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Molecular Characterization and Identification of *Serratia sp*. in Drinking Water Samples in Shatrah District

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Abstract

Serratia species are found in different environments, including water, plants, and animals. This study aims to isolate, molecular characterization, and phenotypically investigation of *Serratia* sp., and identify the antibiotic susceptibility of isolated species to eight antibiotic discs as well. A total of twenty water samples were collected from different regions of Shatrah district in a sterile manner using properly labelled and sterile containers. These samples were then passed through a membrane filter. The colonies were then inoculated on culture media, Gram stain, IMVIC tests, Lysine decarboxylase, catalase, Ornithine decarboxylase tests were done, and the colonies were molecularly characterised by using PCR and *16S rRNA* sequencing methods. Eventually, the isolates were tested against eight antibiotics. The results found that all *Serratia* sp. were negative for the Oxidase Test, Indole Production, Methyl Red (MR), and Voges-Proskauer (VP). In the other flip side, all tested isolates showed positive results for Citrate Utilization, Lysine Decarboxylase, Catalase, and Ornithine Decarboxylase. The results found that 15 isolates were isolated and classified as *Serratia* sp. Also, the antibiotic susceptibility of *Serratia* showed that all tested isolates were susceptible to all tested antibiotics. The results found that all tested pathogenic isolates were affected by the bacteriocin extracted from *Serratia* sp. The drinking water must be free of any microbes as it serves as the primary reservoir for various infectious pathogens.

Introduction:

The preservation of water for drinking safety is of the highest priority in maintaining public health, considering that water that is contaminated may serve as a medium for the transmission of many diseases and opportunistic microorganisms. The genus *Serratia* have attracted considerable attention in drinking water distribution systems due to their possible health implications, given the wide range of microorganisms present in these systems. *Serratia* species have been isolated from different natural environment such as animals' bodies, plants, water, and soil as well [1]. It is a Gram-negative bacterium that has the ability to survive within a broad range of temperature, and it can withstand in many environments including plant surfaces, water, soil, and dietary items like fruit juices, coconut, and fish since it represent a facultative anaerobic genus [2]. Some of *Serratia* spp. is found as colonies in damp coconut such as *Serratia marcescens* and *Serratia rubidaea* which produce red or pink colonies within food products. *Serratia marcescens* produce a unique red pigment known prodigiosin during their replication as a part of metabolic substance. It has been vividly confirmed with antimicrobial features, despite of the absent of their role in the development of bacteria [2]. Extracellular enzymes with hydrolytic proprieties such as chitinase, protease, and lipase are produced from *Serratia marcescens*. It uses these enzymes to breakdown proteins and lipids within the dietary products. *Serratia marcescens* is represented as an opportunistic pathogen that can easily transformed from food such as coconuts to human and causes harmful diseases. A 2% of nosocomial contagion in diverse adult groups was recorded by *Serratia* sp., affecting the bloodstream, lower respiratory system, urinary tract, surgical wounds, and skin and soft tissues [3]. Researchers found that there is an association between the presence of certain strains of *Serratia* in drinking water resources and nosocomial infections which concerned as an evidence of its outbreak [4].

In recent years, the development in molecular studies has made a notable advancement in the area of scientific research, resulting in discover of dependable approaches and techniques that can be easily to use in the molecular study of the components of microbes in complex environmental samples, such as drinking water. It has become easier to discover a new bacterial species or study genetically related microorganisms and to taxonomic characterization of bacterial populations through the application of molecular methods such as polymerase chain reaction (PCR) followed by sequencing and analysis of the sequenced *16S rRNA g*ene [5]. Also the evolutionary links between different types of bacteria can be determined and identify potentially dangerous species in samples from the environment by targeting the highly conserved region of the 16S rRNA gene [6]. While the importance of *Serratia* in the microbiota of drinking water is becoming more recognized, there have been few studies that particularly examine their variety and distribution in this environment. Therefore, it is crucial to carry out comprehensive investigations using molecular techniques to identify and analyze *Serratia* populations in drinking water sources, treatment facilities, and distribution networks. These examinations have the role to provide valuable insights into the occurrence, abundance, and genetic diversity of *Serratia* species, as well as how they may interact with other microorganisms and their role in the spread of causative agents that transmitted by waterborne [7]. This study aims to examine the variety of *Serratia* species found in drinking water, to isolate and analyze the molecular features of *Serratia* sp., and to inquire the susceptibility test of antibiotics of isolated bacteria. Moreover, evaluate *Serratia's* capacity to produce antibiotics.

Materials & Methods:

The present study was carried out at the laboratories of Microbiology Department, College of Medicine Vet, Shatrah University, Iraq, in order to investigate the diversity of *Serratia* in drinking water and molecular characterization and phylogenetic analysis by using PCR and *16S rRNA* sequencing. Twenty water samples were collected from different regions at Shatrah District.

Sample collection

From various sources about 20 water samples were aseptically collected using labeled and sterile containers [7]. Also some information was registered such as (date, location and time collection). In order to eliminate to bacterial growth, the water samples were directly transported to the laboratory using ice and process within 6 hours of collection [7]. Using filter (a volume of sample water 100- 1000 ml) were filtered through membrane filter and by sing a vacuum filtration system (with 0.45 µm the size of pore) [7].

Bacterial Culture

The water samples were inoculated onto MacConkey agar and Tryptic Soy Broth (TSB) supplemented with crystal violet and1% glucose, respectively after being filtered [9]. The petri plates were incubated at 37°C for one –two days [8]. After 24 hours, the subculture of colonies was done for characteristic *Serratia* colonies. These colonies are typically pink to red in color and may have a mucoid or filamentous morphology [8]. Gram stain was performed in order to observe the bacilli morphology of *Serratia* sp. under typical light microscope [10- 11].

Biochemical tests

The biochemical tests were carried out to characterize *Serratia* isolates such as TSI (Triple Sugar Iron agar), and IMVEC (Indole test, Methyl red, Voges–Proskauer, and Citrate utilization) [10]. Also, the ability of *Serratia* sp. to produce hydrolysis enzyme were examined including Catalase, Ornithine decarboxylase, and Lysine decarboxylase. Also, further investigation has been done to distinguish between *Enterobacteriaceae* and *Pseudomonadaceae* by using oxidase test protocol. Using of slide drop technique *Serratia* sp. were examined to produce catalase enzyme using sterile wooden one colony of bacteria was mixed with one drop of 3% of Hydrogen peroxide on clean slide and the result was immediately read depending on the bubbles production [12]. The oxidase test involved applying one drop of the proton donor indicator, namely tetra-dimethyl-para-phenylenediaminedihydrochloride, onto a filter paper. A bacterial colony was then mixed with the indicator. The result was observed within 20 seconds, based on any color change that occurred [13, 14].

Molecular Identification

Primer was designed and the bacterial DNA extracted based on the methods that were mentioned by Wilson, 2001. By using QIAamp DNA extraction and QIAquick PCR purification kits (Germany) and followed the instructions the bacterial chromosomal DNA was isolated and purified.The molecular confirmation of *Serratia* species performed using polymerase chain reaction (PCR) targeting *16S rRNA* gene [15]. A 16S rRNA gene universal primer 9-F {5'-GAGTTTGATCCTGGCTCAG-3'} and 1542-R {5'-AGAAAGGAGGTGATCCAGCC-3} with size product equal to 1540 pb. was designed and applied to amplify the bacterial gene [16]. A 50 µL total volume of PCR mixture was used during the reaction which composed of the following contents (Table 2) [17]. After the extracted bacterial DNA had been purified all our isolates were sequenced using Sanger dideoxy 16S rRNA sequencing method that describes by Sanger in 1977 [18]. After that the PCR reaction product was examined by using 1% agarose gel and electrophoresis method.

Table 1: The PCR reaction components and Program

Materials	Amounts	The multiplication program and temperature cycle			
dNTP	200 µM	Denaturation	94°C	3 minutes	
primers	0.5 µM		94°C	60 seconds	30 cycles
Taq polymerase	2.5 U	Annealing	45°C	60 seconds	
template DNA	100 ng				
Tris-HCl	10 mM				

MgCl2	1.5 mM	Extension	72°C	60 seconds
KCl	50 mM	Final extension	72°C	7 minutes

Antibiotics Profile

The antibacterial susceptibility profile of *Serratia* isolates was determined using the standard disk diffusion method as outlined by CLSI in 2018. The isolates were evaluated against 8 antibiotics [Ciprofloxacin (CRO- 10 µg), Trimethoprim-sulfamethoxazole (TMP-SMX- 25 µg), Imipenem (IMP-10 µg), Meropenem (MEM- 10µg), Gentamicin (GN- 10µg), Amikacin (AK- 30µg), Tigecycline (TGC- 30 µg), and Colistin (CL- 10 µg)].in order to determine the most suitable treatment and to observe any emerging developments in resistance [19].

The Bacteriocin Production

A mix culture of *serratia* sp. was cultivated in 100 ml of nutrient broth (pH 7.0), and incubated 24 h at 35°C in order to acquired a bacteriocin from our isolates. The produced bacteriocins were collected by centrifuging the bacteria growth at 4°C for 15 minutes at 8000 rpm. The pH of bacteriocin was adjusted to 7.0 by adding 1N NaOH. In conclusion the pure bacteriocin broth was collected after the liquid had been filtered through filter membrane [20-21].

Investigation of Bacteriocin antibacterial properties

The antibacterial properties of bacteriocin extracted from *Serratia* sp had been confirmed against some pathogenic isolates were previously isolated including *Staphylococcus aureus, Streptococcus pyogens, Pseudomonas aeuroginosa*, and *Klebsiella pneumonia*. Using a physiological saline the pathogenic bacterial turbidity was separately adjusted to 10^8 CFU/ml, then by using one drop of bacteriocin was added to the Muller Hinton agar inoculated with tested isolates and all petri plates incubated overnight at 35°C. The results were read depending on the diameter of inhibition zones [22].

Results:

Of the twenty water samples collected for this investigation more than 50 isolates had been isolated and based on the biochemical tests, gram staining method and the results of *16S RNA* sequencing 15 isolates have been diagnosed as *Serratia* sp. Including *Serratia odorifera* (2 isolate), *Serratia plymuthica* (3 isolate), *Serratia marcescens* (6 isolates), *Serratia ficaria* (2 isolates), *Serratia nematodiphila* (1 isolate), and *Serratia rubidaea* (1 isolate) were identified through 16S rRNA gene sequencing based on the similarity with reference species in NCBI site. As shown in table (2):

Table 2: *Serratia* sp. Isolated from Drinking water.

Genus	Number	%
S. odorifera	2 isolate	13.3
S. marcescens	6 isolates	40
S. ficaria	2 isolates	13.3
S. nematodiphila	1 isolate	6.6
S. rubidaea	1 isolate	6.6
S. plymuthica	3 isolate	20

The results of this study revealed that all 15 isolates were Gram-negative under the microscope. While it showed negative for IMVEC and oxidase tests; the results for Citrate Utilization, Triple Sugar Iron (TSI) Agar, Lysine Decarboxylase, Catalase, and Ornithine Decarboxylase were positive by all tested isolates. As shown in table (3).

Table.3: Bacterial species, similarities, and biochemical tests of *Serratia* sp. isolates isolated from drinking water.

	Bacteria species					
	marcescens	*S. odorifera*	*S. plymuthica*	*S. ficaria*	*S. nematodiphila*	*S. rubidaea*
Similarity%	99%	97%	97%	99%	97%	99%
Oxidase Test	-	-	-	-	-	-
Indole Production	-	-	-	-	-	-
Methyl Red (MR)	-	-	-	-	-	-
Voges-Proskauer (VP)	-	-	-	-	-	-
Citrate Utilization	+	+	+	+	+	+
Triple Sugar Iron (TSI) Agar	alkaline/alkaline	alkaline/alkaline	alkaline/alkaline, acid/acid, or alkaline/acid reactions	alkaline/alkaline	alkaline/alkaline	alkaline/alkaline
Lysine Decarboxylase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Ornithine Decarboxylase	+	+	+	+	+	+

Antibiotics susceptibility

Eight types of antibiotic discs were tested against all *Serratia* species that were isolated from drinking water previously, and the results found that the growth of all tested isolates was stopped directly after treated with antibiotic discs. The inhibition zones around antibiotic discs ranging from 12 to 27 mm. Meropenem affected all our isolates with different inhibition zones measurements ranging from 16mm to 25mm. As shown in table (4).

Table 4: Bacterial species, antibiotics, and inhibition zones of Serratia sp. isolates isolated from drinking water.

Antibiotics	*S. marcescens KRED 16S*	*S. odorifera NBRC 102598*	*S. plymuthica*	*S. ficaria* DSM 4569	*S. ficaria JCM1241*	*S.nematodiphila DZ0503SBS1*	*S. rubidaea JCM1240*
	Inhibition of the growth of bacteria (mm)						
Ciprofloxacin	22	23	20	19	26	23	18
(TMP-SMX)*	17	18	20	16	17	21	22
Imipenem	23	22	26	18	19	22	23
Meropenem	25	24	23	16	21	19	17
Gentamicin	19	17	22	25	27	21	20
Amikacin	23	19	22	20	23	17	19
Tigecycline	19	24	18	16	19	22	20
Colistin	14	16	17	15	12	13	18

*Trimethoprim-sulfamethoxazole

The results of bacteriocin experiment found that all tested pathogenic isolates were affected by the bacteriocin that extracted from *Serratia* sp. However the inhibition zones around bacterial colonies were varied from one isolate to another. The bacteriocin from *S. marcescens*, *S. odorifera, S. plymuthica, S. ficaria, S. nematodiphila,* and *S. rubidaea* affected *S. aureus* with inhibition zones 17 mm, 18 mm, 20 mm, 16 mm, 18 mm, and 17 mm, respectively. Also, the bacteriocin from *S. marcescens*, *S. odorifera, S. plymuthica, S. ficaria, S. nematodiphila,* and *S. rubidaea* affected *K. pneumonia* with inhibition zones 19 mm, 18 mm, 17 mm, 15 mm, 18 mm, and 17 mm, respectively. However, the inhibition zones around *Pseudomonas aeroginosa* grown colonies were smaller than the others around the rest tested isolates. As shown in table 5.

Table 5: The effectiveness of bacteriocin from *Serratia* spp. on pathogenic tested isolates.

		Bacteriocins Producing Isolates					
		S. marcescens	*S. odorifera*	*S. plymuthica*	*S. ficaria*	*S. nematodiphila*	*S. rubidaea*
		Inhibition zone (mm)					
Bacteria affected by bacteriocins	*S. aureus*	17 mm	18 mm	20 mm	16 mm	18 mm	17 mm
	S. pyogens	13 mm	12 mm	14 mm	15 mm	13 mm	15 mm
	K. pneumonia	19 mm	18 mm	17 mm	15mm	18mm	17mm
	P. aeroginosa	6 mm	5 mm	6 mm	7 mm	5mm	8mm

Discussion:

During this study 20 water samples were collected and tested in order to diagnosis the pathogenic Serratia sp. which can cause some types of diseases to human after swelled such as diarrhea, endocarditis, urinary tract infections, endotoxic shock, Septicemia, wound infections, and occasionally pneumonia have been reported. The findings found that 15 isolates were diagnosed as *Serratia* sp. depending on the biochemical tests, and molecular investigations. Among these isolates were 13.3% *Serratia odorifera* (2 isolate), 20% *Serratia plymuthica* (3 isolate), 40% *Serratia marcescens* (6 isolates), 13.3% *Serratia ficaria* (2 isolates), 6.6% *Serratia nematodiphila* (1 isolate), and 6.6% *Serratia rubidaea* (1 isolate). Previous study isolated 13 isolates from soil and diagnosed as *Serratia marcescens* depending on the MALDI-TOF mass spectrometry assay, in this investigation the antibacterial activity of *Serratia marcescens* pigments tested against other bacterial species [23]. This finding expresses that the primary and secondary metabolic materials (bacteriocins and pigments) from *Serratia* sp. have antibacterial effects against all tested pathogenic isolates. Prior investigation revealed that the antibacterial efficiency of *Serratia* pigments was confirmed against *Pseudomonas aeruginosa, Staphylococcus aureus, and Bacillus cerus*; whereas it was not affect against *Escherichia coli* using pore diffusion technique on Muller Hinton agar [24]. *Serratia aquatilis* was isolated from drinking water in Germany and the taxonomic position was studied in previous study, [25]. Some *Serratia* sp. can be easily transferred to human during tap water such as Serratia marcescens. It is worthy to mention that, some *Serratia* sp. can transport from patients during direct hand touch through wounds in skin. Personal cleanliness and water filtration are crucial preventive measures that must be apply to be safe from pathogenic microorganisms. Although, the health problems that cause by *Serratia* sp., it produces some pigments that may affect other bacteria species. Hence recent researches had paid attention on using *Serratia marcescens* pigments as antibacterial agents against other pathogenic bacterial species. Four isolates of *Serratia fonticola* have been isolated from sputum specimens from respiratory tract patients and considered as a pathogenic species, these isolates showed resistance against CRO, CTX, AZM, LEV; whereas, it was sensitive to GM,AK, and MEM [26].

Conclusion:

Fifteen isolates were isolated and classified as *Serratia* spp. belonging to *Serratia odorifera*, *Serratia plymuthica*, *Serratia marcescens*, *Serratia ficaria*, *Serratia nematodiphila*, and *Serratia rubidaea* based on the similarity with reference species in NCBI site. All these isolates Gram-negative under the microscope, negative for IMVEC and oxidase tests; however, Citrate utilization, TSI agar, Lysine Decarboxylase, Catalase, and Ornithine Decarboxylase were positive. The growth of all tested isolates was inhibited directly after treated with antibiotic discs. The inhibition zones around antibiotic discs ranging from 12 to 27 mm. The bacteriocin from *S. marcescens*, *S. odorifera, S. plymuthica, S. ficaria, S. nematodiphila,* and *S. rubidaea* affected *S. aureus* with inhibition zones 17 mm, 18 mm, 20 mm, 16 mm, 18 mm, and 17 mm, respectively. Also, the bacteriocin from *S. marcescens*, *S. odorifera, S. plymuthica, S. ficaria, S. nematodiphila,* and *S. rubidaea* affected *K. pneumonia* with inhibition zones 19 mm, 18 mm, 17 mm, 15 mm, 18 mm, and 17 mm, respectively. The results of this inquiry are expected to improve our understanding of the microbial ecology of drinking water supplies and offer knowledge for the development of strategies to control and regulate waterborne causes of diseases.

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التوصيف الجزيئي وتحديد ال *Serratia sp.* في عينات مياه الشرب في قضاء الشطرة

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الخلاصة:

تتوجد أنواع السراتيا في بيئات مختلفة، بما في ذلك المياه والنباتات والحيوانات. تهدف هذه الدراسة إلى عزل وتوصيف جزيئي ودراسة المظهر الظاهري لنوع *Serratia* sp. وتحديد حساسية الأنواع المعزولة للمضادات الحيوية لثمانية أقراص من المضادات الحيوية أيضًا. تم جمع عشرين عينة مياه من مناطق مختلفة في قضاء الشطرة بطريقة معقمة وباستخدام أوعية معقمة ومعلمة بشكل صحيح. ثم تم تمرير هذه العينات من خلال مرشح غشائي. تم بعد ذلك تلقيح المستعمرات على أوساط زرعية، تم اجراء مجموعة من الاختبارات البايوكيميائية مثل صبغة جرام، واختبارات IMVIC ، الليسين ديكاربوكسيلاز، والكاتلاز، والأورنيثين ديكاربوكسيلاز، وتم توصيف المستعمرات جزيئيًا باستخدام طرق تسلسل الحامض النووي و كذلك طريقة تفاعل تسلسل البلمرة. وفي نهاية المطاف، تم اختبار العزلات ضد ثمانية مضادات حيوية. وجدت النتائج أن جميع أنواع *Serratia* sp. كانت سلبية لاختبار أوكسيديز، وإنتاج الإندول، والميثيل الأحمر(MR) ، و-Voges Proskauer (VP) وفي الجانب الآخر أظهرت جميع العزلات المختبرة نتائج إيجابية في استخدام السيترات، Lysine Decarboxylase، Catalase، و Ornithine Decarboxylase. توصلت النتائج إلى أنه تم عزل 15 عزلة وصنفت على أنها *Serratia* sp. اعتماداً على تشابه تسلسل الجينات 16 S rRNA والاختبارات البيوكيميائية التي تم إجراؤها. كما أظهرت حساسية السراتيا للمضادات الحيوية أن جميع العزلات التي تم اختبارها كانت حساسه لجميع المضادات الحياتيه التي تم اختبارها. أظهرت النتائج أن جميع العزلات المرضية المختبرة تأثرت بالبكتريوسين المستخرج من ال *Serratia* sp. يجب أن تكون مياه الشرب خالية من أي ميكروبات لأنها بمثابة الخزان الأساسي لمختلف مسببات الأمراض المعدية.