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Molecular and Biochemical identification of gram-negative bacteria associated with gastrointestinal endoscopy

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Abstract
Gastrointestinal diseases affect millions of individuals globally each year, ranking among the most significant health concerns worldwide. These conditions severely diminish quality of life and impose substantial economic burdens on healthcare systems. A major contributing factor to the development of infections linked to healthcare is the cross-contamination of gastrointestinal endoscopes, which frequently occurs as a result of insufficient sterilization practices. This study identifies the bacterial species present, assesses their antibiotic resistance and susceptibility profiles, and investigates into the level of microbial contamination in endoscopes used in Al-Zahraa and Al-Karama teaching hospitals.
A total of 61 gastric biopsy samples were obtained from patients aged 17 to 75 years suffering from gastrointestinal disorders during a six-month study period. These samples were processed using conventional biochemical methods for initial culturing and identification. Molecular techniques, including 16S rRNA gene amplification and Sanger sequencing, were employed for precise bacterial identification. The Kirby-Bauer disk diffusion method was utilized to determine the responsiveness of bacterial isolates to eight antibiotics from six different classes.
Bacterial growth was observed in 54.09% (33/61) of the collected samples. Molecular analysis identified six distinct bacterial species, including *Achromobacter anxifer, Pseudomonas nitroreducens, Shigella flexneri,* and *Stenotrophomonas maltophilia,* which were reported for the first time in Iraqi patients. Additionally, common pathogens such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were also identified. Antibiotic susceptibility testing revealed that amikacin, gentamicin, levofloxacin, and piperacillin-tazobactam exhibited high efficacy, whereas azithromycin, erythromycin, ceftriaxone, and trimethoprim showed significant resistance among the isolates.
This study emphasizes the pressing issue of microbial contamination in gastrointestinal endoscopes, highlighting the necessity for stringent sterilization practices and routine microbiological monitoring. The results underline the critical role of tailored antibiotic treatments and enhanced infection control strategies to reduce cross-contamination, improve patient safety, and address the growing challenge of antimicrobial resistance in healthcare settings.

Introduction

Gastrointestinal disorders affect millions of people each year and represent an important worldwide health burden. These illnesses include severe diseases like stomach cancer and inflammatory bowel disease as well as functional problems like irritable bowel syndrome. They have an adverse effect on patients' productivity and life quality, and elevate medical costs significantly (1). One unique component of the gastrointestinal microecosystem is the stomach. Its distinct microbial community and biological setting are caused by gastric acid (2). Some studies using traditional culture methods confirmed that there are many acid-resistant bacterial strains in the stomach, which are primarily

derived from the transient flora in the mouth and food. Previously, it was thought that gastric acid could kill the bacteria entering the stomach and that the stomach environment was unsuitable for bacterial colonization (3,4).

Medical devices, especially endoscopes, play a crucial role in diagnosis gastrointestinal diseases. These reusable instruments are necessary to be cleaned, sterilized, and disinfected in order to make them safe for use on the following patient (5). Infections at the surgical site may become more common as a result of microbially contaminated instruments (6). Because of that, sterilization and disinfection are crucial to preventing the spread of infectious microorganisms or opportunistic normal flora among patients using surgical and medical equipment. During use, flexible endoscopy, a common diagnostic and therapeutic technique, may become highly polluted with blood, fluids, and infectious agents (7). Endoscopes are the most commonly medical equipment that linked to infections associated to healthcare. These instruments' complex design, with their numerous internal passages and narrow lumens, makes them challenging to clean , disinfect and easy to damage (8,15). Reprocessing flexible endoscopes accurately include cleaning, high level disinfection, washing, and drying before storage (9). Inadequate cleaning of the instruments may affect the drying and disinfection processes and increase the risk of infection transmission from one patient to another (10). Bacteria's ability to create biofilms in the endoscope channels, particularly when they become damaged, can also lead to the decontamination procedure failing (11,12). There are two categories of endoscopy-related infections: exogenous and endogenous. The most frequent outcome of endoscopic procedures is endogenous infection, which is caused by the patient's own microbiota (13,14).

This research examines the frequency of contamination in gastrointestinal endoscope used in Al-Zahraa and Al-Karama teaching hospitals and how it raises the possibility of bacterial infections. Traditional and molecular techniques were used to identify bacterial strains. To our knowledge, several species were identified for the first time in Iraq. Additionally, antibiotics susceptibility testing revealed that the isolated bacterial species showed various resistance and sensitivity patterns, highlighting the effectiveness of particular antibiotics including, Amikacin, Gentamicin, Levofloxacin, and Piperacillin-Tazobactam. The results highly suggested the importance of improving sterilizing procedures, routine microbiological testing, and cutting-edge infection control techniques to reduce the spread of infections and ensure patient safety.

Material and method

Sample collection

Samples were obtained from patients in Al- Zahra Teaching Hospital and Al-Karama teaching hospital in Wasit Governorate/Iraq. Most patients were female, and their ages ranged from 17 to 75 years old. The period of trial was extended From October 2023 to March 2024. Sixty-one gastric biopsy tissues samples were taken from the gastric antrum or gastric corpus of patients who had gastrointestinal disorders and suspected having an H. pylori infection. Biopsy specimens was placed in tubes containing 2mL sterilized brain heart infusion broth (BHI) as a transport medium and were transferred by a cold box to the laboratory within 2 hours to be cultured.

Bacterial culture and storage

Biopsy tissues were homogenized and cultured onto classic Columbia agar. The agar plates were incubated at 37°C in ambient air and a carbon dioxide-humidified environment. Grown colonies were stored for short and long terms by inoculated bacteria on Columbia agar slant tubes and 10% glycerol/BHI tubes, respectively. The slant tubes were stored at 2-4°C while glycerol/BHI tubes were stored at -80°C.

Bacterial Identification

A- Biochemical identification

To identify the grown bacteria, gram stain and biochemical tests were used. The gram stain has been done as described in (16). Catalase test was performed by adding a drop of hydrogen peroxide (H_2O_2) on a glass slide and mixing with a part of bacterial colony. Bubbles formed within 30 seconds indicates the positiveness of the test. On

the other hand, Oxidase test was performed by mixing a drop of the oxidase enzyme reagent with a little of the bacterial colony on filter paper. The color of the colony changed to purple within ten seconds indicates the positive result (17,18). Finally, to do Urease test, several colonies were cultured on the medium of urea agar base and incubated at a temperature of 37 °C for 24 hours. Changing the media color to red or pink indicates the positive result. To validate above tests, positive and negative controls were included (19,24).

B-Molecular identification

DNA Extraction

DNA was extracted using specialized kits (Scientific Research Company). Following the instructions, bacterial isolates were initially activated on Columbia base agar. 1 ml of BHI was inoculated with activated bacteria and incubated overnight at 37ºC. Cells were harvested by centrifugation. Lysis and binding steps were performed using appropriate buffers, and the lysate was processed through a spin-DNA column with washing and drying steps. Purified DNA was eluted in 50 µl of preheated elution buffer and stored at -20ºC.

To check the extracted DNA quantity and quality Quantus™ Fluorometers (Promega, USA) was used. The samples were prepared in 1X TE buffer and loaded into the fluorometer. Manufactures' instructions were followed to measure the DNA integrity.

Polymerase Chain Reaction (PCR)

The PCR technique was used for molecular detection based on the 16SrRNA gene (NCBI; NR_044761.1) with the following primer pairs (22F: GCTAAGAGATCAGCCTATGTCC and 22R: TGGCAATCAGCGTCAGGTAATG). The 25 µl PCR reaction included 12.5 µl of PCR MasterMix (2x) taq polymerase (Promega company, M7822), 1 µl of extracted DNA, 1 µl for each primer, and 9.5 µl DNase-free water. The PCR thermal cycles were carried out as follows: the DNA was first denatured for three minutes at 94°C, followed by forty cycles of denaturing at 94°C for thirty seconds, annealing for thirty seconds at 58°C for joining primers, extension for one minute at 72°C, and final extension for five minutes at 72°C. To examine the PCR product, 0.5% agarose gel with ethidium bromide. The GeneRuler 100 bp DNA ladder was utilized in conjunction with staining to evaluate the amount and caliber of the PCR results (20).

Sanger sequencing

Sequence-based identification of bacteria is more objective and accurate than conventional methods, especially for classifying unusual microorganisms that are emerging pathogens in immunocompromised hosts (21). Two primer pairs (758F, 907R, 27F, and 1492R) employed in this study were targeted 16srRNA gene (Table 1). Sanger sequencing was performance at Macrogen company (Korea) , following their standard protocol.

Table 1: Sanger sequencing primers .

Primer name	Primer sequence (5'-3')	Ref.
758F	GGA TTA GAT ACC CTG GTA	
907R	CCG TCA ATT CMT TTR AGT TT	Macrogen
27F	AGA GTT TGA TCM TGG CTC AG	
1492R	TAC GGY TAC CTT GTT ACG ACT T	

Antibiotic susceptibility test

The antimicrobial susceptibility testing (AST) was performed based on the Kirby Bauer disk diffusion method with the following eight antibiotics (Gentamicin, Amikacin, Ceftriaxone, Levofloxacin, Azithromycin, Erythromycin, Piperacillin-Tazobactam, and Trimethprime). (Table 2). These antibiotics are belonging to six different antibiotic's classes (Aminoglycoside, Cephalosporin, Fluoroquinolone, Macrolide, Pencillin-βlactamase and Sulfonamides)

which are commonly used antibiotics following Clinical Laboratory Standards Institute standards (CLSI, 2020).(22). Shortly, Bacterial colonies freshly cultured on Nutrient agar to performed antibiotic susceptibility test, the isolates were suspended in a sterile normal saline and standardize with 0.5% McFarland (a 0.5 McFarland standard corresponds to approximately 1.5 x 108 CFU/mL). Then, 0.2 mL of culture suspension was spread on the sterile Mueller Hinton (MH) agar plate (Liofilchem, Italy). Antibiotic discs (Liofilchem,Italy) were placed on the plate using sterile forceps under aseptic conditions and incubated at 37°C for 24 h. After incubation, the zones of inhibition were measured in millimeter (mm) (23).

Table 2: Antibiotic information; classification, Abbreviations, and applied discs concentration.

Antibiotic Class	Antibiotic Name	Abbreviations	Disc Concentration (µg)
Aminoglycoside	Gentamicin	CN	10
	Amikacin	AK	30
Cephalosporin	Ceftriaxone	CRO	30
Fluoroquinolone	Levofloxacin	LEV	5
Macrolide	Azithromycin	AZM	15
	Erythromycin	E	15
Penicillin-βlactamase	Piperacillin-Tazobactam	TZP	110
Sulfonamides	Trimethoprim	TM	5

Results and discussion

Isolation and molecular diagnosis :

In this study, 61 patients (male and female) who suffered from gastrointestinal disorder (abdominal pain, diarrhea, vomiting, Anorexia and stomach ulcer) were included and the majority of them were females (58.7%). After the samples, stomach biopsies, were successfully transferred under sterile circumstances and quickly cultivated on Columbia base agar, bacterial growth was confirmed in 54.09% (33/61). The bacteria remained capable of growth and preserved their viability even after an extended period of storage. These samples went through biochemical (traditional) and molecular (modern) identification techniques. All isolates were gram-negative rod-shaped catalase positive, oxidase positive, and 64.0% urease positive, (figure1). (*Achromobacter anxifer*, *Pseudomonas aeruginosa*, and *Pseudomonas nitroreducen*) and 36.0% were urease negative (*Klebsiella pneumonia, Shigella flexneri*, *Stenotrophomonas maltophilia*, and *Uncultured* Enterobacteriaceae bacterium).

The extracted DNA integrity was very good with 15 ng/µl average concentrations for all samples. To identify isolated bacteria with molecular techniques, PCR and Sanger sequencing were used. We identified six distinct types of bacterial species, 8.0% (2/25) isolates were identified as *Achromobacter anxifer*, 16.0% (4/25) as *Klebsiella pneumonia,* 52.0% (13/25) as *Pseudomonas aeruginosa*, 4.0% (1/25) as *Pseudomonas nitroreducen*, 12.0% (3/25) as *Shigella flexneri*, 4.0% (1/25) as *Stenotrophomonas maltophilia*, and 4.0% (1/25) as Uncultured *Enterobacteriaceae* bacterium .(Table 3). Compared with worldwide bacteria on the NCBI by drawing species trees, we identify the following bacteria (*A.anxifer, K.pneumonia, P.aeruginosa, P.nitroreducen, S.flexneri, S.maltophilia*),(figure 2). In the best of our knowledge the following bacterial species (*A. anxifer, P. nitroreducens,*

S. flexneri, S. maltophilia) were identify for the first time in Iraqi patients who had gastrointestinal problems.

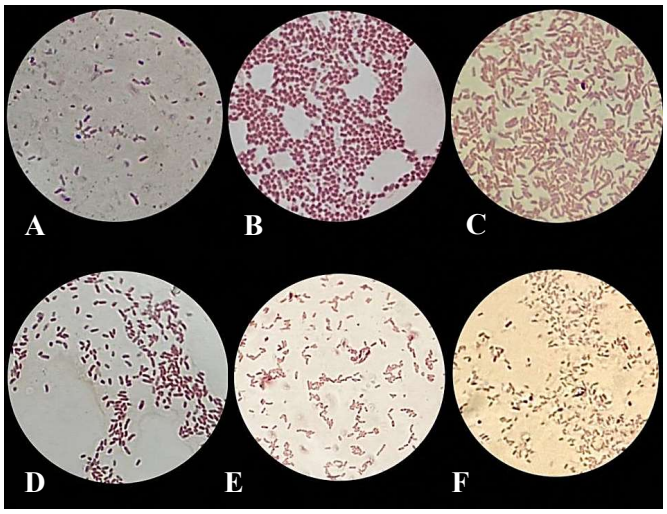
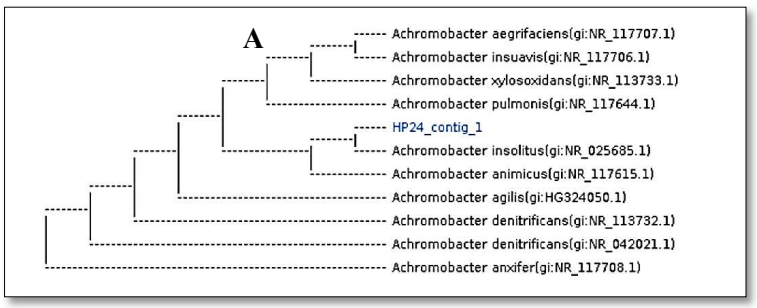


Figure 1: Gram staining results: (A) *A.anxifer, (B) K.pneumonia, (C) P.aeruginosa,(D) P.nitroreducen, (E) S.flexneri, (F) S.maltophilia.*

Table 3: bacterial species with their biochemical tests.

Bacteria Isolate	N	(%)	Biochemical Tests		
			Catalase	Oxidase	Urease
A.anxifer	2	8.0%	+	+	+
K.pneumonia	4	16.0%	+	+	-
P.aeruginosa	13	52.0%	+	+	+
P.nitroreducen	1	4.0%	+	+	+
S.flexneri	3	12.0%	+	+	-
S.maltophilia	1	4.0%	+	+	-
Uncultured *Enterobacteriaceae* bacterium	1	4.0%	+	+	-



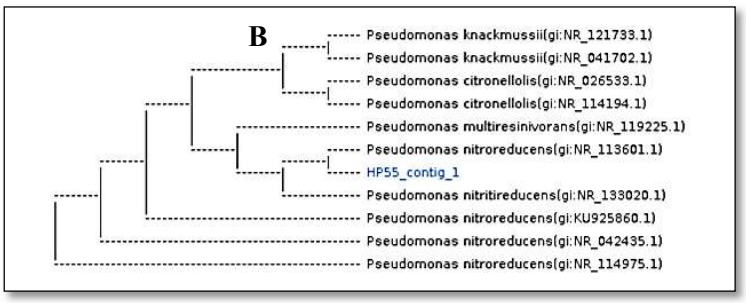


Figure 2: Phylogenetic trees: These trees illustrated the sanger sequence results for isolated and identified bacteria (A) *A.anxifer*, and (B) *P.nitroreducens.*

The study found high contamination rates in gastrointestinal endoscopies at al-Zahraa and al-Karama teaching hospitals, primarily due to inadequate cleaning and sterilization. However, al-Zahraa showed slightly lower contamination rates, likely due to marginally better hygiene and sterilization practices.

A. anxifer, P. aeruginosa, P. nitroreducens, and *S. maltophilia,* are more likely to be considered a bacterial-exogenous contaminants. They are nosocomial pathogens and can also cause opportunistic infections in immunocompromised hosts, such as bloodstream infections, skin infections, urinary tract infections, and respiratory tract infections (25,26). However, it should be kept in mind that each species has its own characteristics with regard to preferred site of colonization, routes and vectors of transmission, and clinical spectrum (27). A bacterial endogenous contaminant is another way to get gastrointestinal endoscope contamination such as *K.pneumonia,* it can be part of the gastrointestinal and nasopharyngeal flora, because it is well known that gastrointestinal endoscopes are grossly contaminated with patient's native flora (28). It may result an outbreak and increases the risk of cross-contamination (29). Similar studies conducted in the United States of America, the United Kingdom, the Netherlands, Germany, and China discovered that gram-negative bacteria, such as *E.coli, K.pneumoniae, P.aeruginosa, S.maltophilia,* were associated with contaminated gastrointestinal endoscopes (30). Other studies carried out in France, reported that *A.anxifer, P.aeruginosa*, and *S.maltophilia* were a bacterial contamination (31).

S. flexneri is an enteric pathogen, it has ability to survive at low acidity of the host's stomach via up-regulating the expression of acid resistance genes (32). Recent evidence supports an alternative model in which *Shigella* primarily infects a much wider range of epithelial cells in gastrointestinal that reside primarily in the colon and small intestine (33), In this study, we identified *Shigella* as a secondary or opportunistic infection in stomach. The process by which *Shigella* infects the gut mucosa is not entirely understood. Shigellosis Infection can have serious consequences, and *S. flexneri* causes more mortality than any other *Shigella* species (34).

Antibiotic susceptibility test

The bacterial isolates showed varied degrees of susceptibility and resistance towards antibiotics were used in this study (Figure 3 and Table 4)

Table 4: Susceptible of bacterial isolates to different antibiotic.

cterial species	Antibiotics															
	AZM		AK		CRO		E		CN		LEV		TZP		TM	
	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)
nxifer	0	100	100	0	0	100	0	100	100	0	100	0	100	0	0	100
neumoniae	0	75	100	0	0	100	0	100	75	25	100	0	100	0	50	50
eruginosa	0	91.6	100	0	8.3	91.6	0	100	83.3	8.3	100	0	100	0	0	100
itroreducens	0	100	100	0	0	100	0	100	100	0	100	0	100	0	100	0
exneri	0	100	100	0	0	100	0	100	100	0	100	0	100	0	0	100
altophilia	100	0	100	0	100	0	0	100	100	0	100	0	100	0	0	100
ultured	0	100	100	0	0	100	0	100	100	0	100	0	100	0	0	100
erobacteriaceae																

NOTE: 25% of *K. pneumonia* had an intermediate response to Amikacin, and 8.3% of *P. aeruginosa* had an intermediate response to Azithromycin and Gentamycin.

Interestingly, our results (Table 4) showed that all bacterial isolates were 100% sensitivity to levofloxacin, piperacillin/tazobactam and Amikacin (except *K.pneumonia.* It was 75% sensitive and 25% intermediate). Levofloxacin belongs to Fluoroquinolone class, interferes with critical processes in the bacterial cell, such as DNA replication, transcription, repair and recombination. It inhibits type II topoisomerases. levofloxacin represents a valid therapeutic option in the treatment of severe Gram-negative nosocomial infections (35).

Piperacillin/tazobactam, belong to Penicillin-βlactamase classes, irreversible inhibitor of bacterial β-lactam/β-lactamase inhibitor combination respectively. These antibiotics are considered to be effective for the treatment of patients with intra-abdominal infections, skin and soft tissue infections, lower respiratory tract infections, and complicated urinary tract infections (36). Amikacin belongs to Aminoglycoside works by attaching to the 30S subunit of bacterial ribosomes, interfering with the decoding of genetic information. This disruption halts protein synthesis, leading to premature termination of polypeptide chains and the incorporation of incorrect amino acids into proteins. Such errors ultimately impair bacterial growth and survival, making Amikacin a potent antibiotic against various bacterial infections (37).

The results of current study are in line with recent researches by Anderson, *et al*.(2008), Gin, *et al*.(2007) and Chen, *et al*.(2021), They found these antibiotics are an effective treatment option for intra-abdominal infections, skin and soft tissue infections, and lower respiratory tract infections. It is well-tolerated with a strong safety profile, making it a dependable choice for the empiric treatment of moderate to severe infections in hospitalized patients (38,39,40). On other hand, Gauba *et al*.(2023) reported that *K.pneumoniae* and *P. aeruginosa* had high-level resistance to the following antibiotics : Amikacin and Piperacillin-Tazobactam (41).

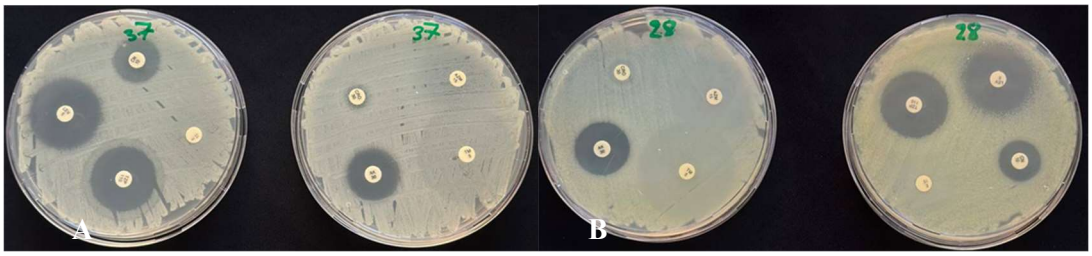


Figure 3: Antimicrobial susceptibility testing performed based on the Kirby Bauer disk diffusion method: (A)Sensitivity of *P.aeruginosa* to Levofloxacin, Gentamycin, Amikacin, Piperacillin-Tazobactam. (B)Sensitivity of

S. flexneri to Levofloxacin, Gentamycin, Amikacin, Piperacillin-Tazobactam.

Bacterial isolates showed 88% resistant to Azithromycin, Ceftriaxone, (except *S.maltophilia*), Azithromycin is a new macrolide antibiotic with a better activity against intracellular gram-negative bacteria, (42), in comparison with Erythromycin (100% resistant). Azithromycin acts by binding to the 50s ribosomal subunit of susceptible microorganism and interfering with microbial protein synthesis (43), while Erythromycin acts by inhibition of protein synthesis by binding to the 23S ribosomal RNA molecule in the 50S subunit of ribosomes in susceptible bacterial organisms (44).

Our results agreed with what Danny *et al.*(2022) found. They concluded patients treated with azithromycin required follow-up endoscopy less frequently than those treated with erythromycin, (45);However, it was stated by Yusuf *et al*.(2021) that revealed Efforts to enhance erythromycin's efficacy focus on modifying its delivery methods or pairing it with agents that counteract bacterial resistance mechanisms, improving its overall antibacterial activity (46).

Bacterial isolates showed 88% resistant to Ceftriaxone, that is belong to cephalosporin. Ceftriaxone works by disrupting bacterial cell wall formation through its interaction with penicillin-binding proteins, These proteins are essential for the cross-linking of peptidoglycan, a key structural component of the bacterial cell wall, and their inhibition leads to the weakening and eventual destruction of the bacterial cell (47).

The results of this research are consistent with the findings of a recent study by Odenholt *et al*.(1998), which reached that gram-negative bacteria resist Ceftriaxone in comparison with gram-positive bacteria (48), But according to bushra *et al*.(2016), it was disclosed that Ceftriaxone highly sensitive against gram-negative bacteria, however progressively decreasing in comparison with last studies (49).

Gentamicin was sensitive 88%, resistance 8%, intermediate 4% against bacterial isolates. Gentamicin belongs Aminoglycoside, act by passes through the gram-negative membrane in an oxygen-dependent active transport (50). Results of this investigation agree with those of a recent study that came to a similar conclusion Charles F *et al*.(1971), (51). However, this result was disprove by Ahmed *et al*.(1989), (52). Bacterial isolated showed 88% resistance and 12% sensitivity to Trimethoprim. Trimethoprim belongs to Sulfonamides and behaves as inhibitors of efflux in Gram-negative bacteria (53). Results of this study are consistent with recent research that yielded a similar conclusion by Heller *et al*.(2017), (54).

However, unlike other bacterial isolates, *S.maltophilia* was 100 % sensitive to Azithromycin and Ceftriaxone. It is typically resistant to many antibiotics due to its intrinsic and acquired resistance mechanisms, but its sensitivity to certain antibiotics like azithromycin and ceftriaxone can occur under specific conditions, may be due to disruption of quorum sensing or biofilm formation, which plays a role in the virulence of *S. maltophilia*, Environmental or clinical factors influence the expression of resistance genes, and Certain strains express lower levels of beta-lactamase enzymes capable of degrading ceftriaxone (55).

Conclusion

Gastrointestinal disorders affect a lot of people each year and represent significant worldwide health problems. Cross- infection/ contamination of gastrointestinal endoscopes is one of the most issue that health care providers have to overcome. In this study, we isolated (54%) bacteria from stomach biopsies taken from the gastric corpus or antrum of patients who had gastrointestinal disorders. By implying biochemical techniques (Gram stain, catalase, oxidase and urease test) and molecular techniques (PCR technique and Sanger sequence).

the following bacterial species were identified for the first time in Iraqi patients : *A. anxifer, P. nitroreducens, S. flexneri* and *S. maltophilia* In addition, *K. pneumonia* and *P. aeruginosa,* The most common contaminated and opportunistic bacteria, were identified as well. Worth mentioned that the molecular identification especially Sanger sequencing were the most accurate and reliable technique to identified the isolated bacteria.

This research highlights the critical issue of contamination in gastric endoscopy procedures, which has been shown

to facilitate the transmission of infections among patients due to use in different patients and insufficient disinfection and sterilization procedures in hospitals. Our findings underscore a concerning lapse in sterilization protocols across various points of contact, including medical staff, operating rooms, and endoscopic equipment. Enhanced focus on infection control, regular microbial assessments, and advanced sterilization practices are crucial to safeguard patient health and prevent the emergence of treatment-resistant pathogens in clinical settings. Despite the significant dangers associated with these microbial infections or contaminants, insufficient attention remains to the types of resistant bacteria that may lead to severe, widespread infections. Antibiotic susceptibility results revealed significant sensitivity and resistance patterns among the tested bacterial species. Amikacin, Gentamicin, Levofloxacin, Piperacillin-Tazobactam demonstrated the highest sensitivity across most bacterial species, while Azithromycin, Erythromycin, Ceftriaxone and Trimethoprim exhibited the highest resistance rates. These findings emphasize the critical need for targeted antibiotic therapies. The emergence and spread of resistance in Enterobacteriaceae are complicate the treatment of serious gastrointestinal infections and threatens to create species resistant to all currently available antibiotics.

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