The 1st International Scientific Conference on Environment and Sustainble Development (ISCESD 2013)29-30 Dec,2013

Detection of Some Pathogenic Water Bacterial Contamination Using PCRtechnique

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ABSTRACT

Water contamination is any change in biological properties of water that have a harmful effect on living things. From the beginning of April 2010 till the end of December 2011, One thousand five hundred sixty seven of tap water samples from different parts of Baghdad city were collected, and examined bacteriologic ally by traditional method for detection of pathogenic bacteria. For further detection the molecular study carried out to detected the virulence genes of pathogenic isolates , five isolates of Salmonella spp.gave positive results for the invA gene and prgKgene , three isolates of non-O1 V.cholera were positive for omp Wgene and yielded negative results for the ctxAB gene and the zotgenes but, one of them gave positive result for the tcp gene. Thirteen isolates of Aeromonashydrophila gave positive results for Fla and laf flagellin genes.

تشخيص بعض البكتريا المرضيةالملوثة للماء بأستخدام تقنية السلسلة المتبلمرة

الخلاصة:

تلوث المياه يعني اي تغير في الصفات البايولوجية . منذ بداية الشهر نيسان للعام 2010 ولغاية نهاية كانون الاول من العام 2011 جمعت 1567 عينة من مياه الشرب المجهز لمعظم مناطق بغداد وفحصت بكتريولوجيا بالطرائق التقليدية لتشخيص بعض البكتريا المرضية. ولغرض مزيد من التشخيص اجريت الدراسة الجزيئية اعتمادا على جينات الضراوة للبكتريا المرضية, اظهرت النتائج وجود 5 عزلات لبكتريا السالمونيلا والتي اعطت نتائج موجبة للموروثان invA gene and prgKgene . في حين اعطت ثلاثة عزلات من بكتريا ضمات الكوليرا غير المتلازنة نتائج موجبة للموروث ompW.ونتائج سالبة للموروثيين ctxAB gene and thezotgenes على التوالي وعزلة واحدة اعطت نتيجة موجبة للموروث tcp gene. في حين اعطت ثلاثة عشر عزلة من بكتريا Aeromonashydrophila نتائج موجبة للموروثيين Fla and laf flagellin genes.

INTRODUCTION:-

Water pollution is any change in the physical, chemical and biological properties of water that has harmful effects on living things. It is the second

https://doi.org/10.30684/etj.32.13A.6
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most important environmental issue next to air pollution (Edema *etal*.,2011). Polluted water consists of industrial discharged effluents, sewage water, rain water pollution and polluted by agriculture or households cause damage to human health or the environment (Roy *etal*.,2011) . This water pollution affects the health and quality of soils and vegetation. Some water pollution effects are recognized immediately, whereas others don't show up for months or years (Abdulhamd, 2010). In fact, the effects of water pollution are recognized to be the leading cause of death for humans across the globe, moreover water pollution affects our oceans, lakes, rivers, and drinking water, making it a widespread and global concern (Scipeeps, 2009).

According to the WHO (2006) , the mortality of water associated diseases exceeds 5 million people per year. From these, more that 50% are microbial intestinal infections, with cholera standing out in the first place. In general terms, the greatest microbial risks are associated with consumption of water that is contaminated with human or animal feces. Wastewater discharges in fresh waters and costal seawaters were the major source of fecal microorganisms, including pathogens (Grabow, 1996 ;WHO , 2008).

Material and Methods:-

Water samples collection and examination:- One thousand five hundred sixty seven drinking water samples were collected randomly from houses in different parts in Baghdad area, from the beginning of April 2010 till the end of December 2011.

Isolation of pathogenic bacteriawhich included Salmonella spp., Vibrio cholera ,Aeromonas spp., through using the membrane filter technique (MF),enrichment with specific broth media ,then cultured on selective solid media, identification by API 20E, Mini API and conformation by serological testesEaton et al., (2005).

DNA extraction:-

Two method were used for extraction:-

- Extraction of DNA from isolated bacteria , carried out by using genomic DNA kit (Gene aid).
- Total genomic DNA was isolated directly from water samples according to Delabre*et al*.,(1998) with few modifications as follow:

1- Water samples were concentrated by filtration through 0.45-µm-pore size nitrocellulose filters.

2- The filters were then vortexes in peptone broth , alkaline peptone for recovering bacteria and then incubated at 37°C for 24 h.

3- Suitable volume(1.5ml) of growth liquid was centrifuged at 4500 g for 20 minute. The pellet was extracted with protocol of genomic DNA extraction kit.

4- Preserved DNA with 50-100µl of Tris-EDTA (TE) solution in ependroff tubes at 20- C°.

Conventional Polymerase Chain Reaction(PCR) :-

Detection of the invA and prgKgenes for conformation the identification of the Salmonella spp., according to Csordaset al,(2004) and Shabanet al.,(2008),these primers synthesized by Cinna gen company Table(1). For sequences of primers were used, one primer for detection of prgK gene (Salm)and three primers for detection invA gene (invA,SEN,Sal) .

Table (1) The sequence and concentration of forward and reverse primers for invA and prgk genes, for Salmonellaspp .isolated from tap water according toCsordas*etal*.,(2004).

Prgk gene	Primers Sequence	Concentration in picomole	Product size
Salm F	CCTTTCTTATTGCGGGCA	28042.52	194 bp
Salm R	GCCGATGTGGATTATGAC	37810.59	194bp
invA gene			
InvA F	GTGAAATTATCGCCACGTTCGGGCAA	31894.93	285 bp
InvA R	TCATCGCACCGTCAAAGGAACC	31919.68	285 bp
Sal F	TATCGCCATTCGTTCGGCAA	33690.39	275 bp
Sal R	TCGCACCGTCAAAGGAACC	35900.10	275 bp
SEN F	TTTCAATGGGAACTCTGC	37165.24	172 bp
SEN R	AACGACGACCCTTCTTTT	28840.82	172 bp

PCR reaction was conducted in 100µl of reaction mixture containing 50 µl of green master mix,5 µl of each primer,10 µl DNA template and 30 µl of deionized water Table (2).

Table(2) :-The mixture of conventional PCR working solution for detection of,invA , prgk genes in **Salmonella spp.**

Working solution	
Water	30 µl
Forward primer	5 µl
Reverse primer	5 µl
DNA	10 µl
Master mix	50 µl
Final volume 100 µl	

Amplification was conducted using a master cycler eppendrof programmed with 30 cycler for initial denaturation 95°C for 3 min. ,denaturation for 94°C for 1min , Anneling 55°C 1min ,Extention72°C and final Extention 70 °C for 2min Table (3).

Table (3) PCR program forfragment*invA* ,*prgk*amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94°C	3 min
Denturation	94°C	1min
Primmer annealing	55°C	1min
Primmer extension	72°C	2 min
Final extend	72°C	2 min
Cycles number : 35 cycle		

Gel Electrophoresis:-

PCR products and the ladder marker were resolved by electrophoresis on 2% w/v agarose gels. DNA samples were loaded in the tray of Gels and 100 bp marker was included in every gel and run in TBE(1X) buffer, Gels were stained with ethidium bromide (0.5 µgml-1) and analyzed using UV eliminator The molecular weight identification of resolved band was based on their correspondence to the ladder bands.

Polymerase chain reaction for detection the*CtxAB*, *tcp* ,*Zot*and *ompW*genes for conformation the identification of the *V. cholera spp*., according to Gole*etal*. (2007) and Sheikh *etal*. (2012),these primers synthesized by Cinnagen company (Table 4).

Table (4) : The sequence and concentration of forward and reverse primers for, CtxAB , tcp, Zot and ompWgenes.

Type of primers	Primer sequence	Concentration in picomoles	Product size
CtxAB F	GCCGGGTTGTGGGAATGCTCCAAG	30205.40	536pb
CtxAB R	GCCATACTAATTGCGGCAATCGCATG	35072.28	
tcp F	CGTTGGCGGTCAGTCTTG	33592.20	805pb
tcp R	CGGGCTTTCTTCTTGTTCG	32252.47	
Zot F	TCGCTTAACGATGGCGCGTTTT	29411.76	947pb
Zot R	AACCCCGTTTCACTTCTACCA	37099.24	
ompWF	CACCAAGAAGGTGACTTTATTGTG	34215.30	588pb
ompWR	GAACTTATAACCACCCGCG	35282.28	

PCR reaction was conducted in 100µl of reaction mixture containing 50 µl of green master mix,5 µl of each primer,10 µl DNA template and 30 µl of deionized water (Table 5) .

Table(5) : The mixture of conventional PCR working solution for detection of Ctx AB, tcp, zot,genesandompW in V. cholera spp.

Working solution	
Water Forward primer Reverse primer DNA Master mix	30 µl 5 µl 5 µl 10 µl 50 µl
Final volume	100 µl

Amplification was conducted using a master cycler eppendroff programmed with 30 cycle for initial denaturation 95°C for 3 min. , denaturation for 94°C1min , gradient PCR with annealing temperature in the range of 52-62 °C to find out the appropriate annealing temperature that did not interfere with annealing of any of the primers .The optimum annealing temperature for the reaction was found to be 59°C for 1min ,extention72°C for 2 min and final extension 72 °C for 7min. (Table 6).

Table (6) : PCR program forfragmentctxAB,tcp,zot and ompWamplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94°C	3 min
Denturation	94°C	1 min..
Primmer annealing	59°C	1 min
Primmer extension	72°C	2 min
Final extend	72°C	7 min
Cycles number : 30 cycle		

PCR for detection of theLaf, Fla genes for conformation the identification of the Aeromonas spp., according to Sen and Rodgers (2004) and Santonsetal. (2010). These primers synthesized by Cinna gen company (Table 7).

Table (7) : The sequence and concentration of forward and reverse primers ofLafandFla genes.

Primer type	Primer size	Concentration in bicomole	Product size
Laf F	-GGTCTGCGCATCAACTC-	37881.12	504bp
Laf R	-GCTCCAGACGGTTGCTG	23592.32	504bp
Fla F	-TCCAACCGTYTGACCTC	38282.63	608bp
Fla R	-GMYTGGTTGCGRATGGT	33707.87	608bp

PCR reaction was conducted in 100µl of reaction mixture containing 50 µl of green master mix,5 µl of each primer,10 µl DNA template and 30 µl of deionized water (Table 8).

Table (8): The mixture of conventional PCR working solution for detection of Laf,Fla genes in Aeromonas spp..

Working solution	
Water	30 µl
Forward primer	5 µl
Reverse primer	5 µl
DNA	10 µl
Master mix	50 µl
Final volume 100 µl	

Amplification was conducted using a master cycler eppendroff programmed with 35 cycler for Initial denaturation 95°C for 5 min. ,Denaturation for 94°C 25 sec., Anneling 55°C 25 sec.,Extention72°C 1min and final Extention 70 °C 5min. (Table 9).

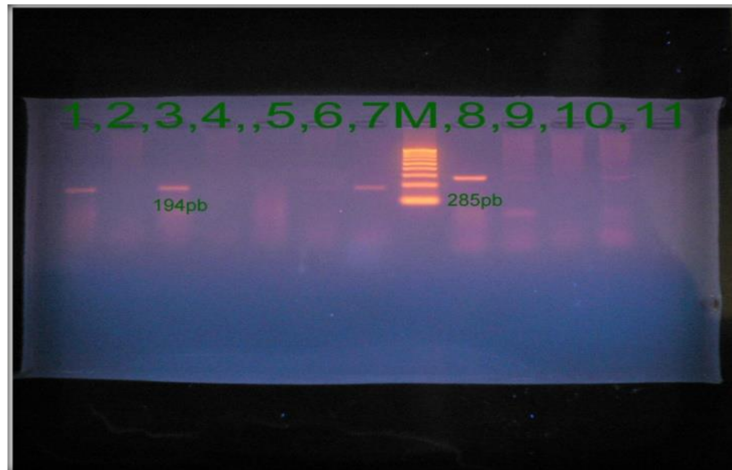
Table (9): PCR program forfragmentLaf,Fla amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94°C	5min.
Denturation	94°C	25sec.
Primmer annealing	55°C	25sec.
Primmer extension	72°C	1 min.
Final extend	72°C	5 min.
Cycles number : 35 cycle		

Results and Discussion:-

Molecular level and PCR technique:-

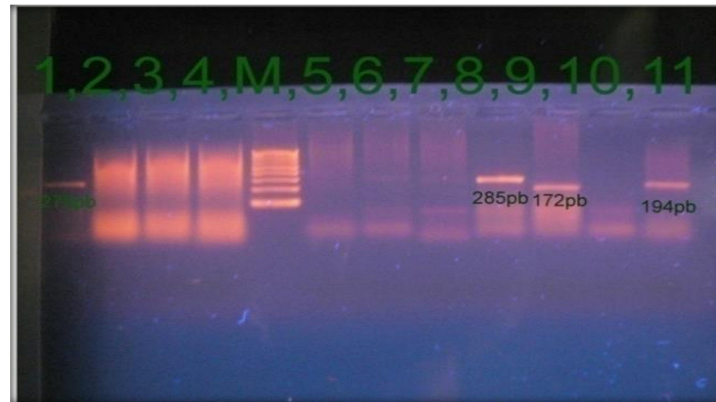
All samples were analyzed for conventional PCR for detection of virulence factors in isolated Salmonella spp., which include invA and prgKgenes .All isolates gave positive results for the two virulence genes with one specific primers(invA) for detectioninvA gene and one primer (Salm) for prgKgene Fig(1).



Figure(1):- Conventional PCR for detection ofinvA (285pb) ,prgK(194pb)genes, Lan:1,3,7 positive forprgK(194pb) using Slam primer and Lan: 8 positive for invA (285pb) usinginvA primer ,Lan:2,4,5,6,9,10,11 were negative for both, M:Marker DNA ladder.

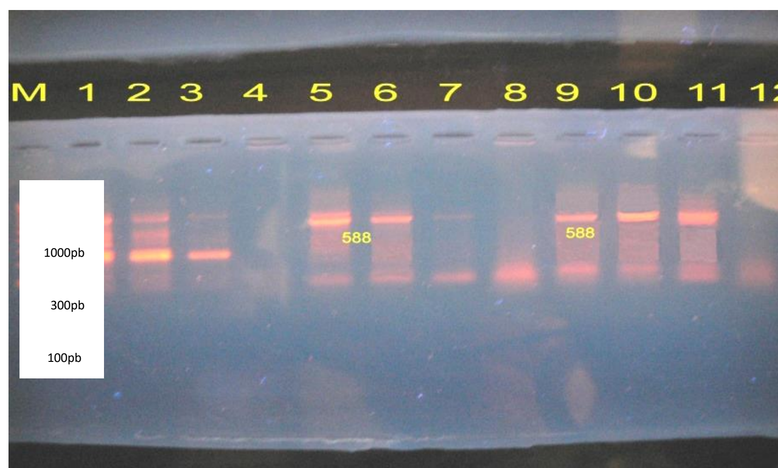
Since detection of bacteria or Salmonella in raw and tap water , needs amplification and detection of specific extracted DNA sequences in one organism to improve the speed and specificity of detection . In our study primers for detection invA (285pb) ,prgK(194pb)genes have been used for conformation the isolation of Salmonella . Salmonella typhimurium possesses at least five such pathogenicity islands (SPI), which confer specific virulence traits and may have been acquired by horizontal transfer from other organisms. These represent the virulence factors needed for invasiveness and survival intracellular in body host. The invA gene was targeted for the diagnosis of Salmonella spp. at the genus level, located on the pathogenicity island 1 of Salmonella spp. is essential for invasion of epithelial cells (Collazo and Gala´n, 1997). It is present in all invasive strains of Salmonella (Gala´n 1996; Murray ,2008). PrgK gene and its homologues are among the most highly conserved type III secretion system (TTSS) proteins and a major components of the needle complex (NC) , are likely to form the basal component of that apparatus.used by many bacterial pathogens to deliver virulence factors to the host cell and interfere with or subvert normal host cell signaling pathways (Marcus et al.2000 ;Kimbrough etal.,2000). All primer sets tested with the Salmonella spp. formed PCR products of the expected sizes , with same degrees of amplification Fig(2) . These results indicated the specificity and sensitivity of using different primers in detecting target gene for avoiding the generated primer dimmers, this may be useful to ensuring the clearing of conventional PCR products for further use in other technique such as real-

time PCR. Other factors such as the extraction and enrichment procedures may improve the sensitivity of PCR, which lead to increase levels of target DNA(Csordasetal.2004) .



Figure(2):- Conventional PCR for detection ofinvA (285pb) ,prgK(194pb)genes, Lan:11 positive forprgK(194pb) using Slam primer and Lan:1,8,9 positive for invA (285pb) using(invA,SEN,Sal) primers ,Lan:2,3,4,5,6,7,10 were negative for both, M:Marker DNA ladder.

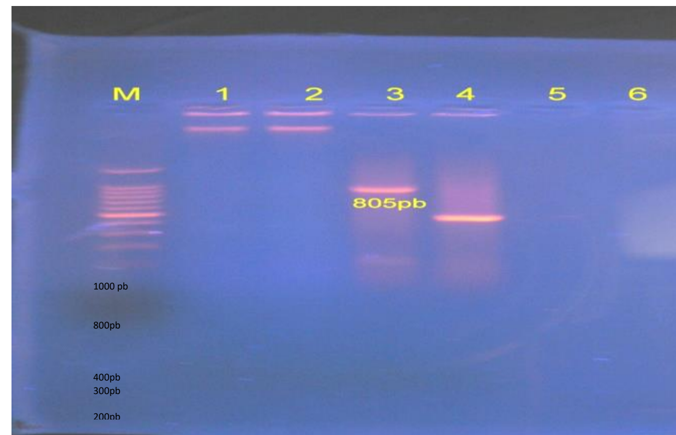
Among the 1567 drinking water samples in this study , three isolates were confirmed as V. cholera spp. by PCR assay with ompWgene (Fig 3),which showed specificity for all V. cholera non- O1 strain tested.



Figure(3): Conventional PCR for detection of *ompW*(588pb)gene, Lan:, 5,6 ,9,10,11 positive for*ompW*primer and Lan:1, 2,3,4, 7,8, negative for it, M:Marker DNA ladder.

The results presented here , go along nicely with the results recorded by Al-Naddawi (2010) and Sheikh et al. (2012),who found that all V.cholera non-O1 isolates were positive for ompW genes. In the PCR assay, all of the non-O1 isolates

yielded negative results for the ctxAB gene and zotgenes. Were as one isolate gave a positive result for the tcp gene (Fig 4).



Figure(4): Conventional PCR for detection of *tcp* (805pb)gene, Lan:3only positive for*tcp*primer and Lan: 1,2,4 negative for it, M:Marker DNA ladder.

Non-O1–non-O139 strains were distinguishable from pathogenic O1 stains, as they did not possess any major virulence genes based on PCR technique.TCP is a single V. choleraepilus that has been demonstrated to date to have a role in colonization of the gut mucosa of humans (Herrington et al., 1998). The species of V. choleraethat carry the tcpgenes on their genome, which are part of the pathogenicity island of their chromosome they can be infected by lysogenic phage CTXø and produced cholera toxin (CT).

The result that was obtained from this study confirmed the presence of tcp gene in one isolate of V. choleraenon O1, may be lead to produce Pilli type IV that are infected by lysogenic bacteriophage CTXø leading to toxin production .

This hypothesis came from the fact upon releasing of V. cholera from human to the environment undergo some physical and genetic changes and produce L –form which lead to cell wall deformity then preplasmic space well be altered too. This explain the stopped the expression of tcp and ctx AB since no location any more for the product of this genes (Brown ,1989; Najdat ,2006).

However, the VBNC state could still be demonstrated in the environment therefore, when a host comes in contact with V. cholerae O1 in the environment it results in clinical cholera or sporadic cases. Combined with rainfall and poor sanitation conditions, this will result in further contamination of water sources, amplification of the organism and hence the beginning of an outbreak (Mishra et al. 2011).

Furthermore, the present study attempted to detected A. hydrophila using Fla and lafflagellin genes to diagnosis motility by polar and lateral flagellum which is responsible for Aeromonas swimming in liquid media and swarming in solid media respectively , most of isolates gave positive results for both these gene (Fig 5).

Figure(5): Conventional PCR for detection of Laf , Flagenes(504 pb) and(608 pb) , Lan:1,2,3, only positive forLafprimer and Lan: 6 was positive for Fla primer and Lan :- 4,5, 7,8,9,10,11,12 were negative for them, M:Marker DNA ladder.

Moreover, swimming motility mediated by the polar flagella is important in attachment to the surface and colonies the intestinal tract in case of clinical samples and constituent of bacterial biofilms in water distribution systems. Lateral flagella permit fast and local colonization , were bacteria multiply to form microcolonies (Scoarisetal., 2007) . Such conclusion was supported by the work of Santons, et al. (2010) whom reported that isolates from environmental and clinical samples exhibit such genes, which have role in biofilm formation. Several studies have shown that mutation in the genes involved in the synthesis of polar and lateral flagella lead to consistent reduction in both adherence and biofilm formation (Kirove,2003).

From foregoing information we can say that molecular methods can characterized the principle genetic virulence of environmental and clinical strains of Salmonella , V.cholera and Aeromonas . One can conclude that potential pathogenic strains are present in the environmental , since all tested isolates possess virulence were from environment.

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