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Genotypic assay to determine some virulence factors of Uropathogenic *E. coli* (UPEC) isolates

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***Abstract*---**A total of 179 urine samples were collected from patients suffering from urinary tract infections were admitted and visit Al-Hilla General Teaching Hospital in Al-Hilla City, during a period from April 2021 to December 2021, from both sex (male and female). Out of 179,123 (68.7%) were positive culture, whereas 56 (31.3%) samples showed no bacterial growth, To confirm the identification of *E. coli* by use selective media (EMB agar medium, biochemical tests, automated Vitek 2 system and *16s RNA* specific primer by the presence of (1492 bp) compared with allelic ladder, it was found that, *E. coli* were deliberated the main an etiological causes UTI to other types bacteria which constitute 56/123 (45.5%), [45/56 (80.4%) from female and 11/56 (19.6%) from male], while 67/123 (54.4%) were related to other types of bacteria. Molecular detection of some virulence factors genes were studied, out of 56 *E. coli* isolates, *hlyA* gene was detected in 21/56 (37.5%) isolates by the presence of (1177 bp) and *sat* gene was detected in 35/56 (62.5%) isolates by the presence of (410 bp) compared with allelic ladder.

***Keywords*---**Gene, PCR, *E. coli*, Urinary tract infections, Hemolysin.

Introduction

Urinary tract infections (UTIs) are one of the most common infectious diseases and resulted from successful colonization of the urinary tract by pathogenic microbes (Wu *et al.*, 2017), commonly caused by *Escherichia coli*, which accounts for 90% of community acquired and 50% of hospital acquired UTIs. *Escherichia coli* infection usually originated from intestinal normal flora or from fecal colonizes at periurethral area which resulting in UTIs known as uropathogenic *E. coli*

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(UPEC). Moreover, the bacteria present in water or food considered as a stool contamination which causes disease in the alimentary tract and urinary tract infection such as (urethra and bladder) (Al-Dulaimi, 2016). (UPEC) has the ability to colonize the urinary tract and persist in face of highly effective host defense so due to possessing a specialized virulence genes in higher prevalence rate than commensal *E. coli* strains (Forsyth *et al.*, 2018), this virulence factors increase the ability to cause symptomatic UTIs, these are located on mobile genetic elements called pathogenicity islands (Wiles *et al.*, 2008). Among most important virulence factors are toxins which secreted from a variety of *E. coli*-mediated diseases. Colonizing *E. coli* produced toxins that may be induced an inflammatory response, a possible pathway for UTIs symptoms, these toxins can alter the host cell signaling cascade and modulate inflammatory responses. UPEC have α-hemolysin is a secreted lipoprotein, strong and ubiquitous cytolysin, belongs to (RTX) family, *HlyA* is a 1024-residue, full-length is 110-kDa in size, multidomain protein that released by a type I secretion system (T1SS) which contains an ATP-binding cassette transporter (Linhartov *et al.*, 2010). In UPEC, (*HlyA*) found in approximately 50% of UPEC, and plays important role in pathogenesis, the *HlyA* gene is located in the operon (*HlyC*, *hlyA*, *HlyB*, and *HlyD), HlyC* is an acyltransferase that activates *HlyA*, and *HlyB* and *HlyD* are involved in *HlyA* secretion. The expression of hemolysin is regulated by several environmental stimuli, involved temperature, oxygen, and osmolarity (Barbieri *et al.*, 2014). The importance of *HlyA* in UPEC isolates is disrupt host nucleated cells by pore forming activity induce apoptosis in epithelium cell of urinary tract and immunity cells, cytotoxic to different cells and causes tissue damage through UTIs (Wiles and Mulvey, 2013) involved in the communication between host and pathogen and lyse other types of host cell involving epithelial cells and leukocytes from a different of human and animal hosts (Wiles *et al.*, 2008). UPEC has strong or weak activity of hemolysin which effect on host cells at lytic and sub lytic doses (Murthy *et al.*, 2019). The mechanism of cytotoxicity by hemolysin (the formation of membrane pores) when targeted erythrocytes cell, loss of intracellular K+ ions to the environment and subsequent influx of cations and water leads to osmotic lysis (Skals *et al.*, 2010). *Escherichia coli* strains derive from different phylogenetic groups; there are four groups: A, B1, B2 and D. The majority of strains responsible for urinary tract infections belong to group B2 or to a lesser degree to group D (Dadi *et al.*, 2020). Members of the Serine Proteases Autotransporters of Enterobacteriaceae (SPATE) family have been described as presenting proteolytic effects against complement proteins. Among the SPATE-encoding genes *sat* (secreted autotransporter toxin) has been detected in high frequencies among strains of *E. coli* isolated from bacteremia (Pokharel *et al.*, 2019). Sat has been characterized for its cytotoxic action, but the possible immunomodulatory effects of Sat have not been investigated (Freire *et al.*, 2022). SPATE is a superfamily of secreted virulence factors highly prevalent in enteropathogens, including *E. coli* and *Shigella*. These proteases are responsible for the degradation of intra or extracellular substrates, and their structure is remarkably similar, composed of three domains: an N-terminal signal peptide, a passenger domain, and a C-terminal translocator domain. The passenger domain, which is entirely secreted to the extracellular milieu, constitutes the mature form of the SPATE proteins and is responsible for their biological activity (Ruiz-Perez & Nataro, 2014).

Aim of study

The aim of this study to detected of some important virulence factors genes (*hlyA* and *sat*) among uropathogenic *E. coli* isolates.

Materials and Methods
Study Design

A total of 179 urine samples were collected from patients suffering from urinary tract infections were admitted and visit Al-Hilla General Teaching Hospital in Al-Hilla City, during a period from April 2021 to December 2021, from both sex (male and female).

Ethical approval

All subjects involved in this work were informed and the agreement required for doing the experiments and publication of this work was obtained from each one prior the collection of samples. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee (at College of Medicine University of Babylon) under the reference No. BMS/0231/016**.**

Clinical specimens

The specimens were generally collected from patients suffering from UTIs. Mid-stream urine samples were collected in sterilized screw-cap containers, then the urine samples were inoculated on culture media and incubated aerobically at 37^{o}C for 24 hours.

Identification of bacteria

Depending on its morphological properties (colony form, size , color, borders, and texture), a single colony from each primary positive culture on blood, MacConkey and nutrient agar and classify it and examine it by light microscope after being stained with Gram's stain. Biochemical tests were performed on each isolate after inspection to complete the final identification according to (Baron *et al*, 1994; McFadden, 2000) and we used the Vitek 2 method for *E. coli* identification.

Identification of bacterial isolates with Vitek2 System

Vitek 2 medical microbiology used as an automatic identification (ID) instrument device.

DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (Korea).

Detection of some of *E. coli* virulence genes

DNA (extract from bacterial cells) was used as a template in specific PCRs for the detection of some of *E. coli* virulence genes. DNA was purified from bacterial cells by using the Geneaid DNA extraction Kit. The primers used for the amplification of a fragment gene were listed in Table (1).

Table 1
The primers, sequences, and PCR conditions

Gene name	Primer sequence (5´- 3´)	Size of Bp	Conditions	References
16Sr RNA	F:AGAGTTTGATCCTGGCTCAG R: GGTTACCTTGTTACGACTT	1492	Step1:94°C, 2min. Step2:98°C, 10 sec. Step3:58°C, 30sec. Step4:68°C, 1.5min. Step5: 68°C, 5min.	Lin *et al.*, (2008)
HlyA	F:AACAAGGTAAGCACTGTTCTG GCT R:ACCATATAAGCGGTCATTCCC GTCA	1177	Step1:94°C, 3 min. Step2: 94°C, 1min. Step3:61°C, 30 sec. Step4:72°C, 3min. Step5: 72°C,7 min	Yamamoto *et al.*, (2009).
Sat	F:CTACAGCTTGATCACCTATGGC R:TCCCTGGTATTTCTTTGTGG	410	Step1: 95°C, 5min. Step2:95°C, 30sec. Step 3:55°C, 30sec. Step 4:72°C, 40sec. Step5:72°C, 5min	Freire *et al.*, (2022)

Results and Discussion

Out of 179,123(68.7%) were positive culture, whereas 56(31.3%) samples showed no bacterial growth (Figure 1), To confirm the identification of *E. coli* by use selective media (EMB agar medium, biochemical tests, automated Vitek 2 system and *16sr RNA* specific primer by the presence of (1492 bp) compared with allelic

ladder, it was found that, *E. coli* were deliberated the main an etiological causes UTI to other types bacteria which constitute 56/123 (45.5%) (Table 2), [45/56 (80.4%) from female and 11/56 (19.6%) from male, Figure 2], while 67/123 (54.4%) were related to other types of bacteria. The results revealed that dominance of *E. coli* (45.5%), which may be due to its natural presence in the normal flora in agreement with the results of Ali Jamal & Rahman, (2020) and Al-Saadi & Abdullah, (2019) (53.85%) and (50%) respectively in their study of isolation of bacterial causes of UTIs.

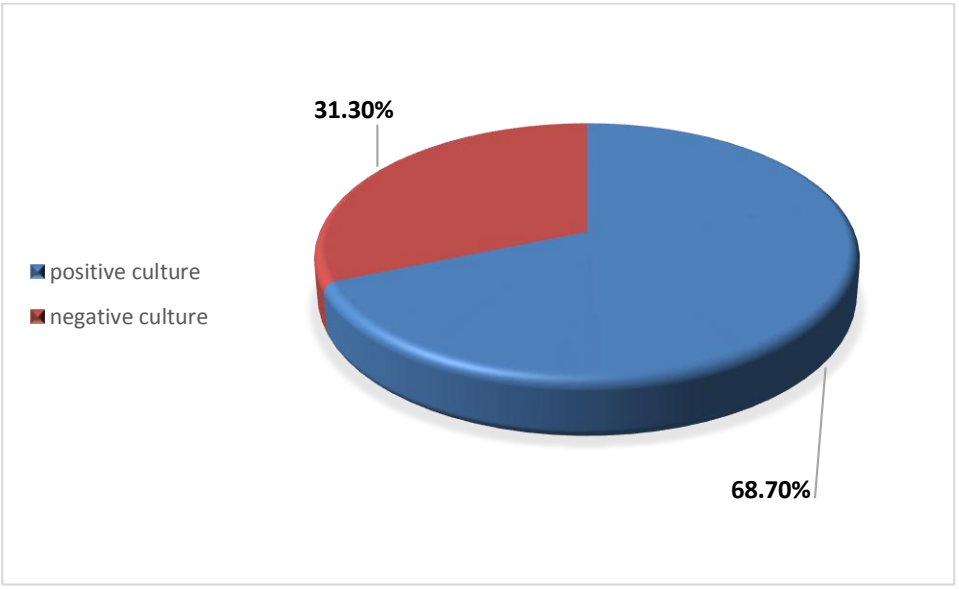

Figure 1: Positive and negative culture of all urine samples were collected from patients suffering from UTIs

Table 2
Identification of *E. coli* isolates depended on the colonial morphology, microscopically, biochemical tests and specific negative cards of Vitek 2 system

Total No. of samples	Positive culture	Negative culture	*E. coli* isolates	Other types of bacteria
179	123 (68.7%)	56(31.3%)	56/123 (45.5%)	67/123 (54.4%)

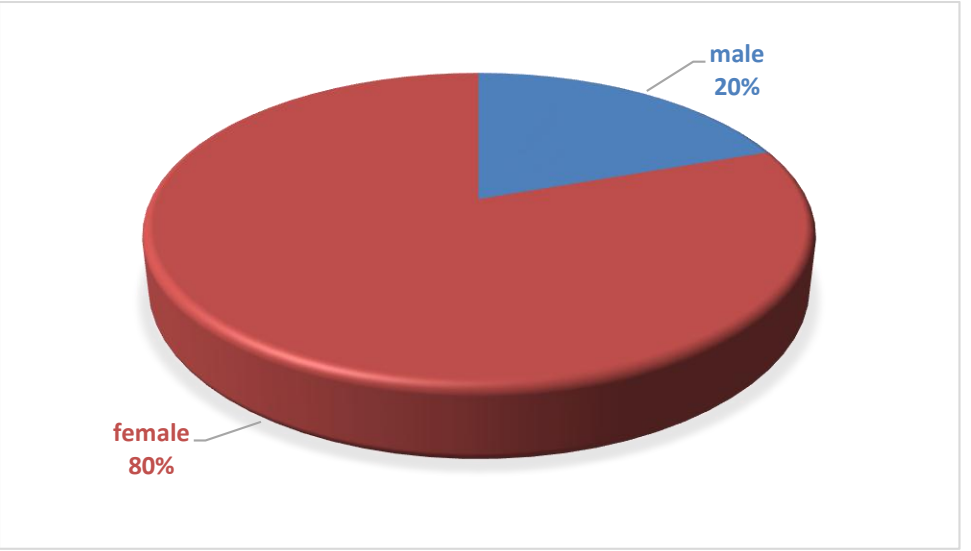

Figure 2: Distribution of *E. coli* isolates according to gender (male and female)

The study showed a difference in the incidence of UTIs among females and males. In female the percentage of UTIs was (80.4%), while males were accounted for (19.6%). These results were quite similar to Ghazvini *et al.*, (2019) which stated that, presence of *E. coli* isolation was (86%) in female and male (14%), while other studies of Tabasi *et al.*, (2019), Ahmed, (2020) and Dhilal, (2021) showed a lower percentage gender differences were recoded (64%), (73.3%), (65%) in female and (36%), (26.7%), (34.8%) in male respectively. Many factors affect UTIs treatment such as gender, age and the severity of the disease in the patient. UTIs more common in female with a high rate of recurrence that due to a different predisposing factors such as anatomical characteristics, hormonal status, pregnancy and lifestyle habits which they lead to much more common in female (Storme *et al.*, 2019). UTIs in males are uncommon due to the longer length of the urethra, antibacterial properties of prostatic fluid and less colonized periurethral, so the problems of infection appear after the 50 age (Shaheen *et al.*, 2019). According to the age, incidence of UTIs in all ages has reached the highest rate of infection (43%) in the age group (22-31) years for males and females, this result was agreement with another studies by Aya, (2019), Sule *et al.* (2016) and Al-Naqshbandi *et al.*, (2019) were determined a high ratio of positive patients between (26-30) and (21-30) years old as shown in Table (3).

Table 3
Distributions of age group to UTIs patients

Age groups	Patients	Percentage (%)
< 10	7	12.5
11-21	9	16
22-31	24	43
32-41	7	12.5
42-51	7	12.5

> 52	2	3.5
Female	45	80.4
Male	11	19.6
Total	56	100

Polymerase chain reaction technique was based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand and, the end of PCR reaction; the specific sequence will be accumulated in billions of copies (Amplicon). DNA was extracted from all suspected isolates that previously identified as *E. coli* by biochemical tests and Vitek system, conventional PCR was carried out using these DNA samples for the amplification of 16sr RNA specific primer gene for *E. coli*; according to the sequences and program listed in Table (1). After that gel electrophoresis showed that, all of 56(100%) samples of *E. coli* by the specific produced (1492) bp DNA fragment when compared with allelic ladder, as shown in Figure (3).

Molecular study of *hlyA* genes was detected in 21(37.5%) out of (56) *E. coli* isolates, the positive results were detected by the presence of (1177 bp) bands when compared with allelic ladder as shown in Figure (4). These results similar to the results obtained by Yazdanpour *et al.*, (2020) who found that, the ratio of the presence of a *hlyA* gene in *E. coli* isolates of urine 26%. Shahbazi *et al.,* (2018) found that, the ratio of the presence of a *hlyA* gene in *E. coli* isolates of urine at rate 41.7% and Moeinizadeh & Shaheli, (2021) found that, *E. coli* isolates of have *hlyA* gene in rate (90.8%).

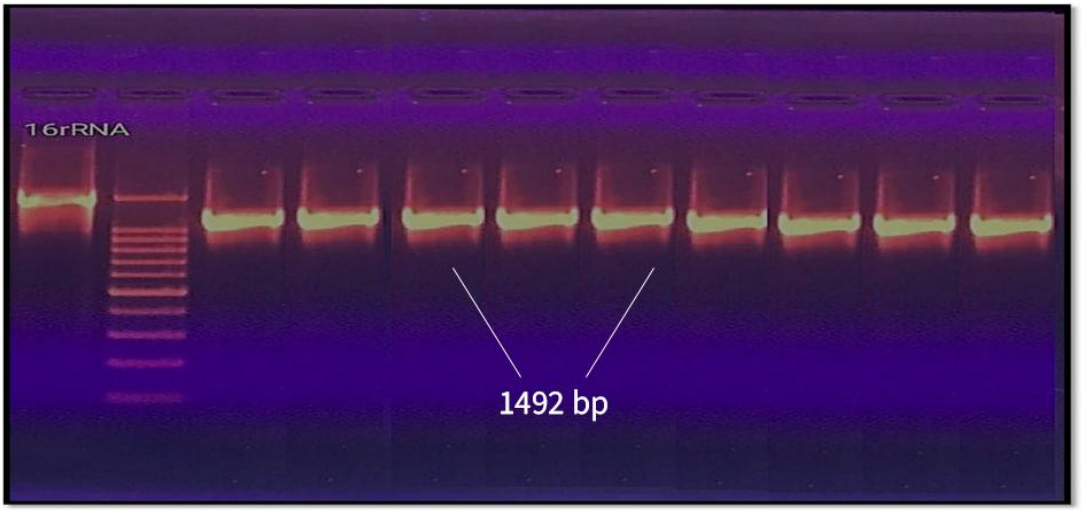

Figure (3): Agarose gel electrophoresis at 70 volt for 50 min for *16srRNA* specific gene products visualized under U.V light at 301 nm after staining with ethidium bromide. Lanes were positive for *E. coli*, the size of product is (1492 bp).

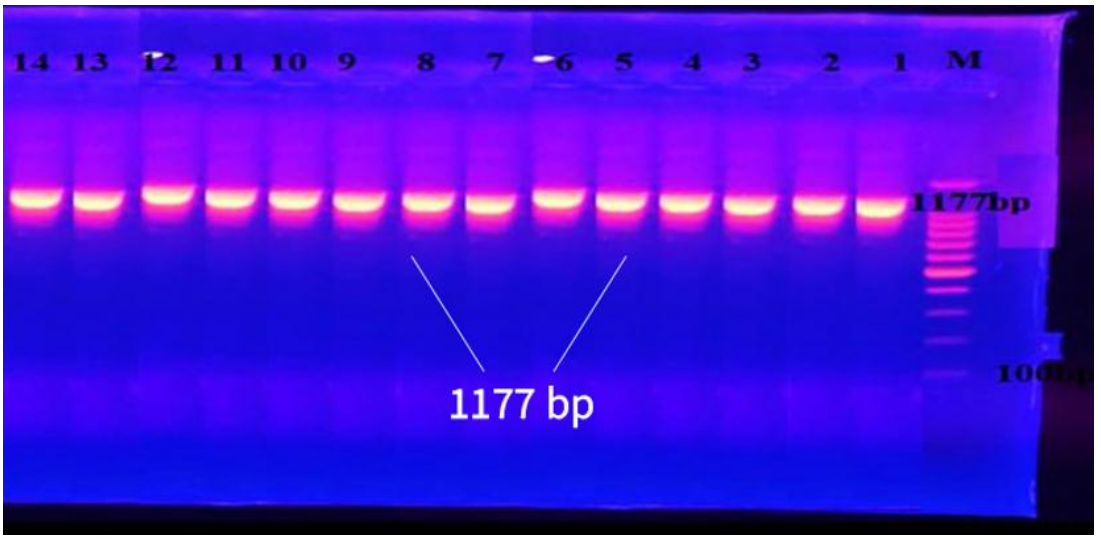

Figure (4): Gel electrophoresis of PCR product of hlyA gene at (1177) bp of E. coli

M: ladder, line (1-14) positive results for amplification, the electrophoresis was at 70 volt for 80 min

Alpha-hemolysin of UPEC, HlyA, is cytotoxic to a wide range of cells and causes serious tissue damage during UTIs (Wiles & Mulvey, 2013). The hlyA gene is located in the operon, including hlyC, hlyA, hlyB, and hlyD. HlyC is an acyltransferase that activates HlyA, and HlyB and HlyD are involved in HlyA secretion (Ristow & Welch, 2016) HlyA is reported to induce kidney inflammation and injury, and a higher percentage of hlyA-positive strains are isolated from pyelonephritis patients (> 70%) than from cystitis patients (31–48%), implying that HlyA is an important virulence factor in pyelonephritis (Wiles & Mulvey, 2013). *In vitro*, HlyA lyses cells by forming pores on cell membrane at high concentrations (Abdulabbas *et al.*, 2019). HlyA disrupts cell adhesion, triggers urothelial cell death, and induces inflammatory cytokines from epithelial cells or monocytes via cell signaling pathways at low concentrations (Dhakal & Mulvey, 2012). Hemolysin is an important virulence factor for many kinds of pathogenic bacteria. Molecular detection of *sat* gene was done for isolates that previously detected as *E. coli.* The results showed that, out of 56 isolates 35(62.5%) gave positive results for this virulence gene. Positive results were detected by the presence of (410bp) bands when compared with allelic ladder as shown Figure (5). These results were similar to results obtained by Freire *et al.*, (2020) who found that, *sat* was the most frequent gene (34.2%), similarly to the frequency observed by others, with *sat* frequencies ranging between 25 to 70% (Takezaki *et al.*, 2019). Further to this, *sat* is among the most frequent SPATE-encoding genes found in uropathogenic *E. coli* (UPEC). The high frequency of *sat* in *E. coli* strains isolated from bacteremia and Sat cytotoxic effects on endothelial and urinary tract cells suggest that this SPATE may be involved in different steps of BSI and sepsis pathogenesis (Freire *et al.*, 2022).

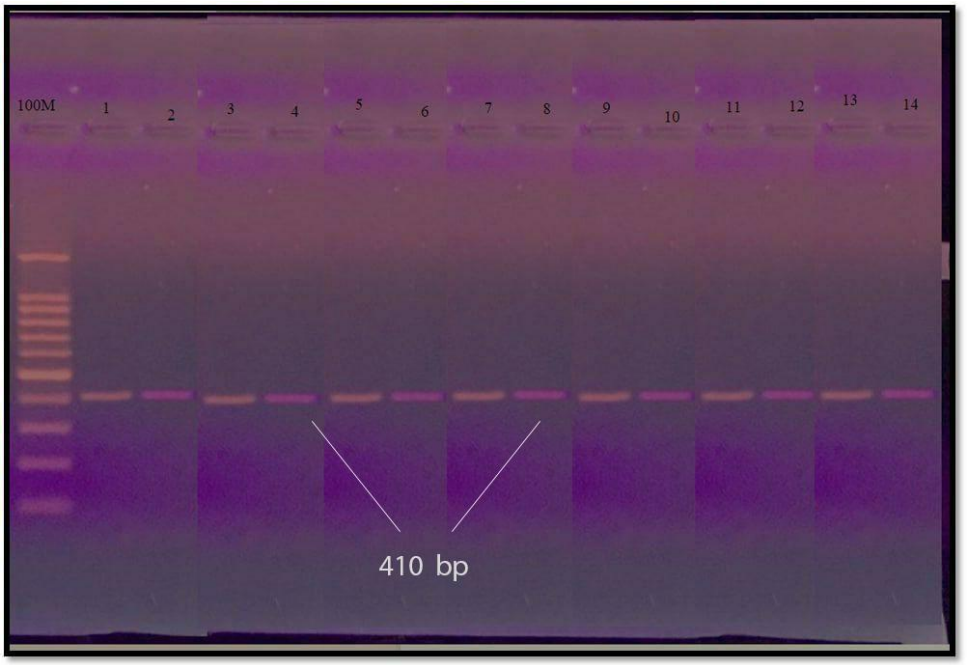

Figure (5): agarose gel electrophoresis (1.5%) of PCR amplified of *sat* gene (410bp) of *E. coli* for (60) min at (100) volt. M: ladder, (1-14) was positive results for *sat* gene

Considering that the complement system is the first barrier of the host innate immune response faced by *E. coli* in the bloodstream, Autotransporters such as Sat possess properties commonly associated with bacterial virulence (Sora *et al.*, 2021). A study of Tapader *et al.*, (2019) demonstrate that, Sat the 107-kDa autotransported serine protease of uropathogenic *E. coli*, elicits cytopathic effects on human bladder, human kidney, and Vero kidney epithelial cells. Based upon the association of the *sat* gene and its protein product with uropathogenic strains, the display of cytopathic activity on various cell lines, autotransporter protein Sat is a virulence determinant which contributes to the pathogenicity of *E. coli* UTI. This is further supported by the ability of Sat to elicit strong antibody response during experimental infection in the CBA mouse model of ascending UTI (Habouria *et al.*, 2019). Although Sat is not required for colonization of the urinary tract, it seems likely that Sat elicits cytopathic activity that damages host tissue and may increase the ability of *E. coli* to propagate. Indeed, one could speculate that specific damage to glomeruli and proximal tubules could facilitate entry of pyelonephritogenic strains into the bloodstream (Sarowska *et al.*, 2019).

Conclusion

UITs are one of the communal infections which are encountered in medical repetition. Significant result of *E coli* infection were found in the age range between (22 to 31) years old. Female were affected higher than men, and it has also shown the production of various virulent factors gene among isolates.

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