



MOLECULAR DETECTION OF *KLEBSIELLA OXYTOCA* ISOLATED FROM CLINICAL SOURCES

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Abstract

The present study is an attempt for detection of *Klebsiella oxytoca* by conventional and multiplex PCR methods using species-specific primers for these *K. oxytoca* that are collected from patients to find out more specific, sensitive and rapid diagnosis method for this bacteria and to detect the *tonB* gene as virulence factor for these *K. oxytoca*. A total of 100 (urine, stool, sputum, CSF and tools of hospitals) samples have been collected from UTI patients in four hospitals of Baghdad City which include Baghdad Teaching Hospital/Medical City, Al-kindi Teaching, Al-Nursing home Hospital, Al-Hariri specialized surgery Teaching Hospital, Al-Yarmuk Teaching Hospital, AL-Abn Al-baladi Teaching Hospital. From the beginning of November 2017 to the end of February 2018, out of 100 (100%) samples, 50 (62.5%) samples have been detected for *K. oxytoca* other bacterial isolate including *Staphylococcus*, *Pseudomonas*, *Proteus*, *Staphylococcus aureus*, *Escherichia*, *Shigella*, *Enterobacter*; *K. oxytoca* isolate identification depending on the morphologic characteristics on the culture media including, blood agar, MacConkey agar, as well as the biochemical tests including the manual biochemical tests that include catalase, oxidase and tests and the automated biochemical tests such as API 20E, VITEK 2 system. Results declared that, generally the genomic DNA of *K. oxytoca* isolates were extracted using wizard genomic DNA purification kit, the extracted genomic DNA was analyzed using 1% agarose gel electrophoresis and then the concentration and purity of the extracted genomic DNA were determined using Nanodrop spectrophotometer device. To detect the *K. oxytoca* isolates by molecular methods, the extracted genomic DNA of these isolates was submitted for amplification to detect the *tonB* gene by the singleplex PCR method using species-specific primers for *K. oxytoca*, to sum up 50 (50%) out of 100 samples were detected for *K. oxytoca* by observing the singleplex PCR product of *tonB* gene with ~700bp, in the agarose gel electrophoresis. all the positive samples of singleplex PCR for these *K. oxytoca* showed positive results.

Key words: Molecular detection, *Klebsiella oxytoca*, clinical sources

Introduction

Klebsiella is Gram-negative, non-motile pathogens, usually encapsulated rod-shaped bacteria, with a mucoid aspect belonging to the family Enterobacteriaceae and the family members are generally facultative anaerobic. Genus *Klebsiella* are increasingly important opportunistic pathogens associated with severe hospital-acquired infections (nosocomial bacterial infections) such as septicaemia, pneumonia and urinary tract infections (Brisse and Verhoef, 2001). The genus *Klebsiella* is classified into four species: *Klebsiella pneumoniae* (*K. pneumoniae*), *Klebsiella oxytoca* (*K. oxytoca*), *Klebsiella terrigena* (*K. terrigena*), and *Klebsiella planticola* (*K. planticola*), with *K. pneumoniae* consisting of three subspecies, *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, and *K. pneumoniae* subsp. *rhinoscleromatis* (Li *et al.*, 2004). *K. pneumoniae* and *K. oxytoca* are the most two common distinguished species within *Klebsiella* genus, while *K. terrigena*, *K. ornithinolytica* and *K. planticola* are less distinguished (Ferlas *et al.*, 2013). *K. oxytoca* produce extended -spectrum beta- lactamase (ESBL), therefore is increasingly resistant to penicillin and ampicillin, in multi-resistant to antibiotics with broad spectrum of activity.

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Materials and Methods

Samples collection

A total of 100 (urine, stool, sputum, CSF and tools of hospitals) samples have been collected from four hospitals of Baghdad City which include Baghdad Teaching Hospital/ Medical City, Al-kindi Teaching, Al-Nursing home Hospital, Al-Hariri specialized surgery Teaching Hospital, Al-Yarmuk Teaching Hospital and

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Abn Al-baladi Teaching Hospital. Samples were taken from the patients under sterile conditions and immediately transferred to the laboratory to inoculate into brain heart infusion broth for 4-6 hours, then inoculated on Sabaroud dextrose agar, Potato dextrose agar and Corn meal agar (Hi media, India) at 37°C for 24 hrs then direct exam by Gram stain under light microscope (40x) followed by biochemical tests API20E and VITEC 3 system (Li *et al.*, 2004).

DNA extraction

Genomic DNA was extracted from the *K. oxytoca* 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 *K. oxytoca* culture grown at 28°C in nutrient broth (Sigma, USA) was transferred to a 1.5 ml micro centrifuge 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 is 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

Table 1: The primers sequences of ton B gene of *K. oxytoca* and their product size (Al- Bayati *et al.*, 2016).

Primer Name	Seq.	Tm	Size of product
ton B -F	AGTCCACTGGGACTGTCCAT	58	~108bp
ton B -R	GCACCACCATACTTGTCCA		

microcentrifuge tube 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.

DNA quantification

The extracted DNA from the *K. oxytoca* isolates was quantified spectrophotometrically at O.D. 260/280 nm with ratios 1.4-1.5. The sensitivity of the *K. oxytoca* -F and *K. oxytoca* -R primers was evaluated by PCR amplification for serial diluted concentrations (10-100 ng) of purified genomic DNA isolated from *K. oxytoca*.

Primers selection

The primers for 16S rRNA gene of *K. oxytoca* as the target gene for this study were selected according to (Al- Bayati *et al.*, 2016). This set of primers was designed based on the conserved region in *K. oxytoca*, primers were synthesized by Alpha DNA, Kanda. The primers sequence of 16S rRNA gene and their size of product are shown in Table 1.

Singleplex PCR master mix

The singleplex PCR reaction of ton B gene detection of *K. oxytoca* was performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 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 MgCl₂, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol *K. oxytoca* -F primer and 2.5 µl of 20 pmol *K. oxytoca* -R primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil Table 2.

Singleplex PCR program

Singleplex PCR was carried out in a thermal cycler (Applied Biosystem, 9902, Singapore) according to the PCR program described by (Ferlas *et al.*, 2013), with some modification. Briefly, the amplification of *tonB* gene of *K. oxytoca* was carried out with initial denaturation at 95°C for 6 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C

for *K. oxytoca* -F and *K. oxytoca* -R primers for 90 seconds, and extension at 72°C for 2 minutes.

The thermal cycles were terminated by a final extension for 10 minutes at 72°C Table 3.

Singleplex PCR products analysis

Table 2: Components of the mixture and their sizes.

Master mix components	Volume
Master Mix	10
Forward primer	1
Reverse primer	1
Nuclease Free Water	5
DNA	3
Total volume	20

Table 3: PCR Program use in this study.

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	58	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

visualized by an image analyzer (ChemImager 5500, Alpha Innotech, USA).

Results

Conventional methods

The conventional methods include culture, Gram staining and biochemical tests showed positive results in 12 (24%) out of 50 (100%) clinically diagnosed with UTI infection, the results of samples culture on blood base agar showed the bacterial isolates were large, mucoid, white to grey and Non-hemolytic colonies (Fig. 1.) and pink colonies, mucoid texture with large size regular edge, round, mucoid texture with large size, were (3-4 mm) in diameter on MacConkey agar. (Fig. 2). The Gram staining of *K. oxytoca* was showed a small straight rods and arranged singly but messily in pairs under the compound light microscope. (Fig. 3). The results of biochemical tests were used for further identification of *K. oxytoca* isolates showed positive reactions for indole, catalase, citrate utilization, urease, capsule stain, voges-proskauer (VP), and motility tests. But was negative for Kligler Iron Agar (KIA) test, motility, oxidase, H₂S production.

Analysis of extracted DNA of *K. oxytoca* isolates

After performing of the DNA extraction from *K. oxytoca* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1% agarose gel at 7 volt/cm for 45 minutes (Fig. 4).

Analysis of singleplex PCR products of *ton B* gene for *K. oxytoca*

On the basis of the *ton B* gene sequence, a product of ~740 bp was amplified by singleplex PCR with *K. oxytoca* -F and *K. oxytoca* -R primers. In 50 clinically

The analysis of singleplex PCR products of *tonB* gene of *K. oxytoca* were performed on 1% agarose gel. The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The singleplex PCR products were stained with ethidium bromide and

diagnosed with UTI infection, the singleplex PCR method detected positive results in 10(83.3%) out of 12(100%) samples that were positive by the conventional methods include culture Gram staining and biochemical tests. The singleplex PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5 µl of the singleplex PCR product were loaded on 1.5% agarose gel and run at 100 volt/ cm for 60 minutes. The gel was stained with ethidium bromide solution (0.5 µg/ml) for 15-30 minutes; finally, bands were visualized on UV transilluminator at 740 wave length and then photographed by using photo documentation system. The singleplex PCR result was considered positive for *K. oxytoca* when there was presence of ~740 bp singleplex PCR product band of *ton B* gene for the *K. oxytoca* on the agarose gel electrophoresis, no amplification was observed with negative control (Fig. 5).

Discussion

Identification of this bacteria by using the conventional methods include culture, Gram staining and biochemical tests which were go together with study conducted by [1] showed that the bacterial cultures were positive in 39(78%) patients versus 11(32%) patients revealed negative bacterial culture, the most common type of bacterial isolated were *Klebsiella* spp. predominant bacteria constitute 19.9 % (56 isolates) of these isolates, and identified as *K. pneumoniae* (40 isolates) 14.2 %, followed by *K. oxytoca* (3 isolates), The other bacterial isolates were *Enterobacter* 94 isolates (9%), *E. coli* 94 isolates (4.54%) and *Proteus* 94 isolates (4.54%). and 1.1 % (3 isolates), respectively. *K. oxytoca* 19(32%) patients was detected by the conventional methods of culture on blood base agar and MacConkey agar plate, Gram staining, API 20 E and VITEC- 2 system . Also the result of *K. oxytoca* culture on blood agar agrees with results of the same study showed that the *K. oxytoca* colonies on blood agar appear . growth of pale to large, mucoid, white to grey and Non-hemolytic colonies of *K. oxytoca* isolates (Don *et al.*, 2005; Qaiser *et al.*, 2011; Kanchana *et al.*, 2013), while culture of *K. oxytoca* on MacConkey agar appear lactose fermenting colonies and gave pink color, regular edge, round, mucoid texture with large size, were (3-4 mm) theses results agree with (Holt *et al.*, 1994). In addition, the result of Gram staining of *K. oxytoca* goes together with result of exhibited that in a typical Gram film, the organism appears as a Gram-negative. Non-motile, small straight rods and arranged singly or in pairs under the compound light microscope results as agree with (Garrity, 2005). The results of biochemical tests were used for further identification of *K. oxytoca* isolates showed positive

production of indole, catalase, citrate utilization, urease, capsule stain, voges-proskauer (VP), and motility tests. but was negative for Kligler Iron Agar (KIA) test, motility, oxidase, and H₂S production. At the species level, the results of indole test differentiate *K. pneumoniae* from *K. oxytoca* it was positive for *K. oxytoca* and negative for other (Atlas *et al.*, 1995). In the indole test, ability to hydrolyze tryptophan to indole is a characteristic of certain enteric bacteria possessing the enzyme tryptophanase, an enzyme that decomposes amino acid tryptophan to indole, pyruvic acid and water. The results of biochemical tests of current study agree with study conducted by (Holt *et al.*, 1994; Collee *et al.*, 1996; Podschun and Ullmann, 1998; Stock and Wiedemann, 2001). And the automated biochemical tests such as Api-20E and VITEK 2 system identification revealed that 17 of *Klebsiella* isolates were belonged to the species *K.*

oxytoca. The manual biochemical tests are largely used for bacterial identification in clinical laboratories, the advantages of conventional methods were non costly but the disadvantages of those methods were consuming time, contamination present, false positive result and require a large amount of sample. While the automated biochemical tests such as Api-20E and VITEK 2 system. The API 20E system is faster still time-consuming to set up and read, requires up to 48 h of incubation, and gives results while VITEK 2 system used in many previous studies was detected bacteria faster, efficient and away from the contamination that may prevent detection of the pathogen. In addition confirmation the biochemical tests.

In current study, the singleplex PCR method was used for detection of *K. oxytoca* by using pair primers targeted the *ton B* gene (~740 bp) showed a positive result in 10(83.3%) out of 12(100%) samples that were positive by the conventional methods. 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



Fig. 1: Colorless colonies of *K. oxytoca* on blood agar plate.

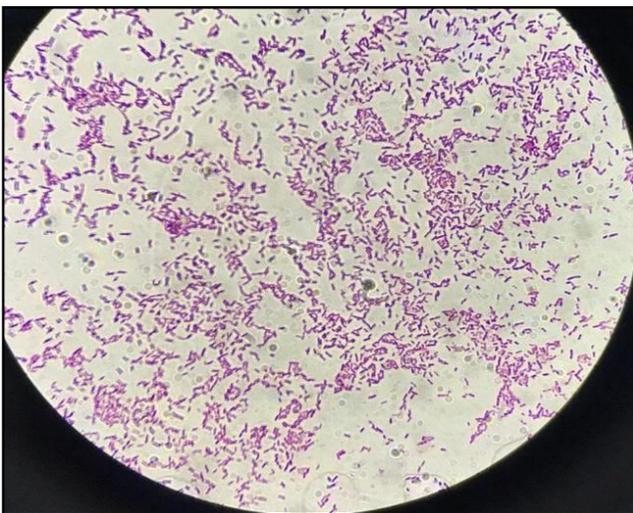


Fig. 3: Gram-negative diplococcus with flattened sides of *K. oxytoca*.



Fig. 2: Grey colonies of *K. oxytoca* on MacConkey agar plate.

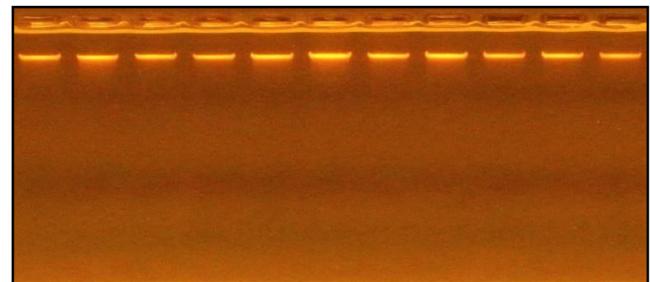


Fig. 4: Gel electrophoresis of extracted DNA of *K. oxytoca* isolates using 1 % agarose gel at 7 volt/mcm for 45 minutes. Lane 1-10: Extracted DNA.

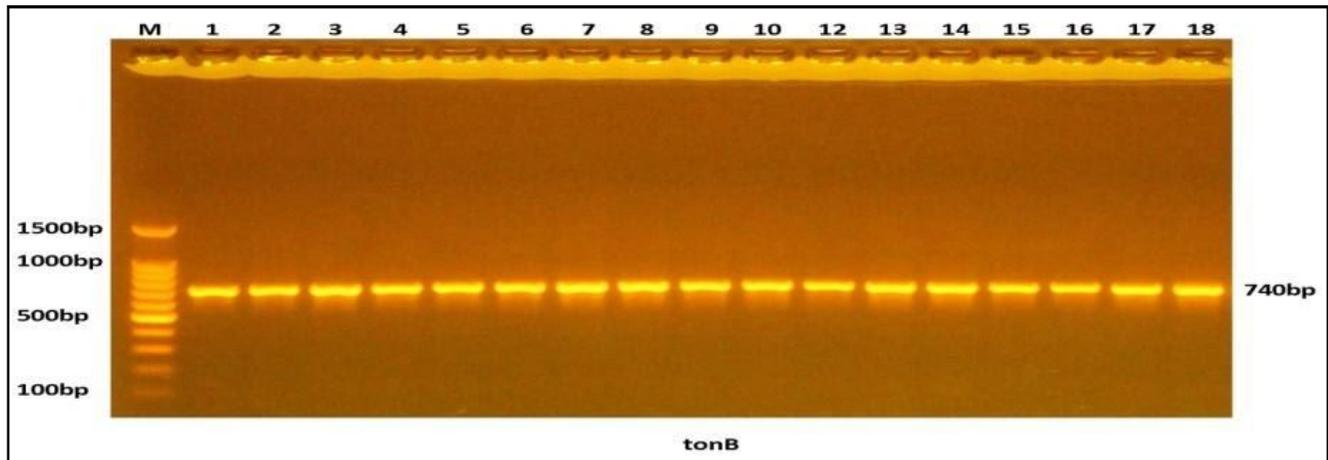


Fig. 5: Gel electrophoresis of singplex PCR product of *k.oxytoca* isolates using 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 1-18: singplex PCR product of *tonB* gene.

methods relatively more accurate than conventional methods (Li *et al.*, 2004). At a comparison between the conventional and molecular methods, we think that 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 results in 2(4%) out of 50(100%) children clinically diagnosed with UTI infection by using these conventional methods, whereas the singleplex PCR method detected only 10(20%) of 50(100%) patients who were also gave positive results by these conventional methods. Conventional studies have highlighted the difficulties in identifying *K.oxytoca* strains based on commercial phenotypic identification systems. Identification of *K.oxytoca* has long been based on phenotypic and biochemical test, these physiological and biochemical tests were performed on selected isolates using API20E, Vitek 2 system and PCR method (Li *et al.*, 2004). This explains that the molecular diagnosis of *K.oxytoca* by the singleplex PCR method was more sensitive and efficiency than the diagnosis of these bacteria by conventional methods. This data agrees with the study by (Ferlas *et al.*, 2013) who confirms the efficacy of the PCR assay compared to conventional methods of diagnosis in the clinical setting.

Conclusion

Many evidence indicate that *K. oxytoca* are based upon inaccurate isolate identification, resulting from inadequate identification conventional methods include culture, Gram staining and biochemical tests that lack the resolution needed to discriminate *K. oxytoca* isolates, on the other hand, 16S rRNA gene appeared to be useful genetic marker for determination of *K. oxytoca* and singleplex PCR using species-specific primers could be

represented rapid, sensitive and specific molecular method for detection of this bacteria in different human infections.

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