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Molecular Discrimination of *Klebsiella oxytoca* using Polymerase Chain Reaction Targeted Polygalacturonase (*pehX*) Gene

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This study aimed to molecular diagnosis of *Klebsiella oxytoca* using polymerase chain reaction. A total of 325 samples were collected from different clinical and environmental sources from six main hospitals in Baghdad province which is capital of Iraq. Out of 282 isolates, fifty six isolates (19.9 %) were isolated and identified as *Klebsiella* spp., forty isolates (12.3 %) were isolated and identified as *K. pneumoniae* (36 clinical and 4 environmental), ten isolates (3.5 %) were isolated and identified as *Klebsiella terrigena* (10 clinical only), three isolates (1.1 %) were isolated and identified as *Klebsiella oxytoca* and *Klebsiella ornitholytica* (2 clinical and 1 environmental) for each one. PCR was performed with the primer that target the *pehX*, Result showed that all the *K. oxytoca* isolate gave a clear band with a molecular weight 344 bp in size and this gene was not found in other *Klebsiella* spp. These results suggest that *pehX* genotype might be a useful marker to promote identification of *K. oxytoca* in Iraq, and used as routine protocol in public laboratories in Iraq.

Introduction

Klebsiella spp. has been first mentioned in 1883 by the German pathologist Karl Friedländer as a causative organism of severe pneumonia. Bacteria of the genus *Klebsiella* was finally described and designated in honor of the German microbiologist Edwin Klebs by Trevisan two years later in 1885 (Weberhofer, 2008). *Klebsiella pneumoniae* (formerly known as Friedländer bacillus) species was also described by Trevisan in 1887 (Zedan *et al.,* 2013).

Klebsiella spp. are gram-negative bacteria, is a significant public health issue which

associated with the majority of human infections causing diseases in human including pneumonia, bloodstream infections, urinary tract infections, and soft tissue infections (Kovtunovych *et al.,* 2003), this genus can live in both anaerobe and aerobe environments. This fact also constitutes the reason why *Klebsiella* spp. on the one hand can survive and proliferate in anaerobe environments such as the gut or the urinary tract (Stojowska-Swędrzyńska and Krawczyk, 2016). On the other hand though, *Klebsiella* spp. are also *Enterobacteriaceae* and, consequently, can cause acute respiratory

diseases such as hospital-acquired pneumonia (Ho *et al.,* 2011; Han *et al.,* 2015). This is also the reason for the infections and inflammations of these organs caused by *Klebsiella* spp. (Podschun and Ullmann, 1998).

Klebsiella pneumonia and *Klebsiella oxytoca* are the most two common distinguished species within *Klebsiella* genus, while *K. terrigena, K. ornithinolytica* and *K. planticola* are less distinguished (Lowe *et al.,* 2012). *K. oxytoca* produce extended-spectrum beta-lactamase (ESBL), therefore is increasingly resistant to penicillin and ampicillin, in addition to 10-20% of this specie in Europe is multi-resistant to antibiotics with broad spectrum of activity such as cephalosporin or ceftazidim because of carrying plasmid-borne beta-lactamases genes and class A chromosomal beta-lactamase (Goossens *et al.,* 2015), However, both species *pneumonia* and *oxytoca* are useful in agriculture due to their ability in fixing nitrogen (Bao *et al.,* 2013).

Accurate diagnosis among *Klebsiella* species is one of the most difficult obstacles in distinction in particular when apply phenotypic methods, improve the accuracy and efficiency of identification might be contribute in understanding the epidemiology spreading (Chander *et al.,* 2011). False results could be happen in identification of the *K. oxytoca* and K. *pneumoniae* as well (Fielding *et al.,* 2012). Furthermore, going further to identify *K. oxytoca* needs long process needing extra effort such as pectate degradation (Chander *et al.,* 2011).

The conventional biochemical tests designed for identification of *Enterobacteriaceae* often fail to differentiate species of *Klebsiella* (Brisse *et al.,* 2009). *K. oxytoca* which is closely related to *K. pneumoniae,* in biochemical differentiation, *K. oxytoca* and *K. pneumoniae* are only distinguished by a positive indole-reaction for *K. oxytoca* and a negative reaction for *K. pneumonae*. Indole testing aims at the detection of the enzyme tryptophanase. Tryptophanase catalyzes the reaction from tryptophan to indole, pyruvic acid and ammonia (Abbott, 2007). However, none of these techniques is completely satisfactory, since the sequences used (*gyrA*, *parC*, *infB*, *rrs* and *bla*) are not exclusively present in *K. oxytoca* (Han *et al.,* 2015). As a consequence, cross-reactions between the probe and DNA sequences from other species of the genus *Klebsiella,* or even other *Enterobacteria*, are possible. In some assays, sequence determination is needed before an identification can be reached, but sequencing is often considered too technically demanding, time-consuming and expensive (Goossens *et al.,* 2015).

It is important to understand their epidemiology and to control their spread in hospitals (Park *et al.,* 2011). The polymerase chain reaction (PCR) is a more objective and reproducible detection method for *K. oxytoca* (Pan *et al.,* 2008). Create a PCR-based identification method for *K. oxytoca* to identify this bacterial strain in a possibly even more sensitive and specific way. The idea was to exploit the pectate degradation capability of *K. oxytoca* which differentiates this species from all other *Klebsiella* species. More specifically, it was used a unique sequence of the gene *pehX* encoding the enzyme polygalacturonase that cleaves a polygalacturonic chain of demethoxylated pectin (Kovtunovych *et al.,* 2003). The *pehX* gene used to increase the specificity and sensitivity of *K. oxytoca* identification (Kline *et al.,* 2009). The PCR method was applied for a PCR amplicon of 344 bp that is typical for *K. oxytoca* (Weberhofer, 2008). This gene was devising a rapid, sensitive and specific test for discrimination of *K. oxytoca* from other *Klebsiella* spp. (Park *et al.,* 2011).

The aim of this study was to molecular diagnosis of *Klebsiella oxytoca* by using polymerase chain reaction. The idea was to exploit the pectate degradation capability of *K. oxytoca* which differentiates this species from all other *Klebsiella* species. More specifically, we used a unique sequence of the gene *pehX* encoding the enzyme polygalacturonase that cleaves a polygalacturonic chain of demethoxylated pectin (Stojowska-Swędrzyńska, K. and Krawczyk, B., 2016).

Materials and Methods

Samples collection

This study was carried out in Central Health Laboratory/Ministry of Health/Baghdad/Iraq, during the period from 1/11/2012 to 7/1/2013.

A total of 325 swab samples were aseptically collected, damped with normal saline in sterile containers and transferred to the laboratory to isolate bacteria. These samples were collected from different Hospital's Environment and patients visiting some Hospitals (Ibn Al-baladi, Central Children, Al-kindy, Al-Wasity, Medical city/Educational Lab.) in Baghdad/Iraq.

Isolation and identification of *K. oxytoca*

Samples were cultured on MacConky's agar plate (Oxoid) and cultured plates were incubated overnight at 37 ˚C. After incubation suspected *Klebsiella* colonies were selected and stored in pure form for further identification.

A number of morphological, physiological and biochemical tests were performed for identifying the bacterial isolates as recommended by Taha (2013). The isolates were confirmed for biotyping by the API 20E system and The VITEK 2 GN ID cards with a

VITEK 2 system were also used as a confirmation of characterization.

DNA extraction

The template DNA prepared from 1.5 ml of fresh cultures of bacterial isolates grown at 37˚C in Luria-Bertani broth (Green and Sambrook, 2012). DNA was extracted using genomic DNA extraction kit/Geneaid according to the manufacture protocol. The extracted DNA solution was stored at -20 ˚C.

DNA concentration and purity measurement

The concentration of DNA was measured by Nanodrop spectrophotometer according to the Nanodrop Optizen/Korea manual, DNA purity was measured depending on the ratio of sample absorbance at wave lengths 260 and 280 nm. A ratio of ~1.8 is considered as "pure" DNA (Green and Sambrook, 2012).

Molecular identification using PCR amplification

The extracted DNA was subjected to amplification with a PCR thermal cycler (Applied biosystems/Singapore) and specific primer (Bioneer/Korea) was used to amplify fragment from the *pehX* gene. Each 20 µl reaction mixture for amplification of *pehX* gene contained 2 µl DNA template, forward and reverse primers 1 µl (10 pmol), 12.5 µl of master mix (2x) (MgCl$_2$ 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM) and 16 µl DNase Free Water (Bioneer, Korea).

Primers used for PCR were, for *pehX* forward, 5- GAT ACG GAG TAT GCC TTT ACG GTG -3 and, for *pehX* reverse, 5- TAG CCT TTA TCA AGC GGA TAC TGG -3 (Park *et al.,* 2011). The PCR conditions for amplification of the *pehX* gene were as follows: 5 min. of initial denaturation at 94˚C,

followed by 30 cycles of 30 s at 94 ˚C, 1 min at 58 ˚C and 1 min at 72 ˚C, with a final extension step at 72 ˚C for 5 min. The amplified DNA was visualized in a 1.5 % agarose gel containing ethidium bromide (0.5 μg/ml). DNA bands were visualized by UV illumination at 302 nm on a UV transilluminator.

Results and Discussion

Isolation and identification

Two hundred and eighty two isolates were obtained from three hundred and twenty five clinical and environmental samples were collected from different hospitals in Baghdad. Fifty six isolates (19.9 %) were isolated and identified as *Klebsiella* spp., forty isolates (12.3 %) were isolated and identified as *K. pneumoniae*, ten isolates (3.5 %) were isolated and identified as *Klebsiella terrigena*, three isolates (1.1 %) were isolated and identified as *Klebsiella oxytoca* and *Klebsiella ornitholytica* for each one, on the basis morphological, physiological and biochemical characteristics (Taha, 2013), and results were confirmed using the API 20E and VITEK 2 system (Robinson *et al.,* 1995).

Two (0.7 %) of these isolates were isolated from clinical sources (urine 1 (33.3 %) and sputum 1 (33.3 %)), 1 (7.1 %) isolates from hospital's environment.

Molecular identification of *K. oxytoca*

In order to molecular identification of *K. oxytoca* isolates DNA was extracted from all isolates of *Klebsiella* spp. Results showed that the recorded range of DNA concentration was 47.4-123.8 ng/μl and the DNA purity was 1.6-2.0.

The obtained quantities and purity of DNA are fair enough for amplification by PCR. Higher amounts of DNA template increase

the risk of generating of Non-specific PCR products. Lower amounts of template reduce the accuracy of the amplification. A pure DNA preparation has expected of 1.8 which are based on the extinction coefficients of nucleic acids at 260 nm and 280 nm (Green and Sambrook, 2012). All *Klebsiella* spp. isolates (*K. pneumoniae*, *K. terrigena*, *K. oxytoca* and *K. ornitholytica*) were subjected to molecular identification through PCR amplification of *pehX* using K peh-F and K peh-R primers which represents specific primers for the PCR amplification of *K. oxytoca pehX*.

Results showed that the amplified fragments were 344 bp in size as shown in figure (1), which the same size is obtained by Park *et al.,* (2011) when they used the same primer. All (3) *K. oxytoca* isolates (1.1 %) gave positive results (344 bp bands), and identified as *K. oxytoca*. It was not obtained amplification from other *Klebsiella* isolates (*K. pneumoniae, K. terrigena* and *K. ornithinolytica*). Results of PCR amplification confirmed that all isolates were *K. oxytoca.*

Figure 1 shows gel electrophoresis for amplification of *pehX* gene using specific primers of *K. oxytoca*. Electrophoresis was performed on 1.5 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane M is a (100 bp) ladder, Line: 1 – T17 (*K. oxytoca* clinical isolate), 2 – T116 (*K. oxytoca* environmental isolate), 3 – T117 (*K. oxytoca* clinical isolate), 4 – DNA-free negative control, 5 – T12 (*K. terrigena* clinical isolate), 6 – T43 (*K. terrigena* clinical isolate), 7 – T47 (*K. terrigena* clinical isolate), 8 – T61 (*K. terrigena* clinical isolate), 9 – T3 (*K. ornitholytica* clinical isolate), 10 – T10 (*K. ornitholytica* clinical isolate), 11 – T16 (*K. ornitholytica* environmental isolate), 12 – T78 (*K. pneumoniae* clinical isolate), 13 – T80 (*K. pneumoniae* clinical isolate), 14 – T105 (*K. pneumoniae* clinical isolate).
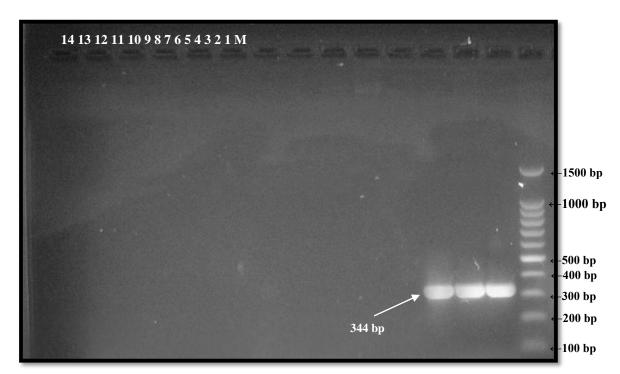
Fig.1 Gel electrophoresis for amplification of *pehX* gene using specific primers of *K. oxytoca*

In common with the findings of Liu *et al.,* (2008), they found that *K. pneumoniae* failed to give this band in the PCR inclusion of the pehX primers. The *pehX* gene provided specific detection of *K. oxytoca* (Turton *et al.,* 2010).

Our results agreed with Kovtunovych *et al.,* (2003) who demonstrated that an amplicon of *pehX* with 344 bp was obtained in all *K. oxytoca* strains. Moreover, they revealed that specific discrimination of *K. oxytoca* from other *Klebsiella* spp. is performed by amplification of a polygalaturonase (*pehX*) gene, which expressed to an enzyme has the ability to cleave a polygalacturonic chain of demethoxylated pectin. This is a unique capability for *K. oxytoca* amongst the *Klebsiella* spp. If a sequence coding for this specific enzyme is amplified with PCR, proper discrimination of *K. oxytoca* from other *Klebsiella* strains can be achieved (Kovtunovych and Kozyrovska, 2000).

K. oxytoca, is closely related to *K. pneumoniae*. In biochemical differentiation, *K. oxytoca* and *K. pneumoniae* are only distinguished by a positive indole-reaction for *K. oxytoca* and a negative for *K. pneumoniae* (Chander *et al.,* 2011). Indole testing is also integrated in the 20 reactions in API 20E test. API testing remains a subjective testing method; therefore it was proposed that PCR is a more objective and reproducible detection method for *K. oxytoca* compared to API. PCR is an objective and well-accepted identification method of bacteria and other pathogens (Nucera *et al.,* 2006). It was created a PCR-based identification method for *K. oxytoca*, in order to identify this bacterial strain in a possibly even more sensitive and specific way than API 20E (Weberhofer, 2008). Park *et al.,* (2011) reported that biochemical test API had only one discriminatory test (IND), between *K. pneumoniae* and *K. oxytoca*. It is wholly dependent on the result of the indole test that may be mistake. Therefore, this dependency of biochemical and API on the indole test is

considered to be inappropriate to differentiate between the two species. Chander *et al.,* (2011) recommended to use PCR technique for distinguish between *K. pneumoniae* and *K. oxytoca* using the PCR technique. Walckenaer *et al.,* (2008) suggested that a *pehX* gene amplification could be distinguished *K. oxytoca* from *K. pneumoniae*; they used pehX primer which is specific for *K. oxytoca*, which might be reliable as routine protocol to follow in Iraqi public laboratories.

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