Research Article

Identification and Isolation of Bacterial Strains and Study of their Ability to Degrade Trimethoprim in the Wastewater of some Kirkuk Hospitals

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Abstract | The current study aimed to identify and isolate bacterial strains that can degrade pharmaceuticals, which will help reduce contamination. From April 2023 to April 2024, 185 samples were taken monthly from three locations, the effluents of Kirkuk City's Hospitals. Kirkuk Teaching Hospital, the Pediatrics Hospital, and the Maternity Hospital. Typically, sampling began at nine in the morning and ended at twelve. Water samples were taken from surface water at a depth of 30 to 40 cm using autoclaved amber bottles that had been pre-washed twice with water samples before being filled. The results demonstrated two categories of isolation: mixed bacterial isolates (78; 70.9%) and single bacterial isolates (32; 29.1%). *Proteus mirabilis*, *Escherichia coli*, *Staphylococcus*, *Enterococcus*, *Streptococcus*, and other isolates had percentages of 8.2%, 31.8%, 19.1%, 35.5%, and 4.5%, respectively. The 16S RNA gene was verified using the multiplex PCR technique regarding the genetic investigation. The existence of a gene was detected by the presence of a single band at a given molecular weight: 232 bp, 1250 bp, 1500 bp, and 791 bp for the 16SrRNA of the marker used for *E. coli*, *Klebsiella spp*., *P.mirabilis*, and *Staphylococcus* spp. Regarding biodegradation, none of the examined microorganisms could break down trimethoprim, and it was clear that the drug exhibited a solid resistance to biodegradation . Although TMP is light-sensitive, the abiotic control demonstrated that during the experimentation period, photodegradation did not occur.

Keywords: Biodegradation, Wastewater, Trimethoprim, Bacteria, Kirkuk City, Hospitals.

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INTRODUCTION

Water is essential for maintaining an adequate food supply and a productive environment for the human population, animals, plants and microbes worldwide Cunningham et al. (2001); Shekha, (2008). Increased water usage coupled with growing populations and economies would affect biodiversity throughout the global ecosystem and drastically reduce per-person access to water Shekha, (2008); Ahmed, (2021). It is estimated that 20% of people on Earth do not have access to clean drinking water, and almost 50% do not have proper sanitation. Over 95% of untreated urban sewage discharged into surface waters occurs in many developing countries, making this a serious issue. Nine Middle Eastern countries lack enough freshwater, including Iraq Pimentel et al. (2004). The water that surface water bodies get via runoff, irrigation, and wastewater (non-point source) is a factor in the problem. The degree of soil contact, the type of water body, the intended use, the hydraulic retention period, and the role that wastewater plays in ecosystems all influence the impacts.

Jimenz, (2006). Hospital wastewaters are extremely complex effluents that potentially contain multidrug-resistant (MDR) genes Emmanuel et al. (2005), as well as bacteria, excrement from patients, disinfectants, and metabolized

pharmaceuticals Guo et al. (2014); Ahmed et al. (2023); Hussein et al. (2019). The interchange of antibiotic-resistance genes is thus thought to occur in hospital wastewaters, hotspots for antibiotic resistance. Multiple bla genes, such as blaNDM, blaKPC, blaCTX-M, and blaSHV, are increasingly observed in hospital-associated gram-negative bacteria Chagas et al. (2011); Zhang et al. (2021). So, the current study aimed to test the ability of isolated bacteria to consume and degrade different pharmaceuticals.

MATERIALS AND METHODS

Sample Collection

At monthly intervals from April 2023 to April 2024, 185 samples were collected from three sites: hospital effluents in Kirkuk City, Kirkuk Teaching Hospital, Pediatrics Hospital, and Maternity Hospital. Sampling typically began at 9 a.m. and ended at 12 p.m. Water samples were collected from surface water (30-40 cm depth) in autoclaved amber bottles pre-washed twice with water samples before filling.

Bacterial Identification

Bacteria were diagnosed based on the following aspects:

Morphological diagnosis and media characteristics

Based on the culturing features of the bacteria, colonies developing on blood agar, EMB, and MacConkey agar were diagnosed after 24 hours of incubation at 37 0C.

Microscopic examination

Using a microscope to examine the morphological characteristics of bacterial cells—specifically, how they contacted the gram stain, which indicates the shape and arrangement of the bacterial cells—colonies were found.

Biochemical reaction and motility test

Numerous biochemical tests, such as methyl red, citrate, urease, Voges-Proskauer, catalase, oxidase, KIA, and indole test, were carried out to identify and diagnose bacteria.

Identification of bacteria isolates via VITEK2

The newest generation of calorimetric technology, VITEK 2, is the gold standard for microbial identification. The processes were completed according to the guidelines provided by the manufacturer, Biomerieux.

Extraction of the bacterial DNA

We extracted the chromosomal DNA from twenty-three *S. aureus* isolates with the highest antibiotic resistance rate and the maximum hemolysin production efficiency. The extraction process complied with the kit's manufacturer's handbook guidelines.

Agarose Gel Electrophoresis

Agarose gel at varying concentrations (about 0.8% for the extracted DNA and 1.2–2% for visual verification of particular PCR results) was utilized to separate DNA fragments. The necessary concentration of agarose powder is achieved by your careful mixing with electrophoresis buffer and heating it in a microwave oven until it melts completely. Then, you pipette the DNA samples into the sample wells after being combined with 1/10 loading buffer. You assemble the device with the lid and power lines attached, and run a current through it. For the PCR product, the gel is typically run at 5 volts per cent for 1.5 hours, and for the plasmid DNA, for 2 hours. Digital cameras are used to capture images of the DNA bands after they become visible using a UV trans illuminator set at 365 nm wave length documentation.

Detection of bacteria by PCR Technique

PCR was utilized to identify bacterial strains using specific primers for the sections of the 16S rRNA gene that are single compared to other eubacteria species and conserved across bacterial strains, as indicated in Table (1).

Biodegradation

Pharmaceutical solution

A stock solution of trimethoprim (Sigma, CAS # 738-70-5) was produced in dimethylsulfoxide (DMSO, Sigma, CAS # 67-68-5) at approximately 12500 ppm and stored under nitrogen. The calibration curves and the biodegradation studies were performed using the stock solution.

Controls in biodegradation experiments

As was already indicated, abiotic degradation is a possibility for medications. Furthermore, throughout their proliferation, microbes emit metabolites that have nothing to do with pharmaceuticals. Three distinct types of controls were prepared for the biodegradation studies: biotic, abiotic, and control-containing dead biomass. Without the medication, the biotic control kept track of the biomass's growth. The preparation was intended to identify metabolites unrelated to the medications. The medicine was present in the media for the second type of control, known as abiotic controls, but no bacterium was present. The degradation of the medications under the experimental conditions was ascertained using the abiotic control. Lastly, autoclaved biomass and medications were introduced to the dead biomass control to check for sorption.

RESULTS AND DISCUSSION

Samples distribution

Three sites provided 185 samples for the current investigation. According to Table (2), 110 (59.5%) of the total samples showed positive results for bacterial growth when cultured on ideal cultured media, such as blood agar, mannitol agar and ManConkey agar.

Identification

The morphology, diameter and shapes of bacterial isolates

Table 1: The specific primer of 16sRNA gene of negative bacteria.

Bacteria	Primer	Sequence	Tm (°C)	GC (%)	Product
E. coli	Forward	5'- ATCAACCGAGATTCCCCCAGT-3'	55	50	232bp
	Reverse	5'- TCACTATCGGTCAGTCAGGAG-3'	55	42.1	
P. mirabilis	Forward	5'-AGAGTTTGATCCTGGCTCAG-3'	55	50	1500bp
	Reverse	5'-CTACGGCTACCTTGTTACGA-3'	55	42.1	
Klebsiella spp.	Forward	5'- AGAGTTTGATCCTGGCTCAG-3'	54.3	50	1250bp
	Reverse	5'- GGTTACCTTGTTACGACTT-3'	49.4	42.1	

Table 2: Distributed of samples based on results

Groups	Number	Percentage
-ve	75	40.5%
+ve	110	59.5%
Total No.(%)	185	100%

on blood agar, mannitol agar, chocolate agar and MacConkey agar were determined. Also, the microscopic and biochemical examinations, which included the specific tests for each type, were used to confirm the results of the biochemical identification. The results of Vitek-2 were identical to the biochemical tests. The results showed two kinds of isolation: single bacterial isolates 32(29.1%) and Mixed bacterial isolates 78(70.9%). Isolates percentages of *E. coli, P. mirabilis*, *Klebsiella spp.*, *Staphylococcus spp.*, *Enterococcus spp*. and *Streptococcus spp*. were 100%, 8.2%, 31.8%, 19.1%, 35.5% and 4.5% respectively, as Tables (3, 4, 5).

Table 3: The present study's bacterial isolate groups

%	No.	Microbial isolates
29.1	32	Single bacterial isolates
70.9	78	Mixed bacterial isolates
100	110	Total

Table 4: Isolates gram-negative bacterial percentages

Bacteria	No.	%
Escherichia coli	110	100
Proteus mirabilis	9	8.2
Klebsiella spp.	35	31.8

Table 5: Isolates gram-positive bacterial percentages

Bacteria	No.	%
Staphylococcus spp.	21	19.1
Enterococcus spp.	39	35.5
Streptococcus spp.	5	4.5

Genetic study

Gram-negative bacteria 16SrRNA gene of *E. coli*

In this study, a multiplex PCR was applied to confirm the presence of the 16SrRNA gene. The existence of the gene was detected by a single band at a given molecular weight, 232 bp, for the 16SrRNA marker used in Figure (1). The current results revealed that 110 110 E. coli isolates harbored 16SrRNA.

These findings are consistent with earlier research and supported by ribosomal RNA sequencing, a more potent method for identifying bacteria. Patel, (2001), effectively employs 16S rRNA gene sequencing in the clinical laboratory to identify bacterial pathogens. Woo et al. (2008), employed 16S rRNA gene sequencing in clinical microbiology labs to identify bacteria and find new species. Fattahi et al. (2013), created a PCR target using 16S rRNA to identify E. coli in rainbow trout. Furthermore, According to Patel, (2001), there are several reasons why the 16S rRNA gene sequence has been widely used in bacterial taxonomy research. These include the following: (i) the 16S rRNA gene is found in nearly all bacteria, frequently forming operons or multi-gene families; (ii) the gene's function has not changed over time, indicating that random sequence changes are a more accurate indicator of time (evolution); and (iii) the gene's length (1,500 bp) makes it suitable for use in informatics.

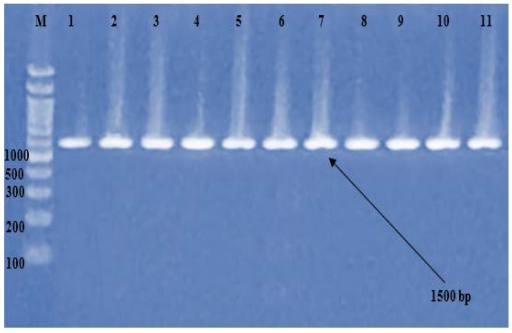

Figure 1: Amplification of a 232 bp 16s rRNA gene of E. coli isolates on agarose gel (2%) electrophoresed in 75 volts for one hr., M: molecular marker (Ladder), lanes 1 - 11 refer to isolates.

16SrRNA gene of *Klebsiella spp.*

In this study, a multiplex PCR technique was applied to confirm the presence of the 16SrRNA gene. The existence of the gene was detected by the presence of a single band at a given molecular weight—1250 bp, for 16SrRNA of marker that be used as in Figure (2). The current results revealed that 33 out of 35 *Klebsiella spp*. Isolates harbored 16SrRNA. *Klebsiella spp*. are commonly found in the environment and human flora. *Klebsiella pneumoniae* is a leading cause of hospital-acquired infections Jones, (2010). The difficulties in *Klebsiella* taxonomy are demonstrative and common to other bacterial genera Martínez et al. (2004). Genome alterations and behavioural changes were seen when a ground-based strain of *K. pneumoniae* was compared to a control strain following spaceflight, providing

insight into the genetic basis of drug resistance Song et al. (2003). Amplification of the 16S rRNA gene is a highly accurate and versatile way to identify bacteria for species identification, even when the species is notoriously difficult to identify using biochemical methods Saleh et al. (2019). It can be concluded that 16S rRNA sequence-based identification reduces the time by circumventing biochemical tests and increases specificity and accuracy. In addition, clinical and environmental samples can be identified and compared, and the source of microbes can be determined.

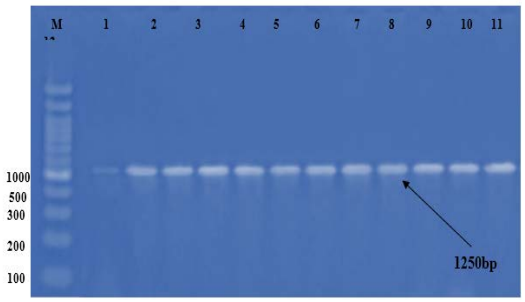

Figure 2: Amplification of a 1250-bp 16s rRNA gene of *Klebsiella spp.* isolates on agarose gel (2%) electrophoresed in 75 volts for 1 hr., M: molecular marker (Ladder), lanes 1 - 12 refer to isolates.

16SrRNA gene of *P. mirabilis*

In this study, a multiplex PCR technique was applied to confirm the presence of the 16SrRNA gene. The existence of the gene was detected by a single band at a given molecular weight—1500 bp—for the 16SrRNA marker that was not used as in Figure (3). The current results revealed that 9 of 9 P. mirabilis isolates harbored 16SrRNA.

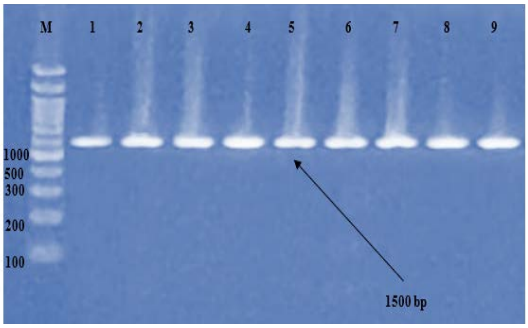

Figure 3: Amlification of a 1500-bp 16s rRNA gene of *P. mirabilis* isolates on agarose gel (2%) electrophoresed in 75 volts for 1 hr., M: molecular marker (Ladder), lanes 1 - 11 refer to isolates.

This study showed that using 16S rRNA in diagnosis is a selective force for *Proteus* recognition. Trending agreed with Saleh et al. (2019) which used a 16S rRNA test to recognize Proteus spp. Isolated from urinary tract infection patients. However, our results conflicted with those of Abed and Shareef, (2021), who carried out research in Iraq and demonstrated that the analysis of the study's gene sequence revealed a match in the sequence of the nitrogenous bases of the 16S rRNA gene of *P. mirabilis* local isolates with P. mirabilis global isolates that were saved in (NCBIGenebank). In contrast, our results produced a unique clone that differed from all eight isolates in the gene bank and could be referred to as mixing with foreign isolates. This could explain antibiotic resistance in many infections caused by the genus Proteus.

Biodegradation of trimethoprim

Three bacteria growing on glucose were exposed to TMP with *E. coli*. (fig; 4), P. mirabilis (fig; 5), and *Klebsiella spp.* (fig; 6). Because the experiment was carried out over a longer length of time, the results of the experiments with *E. coli, P. mirabilis*, and *Klebsiella spp*. are shown separately in figures (4), (5), and (6). Since none of the examined microorganisms could break down trimethoprim, it was clear that the drug exhibited a solid resistance to biodegradation. Although TMP is light-sensitive, the abiotic control demonstrated that photo-degradation did not occur during experimentation. The majority of the samples needed more weight. However, because the concentration of TMP utilized was significantly higher and less impacted by slight changes in volume, the evaporation had less impact than in earlier experiments.

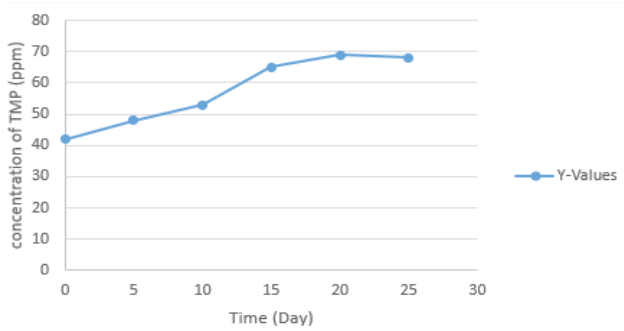

Figure 4: Biodegradation experiment of TMP with *E. coli.*

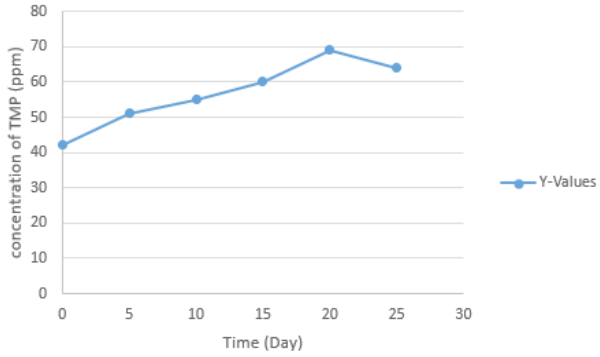

Figure 5: Biodegradation experiment of TMP with *P. mirabilis*

CONCLUSIONS

The current work demonstrated that Trimethoprim has a solid resistance to biodegradation, as none of the bacteria tested were able to break it down. TMP is light sensitive, but the abiotic control showed that even photo-degradation did not occur during the experiments.

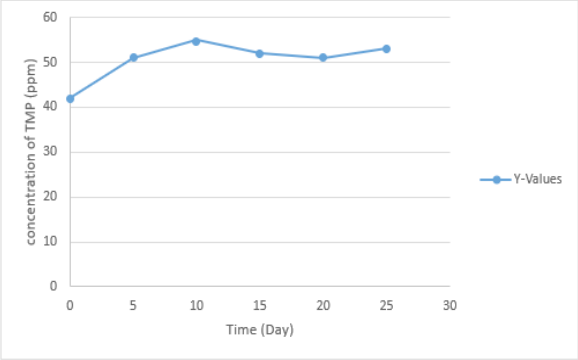

Figure 6: Biodegradation experiment of TMP with *Klebsiella spp*

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Tawoos and Khalid contributed to the conception of the manuscript, acquisition of data, drafting, and final approval of the manuscript. Sameerah contributed to the acquisition of data, revising the manuscript. All authors read and approved the final manuscript.

REFERENCES

Abed M. K., Shareef H. K. (2021). Isolation and Molecular Identification of proteus mirabilis isolated from hospitals in the capital Baghdad. Indian J. Forensic Med. Toxicol., 15(1): 2216-2223.

Ahmed T. M. K. (2021). Detection lipase gene of Pseudomonas aeruginosa from crude oil contaminated soil. In IOP Conference Series: Earth and Environmental Science (Vol. 790, No. 1, p. 012051). IOP Publishing. https://doi.org/10.1088/1755-1315/790/1/012051.

Ahmed T. M. K., Mohammed S. F., Yosif K. M. (2023). Biodegradation of Crude Oil Contaminated Soil in North Oil Refineries/Kirkuk/Iraq by Using Acinetobacter spp. and Streptomyces spp. J. Survey Fisher. Sci., 10(3S): 2286-2293.

Chagas T. P. G., Seki L. M., Cury J. C., Oliveira J. A. L., Dávila A. M. R., Silva D. M., Asensi M. D. (2011). Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. J. Appl, Microbiol., 111(3): 572-581.https://doi.org/10.1111/j.13652672.2011.05072.x

Cunningham W. P., Cunningham M. A., Saigo B. W. (2001). Environmental science: A global concern (Vol. 412). New York: . 6th.Ed. McGraw-Hill. USA. 646pp.

Emmanuel E., Perrodin Y., Keck G., Blanchard J. M., Vermande P. (2005). Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network. J. Hazardous Mat., 117(1): 1-11. https://doi.org/10.1016/j.jhazmat.2004.08.032

Fattahi F., Mirvaghefi A., Farahmand H., Rafiee G., Abdollahi A. (2013). Development of 16s rRNA targeted PCR method for the detection of Escherichia coli in rainbow trout (*Oncorhynchus mykiss*).

Guo Y., Li Y., Su L., Chang D., Liu W., Wang T., Liu C. (2014). Comparative genomic analysis of *Klebsiella pneumonia* (LCT-KP214) and a mutant strain (LCT-KP289) obtained after spaceflight. BMC Genom., 15: 1-11.

Hussein Farhan K., Mahmoud Ashraf J, Yousif Buthainah J. (2020)."Estimation of Immunoglobulin A, Immunoglobulin G, and Immunoglobulin M Antibody Levels in Laboratory Mice Balb/c Infected with Entamoeba histolytica and Treatment with Aqueous Extracts of Cyperus rotundus and Thymus serpyllum," Polytech. J. Vol. 10: Iss. 1, Article 21. https://doi.org/10.25156/ptj.v10n1y2020.pp126-129

Jimenz B. (2006). Irrigation in developing countries using wastewater. Intern. Rev. Environ. Strateg., 6(2): 229-250.

Jones R. N. (2010). Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. Clin. Infect. Dis., 51(Supplement_1): S81-S87. https://doi.org/10.1086/653053

Martínez J., Martínez L., Rosenblueth M., Silva J., Martínez-Romero E. (2004). How are gene sequences analyses modifying bacterial taxonomy? The case of Klebsiella. Int. Microbiol., 7(4): 261-268.

Patel J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Molecul. Diagn., 6: 313-321.

Pimentel D., Berger B., Filiberto D., Newton M., Wolfe B., Karabinakis E., Nandagopal S. (2004). Water resources: agricultural and environmental issues. BioScience., 54(10): 909-918. https://doi.org/10.1641/0006-3568(2004)054[0909:WRAAEI]2.0.CO;2

Saleh, T. H., Hashim S. T., Malik S. N., AL-Rubaii B. A. L. (2019). Down-regulation of flil gene expression by Ag nanoparticles and TiO2 nanoparticles in pragmatic clinical isolates of Proteus mirabilis and Proteus vulgaris from urinary tract infection. Nano Biomed. Eng, 11(4): 321-332. https://doi.org/10.5101/nbe.v11i4.p321-332.

Shekha Y. A. (2008). The effect of Erbil city wastewater discharge on water quality of Greater Zab river, and the risks of irrigation. PhD, university of Baghdad.

Song Y., Liu C., McTeague M., Finegold S. M. (2003). 16S ribosomal DNA sequence-based analysis of clinically significant gram-positive anaerobic cocci. J. Clin. Microbiol., 41(4): 1363-1369. https://doi.org/10.1128/jcm.41.4.1363-1369.2003

Woo P. C., Lau S. K., Teng J. L., Tse H., Yuen K. Y. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin. Microbiol. Infect., 14(10): 908-934. https://doi.org/10.1111/j.1469-0691.2008.02070.x

Zhang X., Lü X., Zong Z. (2012). Enterobacteriaceae producing the KPC-2 carbapenemase from hospital sewage. Diagnost, Microbiol. Infect. Dis., 73(2): 204-206.