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Escherichia coli O104:H4: a New Challenge in Iraq

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Abstract

In recent years, Iraq has been imported quantities of meat and chicken from poor origins. Therefore, this study was conducted in order to ensure that they are free of *E. coli* O104. Samples of meat, chicken and vegetables, as well as samples of stool and urine of children under the age of 15 year were taken. Unfortunately it found in imported meat, stool and urine which are a warning bell for the spread of these bacteria in Iraq . *E. coli* O104:H4 strains were plating onto (CT-SMAC) and chromo agar STEC for isolation ,also attempt to determine the identities of the isolates using Gram staining, preliminary biochemical tests and confirmatory (API 20E and VITEK 2 System) , Presumptive colonies were confirmed by agglutination using latex particles attached to antibodies against sero group O104:H4 were subjected to specific PCR designed to amplify *16srRNA* and Real time PCR O104 antigen-encoding gene (*wzx*O104) and gene expression of *stx*2. The results showed the presence of E. coli O104:H4 in imported meat and in the stool and urine of children suffering from hemorrhagic colitis and hemolytic uremic syndrome while were negative for *Stx*. In conclusion, PCR results indicated that 16SrRNA and *wzx*O104genes were detected in studied samples , while *stx* was not detected. Accordingly, meat products may constitute an important reservoir for O104:H4 and PCR technique is the most sensitive and efficient approach for detection of O104 genes. This study is the first prevalence report for detection of *E. coli* O104:H4 in Iraq.

Keywords: *E. coli* O104:H4 strains, Molecular STEC detection, *wzx* O104 gene, Gene expression of stx2

INTRODUCTION

Escherichia coli (*E. coli*) are known as a versatile bacterial species encompassing both commensal and intraintestinal and extraintestinal pathogenic strains. An *E. coli* genome contains between 4200 and 5500 genes [1]. The O104:H4 was a verocytotoxin producing *E. coli* strain is a hybrid of enterohaemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC). The strain harbours blended EHEC and EAEC virulence genes and expresses phenotypes typical for each pathotype including the production of Shiga toxin (*Stx*) 2a [2], the strain is different from many VTEC strains because it lacks both the locus for the enterocyte effacement (LEE) pathogenicity island and the EHEC virulence plasmid.[3].

In May 2011, an unusual serotype of *E. coli* O104:H4 caused an outbreak of diarrhea-associated hemolytic uremic syndrome (HUS) in Germany. This strain combined the virulence potentials of 2 different pathotypes EHEC with *Stx* 2 production (also called *Stx* -producing *E. coli* or STEC) and enteroaggregating cells [4] .The combination of the prophage encoding *Stx* and multiple resistance factors probably caused the spread of the outbreak and its severity [5]. This strain of *E. coli* causes an illness similar to infection with *E. coli* O157:H7. The STEC *E. coli* O104:H4 responsible for the German outbreak has several genetic factors that contribute to its ability to cause illness. These include its ability to stick to cells in the intestine and to produce *Stx*. The toxin has deleterious consequences as it can affect multiple organs like the intestine that causes bloody diarrhea, impairing kidneys causing kidney failure, and sometimes affecting the nervous system as well. Secondary household transmission from adult patients has also been suggested for O104:H4 infections in Spain [3], France and Netherlands indicating the potential risk of the infection to acquire epidemic proportion[6]. Mora *et al*. [7] detected a significant decrease in the prevalence of STEC in beef sold, chicken and vegetables, and none of the STEC strains isolated from beef belonged to serotype O104:H4, implicated in the German outbreak.

During the course of the O104:H4 outbreak investigation, multi target Polymerase Chain Reaction (PCR) techniques have been used for rapid screening of samples. Molecular methods for STEC detection are used in various nucleic acid based assay formats, the most popular and commercially available being those based on PCR and Real-Time PCR (RT-PCR) techniques. The European Union Reference Laboratory for STEC has proposed a method that aims at the identification of the presence of O104 antigen in stx-positive enrichment cultures. This RT-PCR based assay targets O104 serogroup-associated genes: *wzx*O104, coding for the O-antigen, and the gene encoding the flagellar antigen H4, *fliCH4*. [8].

The aim of the present study was to describe the first isolation of *E. coli* O104:H4 strain in Iraq and determining of *stx2* *wzx*O104 coding for the O-antigen using RT-PCR techniques.

MATERIALS AND METHODS

Sample Collection

This study included **364** clinical and food samples included **226** hospitalized patients (**163** stool samples and **63** urine samples) suffering from hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) comprises children's age (less than 15 years) and from both gender, admitted to Al-Zaafarania hospital and central children's hospital in Baghdad. And **138** food samples included **85** samples of imported meat product and **53** vegetables samples of (potatoes, tomatoes, onion, cucumber, Muskmelon, Cabbage, lettuce, radishes, leeks, celery, Parsley, Crest, basil, and mint) collected from markets in different areas of Baghdad city (AL-Dura, Al-Bayaa, and Al-Karada) during the period from April 2016 to September 2016 .The collected samples were transferred directly to the laboratory in an ice box with a minimum of delay.

Isolation and Enrichment

Each food sample (25g) of meat and vegetables was weighed into sterile container and 225ml of modified tryptic soy broth (m TSB) were added supplemented with vancomysin (0.8 mg/L) or no according to [9] and incubated for 6-24hr. at 42°C . After 24hr. enrichment aliquots of 100µl were plated onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and chromo agar , for 24 hr. at 42°C. For stool and urine samples: Loopfull of each stool and urine samples from humans patients was enriched in mTSB supplemented with vancomycin (0.8mg/L) or no according to[9] then the same procedure of food samples were done.

Conventional Biochemical Tests:

All bacterial isolates were examined morphologically by Gram's stain also Colonies of presumptive O104:H4 were subjected to an important group of tests used IMViC test, indole -methyl red -Voges- Proskauer-citrate [10]. Also oxidase, motility, urea and Kligler's Iron agar test.

API 20E System and VITEK 2 System:

The bacterial isolates suspected as *E. coli* O104:H4according to conventional biochemical tests results were re-examined by API20E system, and VITEK 2 system (bioMerieux). The identification card for gram-negative bacilli (ID-GNB card) for the VITEK 2 system is a 64-well plastic card containing 41

fluorescent biochemical tests [11]. The isolates were analyzed on the VITEK 2 system, in accordance with manufacturer's instructions.

Latex agglutination test :

This test was used for more specific identification of *E. coli* O104:H4 by using commercial kit (*E. coli* O104:H4 latex agglutination kit /Product No.541060, Abraxis, Warminster, PA, USA)

Molecular Study for detection *E. coli* O104:H4

Bacterial DNA Extraction

DNA extraction was done according to manufacturer's instructions by using commercially available DNA extraction and purification kit (Geneaid Genomic DNA extraction Kit) .Primers used for the PCR assays are shown in Table (1) .

PCR amplifications were carried out in total reaction volumes of 20µl containing 1µl of each primer, lyophilized powder Master mix (Accu power® PCR PreMix ,BioNeer ,Korea), 5µl DNA template and then volume completed to 13µl by deionized distal water. All tubes were centrifuged in micro centrifuge for 10 seconds. The PCR tubes were transferred to the thermal cycler to start the amplification reaction according to specific program for primer consisting of initial denaturation at 94°C for 5min, 1 cycle with a denaturation at 94°C for 45 Sec., annealing at 55°C for 1 min and extension at 72°C for 1min, followed by the final extension at 72°C for 5 min. PCR amplicons were analyzed by 1.5% agarose gel electrophoresis, and then visualized under UV transillumination and photographed.

Further analysis for virulence gene *wzx*O104 for the possibility of *E.coli* strain O104:H4 was carried out using quantitative real time qRT-PCR SYBR Green assay and PCR reagent into Green Star™ q PCR Premix tube (Bioneer/Korea) was used. The PCR mixtures contained 5µl DNA sample, 1µl of each primer, DEPC-distilled water Adjust to 20µl. The following thermal profile was used for PCR: 95°C for 2min, followed by 35 cycles of 95°C for 20 s and 60°C for 5min.To determine the dynamic range of the assay, the obtained threshold cycle (CT) values were correlated to the total number of bacteria as gene equivalents for each PCR[15].

Gene Expression of shiga toxin (*stx*2)

Total RNA Extraction using commercially available RNA extraction kit (Bioneer Accuzol™ Total RNA extraction reagent) .The procedure was explained in details in user's manual. And total RNA was reversely transcribed to complementary DNA (cDNA) using Accupower® Rocket Script™ RT Premix Kit. Quantitative real time q RT-PCR SYBR Green assay was used. The mRNA levels of exogenous heterologous internal control-RNA (IC-RNA) were added to spike the reactions to normalize the relative *stx* gene expression in the real time PCR reactions. Primers sequences for *stx2* and *IC-RNA* genes are shown in table (1).

RESULTS AND DISCUSSION

Results showed that out of 364 samples 199 isolates of *E. coli* ferment sorbitol suspected to *E. coli* O104:H4 through pink creamy colonies appearance on sorbitol-MacConkey agar and the result culturing on chromo agar showed light purple or mauve appearance. Result of test performed for isolate *E. coli* O104:H4 showed that latex agglutination test gave positive result 7 isolates constituted 38.9% from total of suspected isolates by VITEK 2 system [18] isolates and only 3(42.9%) isolates from it presence of *wzx*O104 gene in imported meat (minced) and human stool. (Table 2)

In this study the meat products and stool and urine isolates, is a sorbitol fermenter pink creamy .The STEC O104:H4 outbreak strain, as most non-O157 STEC and human fecal E. coli isolates, is a sorbitol fermenter and appears pink on SMAC agar [4]. This result in agreement with Chattaway *et al*. [3] who showed directly plated onto selective CT-SMAC , the O104:H4 colonies grew very well producing a creamy pink morphology.

Chromo agar STEC O104 was successfully used for the isolation of E. coli O104:H4 which gave light purple characteristic feature on chromo agar STEC O104 ,this result in agreement with other authors [17,18] who showed all STEC strains are screened based on one phenotype, mauve. And with Paddock *et al.*[19] who showed light purple characteristic feature on chromo agar STEC and Chromo agar STEC O104. All the isolates were Gram negative rods and therefore satisfied that presumptive characteristic for E. coli. ,also identified by conventional biochemical tests and API20E system(Table 2).The latex agglutination kit was used for more specific identification of *E. coli* O104H4. It is used to detect the somatic antigen O and flagellar antigen H as compared with other methods of diagnosis; results indicated that 97% of isolates were E. coli. The *E. coli* O104H4 latex agglutination kits are available from Abraxis, and its sensitivity and specificity was reported as 100% and 99.0%, respectively. The same antibodies raised in rabbits were attached to both the immune magnetic and the latex beads useful for enhancing the ability to detect *E. coli* belonging to this serogroup

Table 1.The sequences of forward and reverse primers for *E. coli* O104:H4Conventional PCR and RT-PCR based Analysis

Primers	Sequence (5′→3′ direction)	Amplicon size(bp)	References
16srRNA- F	GCGGACGGGTGAGTAATGT	202bp	[12].
16srRNA-R	TCATCCTCTCAGACCAGCTA		
*wzx*O104-F	TGTCGCGCAAAGAATTTCAAC	100 bp	[8].
*wzx*O104-R	AAAATCCTTTAAACTATACGCCC		
*Stx*2- F	GCGGTTTTATTTGCATTAGC	115bp	[13].
*Stx*2- R	TCCCGTCAACCTTCACTGTA		
IC-F	GACCACTACCAGCAGAAC		[14].
IC-R	CTTGTACAGCTCGTCCATGC		

Table 2.Result of test performed for isolate *E. coli* O104:H4

Isolates source	Ferment sorbitol	No. of suspected isolates chromo agar	No. of suspected isolates with API 20E	No. of suspected isolates with VITEK2 system	No.(%)of positive isolates with *E. coli* O104:H4 latex agglutination test	No.(%)of positive isolates with q PCR for *wzx*O104 gene
Meat product	34	22	20	16	5(71.4)	2(5.8)
Vegetables	53	0	0	0	0(0)	0(0)
stool	71	1	1	1	1(14.3)	1(1.4)
Urine	41	1	1	1	1(14.3)	0(0)
Total	199	24	22	18	7	3

Detection of *E. coli 16SrRNA* Gene Using Conventional PCR

Result of the detection of *16SrRNA* gene showed that all 7 samples which suspected as O104:H4 were positive (100%) (Figure 1). In this study 7 samples were positive for O104H4 which agrees with Baranzoni *et al.* [17]. The isolates were confirmed as *E. coli* using PCR Amplification ,PCR has become a very rapid and reliable tool for the molecular biology-based diagnosis of a variety of infectious disease. It has been applied for the detection of microorganisms from microbial cultures and tissues and directly from clinical samples. The PCR diagnostic technique is a DNA-based detection and is one of the most specific and sensitive method for routine confirmatory assay for the bacterium [20]. All 7 tested isolates were confirmed as *E. coli* using *16SrRNA*-PCR shown in fig.1., similar results found by Abu- Duhier, [6]. The pair wise comparison of PCR versus VITEK indicated that there was no difference between these two techniques in the identification of E. coli. This result compatible with Baqer, [21] who showed that CT-SMAC agar gives false positive results at high rates and the using of vitek2 system is more accurate in the diagnosis of bacteria E. coli compared with API 20 and biochemical tests which diagnosed at the genus level only without the strain either PCR technique is the most efficient in the diagnosis of E. coli That depends in the diagnosis with a genetic side (DNA) who has not accepted the error

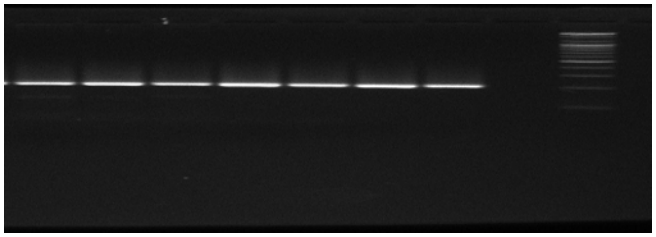

Fig-1: PCR amplification of *E. coli 16SrRNA* gene; (200 bp) specific genomic primer; Lane 1: 100 bp DNA ladder, Lane 1-7 : Positive for *16srRNA*; detected by 1.5% agarose gel electrophoresis and 70 volts for 1hr ,1X TBE buffer.

Detection of *wzx*O104 gene Using qPCR

The result showed that from a total of **7** positive *E. coli* O104H4 samples, only **three** samples positive for *wzx*O104 gene , **2 (5.8%)** meat products and **1(1.4%)** stool, which were latex agglutination-positive colonies. while the other four sample were negative for *wzx*O104 gene. The Ct value shown in (Table 3) the Ct value ranged from [18-22].

Table 3.Ct value of wzx104 gene

Type of sample	The Ct value (Mean ± SD)
Stool	22.11± 1.76
Product meat (minced meat 1)	21.26± 1.82
Product meat (minced meat 2)	18.88± 0.93
LSD value	2.739 *

RT-PCR quantification applied in the present study utilized the SYBR green, a fluorescent dye which recognizes any double stranded DNA including cDNA, the amplification was recorded as a Ct value .The Ct value of wzx104gene ranged from 18-22 (Table 3) .The lower Ct value indicates the presence of higher copies of the target and vice versa [22]. Delannoy *et al*. [15] performed RT-PCR Assay for detecting the *wzx*O104 gene of STEC O104:H4,some of tested samples showed positive for the *wzx*O104 gene, but not all sample ,these result compatible with present study which in line with the results of Trung *et al*. [23] who reported it is important to note that all four typical markers of the outbreak strain (*stx*2, *wzx*O104, *fliC*H4 and *aggR*) were detected, albeit at low numbers, in samples different host populations and Gene *wzx*O104 was observed in one isolate from a farmer (1.1 %).Paddock *et al*. [19] found 15.3% of fecal samples harbored the combination of *wzx*104, *fliC*H4 and *stx*2 genes, which does not necessarily mean that *stx*2, and *fliC*H4 were carried by O104 as the genes could have been carried separately by distinct serotypes. The presence of *wzx*O104 in enriched fecal samples was positively associated with the presence of *fliC*H4 However, there was no significant association between the presence of *wzx*O104 and either of the *Stx* genes from 51 stool samples, only 10 isolates were positive for the O104 serogroup-specific gene (*wzx*104). None of the 10 isolates was positive for *Stx*.

Gene expression (Real time PCR quantification of *stx*2)

All isolates of E. coli O104H4 (n= 7) were negative for shiga toxin. Reverse transcription PCR (RT-PCR) is technique which uses reverse transcriptase enzyme to produce DNA from RNA. RT-PCR has shown great potential for detecting viable pathogens such as when mRNA is detected [24] It is a rapid and sensitive method that can be used for the quantification of microorganisms [25]. It used to examine the presence of virulence genes specific to the pathogenic strain which included *stx*2 [6]. In this study The result for screening *stx*2 in all isolates of *E. coli* O104:H4 (n= 7) were negative for *Stx*, and compatible with Carbonari *et al.* [26] who stated that STEC/EAEC O104:H4 strain isolated during the outbreak in Europe, however, it was negative for the *stx*2 gene. Paddock *et al*. [19] indicated there was no significant association between the presence of *wzx*O104 and either of the *Stx* gene.

CONCLUSION

This study is the first prevalence report for detection of *E. coli* O104:H4 isolated from meat products and vegetables, as well as stool and urine of children under the age of 15 year in Iraq. The current study provides evidence of the onset of the spread of these bacteria in Iraq. This indicated the role of this group of *E .coli* as potentially important food borne pathogen. PCR results indicated that *16SrRNA* and *wzx*O104genes were detected in studied samples, while *stx* was not detected. Accordingly, meat products may constitute an important reservoir for O104 and PCR technique is the most sensitive and efficient approach for detection of O104 genes. Recommend Consumers should understand the importance of adequate refrigeration of meat products, hand washing before preparation and proper cooking of such meat products must be through cooked to permit the internal temperature to be lethal to vegetative bacteria.

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