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PHENOTYPE AND MOLECULAR STUDY FOR SOME BACTERIAL ISOLATES WHICH ISOLATED FROM DIARRHEA PATIENTS IN RAMADI CITY

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ABSTRACT : This study included collected 280 stool samples from patients with diarrhea in the Ramadi Teaching Hospital and the Women's and Children's Hospital to Phenotype and Molecular study. For some bacterial isolates which isolatied from diarrhea pateints DNA was extracted then amplified the LT1, ST1, set 1 and stn genes by polymerase chain reaction through specific primers. The results of positive bacterial culture were 200 bacterial isolated included *E. coli* (77.5%), *Salmonella typhimurium* (5%), *S. muenchen* (2.5%), *Shigella flaxneri* (5%), *Klebsiella* (4%), *Pseudomonas* (3.5%) and *Proteus* (2%). The results of PCR reveled the LT1, ST1 gene were found in *E. coli* isolates with the presence of LT1 gene (85.7%), while ST 1 was not found in *E. coli* isolates. The results of the *Stn* gene analysis were found for all isolates and both *S. typhimurium* and S. *muenchen* in 100%. The results of the detection of the *set1* gene for *Sh. flaxneri* showed contained all isolates *set1* gene (100%). The PCR products were sequenced and detected variation compare with data base in NCBI by using the MEGA7 program, the results for DNA sequencing for LT1 and ST1 showed similarity 100% with NCBI except single tranisition (A-G) in position 233. The results for *stn* gene appear transition polymorphism (T-C, G-A and A-T9) in position 609, 565 and 571, respectively. And transversion (A-T), whereas the result for set1 gene showed 100% similarity with database with single transition (T-C) in position 76.

Key words : Diarrhea, stn gene, LT1, set 1.

INTRODUCTION

Diarrheal disease is a serious disease that is common all over the world, especially in developing and poor countries. The disease is the second leading cause of death in infants and under five years of age worldwide (Nguyen *et al*, 2005). Diarrhea is defined as a condition caused by dysfunction of the bowel or gastrointestinal tract (Davidson *et al*, 2002). Diarrhea is caused by pathological or physiological pathogens. It is defined as the increase in the number of stool times, which is more than the normal rate within 24 hours, with a change in stool consistency, i.e. liquid fluid is loose or watery and leads to loss of water and ions from the body, causing dehydration and increase blood viscosity and thus death (Pawlowski *et al,* 2009) exposed to the intestines of many microorganisms may be bacteria such as *Compylobacter*, *Escherichia coli, Salmonella, Shigella, Vibrio cholerae*. Diarrhea can also occur as a result of parasitic, viral or fungal infections (Haslett, 2000).

The studies confirm that the bacteria belonging to the Gram-negative bacteria group are among the main causes of diarrhea, especially those belonging to the Enterobacteriaceae family and play a role in the occurrence of epidemics (Nweze, 2009). The types of bacteria that cause diarrhea are *E.coli* contains several virulence factors including enterotoxins such as heat-labileLT, heat-stabile (ST) and responsible for watery diarrhea in infants and travelers (Allen *et al*, 2006).

As well as possessing factors to help them to cause infection such as adhesion to epithelial cells and colonization of the region and thus the secretion of extracellular proteins that help in the binding of bacteria to epithelial cells and the occurrence of lysis in adhesion sites (Brooks *et al*, 2001; Aksaray *et al*, 2000; Dalman *et al*, 2012). *Salmonella* plays a role in the occurrence of diarrhea and fever through because of it possess various virulence factors, such as cilia and flagella and Biofilmæ that help bacteria adhere to the epithelial cells of the intestines, resistance to antibiotics and escape from phagocytosis, as well as the possession of heat labile toxins, which helps in the occurrence of diarrhea (Hokcking *et al*, 2003; Jones, 2005; Cavalier, 2006).

Shigella produce numerous virulence factors including plasmid encode group of virulence genes as well as secretion of enterotoxins responsible for secretory diarrhea in *Sh. flaxneri* no 4 such as *Shigella* enterotoxin (ShET1), which is encoded by a set1 gene, while the second toxin *Shigella* entero toxin2 (ShET2) is encoded by the gene sen located on a plasmid and is responsible for epithelial cell inflammation by secreting IL-8 (Vorgas *et al*, 1999; Niyogi *et al*, 2004). *Shigella dysentriae* produces Shiga toxin (stx), which of the potent toxins and causes shigellosis. This toxin of AB5 subunits is associated with glycolipid Gb3 receptors present on epithelial cells of villi and therefore the occurrence of bloody diarrhea (Warnier *et al*, 2006).

MATERIALS AND METHODS

Specimen's collection

A total of 280 samples of patients with diarrheal cases were collected for the Ramadi hospital for gynecology and pediatricians and the Ramadi teaching hospital in Ramadi city. They were stored in clean and sterile plastic containers until they were transferred to the laboratory for testing for 1/9/2017 to 1/4/2018. By the loop and then cultured by streaking on the blood Agar media. The dishes were incubated at 37°C for 24 hours and then the single isolated colonies were taken and cultured on the MacConkey media. The dishes were incubated for 24 hours at 37°C and a number of differential media were used for diagnosis. Diagnosis of bacterial isolates Using biochemical tests and VITEK2 and serological diagnosis of isolates at the Central Health Laboratory of the Iraqi Ministry of Health.

DNA genome extraction

Genomic DNA samples were extracted from the 20 bacterial isolates using the Promega genomic DNA extraction kit and then the electrophoresis was carried out on 1% gel using the 1 Kilobase (Kb).

Detection of some virulence genes

Heat-Labile toxin (Lt1) and Heat-Stabile (ST1) were investigated which the product of enterotoxigenic *E. coli* (ETEC), responsible for the occurrence of watery diarrhea for travelers and infants. The gene *Salmonella* enterotoxin (Stn) related to *Salmonella typhimerium* and *Salmonella muenchen*, which is a gastrointestinal agent that stimulates bowel and diarrheal events. In addition, the gene (set1) is a gene that encodes the toxins of *Shigella* enterotoxin (ShEt1) in the *Shigella flaxneri* no 4 and causes watery diarrhea at an early stage of infection and by using the following primers in Table 1.

The thermocycler was set as in Table 2 to investigate the presence of genes in the selected bacterial isolates as shown in Table 2 and electrophoresis was done with 1% of agarose manufactured by Promega by using 100 pb marker.

DNA sequencing

DNA product results from gene amplification using PCR technology were sent to the Korean company Macrogen, by using the National Center for Biotechnology Information (NCBI) and then reading results according to the Blas Basic Local Alignment search tool.

RESULTS AND DISCUSSION

The results of the bacterial culture were (280) samples, which included 151 males and 129 females. The number of specimens that gave positive culture was 200 (71%). According to the statistical tests, there were high differences at the significant level of $P < 0.01$ for positive bacterial culture.

The percentage of positive bacterial culture of the study was close to the results of one of the researcher's study of AL-Musawi *et al* (2016) in Babil Governorate, with the percentage of Gram-negative bacteria (76.31%). The cause of diarrhea in samples that have been given a negative result of bacterial culture of parasitic or viral pathogens or due to intestinal sensitivity to some foods or milk for infants.

The results of the bacterial isolates diagnosis showed that the *E. coli* isolates were 155.5 (77.5%) of the total isolates, while the remaining isolates *Salmonella* 15 isolates were distributed among the *S. typhimurium* with 10 (5%) respectively. While 5 isolates of *S. muenchen* with 2.5% and (5%) *Shigella flaxneri* no 4 (5%). Other proportions of the other bacteria associated with diarrheal cases were not caused by *Klebsiella* 8 (4%) and 7 (3.5%) *Pseudomonas* spp and 5 *Proteus* (2.5%). The results of the chi-square test showed significant differences in *E. coli* at $P < 0.01$.

The percentage of *E. coli* isolates is closes to the results of a number of researchers in that the *E.coli* highest isolate of the rest of the other types of the total number 200 (77.5%). It came close to the study of Abdel-Kareem and Moussa (2009) were the *E.coli* percentage 76% and was also closed with the results of Suwaatarat *et al* (2008) as the percentage of *E. coli* is (95.82%).

The reason for the isolation of *E. coli*, more prevalent than the rest of the bacteria, may be due to its presence in the gastrointestinal tract and because it is an opportunistic bacteria that may be the cause of diarrhea in the event of immune suppression or malnutrition.

There are other studies that were close to the results

of the isolation of *Salmonella* bacteria in the research carried out by each (AL-karawiy, 2008; Jawad and AL-Hamadani, 2011) with percentages (7.8, 47%), respectively and may be due to this few percentages is that the presence of bacteria *Salmonella* is not normal as in the bacteria *E. coli*, but because of contamination of food and water taken by the infected person led to diarrhea.

For *Shigella* bacteria, their percentages differ from one study to another, for example, our results were consistent with the results of a study (Saeed *et al*, 2014), where they were 22 (6.9%) of the total 361, while there was a difference in the percentage of our study with the results of Jameel (2014), which (0.3%) for *Shigella* isolated from diarrhea in Tikrit and this agrees with most studies that confirm that the most common bacterial strains of diarrhea belong to the Enterobacteriaceae family (*Shigella*, *Salmonella*, *E. coli*) because of possession of entero toxins and other virulence factors (Haque *et al*, 2003). The other bacteria is not directly related to diarrhea were most studies do not refer to their relationship to diarrhea but their presence has differed of our results for example of Jameel (2014) in the study of the percentage of both *Proteus* and *Pseudomonus*, *Klebsiella*, which is 3.83%, 6.1%, 11.1% respectively, indicating the possibility of being a bacterial presence accidentally in the gastrointestinal tract but their presence is associated with the severity of the disease, *i.e.* secondary infection.

Detection of some virulence genes using PCR

Detection of 1LT and ST1 gene in *E.coli* bacteria

The results of the electrophoresis of the PCR products to detect the presence of the gene encoding toxin for heat labile toxin in the *E. coli* isolates and using specialized primers showed that there were 275 bp compared to the 100 bp ladder, giving 6 isolates out of a total of 7 (85.7%) as in Fig. 1.

The results of our study are consistent with the results of the study conducted in Brazil (Spano *et al*, 2008), which showed that all the isolates used in the study were contained in the LT gene, although some studies indicated that not all studied isolates were contained in this gene as in Paniagua *et al* (2007) the percentage was 58%.

In addition, the detection of the ST gene for the isolates (ETEC) and the use of specialized primers for the gene, the electrophoresis did not show any band of the 175 bp ST1 gene in all isolates used in the study. the local studies dealt with ST1 gene refer to decrease percentage of this gene appearance of all local isolates while the study of Lozer *et al* (2013), which dealt with the gene ST, LT in Brazil with the percentage of 2.3% and 0.4%, respectively.

The presence of this high percentage of the LT gene in the ETEC strain is due to the possibility that these strains contain the (LT) heat labile gene and do not possess ST stable heat gene. Several studies of ETEC have shown that this strain may contain these toxins together or contain one of these toxins in its genotype as in the O125 strain containing only the LT1 toxin.

PCR product of the (35) isolate of *E. coli* was selected to determine the sequences of the nitrogen bases of LT1 gene. These were compared with five sequences recorded in NCBI and using BLASTN2.6.4. Results of comparison with the first, second and third sequences were shown with the symbol FN822745. 1, CP023350.11, JX504011.1) belonging to the *Escherichia coli* ETEC strain. There were no mutations in the sequence of the nitrogen bases. The MF512033.1 sequences of the *Escherichia coli* strain EP321a (CP015024) have one mutation transition type in which the A-G base is converted into the 233 position and at the same time the sequences are encoded at the same time the code (CP015024) belonging to other *Escherichia coli* species had a similarity of 100% and no variation was found in the sequences. The results show that the LT1 gene can be repeated between different types of *E. coli* but with genetic variations due to mutations. These results give the impression that, after comparison, there is a similarity between LT1 among types with the same Sequence of nitrogen bases.

Detection of stn gene in *Salmonella*

The PCR was carried out to detect the stn gene and by using specialized primers for detection of Salmonella isolates used in the study, which belongs to the species *S. typhimerium* for 6 isolates and *S. muenchen* 2 isolates. This gene is responsible for the coding of enterotoxins, The results of the electrophoresis revealed the appearance of DNA bands at a size of about 617bp in both types and for the eight isolates used in the study and 100% as shown in Figs. 3-4. The results are consistent with several studies that indicated the presence of the stn gene in all *Salmonella* isolates used in those studies and regardless of the different strains and sources of isolation and by 100% (Sallam *et al*, 2014; Loongyai *et al*, 2007).

This gene is one of the essential genes in Salmonella bacteria. The expression of certain virulence factors for Salmonella bacteria can depend on certain environmental factors. It is expected that certain specific factors were present in the infected patient's environment, thus helping to show the stn gene in all isolates.

PCR products of the isolate of *S. typhimurium* have a number (8) were sent to determine the sequences of

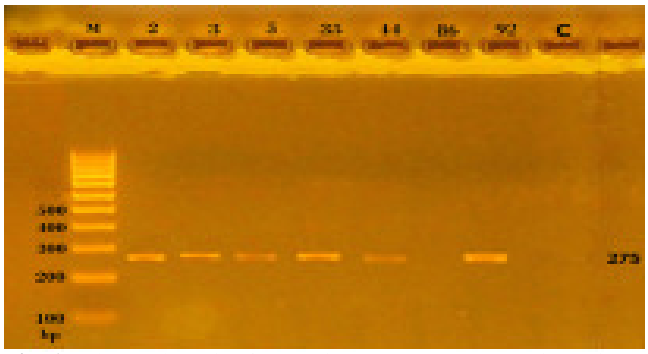

Fig. 1 : Electrophoresis of genomic DNA (100 v, 1%) to investigate the presence of LT gene at molecular size (275 bp) M represents the ladder and the isolates (2,3,5,35,44,86,92) represents *E. coli* bacteria and C represents Negative control.

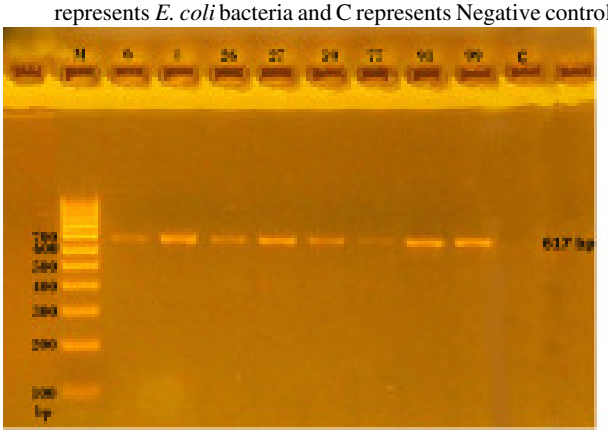

Fig. 2 : Electrophoresis of genomic DNA (100 V, 1%) to investigate the presence of Stn gene at the molecular size (617bp) M represents the ladder representing (6,8,26,27,59,91), *S. typhimurium* isolates, which represented 99.77 isolates and the *S. muenchen* represents C negative control.

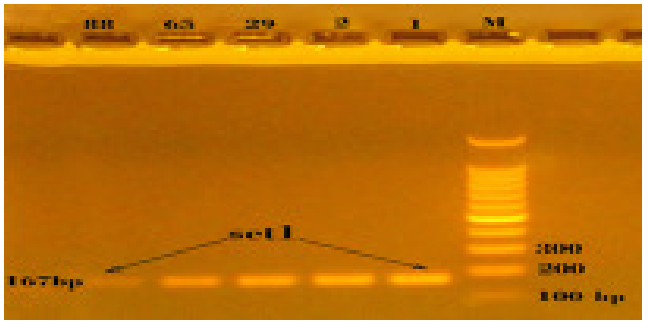

Fig. 3 : Electrophoresis of genomic DNA (100V, 1%) to investigate the presence of a set1 gene at a molecular size (167bp). M represents the ladder representing (1,2,29,65,88) isolates *Sh. flaxneri* no 4 isolations and C represents the negative control.

nitrogen bases of Stn gene, then the sequences were compared with five sequences recorded at NCBI and using BLASTN2.6.4. Results of comparison with the first sequence (CP024619.1) Salmonella strain on *S. entericasero* var *typhimurium* strain BL, the presence of variations in the sequence of nitrogen bases and the presence of two mutations one of which transition type, in which G-A becomes in site 4391669, the second is a transversion type, its A-T at 4391675 site were the matching percentage was 99% when some variations were found when compared with the sequences with symbol KF032246.1 belonging to the strain *S. entericasero* var *typhimurium* appeared 3 mutations of transition type in T-C mutation site 609 and G-A mutation at the site 565 and A-T mutation in the site 571.

For the third gene CP015024.1 belonging to the genus Salmonella and the *S. entericasero* var *Agona* strain where the ratio of similarity between the *Stn* gene was large and by 100% did not show any variations. The cause of some mutations in the same strain due to exposure to mutagenic factors or difference in place of isolation, while the emergence of strains belonging to other species containing the same sequence Stn gene and the same sequence of nitrogen bases is due to the possibility of repeating the gene in the same order of the nitrogen bases between the two mentioned genera.

Detection of gene set1 in *Shigella* bacteria

The PCR test was conducted to detect the presence of the set1 gene, which encoding *Shigella* enterotoxin (ShEt1), an important virulence factors of *S.flaxnerti* no 4, responsible for the early occurrence of watery diarrhea caused by gastroenteritis. The electrophoresis was carried out on the agarose gel to detect the presence of a set1 gene for 5 samples of *Shigella flaxneri* no 4, where the band of this gene was 167 (bp) for all isolates with 100% because of the possession of this strain to this gene.

While the results of the study of Nave *et al* (2016) in Iran the showed that the incidence of gene set1 in the *S. flaxneri* strain about 32.3% of the total number of samples studied. Talukder *et al* (2002) in Bangladesh, where no set1 gene (21) was found to be isolated from the *S.flaxneri* no 4 gene.

Table 1 : Primers and size products.

Primer	Sequence (5'_-3')Primer	Size bp	Reference	Type of bacteria
LT1-F	5'-TTA CGG CGT TAC TAT CCT CTC TA-3'	217	Kong *et al* (1999)	*E. coli*
LT1-R	5'-GGT CTC GGT CAG ATA TGT GAT TC-3'			
Stn-F	5'-TTG TCT CGC TAT CAC CC-3'	617	Chopra *et al* (1999)	*S. typhimerium*
Stn-R	5'-ATT CGT AAC CCG CTC TCG TCC-3'			
Set1-F	5'-GGAATGGCGACATCCATATT-3'	167	Designed based on primer3 plus program	*Shigella flaxneri*
Set1-R	5'-AACACTCTGTGGGGGAACAG-3'			

Table 2 : Thermocycler set for PCR reaction mix of primers.

Step	Temperature(°C)	Time	No. of cycles
Initial denaturation	94	2 min	1
Denaturation	94	1 min.	35 Cycles
Anneling	ST1,LT1 56	1min	
	Stn 59		35Cycles
	Set1 57		
Extension	72	10 min	30 cycles
Final extension	72	7 min	1

The isolation product number 1 of the *Shigella flaxneri* no 4 gene isolated from the diarrheal stool was sent and compared with 5 genes belonging to the site NCBI, the results of the comparison with the CP020339.1 showed that the match ratio 100% (CP020339.1) belonging to the *Shigella flaxneri* 4c strain while the comparison results with the second and third sequence carrying the symbol CP020336.1, CP012140.1 the ratio of similarity was significant with a single-type mutation transition type T-C in location 76.

Results of comparison with the fourth gene, KR822808.1, belonging to the strain *S. dysenteriae* and the fifth gene, CP026793, belonging to the *S.flaxneri 2a* strain, were completely identical to the sequence of the nitrogen bases of gene Set1, no mutation was found in this gene, as they 100% identical. The heterogeneity of the same strain is due to the exposure of bacteria to mutations and differences in the place of isolation, while some strains of other species showed similarity in the sequence of nitrogen bases.

It is possible to show that the gene (set1) can be replicated between different species of *Shigella* but with genetic variations due to mutations. These results give an impression after comparing the strains of *Sh.dysentria* and *Sh. flaxneri* no 4. There is a similar rate of replication of the gene set1 between species and the same sequence of nitrogen bases. This percentage is 100%. The gene can be repeated in the same order of the nitrogen bases between the two genera.

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