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Using of *astA* and *uidA* Genes Characterization in Detection *of Escherichia coli* Prevalence from Human Gallstone

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Abstract: ***A total of 150 Gallstones samples were collected from patients undergoing cholecystectomy at the general teaching Hilla Hospital from November 2011 to June 2012. All samples were surveyed and examined for the presences of the Escherichia coliand differentiate it from other bacterial species. The Bacterial isolates were identified by using morphological and biochemical diagnostic investigations methods; Samples were cultured on Eosin Methylene Blue Agar (EMB), incubated at 37C° for 24 hrs, and then cultured on MacConkey Agar (MAC).50 bacterial isolates were identified as Escherichia coli. By using traditional biochemical tests. The Bacterial isolates were further identified by using Single Polymerase Chain Reaction (SPCR) technique for the presence or absence of 2 genes (astA, uidA) that encode for main virulence factors to diagnose E. coli isolated from Gallstones by using specific primers for these genes. Appearance of ustA and uidA in E.coli that isolated from gallstone confirmed the importance of these genes in gallstone formation through their ability to encoded B-glucuronidase.***

Keywords: gallstone, E.coli, uidA, astA, PCR.

1. Introduction

*E-coli*is one of the most important members of the *Enterobacteriaceae*. Strains predominate among the aerobic commensal bacteria in the healthy human intestine (Colleeet.al*.,* 1996).Bacterial B-glucuronidase produced by *E-coli* is an important enzyme which deconjugates bilirubin diglucuronide, resulting in the release of free bilirubin and glucuronic acid , the former precipitates with calcium ion to form calcium bilirubinate, which is the major component of brown pigment stones. It is assumed that one of the factors playing a role in the pathogenesis of gallstones is *E-coli* (Lee *et.al*., 1999).In the pathogenesis of bacterial infections, great significance is assigned to the adherence properties of bacteria, the permanent attachment of the microorganism to the cells of the host which causes lasting damage (Spitz et. *al.,* 1995).Numerous investigators have proposed a role for bacteria in biliary lithogenesis. We hypothesized that bacterial DNA is present in gallstones, and that categorical differences exist between gallstone type and the frequency of bacterial sequences(Lee ,*et.al*.,1999).We utilized the polymerase chain reaction (PCR) to establish the presence of bacterial DNA, in gallstones, bile juice, and gallbladder mucosa from patients with gallstones(Lee ,*et.al*.,2010).

Strains of entero-aggregative *E. coli* (EAEC), the most recently recognizedcategory of diarrhea genic*E. coli*, adhere to HeLa cells *in vitro* in an aggregative adherence pattern and are associated with watery diarrhea in young children in thedeveloping world. The pathogenesis of EAEC infection is not fullyunderstood; however, a characteristic histopathological lesion and several candidatevirulence factors have been described (Nataro and Kaper, 1998; Scaletsky, et.al., 1984). One of them is a 38-amino-acid protein called entero-aggregative *E. coli* heat-stable enterotoxin 1 (EAST1), encoded by the *ast*Agene, located on plasmids, on the chromosome, or on both of them (Savarinoet.al., 1993; Yamamoto *et.al*., 1997). The role of EAST1 in induction of diarrhea has not been clearly determined, however, the production of this toxin with several human ETEC strains has been demonstrated (Yamamoto *et.al*., 1997).The EAST1 gene was also detected in human ETEC isolated from patients with diarrhea and was mainly found among strains possessing major adherence factors such as colonization factor antigens (CFA) I and II (Yamamoto and Echeverria,1996).

Bacterial DNA sequences are usually present in mixed cholesterol (to 95% cholesterol content), brown pigment, and common bile duct, but rarely in pure cholesterol gallstones. The presence of bacterial B-glucuronidase is also suggested. The role of bacteria and their products in the formation of mixed cholesterol gallstones, which comprise the majority of cholesterol gallstones (Lee, *et.al*., 1999).

The *uidA* gene, which encodes for B-glucuronidase, has been used for detecting *E. coli* in previous studies (Bej*et al*. 1991a; Martins *et al*. 1993; Tsai *et al*. 1993; McDaniels *et al*. 1996; Iqbal *et al*. 1997; Lasalde *et al.* 2003). Although, the *uidA* and *uidR* genes are present in *E. coli* and *Shigella* spp. the activity of the enzyme is limited to *E. coli*. However, some *E. coli* fecal isolates have been shown to be negative for this activity, although the genes for the enzyme are present in these isolates (Martins *et al.* 1993; McDaniels*et al.* 1996; Monday *et al*. 2001). Studies show that many MUG negative *E. coli* strains, including the pathogenic serotype O157:H7, were detected after PCR amplification of the *uidA* gene (Bej*et al*. 1991a,b; Martins *et al*. 1993; Iqbal *et al.* 1997; Monday *et al.* 2001; Rompre*et al.* 2002).

2. Materials and Methods

During the period from November 2011 to June 2012, 150 patients with symptomatic gallstone underwent electivecholecystectomy done in surgical unit at Hilla teaching Hospital. During surgery, gallstones were

collected from different age patients (18-70 years) and different sexes (92 females and 16 males). The stone was washed with normal sterile saline to remove surface contaminants and each core of gallstone was scoped for bacterial culture. A single colony was taken from each primary positive culture on Eosin Methylene Blue Agar(EMB) and it was identified depending on its morphology (shape, size, borders, and texture) and then it was examined under microscope after staining it with Gram stain .After staining , the biochemical tests were done on each isolate to complete the final identification(Collee *et. al*.1996).eight specific biochemical tests (Indol Test ,Urease Test,Citrate Utilization Test ,MethylRed Test, Oxidase test , Catalase test , Motility Test , Haemolysis test). were done to differentiate *E. coli*isolates from other bacteria(Holt*et.al*.,1994).

Detection of selected virulence determinants by PCR

The genes selected for Single PCR(*uidA*, *astA*) were amplified by PCR with the optimized primerpairs listed in Table 1. The PCR reaction of *astA*gene was performed by using a 25µl reaction mixture consisting of 12.5ul green master mixulastA2.5 ,*-F,* 2.5ul *astA-Rul3* , DNA template ,4.5ul nuclease-free water.AndThe PCR reaction of*uidA*,gene was performed by using a 25µl reaction mixture consisting of 12.5ul green master mix ,2.5ul *uidA*-F,2.5ul *uidA*-R,3ul DNA template , 4.5ul nuclease-free water. Thermocycling conditions were as follows: 94°C for 55 min, 30 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 1.5 min, with a final extension at 72°C for 5 min. PCR-amplified fragments (10 µl) were separated on 2.0% (w/v) agarose gels and visualized under UV light after staining with ethidium bromide (Muller *et.al*., 2007).

Table 1: Primer pairs used for detection of marker genes indicative of a particular pathotype

Primers	*Sequence*	*Product Size(Bp)*	*Denaturation*	*Annealing*	*Extension*
uidA F	ATGCCAGTCCAGCGTTTTTGC	102 bp	94^{O}C for 5 min. 1cycle	94^{O}C for 30 sec. 63^{O}C for 30 sec. 72^{O}C for 1.5 min. 30 cycle	72^{O}C for 5 min.1cycle.
uidA R	AAAGTGTGGGTCAATAATCAGGAAGTG				
astA F	TGCCATCAACACAGTATAT CCG	1487 bp	94^{O}C for 5 min. 1cycle	94^{O}C for 30 sec. 63^{O}C for 30 sec. 72^{O}C for 1.5 min. 30 cycle	72^{O}C for 5 min.1cycle.
astA R	ACGGCTTTGTAGTCCTTCCAT				

Table 2: Master mix used in PCR

Master mix 2x	*Source*
Go taq DNA polymerase is supplied in 2x Green tag reaction buffer pH 8.5, 400 µm dATP, 400 µm d GTP, 400 µm dCTP, 400 µm dTTP and 3 µm $MgCl_2$	Promega (USA)

3. Results and Discussion

A total of 150 Gallstones samples were collected from patients undergoing cholecystectomy. .50 bacterial isolates were identified as *Escherichia coli* using traditional biochemical tests .These Bacterial isolates were undergoing a further identification through detection of two specific diagnostic genes by using Single Polymerase Chain Reaction (SPCR) technique.

Detection of *astA*gene

PCR identification of the *astA*gene revealed that among 50 *E. coli* isolates analyzed, 32 (64 %) isolates were positive as determined by the presence of the 102 bp amplified product whereas the other 18 isolates (36%) show negative results for this gene (table 3 ,figure1).

Table 3: Identification of *uidA* and *astA* genes for *E.coli* bacteria isolated from gallstones

Gene	No. of strains	Positive		Negative	
		No.	%	No.	%
astA	50	32	64%	18	36%
uidA	50	43	86%	7	14%

In the present study, the prevalence of the *astA*gene in *E. coli* isolated from patients with gallstone was examined. Several previous studies showed that most *E. coli* strains possessing genesfor at least one enterotoxin type (classified as ETEC) harbored the additional marker encoding the production of EAST1 toxin. Moreover, a close association of the *astA*gene with the presence of porcine fimbria colonization factor F4 was demonstrated (Nagy and Fekete, 1999; Osek, 1999), Therefore, the association of the EAST1 gene with F4-positiveenterotoxigenic strains recovered from piglets with enteric disorders suggests that the entero-aggregative

E. coli heat-stable enterotoxin 1 may be a virulence marker ofisolates pathogenic for these animals (Osek, 2003).

The EAST1 gene was also detected in human ETEC isolated from patientswith diarrhea and was mainly found among strains possessing major adherence factors such as colonization factor antigens (CFA) I and II (Yamamoto and Echeverria, 1996). These authors have alsodemonstrated that the genes for EAST1 and CFA are located on the same plasmidwhereas in porcine ETEC strains the *astA* and F4 virulence factors are carried on separate plasmids (Yamamoto and Nakazawa, 1997). Moreover, the EAST1 gene sequence of porcine F4-positiveETEC isolates was different from that of human CFA+ ETEC strains (Yamamoto *et.al*., 1997). On the otherhand, the pig origin EAST1 gene sequence is identical to that of strain O42 pathogenicfor human volunteers (Yamamoto and Echeverria, 1996).

Recently, Frydendahl, 2002 analyzed 563 *E. coli*isolates from 503 pigs with post weaning diarrhea and showed a high frequency (65.8%) of strains possessing the *astA*gene. The author also observed a close correlation between the presence of theEAST1 toxin marker and F4 fimbrial as well as LTI and STII enterotoxin genes. (Choi *et al.,* 2001) analyzed 720 *E. coli* strains isolated from piglets with entericcolibacillus's for the presence of the *astA*gene and again a close association of theEAST1 gene with the F4 fimbrial marker was found.

In conclusion, the results of the present work indicate that the *astA* gene is widely distributed among *E. coli* strains isolated from patients with gallstone. Therefore, EAST1 toxinmay represent an additional determinant playing a role in the pathogenesis of *E. coli*. Moreover, the presence of the *astA*gene makes the porcine *E. coli* strains similar to human EAST1-positive isolates that are potentiallyable to induce diarrhea (Osek, 2003).

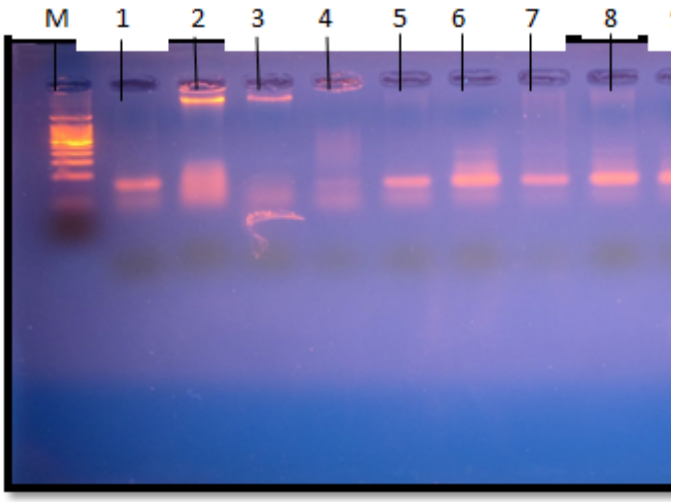

Figure 1: Agarose gel electrophoresis presenting the PCR results obtained with *astA* primers. Lane M, molecular mass marker (100 – 1500 bp range); lanes 1, 5, 6,7,8,9 *astA*-positive*E. coli*; lanes 2,3,4,10,11,12 *astA*-negative *E. coli*

Detection of *uidA*gene

PCR identification of the *uidA*gene revealed that among 50 *E. coli* isolatesanalyzed, 43 (86 %) isolates were positive as determined by the presence ofthe 1487bp amplified product (figure2). The remaining 7 (14%) strains testedwith the PCR method for the *uidA*marker did not generate a PCR amplicon of 1487bp or any other size (table 3).

beta-glucuronidase (GUD) is an inducible enzyme that is encoded by the *uidA*gene in *E. coli*(Jefferson,*et.al.*1986). About 94% of *E. coli* strains andsome *Shigella*spp. (44%) and *Salmonella* spp. (29%) appearto be the only members of the family *Enterobacteriaceae*that produce GUD, except for the pathogenicenter hemorrhagic*E. coli* of serotype 0157:H7, which is4-methylumbelliferyl-beta -D-glucuronide(MUG) negative (Feng *et.al.*1991).

Bacterial beta-glucuronidase causes deconjugation of bilirubin diglucuronide resulting in the precipitation of calcium bilirubinate, which contributes to biliary sludge and stone formation. This processes attributed to enzyme activity produced by the aerobic *Enterobacteriaceae* such as *E. coli* and *Klebsiella* sp. *E. coli* with its higher enzyme activity is more important in the deconjugation of bilirubin diglucuronide (Swidsinski and Lee, 2001).

Polymerase chain reaction (PCR) was used to amplify uidA (encoding *Escherichia coli [E. coli]* beta-glucuronidase) genes in different types of gallstones. PCR products were sequenced.Bacterial DNA sequences are usually present in mixed cholesterol (to 95% cholesterol content), brown pigment, and common bile duct, but rarely in pure cholesterol gallstones. The presence of bacterial beta-glucuronidase is also suggested. The role of bacteria and their products in the formation of mixed cholesterol gallstones, which comprise the majority of cholesterol gallstones, warrants further study.

Numerous investigators have proposed a role for bacteria in biliary lithogenesis. We hypothesized that bacterial DNA is present in gallstones, and that categorical differences exist between gallstone type and the frequency of bacterial sequences.

Bacterial 16S rRNA and *uidA* DNA sequences in *E. coli* were detected in all brown pigment, common bile duct, and mixed cholesterol gallstones (n = 14). In contrast, only one (14%) of seven pure cholesterol gallstones yielded a PCR product. Most (88%) mixed cholesterol gallstones yielded PCR amplification products from their central, as well as their outer, portions. Sequenced products possessed 88–98% identity to 16S rRNA genes of *E. coli* and *Pseudomonas*species (Lee *et.al*., 1999).

Bacterial infection is accepted as a precipitating factor in cholesterol gallstone formation, and recent studies have revealed the presence of *E. coli* species in the hepatobiliary system. We utilized the polymerase chain reaction (PCR) to establish the presence of bacterial DNA, including from *E. coli* species, in gallstones, bile juice,

and gallbladder mucosa from patients with gallstones (Lee, *et.al*., 2010).

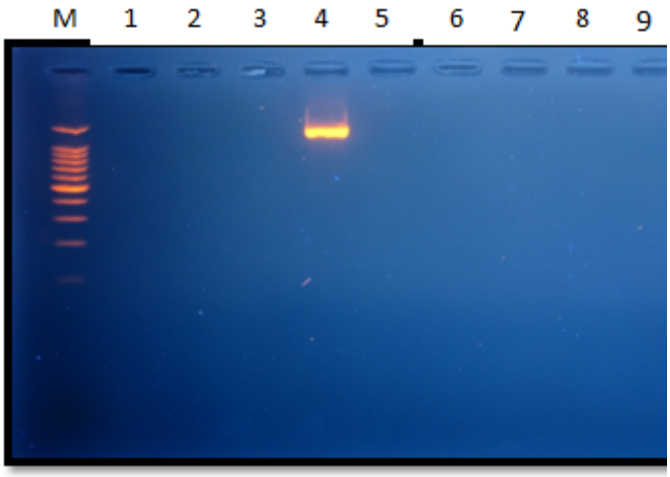

Figure 2: Agarose gel electrophoresis presenting the PCR results obtained with *uidA* primers. Lane M, molecular mass marker (100 – 1500 bp range); lanes **12 ,4** *uidA* -positive *E. coli*; lanes 1,2,3,5,6,7,8,9,10,11 *uidA* - negative *E. coli* .

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