

Biological characterization of some virulence factors of *Stenotrophomonas maltophilia* isolated from urine samples of prostate and bladder cancers patients

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Abstract

Stenotrophomonas infections are becoming more widespread around the world. This study included 120 urine samples collected from AL-Amal National Cancer Hospital and Ghazi AL-Hariri Specialized Surgery Hospital / Medical City Hospital, Baghdad/Iraq, cultured on blood agar and MacConkey, then diagnosed by VITEK compact 2 system and studying the virulence factors of *Stenotrophomonas maltophilia* which included motility, lecithinase production assay, protease activity, lipase activity assay, biofilm formation using tissue culture plate method and siderophore production using M9 medium, and determining the inhibitory effects of Disodium ethylenediaminetetraacetic acid EDTA and curcumin to evaluate the minimum inhibitory concentrations MIC, the results indicated that only 87/120 (72.5%) of urine sample were positive for bacterial growth including different species of bacteria, percentage of bacteria *Stenotrophomonas maltophilia* was 7/87(8%) from the total bacterial growth, all isolates were showed beta hemolysis activity, motility, and able for producing lecithinase, protease, lipase, the ability to produce biofilm and siderophore, EDTA concentration that inhibit the growth of bacteria was 100, 80 mg/ml which gave inhibition zones of 26mm and 15 mm respectively and curcumin showed antibacterial activity, and the curcumin concentration that inhibit the growth of bacteria was 260 mg/ml which gave an inhibition zone of 14 mm.

Keywords: *Stenotrophomonas maltophilia*, Bladder cancer, Prostate cancer.

INTRODUCTION

Urinary tract infections UTIs are caused by microbial pathogens in the urethra or bladder (lower urinary tract). Obstruction of the urinary tract, usage of a bladder catheter, and a reduced in the immune system are all significant risk factors for recurrent UTI 2, and in individuals with obstructed prostate, urinary tract infection reduces their health-related quality of life and general well-being¹. *Stenotrophomonas maltophilia* is one of 20 species in the genus *Stenotrophomonas*, as first isolated from a patient for the first time in 1943, and has since undergone multiple taxonomic upgrades before being awarded its own genus in 1993.

Potential virulence factors include those involved in adherence to plastics, and production of exoenzymes such as elastase and gelatinase and the following risk factors have been linked to *S. maltophilia* infections: Central venous catheterization, cystic fibrosis, ICU stay, malignancy, mechanical ventilation, neutropenia, prior broad-spectrum antibiotic therapy, recent surgery, and HIV infection, Infections in the respiratory or genitourinary tract, bacteremia, and, on rare occasions, wound infections are common, while infections of other body locations do occur 4. So, the aim of current study is the biological characterization of some virulence factors

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like biofilm, enzymes and toxins of *Stenotrophomonas maltophilia* that isolated from urine samples of prostate and bladder cancers patients, by detection their activity in bacterial extract as well as studying the effects of some inhibitors on these virulence factors.

MATERIALS AND METHODS

Patients and sampling

A total of 120 urine samples were collected from AL-Amal National Cancer Hospital and Ghazi AL-Hariri Specialized Surgery Hospital / Medical City Hospital, Baghdad Iraq, in period extended from October 2021 to January 2022. Samples included, 27 urine samples collected from 27 prostate cancer patients, 42 bladder cancer patient, 46 renal failure patients and 5 from UTI infection patients. Urine samples of included subjects were cultured on MacConkey agar, nutrient agar, blood agar, UTI medium, and vancomycin, imipenem and amikacin VIA new selective and differential medium for bacterial growth (incubated at 37°C for 24 hours), in order for further studies. Growth of bacteria was appeared only in cultured urine samples obtained from immunocompromised and nosocomial infection patients. Initial diagnosis of isolates based on morphological characteristics of the colonies that include colony shape, colony texture, color and edges were studied depending on bacterial growth on MacConkey agar, blood agar and VIA media. Bacteria were subjected for using VITEK-2 compact system according to the instructions provided by the manufacturing company.

Biofilm assay using tissue culture plate method

Overnight Bacterial growth was grown ON LB broth at 37 °C for 24hr. The culture was adjusted to 0.01 with McFarland solution of bacterial growth, 50µl was added to 150 µl of LB broth on tissue culture plate wells and incubated at 37 °C for 24 hrs. The culture was removed carefully, and wells were washed two times with 250µl distilled water. Then, 250 µl of (0.2%) of Crystal violet was added and incubated for 10 minutes at 25 °C. Wells were washed with distilled water 2-3 times and dried at room temperature. Finally, 200 µl of 95% ethanol was added to wells. Optical density (O.D) was measured at 630 nm. Interpretation producer according 5.

Siderophore Production

Siderophore production using M9 medium supplemented with glucose 20% and casmino acid 20% was prepared. Bacterial growth turbidity was adjusted to 0.01 with McFarland solution, then cultured in M9 medium supplemented with casmino acid and glucose (2 gm/L each), and incubating at 37 °C for 48hrs. Appearing growth in medium indicates positive results 6.

Enzymatic analysis

Isolates of *S. maitophilia* displaying α - and β - haemolysis were used to examine the production of the following extracellular enzymes.

Protrinase

Casein hydrolysis was tested by dissolved 2g agar agar in 100 ml of distill water and sterilized by autoclave then cooled in 45°C and added 10g of skim milk, make well on agar plate and inoculum 100µl of culture filtratetes of *S. maltophilia* strains and positive controls on to plates and incubating at 37°C for 24 h, the presence of transparent zone around the inoculum spot indicated a positive test 7.

Lipase

Tryptic soya agar supplemented with 1% Tween-80 (polyoxyethylene sorbitan monooleate) served as substrate to 100µl of each culture filtrates of *S. maltophilia*. The appearance of turbid halo around the inoculum spot was taken as a positive test 8.

Lecithinase

Lecithinas was determined utilizing an egg yolk (50%) agar base. 20 µl of the (50%) egg yolk was added to 150 ml of sterilized tryptic soya agar and served as substrate to 100µl of each culture filtrates of *S. maltophilia* 9.

Antibacterial activity

Antibacterial activity for EDTA and Curcumin

Disodium ethylenediaminetetraacetic acid (EDTA) was utilized as the chelating agent (EM Science, Gibbstown, N.J.). Sterile solutions at pH 8.0 were made by dissolving EDTA in sterile distilled deionized water (ddH₂O) and filtering through 0.45-mm filters (Nalge Nunc Intl., Rochester, N.Y.), to achieve concentrations of 200, 100, 80, 40, mg/mL. and for curcumin 260, 200, 100, 80, 40 mg/mL.

Bacterial colonies were inoculated into 10 mL of LB and cultured at 37°C for 24 hours. The bacteria were cultured on muller-hinton agar and wells was formed using clean tips before being incubated at 37°C for 24 hours, to test the antibacterial activity of EDTA and curcumin based on the diameter of the inhibition zone.

RESULTS

Patients Demography

In this study, general demographic criteria including gender, bacterial infection and type of cancer illustrated in figure (1). Age range of patients from (24-86) years,

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 mean age 55.5 ± 5 . Male to female ratio of patients were (76/120) 63.3% and (44/120) 26.7% respectively.

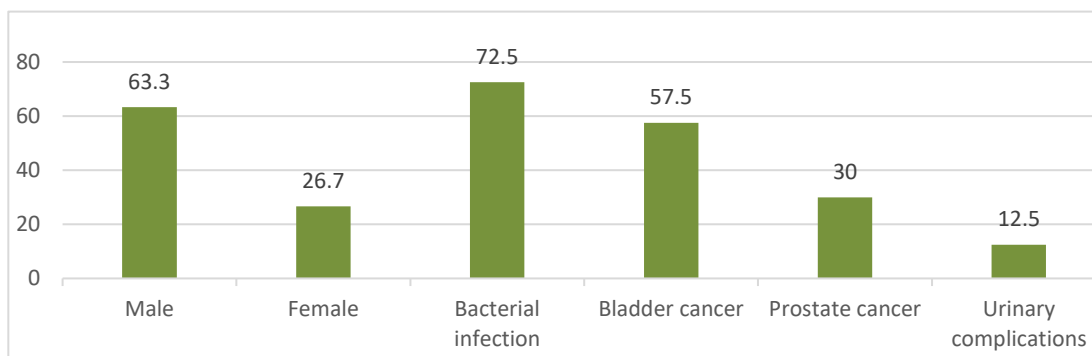


Figure 1: Patients demography

The age of patients with Bladder cancer ranged from 24 years to 86, while other urinary tract complications range from 30 to 83 years. For patients with prostate cancer ranged from 42 to 70, figure (2) reveals age group for all patients.

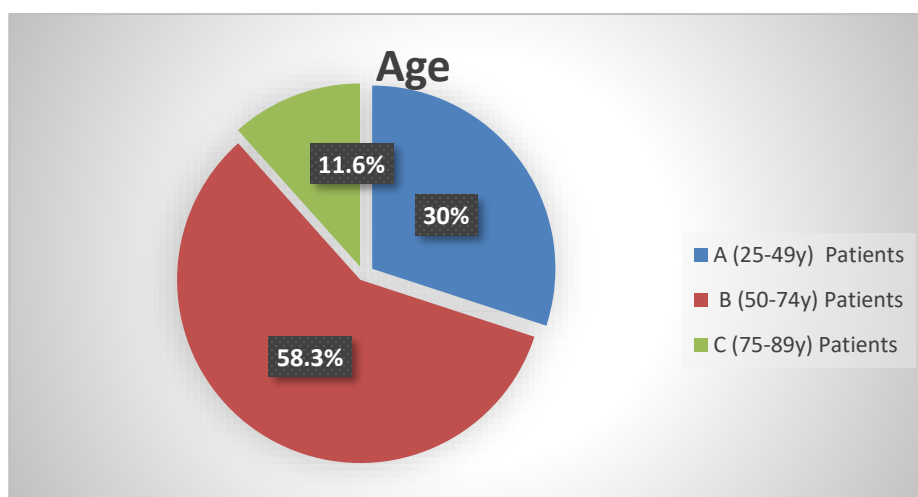


Figure 2: Distribution of patients according to age. Bacterial isolation and identification

Culturing of urine sample obtained from patients revealed that 87/120 (72.5%) samples were positive for bacterial growth including different species of bacteria. Bacterial growth was obtained from ordinary culture media including blood agar media, nutrient agar media, MacConkey agar Media and chromogenic agar UTI media. Primary diagnosis of bacteria depending on the morphological characteristics of colonies, hemolysis of blood, ferment lactose, then staining with gram stain and biochemical tests including Catalase, oxidase, IMViC, motility for further identification of bacteria and finally diagnosed by vitec 2 compact system which confirm the identification of bacteria depending on 49 biochemical tests.

After incubating aerobically on MacConkey agar, blood agar, and UTI agar plates at 37 Co for 24-48 hours, bacterial isolates were identified. *Stenotrophomonas maltophilia* colonies on MacConkey agar were non-lactose, with a regular edge, round, flat colonies, and a fruity odor, whereas blood agar gave beta hemolysis and pale colonies on Hichrome Uti medium, rods and negative staining with gram

stain under microscope.

This bacterium was identified using a selective enrichment procedure that included cultivating samples on MacConkey agar. The presence of bile salts and crystal violet in this medium stimulates the development of Enterobacteriaceae and related enteric Gram-negative rods while suppressing the growth of Gram-positive bacteria and some fastidious Gram-negative bacteria. Lactose is the sole carbon source in this medium that distinguishes lactose fermenting bacteria from non-lactose fermenting bacteria. The first is distinguished by the formation of transparent or colorless colonies. This technique was utilized to confirm a definitive *S.maltophilia* diagnosis. This method discovered germs faster, more efficiently, and away from contaminants that could inhibit pathogen identification. The findings of the tests utilized in this system confirmed the morphological and biochemical results. As a result, all 7 previously discovered *Stenotrophomonas* isolates have been proven to be *Stenotrophomonas*.

Virulence factors

Motility

Stenotrophomonas isolates were motile. The bacteria are motile if they move away from the stab line or have a hazy

appearance in the semisolid media, figure (3).

Hemolysin

Hemolysis was measured using trypticase soy agar with 5% blood, all isolates were giving beta-hemolysis for blood, figure (3).

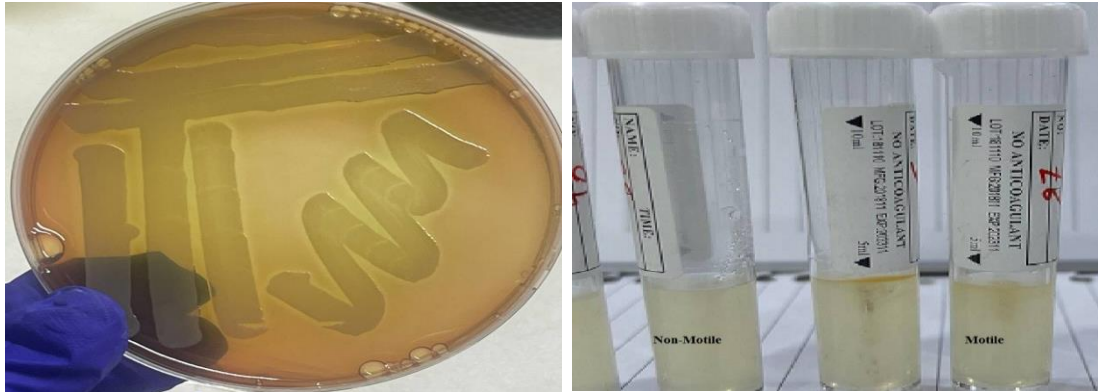


Figure 3: left: hemolytic activity of bacteria, right: motility test

lecithinase

Egg yolk was mixed with sterilized tryptic soya agar to investigate the production of lecithinase. A white precipitate around or beneath an inoculum site indicated the production of lecithinase, figure (4a).

Mueller-Hinton agar containing 3% (w/v) skimmed milk. A positive test was indicated by the existence of a transparent zone surrounding the inoculum site, figure (4b).

Protease

Proteinase activity was determined by hydrolyzing casein on

Lipase

Lipase activity was measured by the formation of a turbid halo surrounding the inoculum on trypticase soy agar plates supplemented with 1% Tween 80, figure (4c).

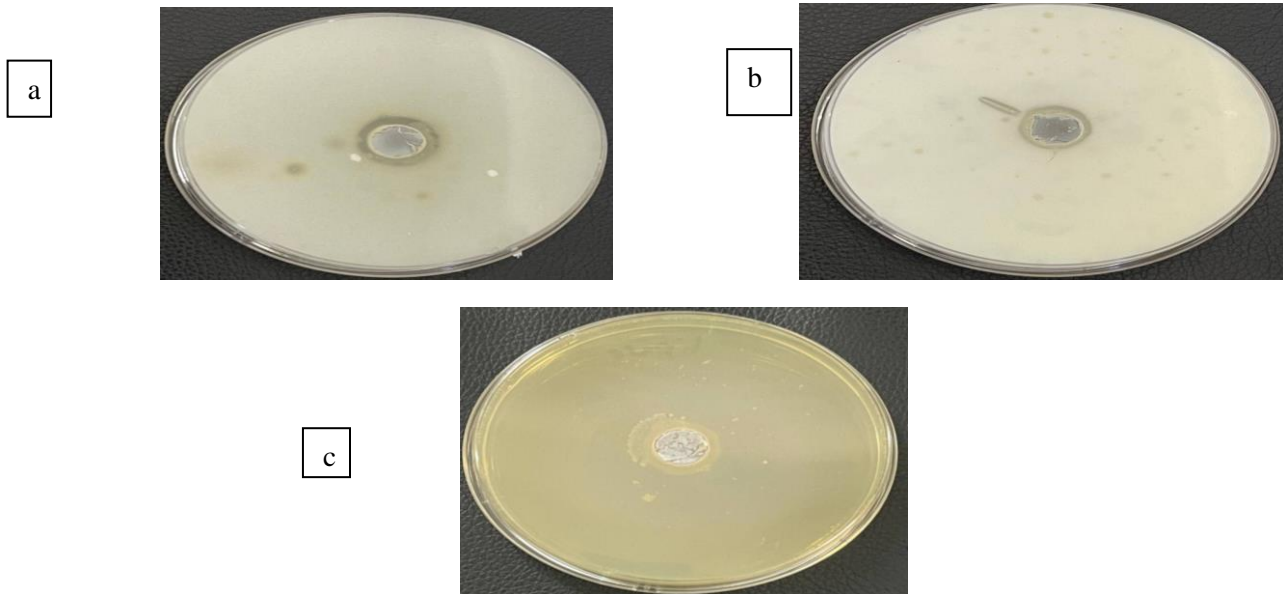


Figure 4: a- production of lecithinase, b- Protease activity, c-lipase production assay

Biofilm formation

The results of detection of biofilm formation of 7 isolates using TCP methods revealed that all isolate have the ability for biofilm formation as 5/7 (71%) isolates were moderate biofilm producer and 2/7 (29%) isolates were strong producer for biofilm, this test is a quantitative test to detect the production of biofilms gives a numerical value for absorption along the 570nm wavelength using ELISA Reader to determine the number of biofilms formed, figure

(5a).

Siderophore production

Siderophore production capabilities of 7 isolates utilizing M9 medium (supplemented with vitamin B12 Casamino Acids and glucose) revealed that 7/7 (100%) isolates were producers (growing on M9 medium) figure (5b).

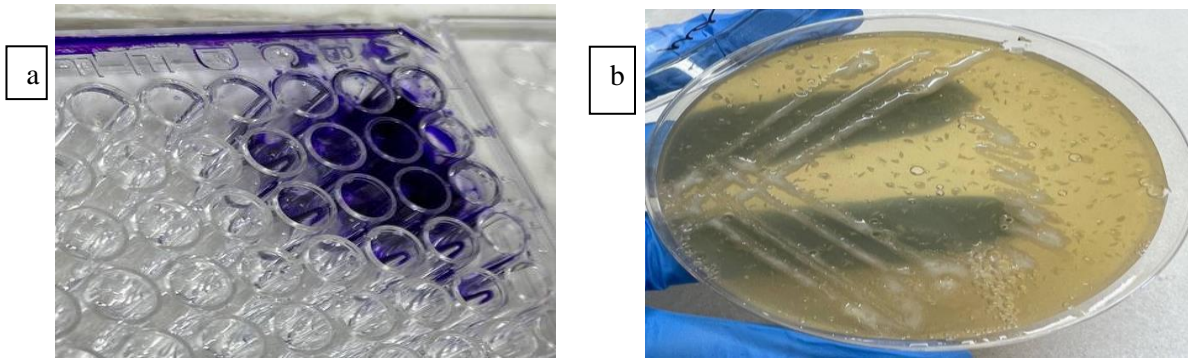


Figure 5: a- biofilm formation using TCP methods, b-Siderophore production using M9 medium

The effects of inhibitors against virulence factors of bacteria

Antibacterial activity of EDTA

The antibacterial activity of the EDTA, with concentrations ranging from 100mg/mL to 6.25 mg/mL, the concentrations that inhibit the growth of bacteria were 100, 80 mg/ml which give

inhibition zone 26mm and 15 mm respectively, figure (6a).

Antibacterial activity of Curcumin

The antibacterial activity of the curcumin, with concentration ranging from 260 mg/mL to 40 mg/mL, the concentration that inhibits the growth of bacteria was 260 mg/ml which gave an inhibition zone 14 mm, figure (6b).

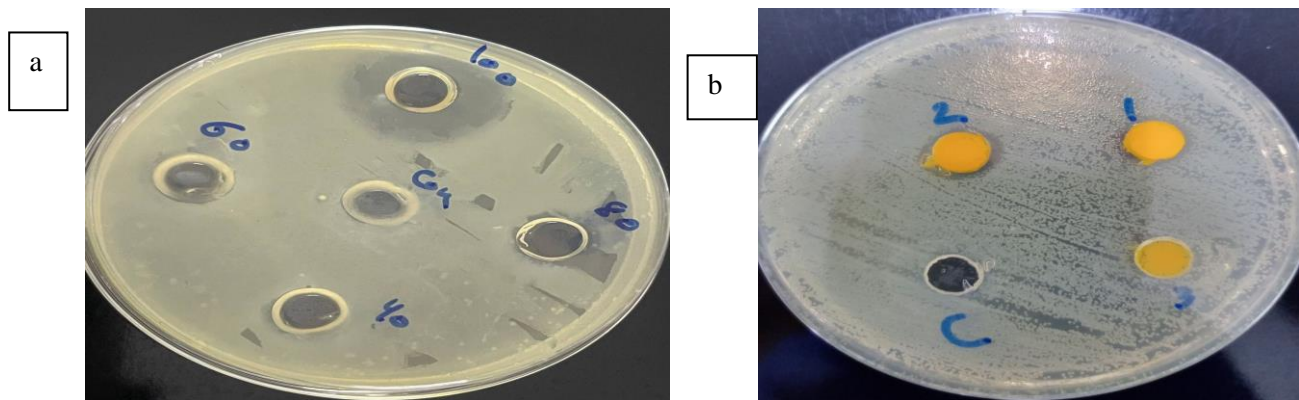


Figure 6: a- Antibacterial activity of EDTA toward *Stenotrophomonas melophilia*, b-Antibacterial activity of curcumin toward *Stenotrophomonas malophilia*.

DISCUSSION

Bladder cancer ranks tenth among all malignant diseases. It is projected that the number of infected people would rise in the future because the majority of new cases are discovered in those over 65 due to an increase in life expectancy 10. patients with prostate cancer ranged from 42 to 86, while other urinary tract complications range from 30 to 70. This ratio likely explains the gender differences in bladder cancer incidence, stage at diagnosis, and outcomes like smoking status. Potential molecular mechanisms include different hepatic enzyme metabolism of carcinogens in men and women, who leads to different exposure of the urothelium to carcinogens, as well as activity of the sex steroid hormone pathway may play a role in this ratio. When urine samples from patients were cultured, it was discovered that 87/120 (72.5 percent) of the samples tested positive for bacterial growth, including various bacterial species. Gram negative bacteria made up 53/87 (61%) of the isolated microorganisms, while Gram positive bacteria made up 34/87. (39 percent). A study in United States at 2022 was referred that UTIs are increasingly being linked to *Stenotrophomonas maltophilia*. Understanding the disease's risk factors is essential since people with specific comorbidities may experience a more severe sickness. To design a suitable antibiotic regimen to treat the infection, it is important to comprehend the mechanisms underlying antibiotic resistance 11.

The results of the current study show, all bacterial isolates were excreting enzymes (Proteinase, Lipase, Lecithinase). This result occur due to these enzymes have main role in pathogenicity of this bacterium, so this is a proof that all isolates were pathogenic. Also *S. maltophilia* benefits from the ability to manufacture proteolytic enzymes by surviving, growing, and spreading in unfavorable environmental conditions. While the presence of lipase help bacteria for survival in carbohydrate restricted environment where lipids are the sole source of carbon. Thomas *et al.*, demonstrated that all clinical isolates produced significant amounts of the tested enzymes, produced melanin, and demonstrated a swimming and swarming motility pattern. These findings imply that *S. maltophilia* clinical isolates serve as a source of potentially harmful enzymes, Invasiveness, host tissue damage, and evading host defense all depend heavily on proteinases. Extracellular lipases facilitate bacterial adhesion to host tissue and support bacterial growth in a carbohydrate-restricted environment when lipids are the only carbon source. As observed in *Listeria monocytogenes* pathogenicity, the lecthinase enzyme has functions in cell-to-cell transmission and regulates the host immune system 12.

Other study revealed by Figueire [^]do *et al.*, the purpose of this study was to identify potential virulence factors produced by *Stenotrophomonas maltophilia* clinical isolate culture supernatants. This study showed all strains of *S. maltophilia* had enzymatic activity such as lipase, proteinase and lecithinase activity 13.

Trifonovaa and Strateva Showed the high number of virulence factors and the broad spectrum of infections caused by *S. maltophilia* are clear indications that the pathogenesis of these illnesses is complex. The global regulatory Quorum sensing (QS) system regulates the synthesis of several extracellular and cell-associated virulence components as well as the development of biofilms 14. Several extracellular enzymes involved in the development of infections are encoded in the *S. maltophilia* genome. Cytotoxic activity has been reported in some clinical isolates. To further our understanding of the pathogenesis of diseases and to aid in the creation of novel therapeutic strategies, it is crucial to examine the existence and expression of virulence genes and provide a thorough explanation of their function 14,15,16.

The result in current study shows that all bacterial isolates 100% (7/7) in this study produce biofilm. The result of current study agrees with several recent studies, Bostanghadiri *et al.*,2021 showed that all isolates (100%) form biofilm. It is believed that *S. maltophilia*'s ability to form biofilms is a significant virulence factor and contributes significantly to the survival of *S. maltophilia* infections in hospital settings 17,18,19,20,21.

Extracellular enzymes, bacterial motility, and biofilm formation are among the virulence factors involved in the pathogenesis of *S. maltophilia* infections 14. Pathogenic bacteria have several significant virulence-related traits, one of which is the capacity to develop in matrix-enclosed biofilms. The growth of bacterial persistence within the host is facilitated by biofilms, which also increase resistance to the host immune system and antimicrobials, such as the last-resort antibiotic colistin 22.

In a local study by Saleh *et al.*, the purpose of this study was to identify the characteristics of *S. maltophilia* isolates in Iraq with reference to phylogenetic type, virulence factors, and multidrug resistance determinants. The results in this study showed that 51% and 33% of *S. maltophilia* produced strong- and moderate-level biofilms, respectively 23. Pompilio *et al.*, demonstrated that the majority of strains (91.7%) were able to create biofilm, but only blood borne strains and those classified as "definite" pathogens produced biofilm levels that were significantly larger than those caused by hospital-rather than community-acquired illnesses. The clinical strains of *S. maltophilia* exhibit a highly conserved capacity for biofilm formation, and this capacity may allow *S. maltophilia* to maintain its ecological niches as commensal microorganisms. This capacity for biofilm development may also be a significant virulence factor with significant clinical implications 24.

The present study shows that all bacterial isolates were siderophore producers (100%). This result occur due to the bacterial isolation was high virulent in this study, because siderophore considered to be important virulence factor especially in pathogens which encode multiple siderophores. Alcaraz *et al.*, found in research that all isolates were siderophore producers. In order to survive in the host's iron-restricted environment, siderophores are thought to be

crucial virulence factors for many diseases 25. In contrast, pathogenic strains unable to secrete siderophores have decreased virulence and fitness during infection and colonization. Hypervirulent pathogenic strains are those that are capable of creating excessive amounts of siderophores. Siderophore production in *S. maltophilia* has been thoroughly researched recently, which is a cause for serious worry 26.

The result of current study shows that the EDTA inhibit the growth of *S. maltophilia* at 100 mg/ml. EDTA has bacteriostatic activity which acts on cell surface by lysing the cell wall and releasing polysaccharide with lose of other cell components. The EDTA action leads to change on membrane permeability of bacteria to agents.

A France study at 2018 conducted to analyze the antibacterial properties of an antimicrobial peptide, the arenicin-3 derivative AA230, ethylenediaminetetraacetic acid (EDTA) and the combination of the two substances against Gram-negative bacteria. Antibacterial susceptibility tests revealed that, regardless of the resistance patterns of the investigated bacterial strains, AA230 exerted strong inhibitory effects. The fact that AA230 displayed a bactericidal mechanism of action raises the possibility that it may be utilized therapeutically to treat bacterial infections, particularly those brought on by strains that are resistant to antibiotics. Additionally, EDTA enhanced AA230's antibacterial activity. Therefore, the inclusion of EDTA might make it possible to employ AA230 at lower concentrations 27. Other study demonstrated by Hamoud *et al.* who proving that EDTA only has bacteriostatic effects on microorganisms that are Gram-negative. Additionally, it has been demonstrated that EDTA has a bacteriostatic impact on Gram-positive bacteria 28.

Egyptian study at 2021 was included in the Dakahlia governorate of Egypt, hospitals affiliated with Mansoura University produced a total of 130 clinical isolates of Gram-negative bacteria. Combinations of EDTA/with antibiotic may be beneficial in treating gram negative infections because EDTA improves the action of antibiotics against bacterial isolates, particularly when paired with gentamicin. Additionally, EDTA functioned as an efflux pump inhibitor at small concentrations (1 and 2 mM) in bacterial isolates, reducing their antibiotic resistance as a result 29.

While the present study shows that curcumin inhibit growth of *S. maltophilia* at 14 mg/ml. Curcumin had antibacterial activity which act on virulence factor, biofilm production, quorum sensing (QS) system, cell wall, cell membrane and other bacterial activity, so the result explain that curcumin acts on bacterial growth.

A study in Switzerland at 2022 was designed on the antibacterial, antifungal, and antiviral activities of curcumin in various formulations to increase its bioavailability, highlighting the potential role of curcumin in orthopedics due to its antimicrobial and osteogenic effects as well as limitations and the upcoming insights for the management of

infectious diseases in medical care. It has been shown to contain a potent combination of antioxidant phytonutrients called curcuminoids and to possess potent antibacterial activities. It works against a variety of germs and has broad-spectrum antibacterial effect. Based on the results of both in vitro and in vivo studies, it can be said that curcumin has become a potent broad-spectrum antibacterial agent. When administered as an adjuvant therapy, it also has an additive effect with some antibiotics 30.

Other study in Poland at 2020 was evaluated the effectiveness of curcumin against more than 100 pathogen strains from 19 different species. The minimal inhibitory concentration and the broth microdilution method were used to determine this activity (MIC). Consequently, curcumin is a promising antibacterial drug with highly selective efficacy. The findings demonstrate the health advantages of employing curcumin as a significant food additive and spice, not only for its color, taste, and preservation qualities but also for its antibacterial activity against human infections 31.

A study in India at 2015 was concentrated on the antibacterial activity of curcumin, a key ingredient in commercial curcumin, against genera of bacteria, including both Gram-positive and Gram-negative bacteria. A study demonstrates curcumin's significant antibacterial ability against all of the examined microorganisms from the Gram-positive and Gram-negative groups. Propidium iodide and calcein, two fluorescent markers that indicate differential permeabilization, were used to assess the integrity of the bacterial membrane. Gram-negative and Gram-positive bacteria both exhibited membrane leakage after being exposed to curcumin, according to both membrane permeabilization assays. The current investigation supports curcumin's broad-spectrum antibacterial properties as well as its ability to damage membranes 32

CONCLUSIONS

Immunocompromised patients are more likely to become infected with the opportunistic pathogen *Stenotrophomonas maltophilia*, which has the potential to produce a wide spectrum of antigens. EDTA and curcumin have antibacterial activity against this bacterium.

Compliance with Ethical Standards statements

Ethical approval: Al-Iraqia University-College of Medicine-Medical Microbiology Department, Baghdad/ Iraq, certifies the ethical approval, Funding details (In case of Funding): I am responsible for paying the financing, Conflict of interest: There is no conflict of interest, Informed Consent: Al-Iraqia University-College of Medicine- Medical Microbiology Department, Baghdad/ Iraq.

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