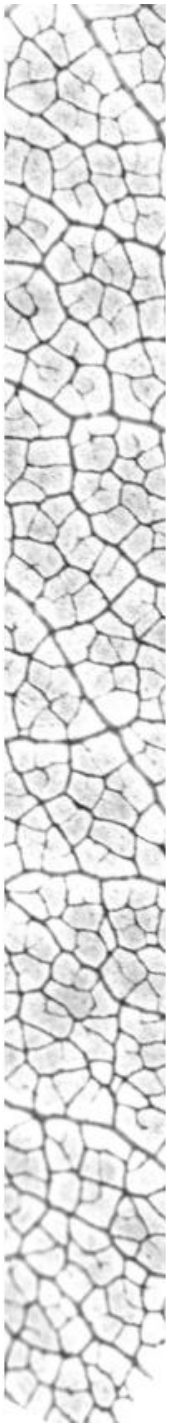

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Determination of genes responsible for some virulence factors of bacteria isolated from contaminated groundwater

Israa Hammood Abed AlDoori [1], Jihad Diab Mahal [1], Mohammed Nadhir Maaroof [1*]

[1] College of Education for Pure Science, Tikrit University, Tikrit, IRAQ
*Corresponding author: dr.mohammed78@tu.edu.iq

Abstract
The current study is conducted to detect bacterial contamination of groundwater in Al-Dour district within Salah al-Din Governorate for a distance extending about 35 km by eight wells with depths ranging between (65-90) meters during the study period extending from spring 2018 to winter 2019. The study includes isolation and identification of the most important species of bacteria contaminating well water and investigating its virulence factors with molecular diagnosis. The Isolates were detected as being able to produce some virulence factors, which included hemolycin, lipase and protease, lecithinase, biofilm, urease, and beta-lactamases, is detected. The results show that *Klebsiella pneumoniae* produces biofilm, hemolysin, lipase and lecithinase by 100%, while beta-lactamase and both urease and protease enzymes are not produced. As for *Enterobacter cloacae*, it produced hemolysin, protease and lipase by 100%, while it produced the enzymes beta-lactamase and lecithinase by 50%, and did not form or produce the biofilm and the urease enzyme. Concerning *Aeromonas veronii*, it produced beta-lactamase, lipase, biofilm, protease and lecithinase by 100% and hemolysin by 50% and did not produce urease enzyme. *Enterococcous cecorum* produced beta-lactamase, urease, protease and biofilm by 100%, and produced hemolysin and lipase enzymes by 66.7% and lecithinase enzyme by 33.3%, while *Enterococcous columbae* produced beta-lactamases, urease and biofilm by 100% but unable to produce lipase, lecithinase, hemolysin and protease. *Granulicatella elegans* were shown to be producing beta-lactamase, lecithinase, biofilm and protease by 100%, but did not produce hemolysin, urease and lipase enzymes. As for *Aeromonas hydrophila*, they appeared to be producing lipase, urease and hemolysin by 100%, but produced protease, lecithinase and beta-lactamase by 50% but did not produce the biofilm. The results of Polymerase Chain Reaction (PCR) show the use of specialized primers of some genes of virulence factors in the isolated bacteria. The protease gene encoded *ProA* and beta-lactamases gene encoded *MOXM* are found in *Enterobacter cloacae*, the hemolysin gene encoded *HemK* and the biofilm gene encoded *LuxS* found in *Klebsiella pneumonia*, the urease gene encoded *UreA* and lipase gene encoded *lip* found in *Aeromonas hydrophila*. As for *A.veronii*, it has been detected holding the protease gene encoded *ProA* and the lipase gene encoded *lip* the. With respect to the genera *Enterococcus sp.*, *Tuf* biofilm encoded is found in both *Enterococcous columbae* and *Enterococcous cecorum*. In *Granulicatella elegans*, the presence of the *RpoB* gene is detected.

Keywords: groundwater, bacterial species, virulence factors, specific primers, PCR

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INTRODUCTION

Groundwater is considered one of the most important water resources since it constitutes (71.7) % of the drinking water in the world. Groundwater includes wells and springs which arise mainly from rain water, and irrigation water that is filtered into the ground and stored under its surface in non-porous layers forming groundwater reservoirs (AlKulaby, 2016).

The bacterial contamination of groundwater is less than that in surface water, but this does not mean that it is suitable for drinking. Recent studies conducted by scientists in the field of environment indicate that in many cases the wells used are close to the surface of the earth, which increases the chance of exposure to biological pollution from many sources, such as pollution due to human activities that lead to pollutants leaking into wells from wastewater purification plants as well as animal husbandry stations and the resulting fecal pollution (Fadi, 2011). In addition, pollution also occurs as a result of the interference between groundwater and

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wastewater, especially in villages and cities where modern sewage systems are not available (Alexander, 2008). The virulence factors that microorganisms possess help it to stay better in the host and enable it to perform specific functions as they enable it to cause damage to the host cells and tissues. These factors are regulated by specific triggers (Willey et al, 2014). The bio-membrane is the grouping of microorganisms inside a closed structure consisting of extracellular polymeric substance that is self-excreted by the organisms themselves. These polymers are a complex mixture of biopolymers (Vu et al, 2009), while beta-lactamases as defensive enzymes have the ability to protect the cell from decomposition and which is excreted by some bacterial species and directly affect the action of killing inhibiting antibiotics forming a cell wall through the ability of enzymes to break down the amide bond in the beta-lactam loop found in beta-lactam antigens such as cephalosporins, penicillins, carbenamobes, and carbapamons This makes these antagonists useless and extracellularly biotic (Husu et al, 2003; Finlay et al, 2003).

As for the Urease enzyme, it is a main focus in recent studies, as it is one of the most important factors of virulence and pathogens, which is produced by many bacterial species (Dattelbaum et al, 2003). The Urease enzyme is produced by approximately 200 gram negative and gram positive bacteria species. The infection with bacteria species such as Pseudomonas spp., Micrococcus spp., and Proteus spp. leads to urinary tract infections and kidney stones formation, by raising the pH of the environment in which it operates from 5 to 9 and thus precipitation of salts (Torzewska et al, 2003).

The hemolysin enzyme is of great importance in spreading bacteria and increasing its pathogenicity. Hemolysin is an important virulence factor for many gram-negative and gram-positive bacterial species, and it belongs to a group of proteins that analyze erythrocytes. Its genetic makeup varies from one bacterium to another and plays a fundamental role by its toxic effect on the cells of the host body and leads to the destruction of tissues, as well as a toxic effect on granular white blood cells and non-granular white blood cells (Liaw et al, 2003). Concerning the protease enzyme, it plays a large role in causing pathogenicity in humans by the bacterial species producing it, as it is considered one of the most important factors of ferocity that play its role in breaking down host cell proteins (Omer and Humadi, 2013).

The lecithinase is a type of enzyme called phospholipases, which is one of the important enzymes produced by some bacteria species for its role in increasing its pathogenicity. It is characterized by its ability to destroy tissues by breaking down the phosphoryl (lecithin) in the cell membrane, which results in the formation of disosphorylcholine and diglyceride and leads to hemolysis. It causes hematopoiesis (Sharaf et al, 2014), whereas lipase enzyme is an aqueous analysis enzyme, that is, it is in need of watery conditions in order to break down the ester bonds of the carboxyl in the triacyllglycerols, leading to the release of fatty acids and glycerol (Gupta et al, 2004). In addition, it is considered one of the important virulence factors in human pathogenic bacteria, especially in bacteria that cause skin infections (Stehr et al, 2003). Our current study aims to investigate the most important virulence factors produced by isolated pathogenic bacteria and molecularly detect the most important gene encoders for producing virulence factors by PCR using specific primers.

MATERIALS AND METHODS

Collection of Samples

A total of 32 samples are collected from groundwater in Al-Dour district within Salah-Al-Din governorate for a distance of about 35km, and by eight wells with depths ranging between (65-90) meters in order to know the suitability of well water for different uses and monitor the seasonal and site changes during the study period extending from spring 2018 to winter 2019.

Isolation and identification of bacteria

Samples are cultured on blood agar and MacConkey agar, and incubated at 37 ° C for 24 hours. The bacteria are initially identified depending on the morphological characteristics of the colonies and their growth on selective media such as Mannitol salt agar, Cetrimide agar and Eosin methylene blue agar. Then, they are examined microscopically after being dyed with Gram stain. Various biochemical tests are carried out to identify the isolated bacteria, and this includes IMViC, Oxidase, Catalase, coagulase, and motility tests, to test its ability to produce H2S and gas on Kligler iron agar and fermentation tests for different saccharides. In addition, its ability to produce various virulence factors are also tested such as hemolysin,Biofilm, urease, lipase, lecithinase protease, and B-lactamase (Leber, 2016), and the diagnosis is confirmed using a Vitek Compact System2 (Biomerieux, France).

Morphological detection of virulence factors

Biofilm formation is detected by two methods, the first is Congo red agar. In this, the medium is prepared using the method described by Freeman et al (1989). The medium is composed of 37 gm /L of Brain Heart Infusion agar, 50 gm/L of sucrose, 10 gm /L of agar and 0.8 gm /L of congo red stain. The congo red reagent is prepared as a concentrated water solution, sterilized by autoclave separated from the other components at 121°C for 15 minutes, and then added to the rest of the medium when the agar reached 50°C, and the medium is poured into petri dishes. The bacteria are cultured on the medium and incubated for 24 hours at 37°C. The results are considered positive when black colonies with dry

Table 1. Oligonucleotide Primers Used for Virulence Genes Amplification

Bacteria species	Primer		Nucleotide Sequence 5-3'	Product (bp)	Reference
Enterobacter cloacae	*ProE*	F	GCA TGT TCG CTT CTG CCT TG	121	Design
		R	GTG GGC AAA CCA GAC TAC CC		
	MOXM	F	GCTGCTCAAGGAGCACAGGAT	520	Seo & Lee. (2019)
		R	CACATTGACATAGGTGTGGTG		
Klebsiella pneumonia	*HemK*	F	GCT ACT TAT CCC GAC AGC CC	414	Design
		R	GTC GTG TGG ACC GAA GAA CT		
	LuxS	F	GCCGTTGTTAGATAGTTTCACAG	447	Hashemi and Hemat(2016)
		R	CAGTTCGTCGTTGCTGTTGATG		
Aeromonas hydrophila	*UreA*	F	AGG AGC CCA GCT TCA CTT TC	131	Design
		R	CCT GCG CGA CTC GTA ATT TG		
	Lip	F	GACCCCCTACCTGAACCTGAGCTAC	155	Hayati (2015)
		R	AGTGACCCAGGAAGTGCACCTTGAG		
Aeromonas veronii	*ProA*	F	GCT GAG TGT GAC GGT GCT	317	Design
		R	GAA ATT TAC GGG CCG CTT CG		
	Lip	F	GACCCCCTACCTGAACCTGAGCTAC	155	Hayati (2015)
		R	AGTGACCCAGGAAGTGCACCTTGAG		
Granulicatella elegans	*RpoB*	F	TGTAACTCTAACACTTGTCCGA	146	Lévy & Fenollar, (2012)
		R	GGACGTCACGGTAATAAAGGG		
		R	TGAAGTAAGTGACCAGAATC		
Enterococcus columbae	*Tuf*	F	TACTGACAAACCATTCATGATG	112	Jung *et al.* (2007)
		R	AACTTCGTCACCAACGCGAAC		
		R	TGAAGTAAGTGACCAGAATC		
Enterococcus cecorum	*Tuf*	F	TACTGACAAACCATTCATGATG	112	Jung *et al.* (2007)
		R	AACTTCGTCACCAACGCGAAC		

crystalline are found, while the colonies that appear pink are considered as negative (Cotter et al., 2009). The second method used in detection of biofilm production is the Tubes method. In this method, the bacteria are cultured in tubes containing trypticase soy broth with 1% glucose and incubated at 37°C. After 24 hours, the tubes are emptied and washed carefully with distilled water and left to dry and then stained with 1% crystal violet stain for 30 minutes and the excessive stain is eliminated. After the tubes become dry, the formation of biofilms is observed and the result is considered positive when thin films appear on the walls and bottom of the tubes (Christensen et al, 1982).

Hemolysin is detected by the emergence of halos around colonies of bacteria cultured on blood agar and incubated for 24 hours at 37 ° C, while urease is detected by culturing the bacteria in tubes containing urea agar and incubated at 37 ° C. The result is positive when the agar turns to pink, and for detection of lecithinase and Lipase the bacteria are cultured on egg yolk agar (EYA) and incubated for 24 h at 37 ° C. Lecithinase production is found by the emergence of halos around the colonies. The lipase enzyme is also inferred through the appearance of iridescent sheen (Leber, 2016) and protease is detected by the emergence of halos around wells of bacteria cultured on skimmed milk agar and incubated for 24 hours at 37 ° C (Collee et al, 1996). Finally, B-lactamase is detected according to Koneman *et al*., 1988), by using Pencillin G powder and filter paper. The result is positive when a white spot in the center of the filter paper appears.

Plasmid DNA extraction

Plasmid DNA is extracted for *Aeromonas veronii*, *Enterobacter cloacae, Aeromonas hydrophila, Enterococcus cecorum, Granulicatella elegans, Enterococcus columbae,*and *Klebsiella pneumonia* by using the miniprep kit for plasmid DNA extraction (Pomega, USA).

Detection of virulence genes

The polymerase chain reaction is performed to detect the presence of the genes (*proE*, *MOXM)*) in the *Enterobacter cloacae,* (*HemK*, *LuxS*) in *Klebsiella pneumonia*, (*UreA*, *Lip*) in *Aeromonas hydrophila,* (*ProA,Lip*) in *Aeromonas veronii,(RpoB)* in *Granulicatella elegans* and gene (*Tuf*) in the *Enterococcus columbae* and *Enterococcus cecorum*. The reaction is performed for each separately, and the mixture contains 10µl of GoTaq® Green Master Mix (promega, USA), 3µl of DNA template, 1µl of forward primer, 1µl of reverse primer and 5µl of nuclease-free water. The primers sequence, PCR product size and reaction conditions for each primer are shown in **Tables 1** and **2**. The PCR products are tested by electrophoresis in a 1.5% agarose gel.

RESULTS AND DISCUSSION

Isolation of contaminating bacteria from wells under study

Thirteen polluted samples of groundwater are collected from eight wells throughout the four seasons during the study period. The isolation results show that 12 samples obtain different bacterial growth on the primary isolation media which are Blood agar, Manitol agar, and MacConkey agar. The wells numbered (2,3,4,6) are the most contaminated by bacteria, where two isolates are obtained, while the rest of the wells numbered (1,5,7,8) are contaminated with one bacterial isolation as in **Table 3**. The reason for the variation in the contamination of the wells under study may be due to their proximity to the sources of domestic sanitation or

Table 2. PCR Conditions for each Primer

Steps	Temperature °C	Time	Number of cycles	Primer Name
Enterobacter cloacae				
Initial denaturation	95	5 min	1	***ProE***
Denaturation	95	35sec	33	
Annealing	58	35 sec		
Extension	72	35 sec		
Final extension	72	7 min	1	
Enterobacter cloacae				
Initial denaturation	94	3 min	1	***MOXM***
Denaturation	94	30sec	25	
Annealing	64	30 sec		
Extension	72	1 min		
Final extension	72	7 min	1	
Klebsiella pneumonia				
Initial denaturation	95	5 min	1	***HemK LuxS***
Denaturation	95	35sec	33	
Annealing	52	35 sec		
Extension	72	35 sec		
Final extension	72	7 min	1	
Aeromonas hydrophila				
Initial denaturation	94	3 min	1	**UreA** ***Lip***
Denaturation	94	1 min	35	
Annealing	64	30sec		
Extension	72	45sec		
Final extension	72	7 min	1	
Aeromonas veronii				
Initial denaturation	95	5 min	1	**ProA** **Lip**
Denaturation	95	35sec	33	
Annealing	60	35 sec		
Extension	72	35 sec		
Final extension	72	7 min	1	

Table 3. Number of contaminating bacteria for wells under study

Well No.	No. of contaminating bacteria
1	1
2	2
3	2
4	2
5	1
6	2
7	1
8	1

the presence of other contamination sources such as health centers and hospitals.

By reference to the location of the sample taken which show bacterial growth, the results of the diagnosis reveal that the largest percentage among all isolated bacterial species is *Enterococcus cecorum* with (3) isolations (25%). This is followed by *Aeromonas hydrophila* with (2) isolates (16.67%), *Enterobacter cloacae* with (2) isolates (16.67%), *Aeromonas veronii* with (2) isolates (16.67%), *Klebsiella pneumonia* by (1) isolation by (8.33%) and *Enterococcus* columbae with (1) isolate (8.33%) and *Granulicatella elegans* with (1) isolate (8.33%) as shown in **Table 2**.

These results are in agreement with Al-Rawi and Al-Taei (2019), with isolates of *A. hydrophila* by 17.9% and *K*. *pneumonia* by 10.7% from domestic well water in Nineveh governorate.

The presence of bacterial contamination may occur as a result of the interference between groundwater and wastewater or from rain water that may have a role in the well contamination. The rain water running on the soil may be contaminated with pesticides, herbicides, fertilizers, manure used as fertilizer and residues of residential neighborhoods which may end to be in the groundwater. These pollutants reach the wells as a result of poor construction of the well and the presence of cracks in it or contamination may occur from the fluids resulting from burying the garbage due to the decomposition of organic materials, rain water that falls directly on the garbage or torrents water. This water is saturated with microorganisms and other contaminants and enters into the soil carrying pollutants to the groundwater (U.S.EPA, 2015). Other reasons for the presence of pollutants are related to the well itself, as the well may contain cracks and holes in the cover or the wells are not covered tightly and also the presence of defects in the well design with the sides of the well not built from the inside with rocks and cement to prevent surface water and pollutants from entering the well (UNESCO, 2012).

Virulence Factors Detection

The fact that bacteria possess different virulence factors indicates that it has different mechanisms of infection and may enhance its ability to cause diseases in humans and other organisms. Therefore, some important virulence factors are detected in bacteria isolated from wells under study as shown in **Table 5**.

The results of the virulence factors detection of *K. pneumoniae* show that it produces the gene encoded for Haemoalysin, Biofilm, Lecithinase, and Lipase by 100% and does not produce the gene encoded for protease, Urease, and B-Lactmase. For *E*. *cloacae* bacteria. It produces the gene encoded for Lipase., Hamoalysin,

Table 4. Numbers and percentages of bacterial isolates isolated from the water of the study wells

No.	Isolates	No. of isolates	Percentage%
1	*Aeromonas hydrophila*	*2*	16.67
2	*Klebsiella pneumonia*	*1*	8.33
3	*Enterobacter cloacae*	*2*	16.67
4	*Aeromonas veronii*	*2*	16.67
5	*Enterococcus cecorum*	*3*	25
6	*Enterococcus columbae*	*1*	8.33
7	*Granulicatella elegans*	*1*	8.33

Table 5. The ability of isolated bacterial species to produce virulence factors

Bacteria Species	Protease	Haemolysin	Urease	Biofilm	Lecithinase	Lipase	B- Lactmase
Enterococcus Columbae (1)	0%	0%	100%	100%	0%	0%	100%
Klebsiella pneumonia(1)	0%	100%	0%	100%	100%	100%	0%
Granulicatella elegans(1)	100%	0%	0%	100%	100%	0%	100%
Enterobacter cloacae(2)	100%	100%	0%	0%	50%	100%	50%
Aeromonas hydrophila(2)	50%	100%	100%	0%	50%	100%	50%
Aeromonas Veronii(2)	100%	50%	0%	100%	0%10	100%	100%
Enterococcus cecorum(3)	100%	66.7%	100 %	100 %	33.3%	66.7%	100 %

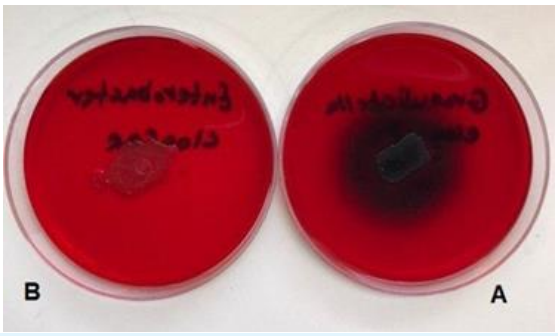

Fig. 1. Biofilm test on Konko Reed medium: Granulicatella elegans (A) producing the biofilm of *Enterobacter cloacae* (B) unproductive

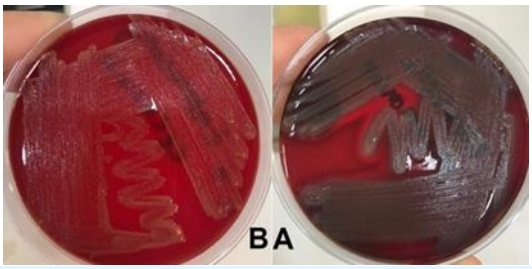

Fig. 2. Hemolysin test on the blod agar medium of the *Enterobacter cloacae* (A) hemolytic of the type β with complete lysis *Granulicatella elegans* (B) (non- hemolytic)

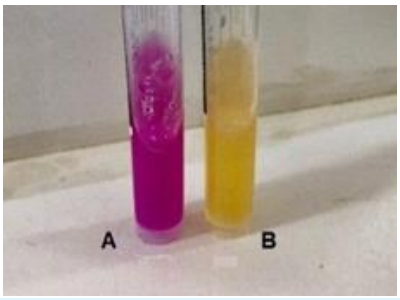

Fig. 3. Urease test of *Enterococcus cecorum* (A) producing urease enzyme of *Aeromonas veronii* (B) unproductive

and protease by 100%, and by 50% for each of B-Lactmas, Lecithinase, and unproducing for Urease and Biofilm. As for *A.veronii* bacteria, it produces a gene encoded for protease, Biofilm, Lipase, B-Lactmase, and Lecithinase by 100% and 50% for Hamoalysin and does not produce for Urease. In *E. cecorum* bacteria, it produces the gene encoder for protease, Urease, Biofilm, B-Lactmase by 100% and 66.7% for Hamoalysin and Lipase and 33.3% for Lecithinase. For *E. columbae* bacteria, it produces the gene encoded for Biofilm, Urease, and B-Lactmase by 100% and nonproductive for Protease, Haemolysin, Lecithinase, and Lipase. In *G.elegans*, it produces gene encoded for protease, Biofilm, Lecithinase, and B-Lactmase by 100% and unproductive for Lipase, Urease, and Haemolysin. For *A. hydrophila*, it has been shown to produce genes encoded 100% urease, lipase and Haemolysin by 100% and 50% for Lecithinase Protease, and B-Lactmase and nonproductive for biofilm.

The results are in agreement with Al-Ukaili *et.al* (2017) who found that all *A. hydrophila* isolates which are isolated from the Tigris river water produce both protease and hemolysin. As for Bhowmik *et. al.* (2009), they found that it is hemolytic by 71%, and also found that most of the isolates produce the lipase enzyme and some produce protease enzyme. The reason for the variation in the production of virulence factors may be due to the difference in environmental conditions. Abreu *et.al.* (2018) found that the concentration of pH and ammonia in the environment in which *A. hydrophila* lives affected its susceptibility to the production of virulence factors. The results are also in agreement with Sun *et.al.*(2016) who found that *A. veronii* isolates produce lecithinase enzyme, but differed in the fact that their isolates produce hemolysin of the beta type.

Agarose gel electrophoresis of Plasmid DNA

The DNA is extracted for bacteria isolated from well water, which include four gram-negative bacteria isolates and three gram-positive bacteria isolates. The extraction is made using a ready-made extraction kit, and the presence of DNA segments is confirmed by the appearance of DNA product after conducting the electrophoresis of the samples as shown in **Fig. 4**. The

Table 5. Purity and concentration of extracted plasmid DNA using Nanodrop device

260/280 purity	Nucleic acid conc (ng/ml)	Bacteria species
1.8	57	*Enterobacter cloacae*
1.81	63	*Klebsiella pneumonia*
1.78	83	*Aeromonas hydrophila*
1.81	88	*Aeromonas veronii*
1.9	83	*Granulicatella elegans*
1.84	60	*Enterococcus columbae*
1.79	74	*Enterococcus cecovum*

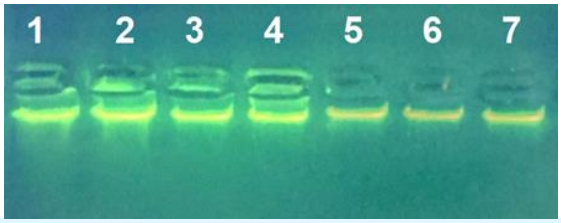

Fig. 4. Agarose gel electrophoresis of Plasmid DNA by 1% concentration at 5 volts/cm for one hour "(1) *Aeromonas veronii*; (2) *Enterobacter cloacae*; (3) *Aeromonas hydrophila*; (4) *Enterococcus cecorum*; (5)*Granulicatella elegans*; (6) *Enterococcus columbae*; (7) *Klebsiella pneumonia"*

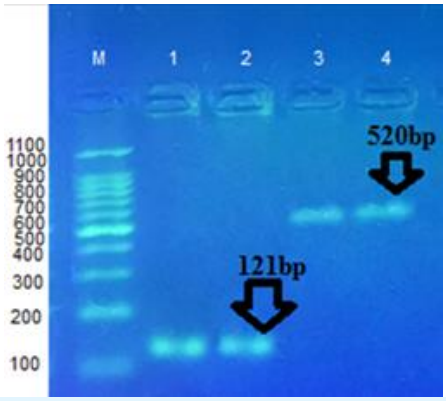

Fig. 5. PCR reaction result for protease gene encoder ProE, product size 121bp, and beta-lactamase enzyme gene encoder MOXM encoding with product size 520bp in *Enterobacter cloacae* bacteria, and the result is electrophoresed on agarose gel at a concentration of 1.5% ladder100

purity and concentration of the extracted DNA is measured using the Nanodrop device, and the purity of the samples ranged between 1.78 and 1.9 which confirms the high purity as shown in **Table 4**.

Detection of Virulence factor genes

The PCR is used to detect the presence of genes responsible for the encoding of some virulence factors in bacteria isolated from the water of the studied wells. The presence of genes responsible for protease genes encoder proE in the *Enterobacter cloacae* is detected by the emergence of a product at size of 121 bp base pair, and the presence of the MOXM gene responsible for producing beta-lactamase by showing a 520 bp-size of 520 bp base pair product as shown in **Fig. 5**.

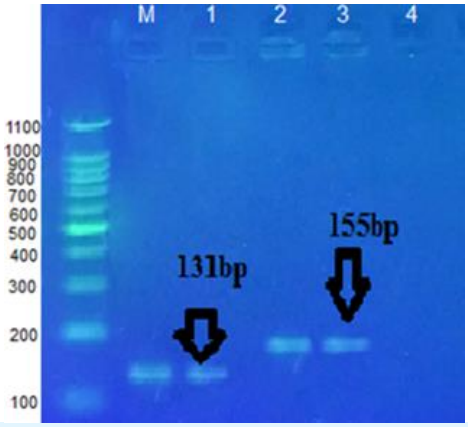

Fig. 6. The PCR reaction of the urease gene encoder *UreA*, with product size 131bp, and lipid gene encoder *lip*, with product size 155bp in *Aeromonas hydrophila*. The reaction output is electrophoresed on the agarose gel at a concentration of 1.5%. ladder (100)

As for the *Klebsiella pneumonia* bacteria, the presence of the hemolysin gene encoder *HemK* is detected by the emergence of a product with the size of 414bp base pair, and the presence of the biofilm gene encoder *LuxS* responsible for encoding the production of the biofilm by the appearance of a product of 447bp basal pair.

Some virulence genes have also been investigated in *Aeromonas sp*. Bacteria, The results show the presence of the *lip* gene responsible for the production of the lipase enzyme through the emergence of a product with the size of 155bp base pair and the gene responsible for producing the enzyme UreA through the appearance of a product with the size of 131bp base pair in *Aeromonas hydrophila* bacteria as shown in **Fig. 6**. This finding is consistent with the study of Abu - Elala *et.al*.(2015) and Mansour *et.al*. (2019), as they revealed the presence of a *lip* gene in all *A*. *hydrophila* isolated from fish.

As for *Aeromonas veronii* bacteria, it is detected that the gene responsible for the production of the protease enzyme ProA by the emergence of a product with the size of 317bp base pair and the gene responsible for the production of the lipase through the emergence of a product with the size of 155 bp base pair as shown in **Fig. 7**.

The possession of bacteria for these genes indicates its ability to cause disease when infecting living organisms. Nawaz *et.al.* (2010) found that the *lip* gene is present in 85% of *A*. *veronii* isolates, while Abu - Elala *et.al.* (2015) found that the *A*. *veronii* isolates do not contain protease and lipase enzyme genes encoders**.**

For *Enterococcus* sp. Species, the presence of the *Tuf* gene responsible for biofilm production is detected by the emergence of a 112bp base pair in *Enterococcus columbae* bacteria.

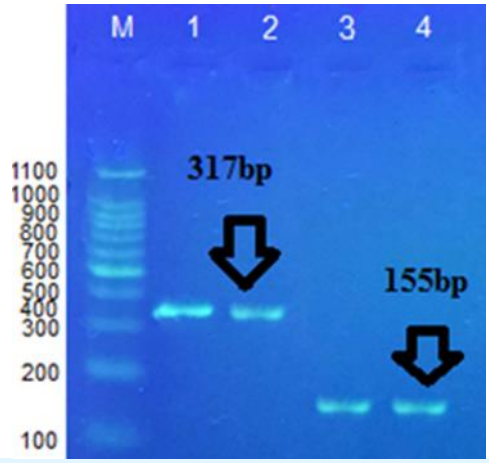

Fig. 7. The PCR reaction result of protease gene encoder ProA, with product size 317 bp, and lipase gene encoder *Lip*, with product size 155bp in *Aeromonas veronii*. The reaction result is electrophoresed on the agarose gel at a concentration of 1.5% ladder (100)

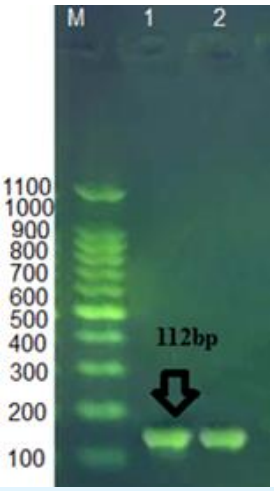

Fig. 8. The PCR reaction of the *Tuf* gene responsible for biofilm production with 112bp product size in *Enterococcus cecorum* bacteria. The reaction result is carried ou by the electrophoresis agarose gel at a concentration of 1.5%. ladder (100)

In *E*. *cecorum*, as shown in **Fig. 8**, the detection of the presence of *Tuf* gene responsible for biofilm production with the product size 112bp base pair.

In *Granulicatella elegans* bacteria, the presence of the *RpoB* gene is detected by the appearance of a 146bp product size as shown in **Fig. 9**.

The spread of bacteria that possess genes encoding some virulence factors, and antibiotic resistance genes

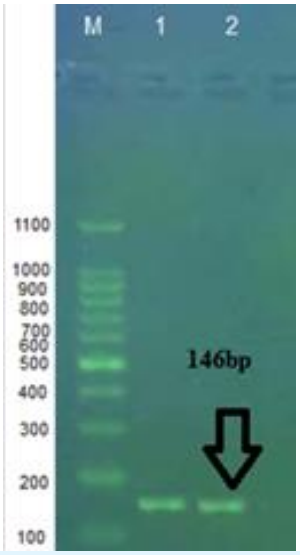

Fig. 9. The PCR reaction result of RpoB gene, with 146bp base pair product size, in *Granulicatella elegans* bacteria, and carried out on the electrophoresis agarose gel at 1.5% concentration. ladder (100)

in the water can be a major concern for human and animal health due to the the possibility of the spread of these bacteria and the occurrence of infections in humans and other organisms. The high prevalence of virulence factors in isolates indicates that these factors may play an important role in the pathogenic mechanisms of these microorganisms (Abu - Elala et al, 2015).

CONCLUSION

This study indicated a difference in the ability of bacterial species isolated from contaminated groundwater w to produce some virulence factors,which included hemolycin, lipase and protease, lecithinase, biofilm, urease, and beta-lactamases.

This variation in the production of virulence factors was associated with the isolated bacterial species having the genes responsible for coding for these virulence factors, such as the ProA and MOXM are found in Enterobacter cloacae, HemK and LuxS found in Klebsiella pneumonia, UreA and lip found in Aeromonas hydrophila. ProA and lip found in Aeromonas veroni., Tuf is found in Enterococcous cecorum and Enterococcus columbae and Granulicatella elegans, the presence of the RpoB gene.

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