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Molecular Prevalence of *E. coli* and *Enterobacter cloacae* Caused Urinary Tract Infections in Iraqi Patients

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Abstract: ***The prevalence of antibiotic resistance in Enterobacteriaceae has increased sharply in recent years. Extended-spectrum ß-lactamase-producing Enterobacteriaceae include E. coli and E. coli and Enterobacter cloacae have become especially common. Although traditionally linked to risk factors such as prior hospitalization and antibiotic use, these bacteria have become increasingly recognized in the community, especially as pathogens in urinary tract infections (UTIs). This pattern of community-acquired resistance is concerning, as it may dictate a need for use of broader-spectrum antibiotics when initiating empiric therapy for routine infections. Aims of present study were to review the impact of E. coli and Enterobacter cloacae in urinary tract infections, and study the role of chuA and yjaA genes and anonymous DNA fragment TspE4C2 in the molecular detection of E. coli and Enterobacter cloacae in the Iraqi patients clinically diagnosed with urinary tract infection. In this study urine samples from 75 patients clinically diagnosed with urinary tract infection were used for Gram staining, culture, API 20 E, singleplex PCR and multiplex PCR methods. Singleplex and multiplex PCR were performed with primers targeted to chuA and yjaA genes and anonymous DNA fragment TspE4C2 of E. coli and Enterobacter cloacae. The results of the singleplex and multiplex PCR were compared with conventional methods of Gram staining, culture and API 20E test. The positive singleplex and multiplex PCR products were identified by presence of ~279 bp, ~211 bp and ~152 bp amplicons of chuA and yjaA genes and anonymous DNA fragment TspE4C2 for both of E. coli and Enterobacter cloacae. Conventional methods of Gram staining, culture and API 20E test showed positive result for E. coli in 14(18.6%) out of 75 patients clinically diagnosed with urinary tract infection and positive result for Enterobacter cloacae in 10(13.3%) out of 75 patients. PCR detected 13(17.3%) out of the 14(18.6%) samples that were positive for E. coli and detected 9(12%) out of the 10 (13.3%) samples that were positive for Enterobacter cloacae by using these conventional methods. The majority of urinary tract infections (UTIs) caused by spectrum ß-lactamase-producing Enterobacteriaceae include E. coli and Enterobacter cloacae were acquired in the community, so rapid, specific and sensitive molecular method such as multiplex PCR method is urgently needed to better prevalence, prevent and treat these infections in Iraq.***

Keywords: UTIs, *E. coli*, *Enterobacter cloacae*, *ChuA* gene, *yjaA* gene, *TspE4C2* fragment

1. Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections in human, both in community and hospital sittings. UTIs cause serious health problem, which affect millions of people every year. Infections of the UTIs are the second common type of infection in the body (Salvatore *et al.*, 2011). Prevalence of infections may differ with age, sex, and certain predisposing factors, the risk factors associated with UTIs include, immunosuppression, trauma, foreign body, broad spectrum antibiotic, use infused body fluids such as saline irrigations and also urinary catheterization, UTIs may affect 10% of people during childhood (Al-Araji, 2015). Bacteria are the most frequent cause of UTIs and aerobic Gram negative bacilli predominant (El-Naggar *et al.*, 2010). Enteric bacteria particularly *Enterobacter cloacae* are common gram negative opportunistic type of bacteria that cause disease after the host immune system has been weakened by another infection or injury and is associated with nosocomial infections, the infection may be contracted through the skin, GIT, UT, or cross contamination (Anabazhagan *et al.*, 2010).

The organism affects mostly the vulnerable age group, such as the elderly and the young and can cause prolonged hospitalization in intensive care unit (ICU) (Nishiijima, 1999; Francine and Grimont, 2006). Identification of bacterial isolates is an essential task of clinical microbiology laboratories, and its` relies on phenotypic tests. Traditional phenotypic identification is difficult and time consuming, while genotypic identification is emerging as an alternative or complement to established phenotypic methods. Typically, genotypic identification of bacteria involves the use of conserved sequences within phyelogenetically informative targets (Noor *et al.*, 2013). Phylogeny is the study of the evolutionary history and relationships among individuals or groups of organisms. Molecular phylogeny has also revealed that horizontal transfer plays an important and unexpected role in evolution (Wertz *et al.*, 2003).

2. Materials and Method

2.1 Samples Collection

Mid-stream urine samples were obtained from patients clinically diagnosed with urinary tract infection in Al Kadhmiya Teaching Hospital, Baghdad, Iraq for a period of 3 months. Seventy cases of urinary tract infection were investigated in this study. Patients' ages ranged from 20 to 30 years and for both gender. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq.

2.2 Identification of the bacteria

The urine sample was obtained from 70 patients clinically diagnosed with urinary tract infection and directly processed

for bacterial isolation and identification using standard methods according to (Forbes *et al.*, 2007), the urine samples were cultured on nutrient agar and incubated at 37°C for 24 hrs. The bacterial isolates from positive samples were activated using brain heart infusion broth and incubated at 37°C for 18 hrs, then the Gram stains were performed and cultured on nutrient agar and MacConky agar (Merck, Germany) at 37°C for 24-48 hours under aerobic condition as enrichment and deferential media for *E. coli* and *Enterobacter cloacae* from other *Enterobacteriaceae* members. API 20 E (Biomeriux, France) was used as a further conventional diagnosis for the *E. coli* and *Enterobacter cloacae* isolates from other *Enterobacteriaceae* isolates (MacFaddin, 2000).

2.3 DNA extraction

DNA was extracted from the *E. coli* and *Enterobacter cloacae* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of an overnight *E. coli* and *Enterobacter cloacae* culture grown at 37°C in brain heart infusion broth (Sigma, USA) was transferred to a 1.5 ml microcentrifuge tube. The microcentrifuge tube was centrifuged at 14,000 rpm for 3 minutes to pellet the cells and the supernatant was removed. 600 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added and gently pipet until the cells are resuspended. The microcentrifuge tube was incubated in water bath at 80°C for 5 minutes to lyse the cells; then cool to room temperature. 3 µl of RNase solution (wizard genomic DNA purification kit) was added to the cell lysate and the microcentrifuge tube was inverted for 5 times to mix. The microcentrifuge tube was incubated at 37°C for 60 minutes and cool to room temperature. 200 µl of protein precipitation solution (wizard genomic DNA purification kit) was added to the RNase-treated cell lysate and vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate. The microcentrifuge tube was incubated on ice for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The microcentrifuge tube was gently mixed by inversion until the thread-like strands of DNA form a visible mass and centrifuged at 14,000 rpm for 5 minutes. The supernatant was carefully pour off and the microcentrifuge tube was drained on clean absorbent paper. 600 µl of room temperature 70% ethanol was added and then the microcentrifuge tube was gently inverted several times to wash the DNA pellet. The microcentrifuge tube was centrifuged at 14,000 rpm for 2 minutes and the ethanol was carefully aspirated. The microcentrifuge tube was drained on clean absorbent paper and the pellet was allowed to air-dry for 15 minutes. 100 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added to the microcentrifuge tube and the DNA was rehydrated by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the microcentrifuge tube and the DNA sample was stored at -20°C until use.

2.4 DNA quantification

The extracted DNA from *E. coli* and *Enterobacter cloacae* isolates was quantified by spectrophotometer at O.D. 260/280 nm with ratios 1.4-1.5. The sensitivity of the chuA F, chuA R, yjaA F, yjaA R, TspE4C2 F and TspE4C2 R primers were evaluated by PCR amplification for serial diluted concentrations (10-100 ng) of purified DNA isolated from *E. coli* and *Enterobacter cloacae* isolates.

2.5 Primers selection

The primers for *ChuA* and *YJa* genes and anonymous DNA fragment TspE4C2 of *E. coli* and *Enterobacter cloacae* as the target genes for this study were selected according to (Clermont *et al.*, 2000). These sets of primers were designed based on the conserved regions in *E. coli* and *Enterobacter cloacae*, primers were synthesized by Alpha DNA, Kanda. The name, sequence and expected product size of these primers are shown in (Table 1).

Table (2-1): Primer name, sequence and expected product size of *ChuA* and *YJa* genes and anonymous DNA fragment TspE4C2 of *E. coli* and *Enterobacter cloacae*.

Primer name	Primer sequence (5'→3')	Expected product size (bp)
chuA F	GACGAACCAACGGTCAGGAT	~279
chuA R	TGCCGCCAGTACCAAAGACA	
yjaA F	TGAAGTGTCAGGAGACGCTG	~211
yjaAR	ATGGAGAATGCGTTCCTCAAC	
TspE4C2 F	GAGTAATGTCGGGGCATTCA	~152
TspE4C2 R	CGCGCCAACAAAGTATTACG	

2.6 Singleplex PCR Master Mix

The singleplex PCR reactions for detection of *ChuA* and *YJa* genes and the anonymous DNA fragment TspE4C2 of *E. coli* and *Enterobacter cloacae* were performed individually for each one of these genes in 20 µl volumes containing 4 µl of nuclease free water, 5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 3 µl of 10 pmol of forward primer (chuA F, yjaA F, TspE4C2 F) and 3 µl of 10 pmol reverse primer (chuA R, yjaA R, TspE4C2 R), and 5 µl of DNA sample. The mixes were overlaid with 2 drops of mineral oil.

2.7 Singleplex PCR program

The singleplex PCR reactions were carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Clermont *et al.*, 2000). Briefly, the Amplification of *ChuA* and *YJa* genes and anonymous DNA fragment TspE4C2 of *E. coli* and *Enterobacter cloacae* was carried out with initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds (for forward and reverse primers), and extension at 72°C for 30 seconds. The thermal cycles were terminated by a final extension for 7 minutes at 72°C.

2.8 Multiplex PCR Master Mix

The multiplex PCR reaction for detection of *ChuA* and *YJa* genes and the anonymous DNA fragment *TspE4C2* of *E. coli* and *Enterobacter cloacae* was performed in 50 µl volumes containing 12 µl of nuclease free water, 15 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 3 µl of 10 pmol of each chuA F, yjaA F and TspE4C2 F forward primers and 3 µl of 10 pmol of each chuA R, yjaA R and TspE4C2 R reverse primers, and 5 µl of DNA sample of *E. coli* or *Enterobacter cloacae*. The mixes were overlaid with 2 drops of mineral oil.

2.9 Multiplex PCR program

The multiplex PCR reaction was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Clermont *et al.,* 2000), which was used in the singleplex PCR reactions.

3. Results

3.1 Conventional methods

The result of conventional methods showed positive result for *E. coli* in 14(18.6%) out of 75 patients clinically diagnosed with urinary tract infection and positive result for *Enterobacter cloacae* in 10(13.3%) out of 75 patients. PCR detected 13(17.3%) out of the 14(18.6%) samples that were positive for *E. coli* and detected 9(12%) out of the 10 (13.3%) samples that were positive for *Enterobacter cloacae* by using Gram staining, culture on nutrient agar as shown in figure (3-1) and figure (3-2), and culture on MacConcky agar as shown in figure (3-3) and figure (3-4), respectively and API 20E test.

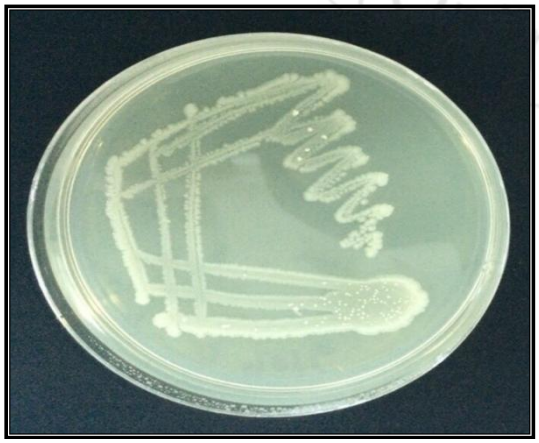

Figure (3-1): Growth of creamy colonies of *E. coli* on nutrient agar plate.

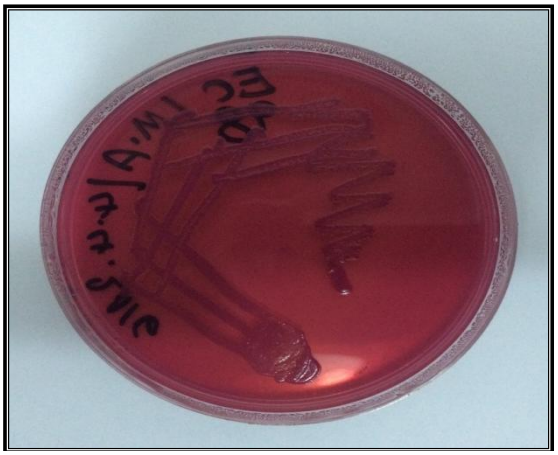

Figure (3-2): Growth of pink colonies of *E. coli* on MacConcky agar plate

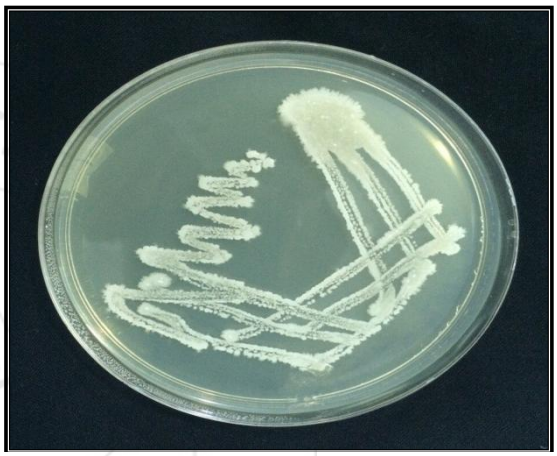

Figure (3-3): Growth of creamy colonies of *Enterobacter cloacae* on nutrient agar plate

Figure (3-4): Growth of pink colonies of *Enterobacter cloacae* on MacConcky agar plate.

3.2 Analysis of extracted DNA of *E. coli* and *Enterobacter cloacae* isolates

After performing of the DNA extraction from *E. coli* and *Enterobacter cloacae* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1% agaros gel at 7volt/ cm for 1 hour as shown in figure (3-5) and figure (3-6), respectively.

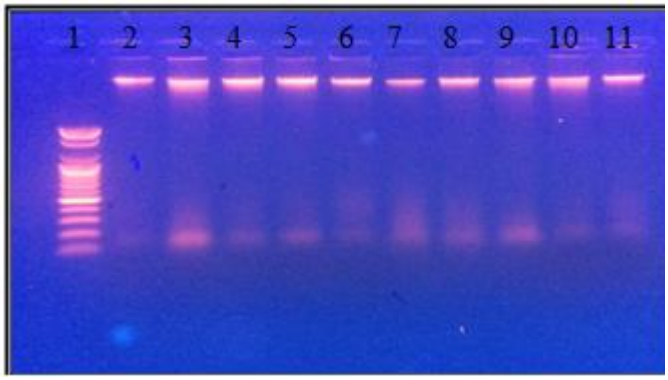

Figure (3-5): Gel electrophoresis of extracted DNA of *E. coli* isolates using 1% agarose gel at 7volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-11: Extracted DNA.

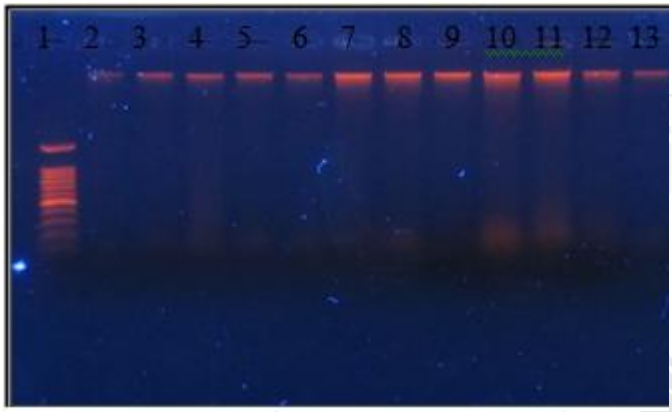

Figure (3-6): Gel electrophoresis of extracted DNA of *Enterobacter cloacae* isolates using 1% agarose gel at 7volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-13: Extracted DNA.

3.3 Singleplex PCR products analysis of *ChuA, YJa* and TspE4C2 genes of *E. coli* and *Enterobacter cloacae*

The result of *ChuA* and *YJa* genes and anonymous DNA fragment TspE4C2 amplification by singleplex PCR using ChuAF, ChuAR, YJaF, YJaR, TspE4C2F and TspE4C2R primers, respectively, that in 75 patients clinically diagnosed with urinary tract infection, a positive result for *E. coli* was detected in 13(17.3%) out of the 14(18.6%) samples and a positive result for *Enterobacter cloacae* was detected in 9(12%) out of the 10 (13.3%) samples who were gave positive result by the conventional methods. The singleplex PCR products and 100 bp DNA ladder were resolved by electrophoresis, 5 µl of signleplex PCR product were loaded on 1% agarose gel and run at 100 volt/ cm for 45 minutes. The gel was stained with ethidium bromide solution (0.5 µg/ ml) for 15-30 minutes, finally, the DNA bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system. Singleplex PCR result was considered a positive for *E. coli* isolates when there were presence of ~279, ~211 and ~152 bp PCR product band on agarose gel electrophoresis as shown in figure (3-7), figure (3-8) and figure (3-9), respectively. In addition the Singleplex PCR result was considered a positive for *Enterobacter cloacae* isolates when there were presence of ~279, ~211 and ~152 bp PCR product band on agarose gel electrophoresis as shown in figure (3-10), figure (3-11) and figure (3-12), respectively. No amplification was observed with negative control.

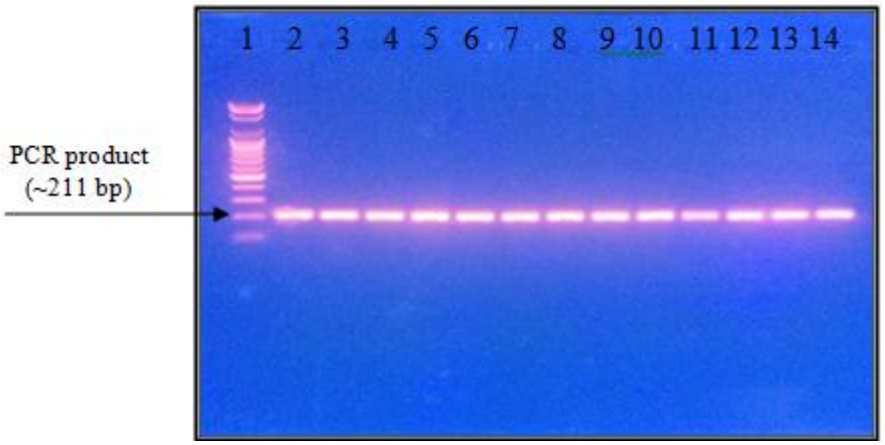

Figure (3-7): Gel electrophoresis of singleplex PCR products of *ChuA* gene for *E. coli* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-10: PCR products of *ChuA* gene.

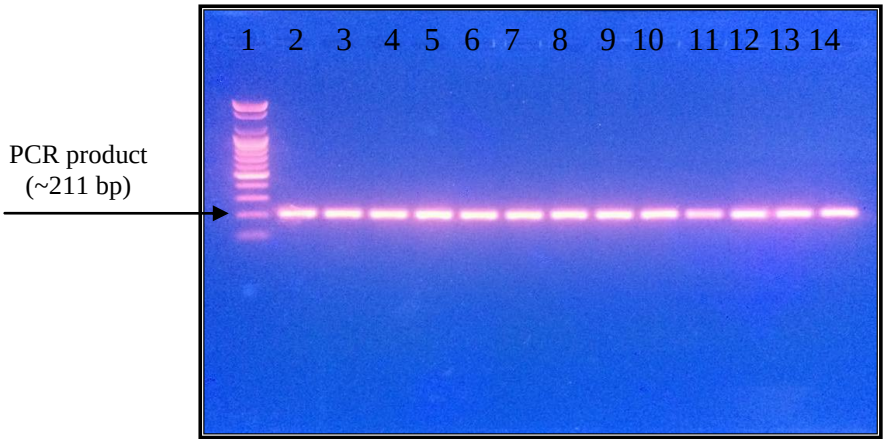

Figure (3-8): Gel electrophoresis of singleplex PCR products of *YJa* gene for *E. coli* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-14: PCR products of *YJa* gene.

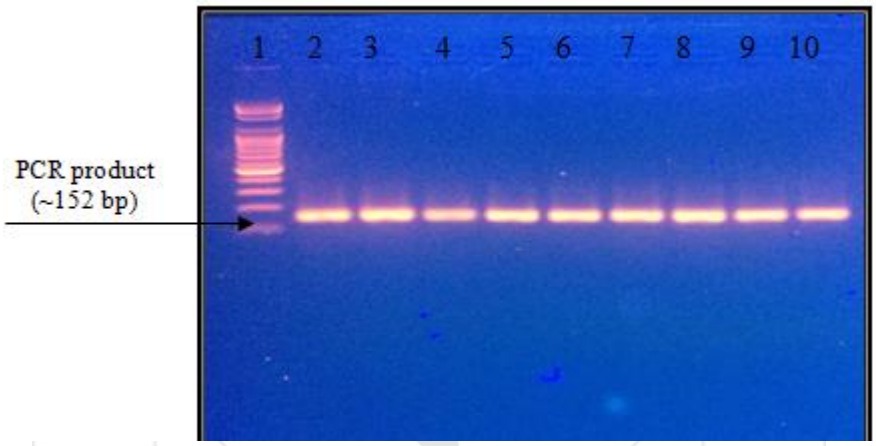

Figure (3-9): Gel electrophoresis of singleplex PCR products of TspE4C2F DNA fragment for *E. coli* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-10: PCR products of TspE4C2F DNA fragment.

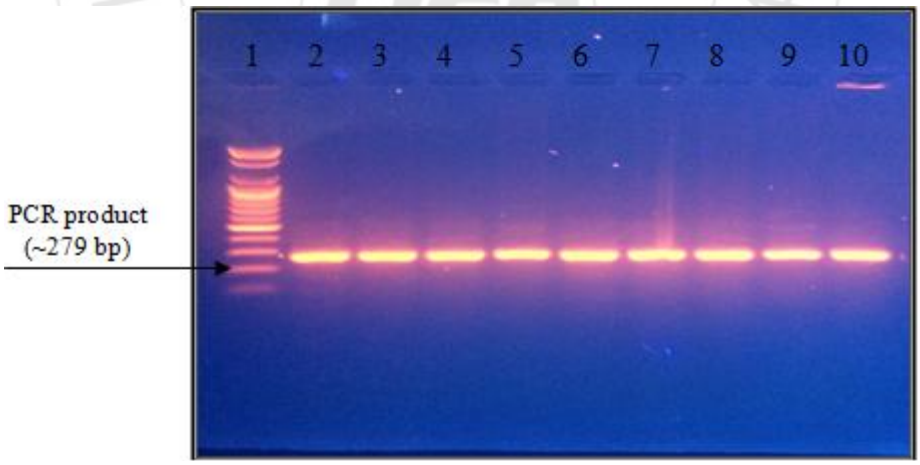

Figure (3-10): Gel electrophoresis of singleplex PCR products of *ChuA* gene for *Enterobacter cloacae* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-10: PCR products of *ChuA* gene.

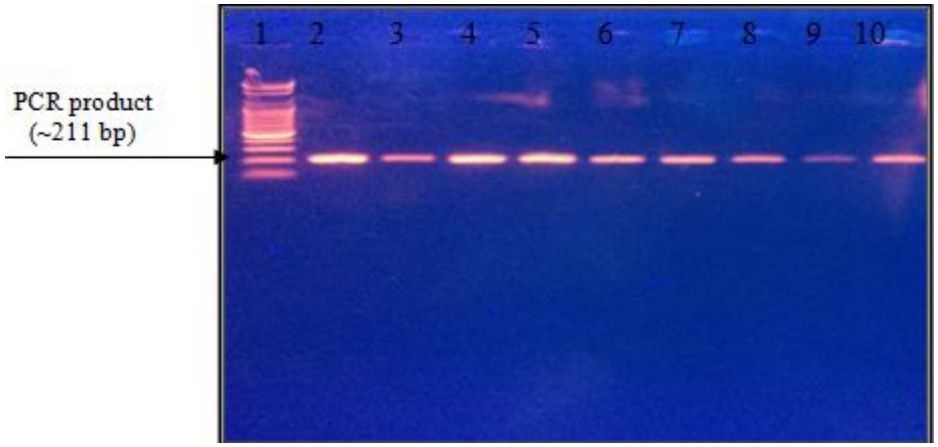

Figure (3-11): Gel electrophoresis of singleplex PCR products of *YJa* gene for *Enterobacter cloacae* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-10: PCR products of *YJa* gene.

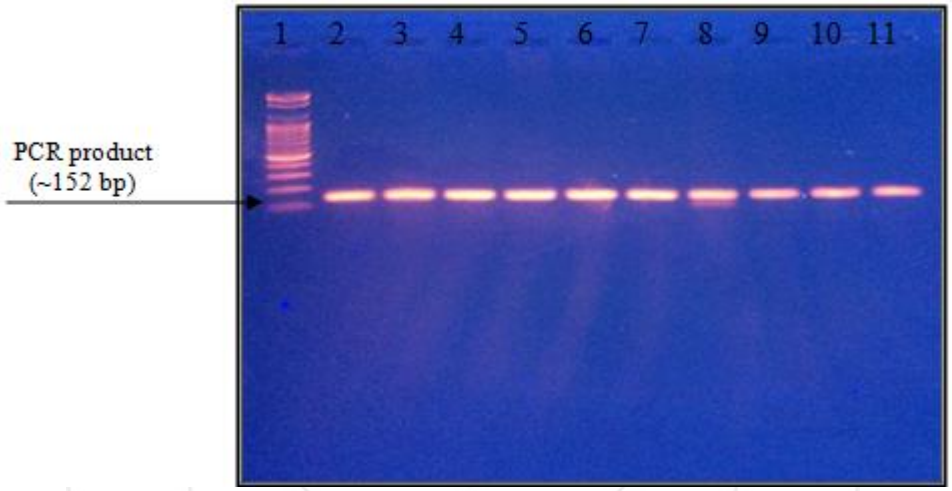

Figure (3-12): Gel electrophoresis of singleplex PCR products of TspE4C2F DNA fragment for *Enterobacter cloacae* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-11: PCR products of TspE4C2F DNA fragment.

3.4 Analysis of multiplex PCR products

In successful multiplex PCR reaction, the *ChuA* and *YJaF* genes and TspE4C2F DNA fragment products of ~ 279 bp, ~211 bp and ~152 bp molecular weights for both of *E. coli* and *Enterobacter cloacae* isolates were observed as shown in figure (3-13) and figure (3-14), respectively.This was considered as mandatory sign of successful reaction, upon gel electrophoresis, the bands were located between 300 to 400 bp, 200 to 300 bp and 100 to 200 bp bands of the 100 bp DNA ladder respectively, indicating the presence of these genes in *E. coli* and *Enterobacter cloacae* isolates. In the 75 patients clinically diagnosed with urinary tract infection, positive result for *E. coli* was detected in 9(12%) patients and positive result for *Enterobacter cloacae* were detected in 13(17.3%) patients by using multiple PCR. The multiplex PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5 µl of the multiplex PCR product were loaded on 1.5% agarose gel and run at 7volt /cm for 1 hour. The gel was stained with ethidium bromide solution (0.5µg/ml) for 15-30 minutes, finally, the DNA bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system.

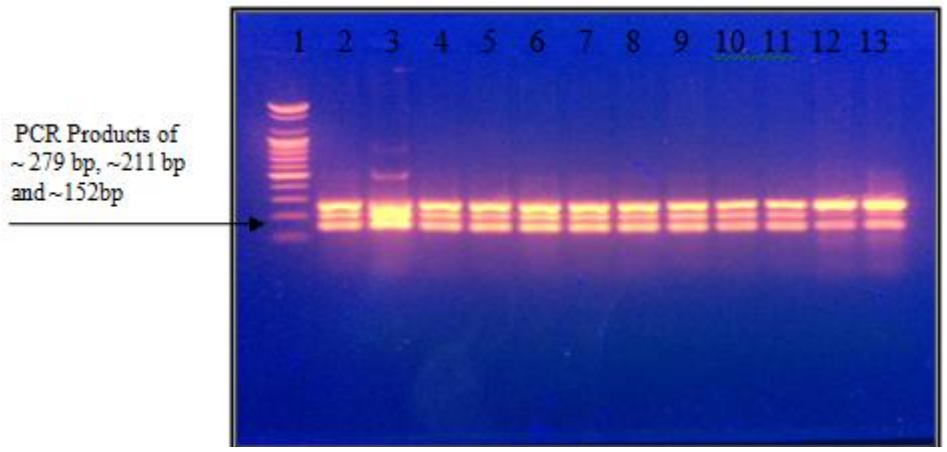

Figure (3-13): Gel electrophoresis of multiplex PCR products of *ChuA* and *YJaF* genes and TspE4C2F DNA fragment for *E. coli* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-13: Multiplex PCR products of *ChuA* and *YJaF* genes and TspE4C2F DNA fragment.

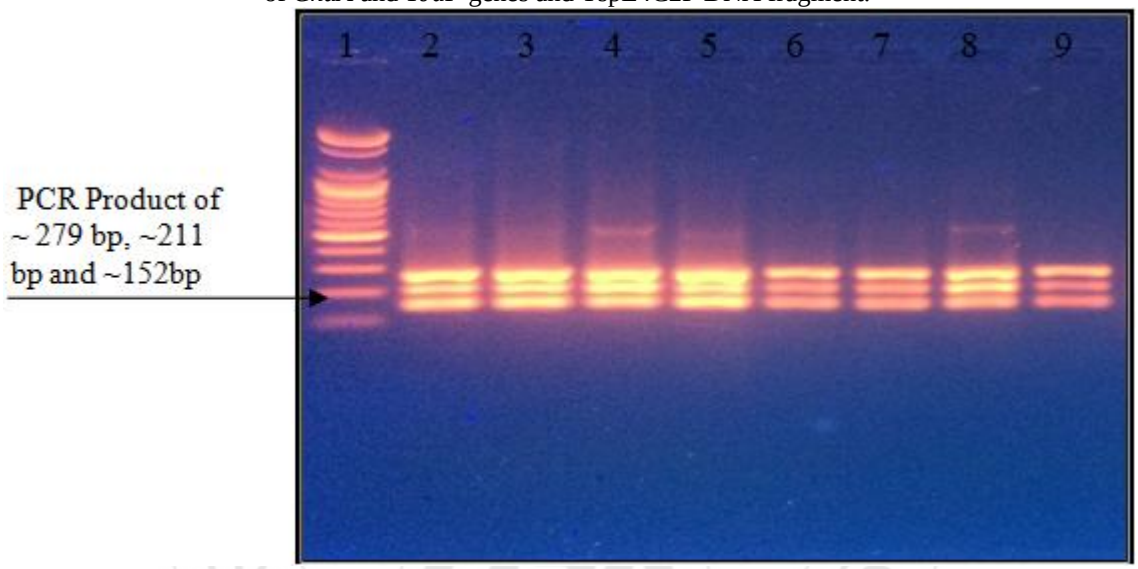

Figure (3-14): Gel electrophoresis of multiplex PCR products of *ChuA* and *YJaF* genes and TspE4C2F DNA fragment for *Enterobacter cloacae* isolates using 1.5% agarose gel at 7 volt/ cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-9: Multiplex PCR products of *ChuA* and *YJaF* genes and TspE4C2F DNA fragment.

4. Discussion

The result of conventional methods showed positive result for *E. coli* in 14(18.6%) out of 75 patients clinically diagnosed with urinary tract infection and positive result for *Enterobacter cloacae* in 10(13.3%) out of 75 patients, this results agree with data from various medical records showed that the epidemiology of the pathogens of UTI is represented by *Escherichia coli* the most common causative agent which was 51.70%, whereas the *Klebsiealla pneumonia, Proteus spp., Acinetobacter spp., Enterobacter spp., Citrobacter spp.* and *Pseudomonas aerogenosa* were 10. 88%, 16.12%, 5.44%, 4.08%, 3.40% and 2.04%, respectively (Tajbakhsh *et al.*, 2015). In Iraq, Al-Dahmoshi (2015) found that the most prevalent bacteria in the urinary tract infections were *E. coli*, *Klebsiealla pneumonia*, *Proteus vulgaris*, and *Enterobacter spp*. which were represent 54.72%, 16.9%, 11.32% and 3.77% of UTIs, respectively (Al-Dahmoshi, 2015). Another study by Basima *et al.*, (2015) on UTIs in children, revealed that the *E. coli* was (52.2%) which represent the most common bacterial isolates followed by *Enterobacter spp*. (23.9%), *Proteus spp*. (14.9%) *Klebsiealla spp*. (4.7%), *Pseudomonas spp*. (1.5%), and *Staphylococcus spp*. (1.5%).

In current study, the singleplex and multiplex PCR methods that were performed with primers targeted to *chuA* and *yjaA* genes and anonymous DNA fragment TspE4C2 of *E. coli* and *Enterobacter cloacae* referred to detect 13(17.3%) out of the 14(18.6%) samples that were positive for *E. coli* by using the conventional methods and detected 9(12%) out of the 10 (13.3%) samples that were positive for *Enterobacter cloacae* by using the same conventional methods. At a comparison between the conventional and molecular methods, the incubation period is uncertain and inappropriate growth media, in addition to the contamination of culture in identification methods could be the reason for false positive results, this may explain the false positive result of *E. coli* in 1(7.1%) out of 14(18.6%) samples were detected by the conventional methods and may explain the false positive result of *Enterobacter cloacae* in 1(10%) out of 10 (13.3%) samples were also detected by the same conventional methods from the patients clinically diagnosed with urinary tract infection.

The benefits of molecular methods are more sensitive, more qualitative for results, materials available, but the drawback of

molecular methods is costly. These explanations made molecular methods relatively more accurate than conventional methods (Kim *et al.*, 2013). The data of present study agrees with the study conducted by Cheng *et al.*, (2013) that confirms the efficacy of the PCR assay compared to conventional methods of diagnosis in the clinical setting (Cheng et al., 2013).

5. Conclusion

This study has demonstrated the efficacy of the *ChuA* and *YJaF* genes and TspE4C2F DNA fragment as molecular markers to detect both of the *E. coli* and *Enterobacter cloacae*. In addition, the present study referred to possibility of using the multiplex PCR-based test as a rapid, specific and sensitive method to prevalence the *E. coli* and *Enterobacter cloacae* in the urinary tract infections or other possible clinical infections.

6. Acknowledgements

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