Detection and identification of *klebsiella* species in chicken in Assiut Governorate by using polymerase chain reaction (PCR)

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Abstract
Bacteria of the genus *Klebsiella* are important opportunistic pathogens responsible for infections that are increasingly resistant to antimicrobial agents. A total of 120 chicks (3-40 days old) were examined bacteriologicaly and biochemicaly and results showed that, 34 were positive for *Klebsiella* infection. 18 out of 34 were *K. pnemonia* and the rest 16 were *K. oxytoca* with a percentage of 52.95% and 47.05% respectively. Discrimination of *K. oxytoca* from other species of the genus *Klebsiella* was done by using specific PCR assay targeting the polygalacturonase pehX gene. Ten representing isolates were confirmed by PCR amplification of the polygalacturonase pehX gene using the primers pair to identify *K. oxytoca* from other *klebsiella* species. The obtained PCR amplicon was 344 bp indicating that it belongs to *K. oxytoca* with an isolation rate of 40% (4/10). The experimental infection was made on 50 birds aging 3 days classified into three groups: the first group was infected by *K. oxytoca* through subcutaneous route (S/C) and the second group was infected orally while the last group was used as a control group. The three groups were observed for 15 days. The S/C route was highly effective with mortality rate 40%, while oral route caused 20% mortality. The antibiotic sensitivity test for all of 18 *K. pneumonia* revealed that, it was highly sensitive to norofloxacin, lincomycin/spectinomycin, sensitive to flumequine, sulphamethaxazole/trimethoprim, and less sensitive for spiromycin, gentamycin, neomycin and streptomycin. However, *K. oxytoca* was highly sensitive to norofloxacin, gentamycin, sensitive for sulphamethaxazole/trimethoprim, less sensitive for spiromycin, neomycin and streptomycin, but it was resistant for lincomycin/spectinomycin and flumequine.

Key words: *klebsiella, chickens, polygalacturonase (pehX) gene, Antibiotic resistance.*

Introduction

The genus *Klebsiella* belongs to the tribe *Klebsiellae*, a member of the family *Enterobacteriaceae*. The organism is named after Edwin Klebs, a 19th century German microbiologist (**Trivesan, 1885**). The genus *Klebsiella* was originally divided into 3 main species based on biochemical reactions. Nowadays, 7 species with demonstrated similarities in DNA homology are known. These are *(1) K. pneumoniae, (2) K. ozaenae, (3) K rhinoscleromatis, (4) K. oxytoca, (5) K. planticola, (6) K. terrigena, and (7) K. ornithinolytica. K. pneumoniae* is the most medically important species of the group. However *K. oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiellae* have become important pathogens in nosocomial infections (**Nordmann *et al.*, 2009**).

Klebsiellae are non-motile, rod-shaped, gram-negative bacteria with a prominent polysaccharide capsule (**Brooks *et al.*, 2004**). This capsule encases the entire cell surface, accounts for the large appearance of the organism on gram stain, and provides resistance against many host defense mechanisms. *Klebsiella* is among the five gram-negative pathogens most commonly encountered in hospital-acquired infections (**Horan *et al.*, 1988**). *K. pneumonia*

is the most frequently occurring species, accounting for 75 to 86% of *klebsiella* species reported (**De La Torre *et al*., 1985; Hansen *et al*., 1998; Watanakunakorn and Jura, 1991).** *K oxytoca* is the other well-established species, accounting for 13 to 25% of isolates (**De La Torre *et al*., 1985; Hansen *et al*., 1998; Watanakunakorn and Jura, 1991**).

Klebsiellae are considered as an important cause of nosocomial infections. The two clinically relevant species, *K. pneumoniae and K. oxytoca*, are differentiated by the ability to produce indole from tryptophan, *K. oxytoca* being indole positive (**Maslow *et al*., 1993; Hausler and Sussman 1998; Nordmann *et al*., 2009**).

Brisse and van Duijkeren (2005) Resistance in *Klebsiella* isolates was common against ampicillin (99%) but not against tetracycline, enerofloxacin, gentamycin and trimethoprim-sulfamethoxazole.

DNA-based techniques, which are rapid, specific and highly sensitive, are efficiently employed for early detection and differentiation of various strains of different microrganisms from single/different outbreaks (**Blackall and Miflin, 2000; Biswas *et al*., 2002**). The discrimination of *K. oxytoca* from the other species of the genus *Klebsiella*, is based on the PCR amplification of the polygalacturonase (*pehX*) gene. A PCR amplicon of 344 bp was obtained in all tested *K. oxytoca* strains (**Kovtunovych *et al*., 2003**).

Aim of the work:

1- Investigation of clinical signs and PM lesions caused by *Klebsiella* infections.
2- Identification of *K oxytoca* by using PCR amplification of the polygalacturonase (*pehX*) gene.
3- Study the sensitivity of the microorganism to antimicrobial agents using antibiotic sensitivity test.

Materials and methods

Samples collection:

Samples from internal organs of one hundred and twenty diseased broiler chickens (suffering from diarrhea and septicemic picture) were collected from farms in different localities in Assiut province. Birds were necropsied at Department of Poultry Diseases, Faculty of Vet. Med., Assiut University and Assiut Provincial Laboratory. Samples included liver, spleen, kidney, heart, blood, unabsorbed yolk sac, tracheal exudate, lungs, bloody exudate of subcutaneous tissue in the head region. Clinical signs and post mortem examination of all selected birds were recorded and subjected to bacteriological examination.

Culture procedure:

Samples were aseptically cultured into nutrient broth or lactose broth for 18-24 hours at 37°C. Subsequently, a loopfull of each broth was streaked on surface of MacConky´s agar plates, eosin methylene blue agar plates and methyl violet lactose agar for 24-48 hours at 37°C (**Campbell and Roth, 1975**).

Biochemical tests:

Klebsilla typical colonies on the plates were picked up and subjected to further examination based on colonial morphology, sugar fermentation and other biochemical tests according to (**Cruickshank *et al*., 1975; Wilson and Miles, 1984**).

DNA Extraction from culture cell:

DNA extraction was made in moleculer biology Research Unit by using diagram DNA extraction kit (USA). *Klebsiella*-typical colonies on the plates were confirmed with standard biochemical tests and procedures for *Klebsiella* spp. (**Campbell and Roth, 1975**). Lactose broth inoculated with *Klebsiella*-typical colonies for 18-24 hours at 37°C (Difco) with the addition of 20% (v/v) glycerol. Presumptive *Klebsiella* colonies were kept at -70°C. An aliquot of this storage solution was taken and incubated in 5 ml phosphate-buffered peptone water for 24 h at 37°C prior to PCR for further confirmation. Primer sequences; PEH-C (5_-GATACGGAGTATGCCTTTACGGTG-3_) and PEH-D (5_-TAGCCTTTATCAAGCGG-ATACTGG-3_) are used for identification of *K oxytoca* **(Kovtunovych *et al*., 2003)**. PCR amplifications were performed in a final volume of 2.5 µl, containing 4 µl of template DNA, 6.5 µl Enzyme (DNA polymerase, Tag) dNTPs (A,

T, G, C) Buffer (50 mMKCL; 10mM Tris-HCL; 1.5 mM MGCL2), 2x PCR Master Mix 0.5µl F and 0.5µl R Primers (forward and reverse) 1 µl Water DNAase free water 12.5 µl total reaction volume. The samples were subjected to an initial denaturation step of 3 min at 95 ◦C, followed by 40 amplification cycles (94 ◦C, 30 s; 59 ◦C, 30 s; 72◦C, 30 s) and a final elongation step of 10 min at 72 ◦C. PCR products were analyzed by electrophoresis in 1.2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

Experimental birds:

Fifty, 3 day old chicks obtained from baby chicks production farm in Assiut were used for pathogenicity test. The experimental infection was made on 50 birds classified into three groups: the first group was infected by *K. oxytoca* through (S/C) route and the second group was infected orally while the last group was kept as control. All groups were under observation for 15 days, after that, mortality rate, clinical symptoms and PM lesions were recorded.

Antibiotic sensitivity test:

Klebsiella typical colonies on the plates were cultured into lactose broth over night at 37°C and cultured fluently over the entire surface of nutrient agar (Difco) with sterile cotton swab. Commercial antibiotic disks containing single concentrations of each antibiotic were then placed onto the inoculated plate surface. The zone of growth inhibition around each disk after overnight incubation at 37°C, was measured in millimeters. The zone diameter was interpreted using a zone size interpretation chart. The used antibiotics included norofloxacin (10 mcg), lincomycin/spectinomycin (9/ 100mcg), flumequine (30ug), sulphamethaxazole / trimethoprim (25ug), spiromycin (100 mcg), gentamycin (10mcg), neomycin (30mg) and streptomycin (10mcg).

Results

Isolation of *Klebsilla* from the examined samples:

Samples were collected from a total of 120 birds obtained from Assiut governorate. They included dead chicks, broilers, and diseased adult birds. Samples were collected from liver, lung, spleen, heart, and yolk as well as cloacal swabs from diseased birds. These samples were examined for the presence of *Klebsiella* isolates. Isolation trials of *Klebsilla* from collected samples yielded overall isolation rate (34/120). Using biochemical reactions, 18/34 belonged to *K. pnemonia* while the rest 16 belonged to *K. oxytoca* with a percentage of 52.95% and 47.05% respectively.

Experimental infection:

Clinical symptoms were in the form of depression, weakness, coma, ataxia and congestion of the head region and abdomen, some bird showed signs of lameness. PM lesions of dead birds revealed a typical septicaemic picture as shown in **Fig (1).** High mortality rate (40%) was encountered in s/c infected birds, while mortality rate was low (20%) in orally infected birds **(Table 1)**.

Table (1). Results of experimental infection of chicks with *Klebsiella* isolates.

No. of infected chicks	Route of inoculation	Inoculated Dose	Daily death post-infection											Total no of death	Mortality rate
			1	2	3	4	5	6	7	8	9	…	15		
20	S/C	10^7	-	-	3	2	1	1	-	1	-	-	-	8	40%
20	Oral	10^9	-	-	-	1	1	1	1	-	-	-	-	4	20%
10	Control	0	-	-	-	-	-	-	-	-	-	-	-	-	0%

Specificity of the polymerase chain reaction:
Ten representing isolates were confirmed by PCR amplification of the polygalacturonase *(pehX)* gene using the two primers pair. The isolation rate of *K. oxytoca* was (4/10) with a percentage of 40%. A PCR amplicon of 344 bp was obtained in all *K. oxytoca* **(Fig. 2).**

Sensitivity test:
Fourteen *K. Oxytoca* and eight *K. pneumonia* were examined to determine their sensitivity to different chemotherapeutic agents using paper disc technique **(Kolmer *et al*., 1951)**. *K. pneumonia* was highly susceptible to norofloxacin (10 mcg), lincomycin/spectinomycin (9/100 mcg), sensitive to flumequine (30 ug), sulphamethaxazole/trimethoprim (25 ug), and less sensitive for spiromycin (100 mcg), gentamycin (10 mcg), neomycin (30 mg) and streptomycin (10 mcg). While *K. oxytoca* was highly sensitive to norofloxacin (10mcg), gentamycin (10mcg), sensitive for sulphamethaxazole/ trimethoprim (25ug), less sensitive for spiromycin (100mcg), neomycin (30mg) and streptomycin (10mcg), but it was resistant for lincomycin/spectinomycin (9/100mcg) and flumequine (30ug) (table 2). Results were recorded according to the measures of **Yousseff (1976)**.

Table 2: Results of sensitivity test of *Klebsiella* species to various antibiotics.

Antibiotics	Sensitivity of *K. Oxytoca*	Sensitivity of *K. pneumonia*
Norofloxacin (10mcg)	++++ ve	+++ve
Lincomycin/Spectinomycin (9/100mcg)	+++ ve	-ve
Flumequine (30ug)	++ve	-ve
Sulphamethaxazole / Trimethoprim (25ug)	++ve	++ve
Spiromycin (100mcg)	+ve	+ve
Gentamycin(10mcg)	+ve	+++ve
Neomycin (30mg)	+ve	+ve
Streptomycin (10mcg)	+ve	+ve

++++ve =Highly sensitive ++ve= Moderate sensitive
+ve= Low sensitive -ve = Resistant

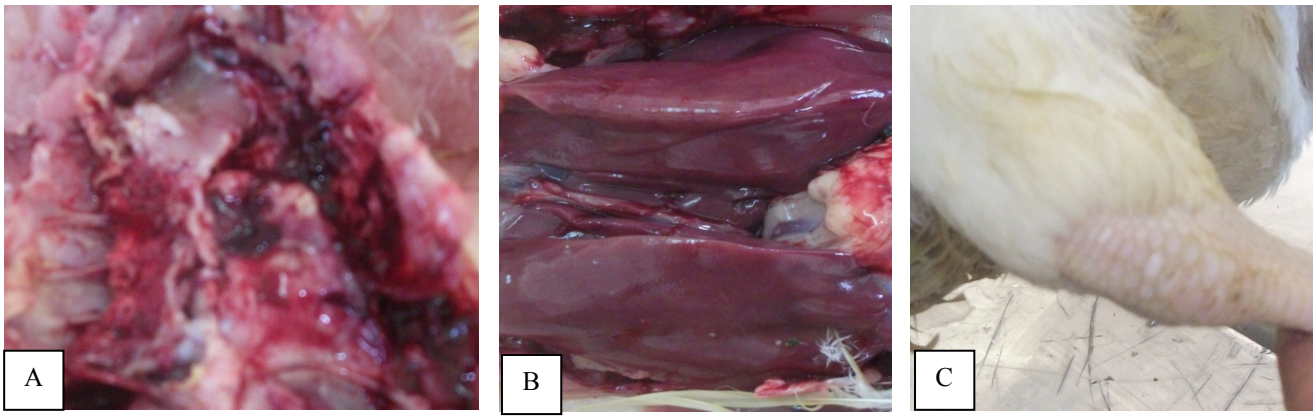

Fig (1). Congested lung (A), liver (B) and oedema in the leg (C) of chicks experimentally infected with *Klebsiella* species.

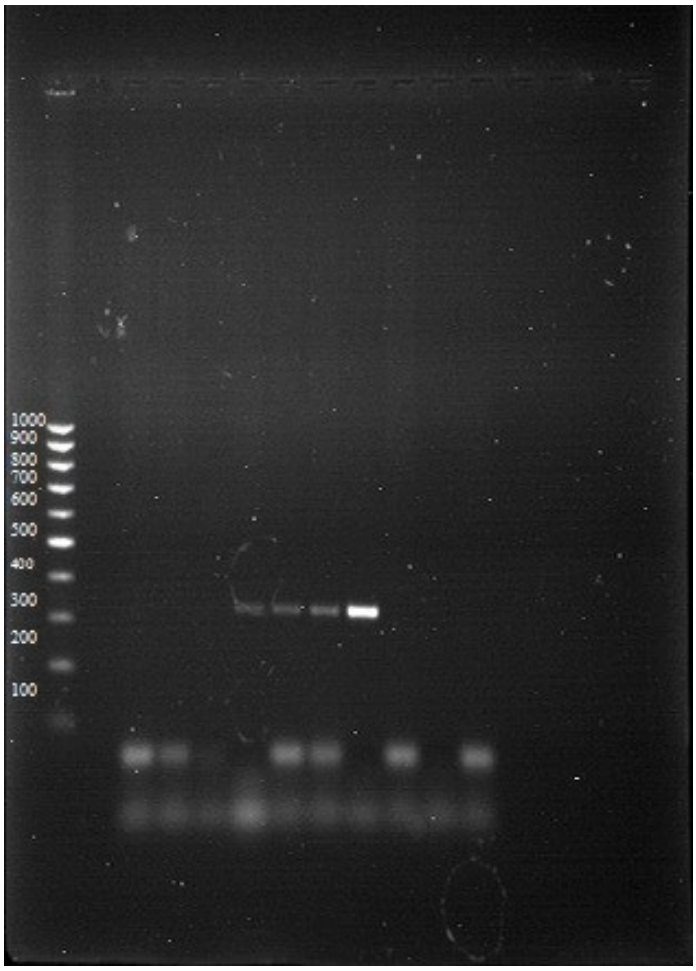

Figure (2). Results of PCR amplifications of the (*pehX)* gene.

M1: 100kb plus ladder marker. Lanes 4, 5, 6, 7 are *K. oxytoca (possessing a 344bp).*

Discussion

The present study revealed that the morphological appearance and biochemical reaction are typical to *Klebsiella* species as stated by **Horan *et al.* 1988; Holt and Bergey (1994) and Brooks *et al.* 2004)**. *K. pneumonia* is the most frequently occurring species, accounting for 75 to 86% of reported *klebsiella* species (**De La Torre *et al.*, 1985; Watanakunakorn and Jura 1991 and Hansen *et al.*, 1998).** The two clinically relevant species, *K. pneumoniae and K. oxytoca*, are differentiated by the ability to produce indole from tryptophan, *K. oxytoca* being indole positive (**Maslow *et al.*, 1993; Hausler and Sussman 1998** and **Nordmann *et al.*, 2009**).

The genus *Klebsiella* is an opportunistic pathogen difficult to identify and is often misclassified in clinical microbiology laboratories (**Monnet and Freny 1994; Hansen *et al.*, 2004** and **Alves *et al.*, 2006**). In present study clinical symptoms of diseased birds and PM lesions agree with findings of **Abd-El-Gwad and Mohamed (2004**), who isolated and identified *K pneumonia* from broiler chicken in Assiut governorate, Egypt. The clinical signs of the infected broiler chickens were severe weakness, depression and growth retardation. PM lesions were severe congestion of the parenchymatous organs and oedema of the subcutaneous tissue in the head region and abdomen.

Current biochemical methods of identification are time consuming and are often inconclusive because *K pneumonia and K oxytoca* or even *Enterobacter aerogenes*, often present similar biochemical patterns (**Lopes *et al.*, 2007**).

The specific PCR test used in the present study is useful in clinical microbiology laboratories and for ecological monitoring of *K oxytoca* as it is a rapid and simple method for detection of *K oxytoca*. PCR amplicon of 344bp was obtained in all *K oxytoca* strains tested in this study. Ten representing isolates were confirmed by PCR amplification of the polygalacturonase (*pehX*) gene, these results agree with findings of **Brisse and verhoef (2001); Kovtunovych *et al.* (2003) and Chander *et al.* (2011)** who found that PCR amplicons of 344 bp were absent in Klebsiella species other than *K. oxytoca*.

The antibiotic sensitivity test of the isolated Klebsiellae revealed that *K. pneumonia* species was highly susceptible to norofloxacin (10 mcg), lincomycin/spectinomycin (9/100 mcg), sensitive to flumequine (30 ug), sulphamethaxazole / trimethoprim (25 ug), and less sensitive for spiromycin (100 mcg), gentamycin (10 mcg), neomycin (30 mg) and streptomycin (10 mcg). While for *K. oxytoca* was highly sensitive to norofloxacin (10 mcg), gentamycin (10 mcg), sensitive for sulphamethaxazole / trimethoprim (25 ug), low sensitive for spiromycin (100 mcg), neomycin (30 mg) and streptomycin (10 mcg) and resistant for lincomycin/ spectinomycin (9/100 mcg) and flumequine (30ug). These results are concomitant with those of **Niazi (1976); Ashgan (1988); Abd El -Motelib and El-Zanaty (1993); Ayan *et al.* (2003); Abd-El-Hafez (2011)** and **Fielding *et al.* (2012**).

The experimentally infected birds by *K. oxytoca* induced high mortality in S/C infection (40%) and low (20%) in oral infection. This is consistent with **Picoff (1966) and Abd-El-Hafez (2011)** and partly consistent with those of **Abd-El Gawad and Mohamed (2004**), but disagree with the result reported by **Abd El-Motelib and El-Zanaty (1993**) who proved the pathogenic nature of *K pneumoniae* isolates that produced mortality rate of 2%.

In conclusion, the specific PCR test used in the present study should be applied in clinical microbiology laboratories and for ecological monitoring of *Klebsiellae* as it is a rapid, simple accurate method for diagnosis of infections caused by this organism.

Abd-El-Gwad and Mohamed (2004) conducted a study to isolate and identify *K. pneumonia* from broiler chicken from Assiut governorate, Egypt. A total of 75 freshly slaughtered broiler chickens (3-40 days old) were collected from different farms. The clinical signs of the infected broiler chickens were severe weakness, depression and growth retardation. PM lesions were severe congestion of the parenchmatous organs and oedema of the subcutane-

ous tissue in the head region and abdomen.

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الكشف والتعرف علي معزولات الكليبسيلا في الدجاج في محافظه أسيوط بإستخدام تفاعل أنزيم البلمرة المتسلسل

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الملخص العربي
تعتبر البكتريا من جنس الكلبسيلا من المسببات المرضية المسئولة عن عدوي مقاومة باستمرار للمضادات الحيوية. استخدم في هذه الدراسة 120 كتكوت من عمر 3-40 يوم والتي تم فحصها باستخدام اختبارات بكتريولوجية وبيوكيميائية . 34 من هذه الطيور كانت ايجابية لعدوي الكلبسيلا حيث كان 18 منهم من نوع كلبسيلا نيومونيا و 16 من نوع كلبسيلا أوكسي توكا بنسبة مئوية 52.95%، 47.05% علي التوالي. اضافة الي ذلك تم استخدام طريقة خاصة لتمييز الكلبسيلا أوكسي توكا من باقي الأنواع الأخري من الكلبسيلا باستخدام أختبار البلمرة المتسلسل للكشف عن البولي جلاكتويورنيز بهكس جين . حيث أستخدم 10 معزولات ممثلين للمعزولات المختلفة في الكشف عن هذا الجين باستخدام زوج من البادئات. هذا وقد أظهر الأختبار وجود بي سي أر أمبليكون بوزن 344 زوج من القواعد والذي يخص الكلبسيلا أوكسي توكا في 4 حالات من المعزولات العشرة بنسبة 40%. أضافة الي ذلك فقد تم عمل عدوي تجريبية علي عدد 50 طائر عمر ثلاثة أيام قسمت الي 3 مجموعلت حقنت الأولي تحت الجلد والثانية تم عمل عدوي لها عن طريق الفم والثالثة تركت كمجموعة ضابطة وتم تسجيل نسبة الوفيات للمجموعات الثلاثة لمدة 15 يوم وقد أسفرت الدراسة عن نسبة وفيات 40% للطيور المعدية عن طريق الحقن تحت الجلد و 20% للطيور المعدية عن طريق الفم. تم اجراء اختبار مدي حساسية المعزولات للمضادات الحيوية وأظهرت النتائج أن ميكروب الكلبسيلا نومونيا ذو حساسية عالية لكل من النوروفلوكساسين واللنكومايسين/ سبيكتومايسين وحساسة للفلوموكوين والسلفاميثازول/ترايميثوبريم وأقل حساسية للسيبروميسين والجينتاميسين والنيومتسين والستربتوميسين بينما كان ميكروب الكلبسيلا أوكسي توكا ذو حساية عالية للنوروفلوكساسين والجينتاميسين وحساس للسلفاميثازول/ترايميثوبريم وأقل حساسية للسيبروميسين والنيومتسين والستربتوميسين بينما كان مقاوم للنكومايسين/ سبيكتومايسين و للفلوموكوين.