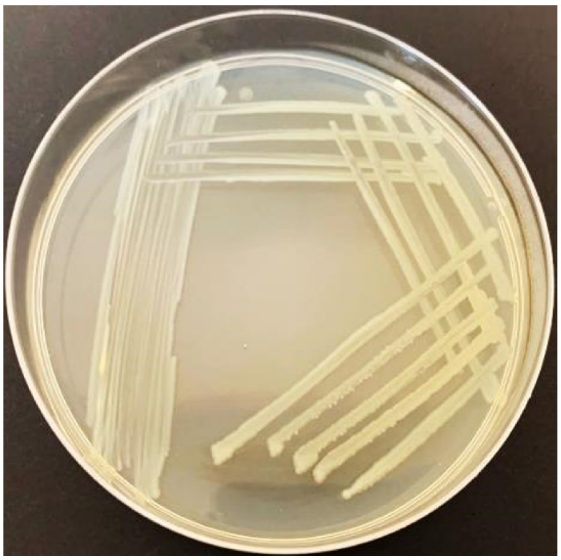

Figure 4. Detection of siderophore production from *S.maltophilia*.

Identification of *S. maltophilia* by amplification of 23S rRNA gene: Using specific primers designed in a current study for detecting 23S rRNA genes of *S. maltophilia*. The result gives an amplification of 11 isolates from 14 isolates under study at 816 bp in 2% agarose gel after electrophoresis (figure 4).

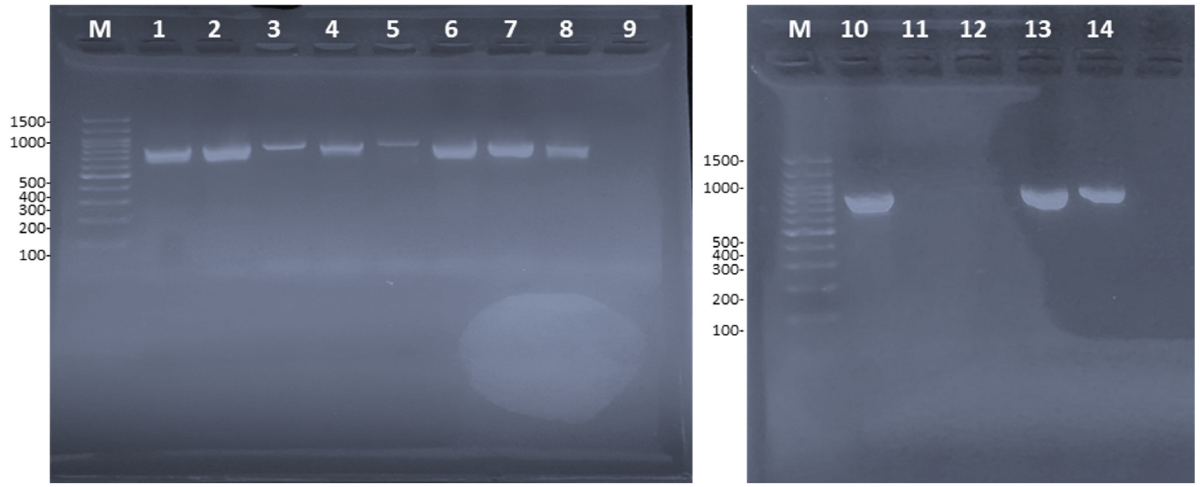

Figure 5. Specific 23S rRNA gene bands at 816 bp in 2% agarose gel electrophoresis at 70 volts for 50 min, the bands visualized under UV light. compared to a 1500 bp ladder.

Sequencing analysis: Sequencing was performed for the 23S rRNA gene. Results of nucleic acid sequencing for 23S rRNA gene for isolate were compared within the National Center for Biotechnology Information NCBI using the Basic Local Alignment Search Tool (BLAST) program that shows a high similarity rate to *S. maltophilia* strain ranging from range (91-99). The three isolates that did not give amplification was suspected as *S. maltophilia* from the cultural characteristics and oxidase test, these isolates appeared as transparent mucoid colony with pink colour and identify as *Enterobacter hormaechei*, this colour also appeared for some *S. maltophilia* strains found during the current study. All sequences were submitted to the gene bank (NCBI) and were given the accession numbers (OQ693702- OQ693710) (Table 4).

Table 4. Identification of *S. maltophilia* by 23S rRNA gene sequencing.

N	Isolate symbol	Accession number
1	BE24	OQ693709
2	BE25	OQ693705
3	BE26	OQ693704
4	BE27	OQ693706
5	BE28	OQ693710
6	BE29	OQ693708
7	BE30	OQ693707
8	BE31	OQ693703
9	BE32	OQ693702

Studying the phylogenetic relationship between the strains by using the Clustal W program and the UPGMA method within the Mega 10 program (19) (Figure 6), the *S. maltophilia* clustered in two clusters A and B as shown in the tree diagram. Seven isolates linked on cluster A (BE32, BE31, BE26, BE 25, BE27, BE30, BE29) with a bootstrap value of (99.99%) and two isolates on cluster B (BE 24, BE28) with the bootstrap value (98.88%).

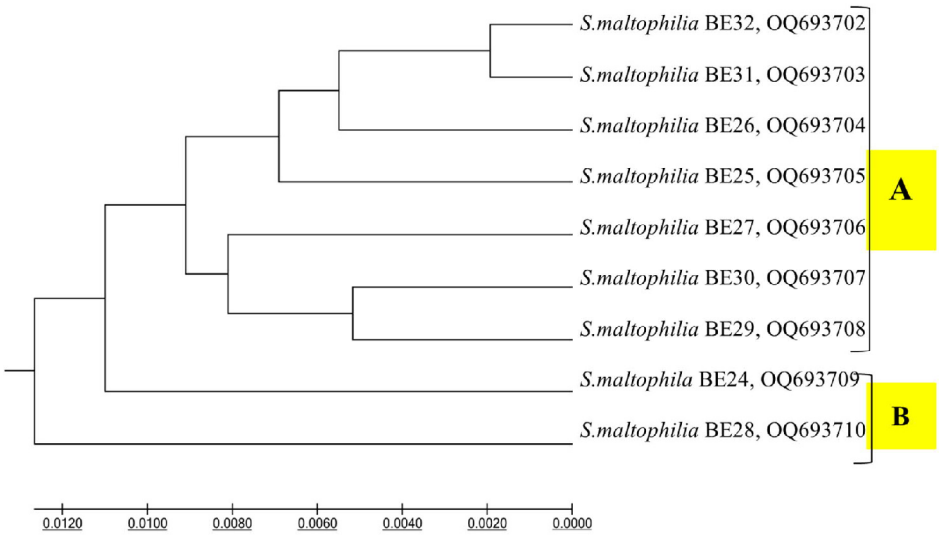

Figure 6. Phylogenetic tree analysis of the *S. maltophilia* based on specific 23S rRNA gene.

Discussion

WHO identified *S. maltophilia* as one of the underappreciated significant multi-drug resistant organisms in hospitals; British microbiologists ranked it as the ninth most significant pathogen and one of the most difficult to treat. This pathogen is widely recognized as an opportunistic bacteria linked to high rates of morbidity and mortality among immune-compromised patients (20). Biochemical tests, Vitek-2, and API-20NE mainly used to identify *S. maltophilia* occasionally misidentify this pathogen with other Gram-negative non-lactose fermenter bacilli (21, 22).

In the local study presented by AL-Moomen (2022), 21 isolates of *S. maltophilia* were obtained from a total of 625 samples, and the highest percentage of isolation was from UTI patients (23). As for the study presented by AL-Khafajy (2022), 20 isolates were obtained, and the highest isolation percentages were also from UTI patients (24). According to the phenotypic characteristics and the Regnum Prokaryotae program, 31 isolates of *S. maltophilia* were obtained in the local study in Mosul City presented by Sulaiman (2020) (25).

S. maltophilia are resistant to a variety of antibiotics, including macrolides, aminoglycosides, β-lactam antibiotics, and fluoroquinolones. The altered penicillin-binding protein (PBP) and reduced permeability of the membrane which results in resistance to β –lactam antibiotics, as well as the existence of multidrug-resistant efflux pumps that are usually chromosomally encoded, antibiotic-modifying enzymes, β-lactamases that all contribute to the intrinsic antibiotic resistance of *S. maltophilia* (26, 27). Drug-resistant mechanisms are acquired by horizontal gene transfer that is carried out by plasmids, integrons, transposons, integron-like elements, and insertion elements (28, 29).

The study of Alcaraz *et al*. (2021), discovered that all isolates produced siderophores in Najaf governorate during the period between November 2020 to May 2021 (30). Siderophores are thought to be critical virulence factors for many diseases to survive in the host's iron-restricted environment (11).

Pathogenic strains that are unable to secrete siderophores, on the other hand, have lower virulence and capacity during infection and colonization. Hypervirulent pathogenic strains can create an overabundance of siderophores. Recent studies have focused on S. *maltophilia's* production of siderophores, which is extremely concerning (31, 32).

In the study of (30) only three isolates were found to generate siderophores (3/11). Gallo *et al*. (2013) intended to develop a specific method to identify *S. maltophilia*, by choosing the 23S rRNA gene for targeting by conventional PCR and real-time PCR assays (33). This is due to the high variability of the 23S region among species of *Stenotrophomonas* sp as compared to the 16S rRNA region. Also indicated in their studies that the primer pair was specific for *S. maltophilia* but not with other species of *Xanthomonas* and S. *rhizophila* however the primers designed in their studies are crucial alternatives for the specific detection of S. maltophilia as in their study by using rapid molecular methods, which can make it possible to choose the proper antimicrobial strategy for treating illnesses successfully caused by this microorganism.

Whitby *et al*. (2000), identified more than one hundred clinical isolates of *S. maltophilia* gathered from different places by species-specific PCR primers directed to amplify the 23S rRNA gene and checked to identify *S. maltophilia* from sputum specimens (34). This study could specifically find application in the early detection of infection or protect critically ill patients, such as patients with renal dialysis (35, 36), multiple sclerosis (37), asthma (38), diabetic foot (39), cancer (40), and cardiac septal defects (41).

Conclusion

Antibiotic-resistant bacteria have become a growing concern in hospitals worldwide, and the discovery of opportunistic antibiotic-resistant S. maltophilia bacteria in Mosul hospitals over six months has raised concerns about the effectiveness of current treatment methods. This study revealed the isolation of these bacteria using both phenotypic and molecular diagnostic techniques, including the S rRNA23 gene. The results of this study highlight the need for increased vigilance in hospital settings to prevent the spread of antibiotic-resistant bacteria and the importance of developing new treatment strategies to combat these infections. The identification of the S rRNA23 gene as a marker for antibiotic-resistant S. maltophilia bacteria could aid in the early detection and treatment of these infections, leading to better outcomes for patients. Overall, this study underscores the importance of ongoing surveillance and research into antibiotic-resistant bacteria and the need for continued efforts to develop new and effective treatment options.

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Adherence to Ethical Standards

The study was registered and approved by the Department of Biology, College of Science, University of Mosul (letter No. 2483 on 28.04.2022).

Conflict of interest

The authors declare no conflict of interest concerned in the present study.

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