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Identification and Antibiogram of *Klebsiella pneumoniae* Isolated from Camels with Molecular Recognition of Some Virulence and Antibiotic Resistance Encoding Genes

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Authors' Contribution
AAM, AMA, AND and DAMR performed the experimental work. AAM and AMA designated the plan of study and supervised the study steps. DAMR collected the samples and performed the cultural isolation and biochemical identification. AND and DAMR shared in antibiogram, and molecular identification besides writing and publication processes.

Key words
Antibiotic resistance, Camel, *Klebsiella pneumoniae*, Respiratory, Virulence, Antibiotic resistance genes, *bla*TAM, *aad*B, *mag*A, *rmp*A, Virulence gene

ABSTRACT

Respiratory diseases are emerging concerns of animals including camels which result in considerable loss in production, elevated mortalities and increased cost of treatment. *K. pneumoniae* is a substantial opportunistic pathogen that induces a wide spectrum of respiratory infections in human and animals. In the present study, a total of 116 nasal swabs and 89 lung tissue samples were collected from 33 apparently healthy and 83 respiratory ill camels. Samples were screened for the isolation of bacteria, and positive samples were subjected to classical and API 20 E biochemical-based characterization of *Klebsiella pneumoniae*. Additionally, obtained isolates were assessed for antibiotic susceptibility, the existence of antibiotic resistance genes (*bla*TEM and *aad*B) and virulence factors (*mag*A, *rmp*A). Out of 205 examined samples, 15 isolates (7.31%) were culturally and biochemically confirmed as *K. pneumonia*. All isolates appeared to be resistant to amoxicillin, ampicillin and gentamycin, however, showed variable susceptible to levofloxacin, imipenem (100%), norfloxacin (93.3%) and ceftriaxon (73%). The application of uniplex PCR on the selected *K. pneumoniae* isolates revealed the detection of antibiotic resistance genes (*bla*TAM and *aad*B) in all isolates. The virulence genes including *mag*A and *rmp*A were found in 40% and 0% of samples, respectively. In conclusion, the data highligh the existence multidrug resistant *K. pneumoniae* among Egyptian camels and may represent a theat to public health.

INTRODUCTION

Camelids are unparalleled group of mammals with distinguished physiological and immunological statuses. The members of this family have developed features to accommodate diverse and challenging environments. In Africa and Middle East, camels are nursed, and contribute significantly to the economy of certain communities which rely on camels for meat, milk, sport and transportation (FAOSTAT, 2016). In spite of resilience to the environment, camels are susceptible to numerous bacterial pathogens. A group of the most common infectious diseases affect camels are the respiratory illnesses which are usually attributed to multifactorial etiology (Zhu *et al*., 2019).

Respiratory sickness in camels is a growing concern and is leading cause for significant production losses, higher mortalities, and costly preventive and curative efforts (Kane *et al*., 2005). One of the most prevalent bacterial pathogens impact respiratory illnesses is *Klebsiella pneumoniae* which is a Gram's negative, facultative anaerobic, nonmotile and opportunistic pathogen belonging to the family Enterobacteriacae. The *K. pneumoniae* cause a wide range of symptoms in both humans and animals including pneumonia, septicemia, diarrhea, liver abscesses, meningitis and urinary tract infections (Guo *et al*., 2017; Navon-Venezia *et al*., 2017).

Recently, mucoid-hypervirulent *K. pneumonia* (hvKP) strains have emerged worldwide and found to harbor genes that coding several virulence features that

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help in the microbial pathogenicity (Shon *et al*., 2013). Two important genes found to be implemented in the virulence of these hvKP include *rmp*A gene (regulator of mucoid phenotypes) and *mag*A gene which is associated with K1 capsular serotype. Both genes have been reported to enhance the mucoviscosity and pathogenicity, resulting in severe septicemia and death (Yeh *et al*., 2006; Hsu *et al*., 2011).

Furthermore, the global concern of increasing antimicrobial resistance among microbes was extended to *K. pneumonia* strains. Antibiotics of the β-lactam and aminoglycosides groups are broadly prescribed worldwide. The β-lactams which include cephalosporins, carbapenems, penicillin, amoxicillin and ampicillin are mainly neutralized by production of beta-lactamase. *K. pneumonia* is considered as one of extended-spectrum beta-lactamase (ESBL) producing bacteria (WHO, 2014; Rahman *et al*., 2018). On other side, aminoglycosides are potent natural or semisynthetic broad-spectrum antibiotics derived from actinomycetes, which act via prohibition of protein synthesis and widely used for treatment of diseases caused by Enterobacteriaceae.

The *K. pneumonia* strains may harbor genes responsible to establish resistance against many antibiotics. These include *bla*TEM and *aad*B which are responsible for amoxicillin and gentamicin/kanamycin/tobramycin resistence, respectivley (Cameron *et al*., 1986).

In the current study, the living and slaughtered camels in Gharbia Governorate were screened for the prescence of *K. pneumoniae* by traditional and advanced molecular techniques. The chracterized isolates were analysed for the prescence of virulence associated (*rmp*A and *mag*A) and antibiotic resistant genes.

MATERIALS AND METHODS

Sample collection

A total of 205 samples (116 nasal swabs and 89 lung tissues) were obtained from apparently healthy (n≈33) as well as respiratory manifested camels (n≈83) from established farms and abattoirs in Gharbia Governorate during 2018. Nasal samples were incubated in nutrient broth while lung tissues were processed for the bacteriological examination.

Isolation of Klebsiella pneumoniae

Bacterial isolation was carried out following standard techniques described by Gundogan and Avci (2013). Nasal swabs and tissue samples were primarily inoculated in to blood agar plates (HiMedia, India) and incubated for 24-48 h at 37ºC. Thereafter, the suspected colonies were streaked on MacConkey bile salt lactose agar (Oxoid Basingstoke, UK), XLD agar media (Oxoid Basingstoke, UK) and Eosin methylene blue agar (HiMedia, India) and incubated aerobically at 37ºC for 24-48 h. After incubation, the plates were examined for the nature of growth and appearance characters of bacterial colonies. The preliminary morphological identification was performed based on Gram's staining.

Biochemical identification of Klebsiella *spp.*

The pure colonies were used for biochemical identification through classical biochemical tests, including catalase, oxidase, indole, methyl-red, Voges-Proskauer, urease production and citrate utilization, string test for mucoviscosity, gelatin liquefaction, hydrogen sulphide production on TSI agar and carbohydrates fermentation. Furthermore, the purified colonies were confirmed by application of API 20E system (BioMerieux, Marcy l'Etoile, France) following manufacturer's guidelines.

Antimicrobial susceptibility assay

The sensitivity of the obtained isolates to ten commonly used antibiotics was performed using disk diffusion technique on Mueller-Hinton agar (Oxoid Basingstoke, UK) and interpreted accordingly CLSI (2018). Antibiotic discs (Oxoid Basingstoke, UK) listed in the Table III were used to assess antibiotic sensitivity.

Genotypic characterization of K. pneumonia *isolates*

DNA was extracted from the *K. pneumoniae* isolates using QIAamp DNA mini kit following the manufacturer's guidelines. A total of five isolates from pneumonic lung tissues were used for genotyping. The extracted DNA was subjected to uniplex PCR assay using oligonucleotide specific primers for tested genes as mentioned in Table I. PCR master mix and cycling protocol was adapted according to EmeraldAmp GT PCR Master Mix (Takara, Japan). Amplification was carried out using Biometra thermal cycler.

The reaction was performed under different temperature and duration conditions as described in Table II. Aliquots of amplified PCR products were electrophorised on agarose gel of 1.5% (AB gene) in 1x TBE buffer at 25ºC. For gel analysis, 15 µl of PCR products were loaded in each gel slot. A 100 bp DNA ladder (QIAGEN Inc, CA, USA) was used to define the fragment sizes. The gel was photographed using a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed via different softwares.

RESULTS

A total of 8 isolates of *K. pneumoniae* from pneumonic camels' nasal swabs, and 5 isolates obtained from pneumonic lung tissues. The overall incidence of

Table I. Primers' sequences used for amplification of virulence and antibiotic resistance genes.

Primer	Sequence 5`→3`	Amplified product	Reference
aadB	GAGCGAAATCTGCCGCTCTGG	319 bp	Frana *et al*., 2001
	CTGTTACAACGGACTGGCCGC		
bla_{TEM}	ATCAGCAATAAACCAGC	516 bp	Colom *et al*., 2003
	CCCCGAAGAACGTTTTC		
*mag*A	GGTGCTCTTTACATCATTGC	1282 bp	Yeh *et al*., 2007
	GCAATGGCCATTTGCGTTAG		
rmpA	ACTGGGCTACCTCTGCTTCA	535 bp	
	CTTGCATGAGCCATCTTTCA		

Table II. Cycling condition of the various primers during PCR.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
aadB	94˚C 5 min.	94˚C 0.5 min.	58˚C 0.5 min.	72˚C 0.5 min.	35	72˚C 10 min.
bla_{TEM}	94˚C 5 min.	94˚C 0.5 min.	54˚C 40 sec.	72˚C 45 sec	35	72˚C 10 min.
magA	94˚C 5 min.	94˚C 45 sec.	50˚C 1 min.	72˚C 1.2 min.	35	72˚C 12 min.
rmpA	94˚C 5 min.	94˚C 0.5 min.	50˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

Klebsiella was 8.96%. As regards to apparently healthy camel, one isolate was recovered from nasal swabs and also one isolate from lung tissues. The overall resulting incidence of isolates was 3.33%.

The results were based on the cultural appearance of colonies, Gram's staining, classical and confirmatory biochemical tests. On blood agar, *Klebsiella* species appeared as greyish white non hemolytic colonies, whereas on MacConkey's agar the colonies appeared mucoid, slimy, rose-pink colonies due to lactose fermenter. Furthermore, *Klebsiella* spp. appeared purple colonies on EMB agar while bright yellow colonies on XLD agar.

All *Klebsiella* isolates were identified as *Klebsiella pneumoniae* according to their classical and API 20 E biochemical reactions. The *K. pneumoniae* shows yellow slants, yellow butt with gas production in triple sugar test. The isolates were negative for oxidase activity, and showed positivity with indole, methyl red and hydrogen sulfide test. The bacterial isolates showed positive results for catalase activity, Voges Proskauer test, Simmons citrate tests, and were found to be positive for urease, lactose, sucrose, maltose, raffinose, trehalose, D-mannitol, D-sorbitol, L-arabinose and L-rhamnose

The *in vitro* susceptibility of obtained 15 isolates of *K. pneumoniae* exhibited all the isolates as sensitive to levofloxacin, imipenem (100%) followed by norfloxacin (93.3%) and ceftriaxon (73%). On the other side, all isolates were resistant to amoxicillin, ampicillin and gentamycin (100%) as outlined in Table III.

Table III. Results of antimicrobial susceptibility of recovered *Klebsiella pneumonia* isolates.

Antibiotic	Sensitive No. (%)	Intermediate No. (%)	Resistance No.(%)
Imipenem (10µg)	15 (100)	0	0
Levofloxacin (10µg)	15 (100)	0	0
Ampicillin (10µg)	0	0	15 (100)
Tobramycin (15µg)	7 (46)	0	8 (53)
Gentamycin (10µg)	0	0	15 (100)
Tetracycline (30µg)	5 (33.33)	6 (40)	4 (26.6)
Kanamycin (30µg)	9 (60)	6 (40)	0
Imipenem (10µg)	15 (100)	0	0
Ceftriaxone (30µg)	11 (73)	4 (26.66)	0
Norfloxacin (10µg)	14 (93.33)	1 (6.66)	0

Uniplex PCR was applied on the selected five *K. pneumoniae* isolates to detect the virulence genes (*mag*A,

*rmp*A). Analysis indicated a detection of two and zero isolates by a percentage of 40% and 0%, respectively. On the other hand, the both genes responsible for antibiotic resistance (*bla*TAM, *aad*B) were found in all five isolates by a percentage rate of 100%.

DISCUSSION

The role of *K. pneumoniae* as a significant etiological pathogen for causing pneumonia especially interstitial and chonic pleuropneumonia with mortalities in neonatal dromedary camels was reported (Al-Tarazi, 2001; Narnaware *et al*., 2020).

Our data revealed eight isolates of *K. pneumoniae* from pneumonic camels' nasal swabs, and 5 isolates obtained from pneumonic lung tissues. Therefore, the overall incidence of isolates was 8.96%. As regards to apparently healthy camel, one isolate was recovered from nasal swabs and also one isolate from lung tissues, reulting an overall incidence of 3.33%.

Lower similar incidences (6.3%) have also been mentioned by Abubakar *et al*. (2008) from Nigerian normal and diseased camel lungs and Ismail *et al*. (2014) from Egypt (0.5%) in apparently healthy camels. Ahmed and Musa (2015) have recorded a lower rate (0.1%) among pneumonic camels in Sudan. Also, the low prevalence of *K. pneumonia* in camel was noticed as 16 out of 232 camels (6.9%) in Tunisia (Saidani *et al*., 2019).

On the other side, higher isolation rates (25.0%) were reported by Azizollah *et al*. (2009) among healthy dromedaries in central Iran. Sharma *et al*. (2013) have identified *K. pneumoniae* isolates as high as 26.9% and 49% in nasal swabs obtained from apparent healthy as well as acute respiratory infected camels, respectively. A total of 70 pneumonic lung tissues obtained from abattoirs in Cairo, Egypt revealed isolation of *K. pneumoniae* in 26.7% of samples (Wareth *et al*., 2014). A moderate incidence was reported in cross-sectional bacteriological study performed in Afar Region, Ethiopia among 74 examined camels. The *K. pneumoniae* was isolated from pneumonic tracheas and lungs by 17.3% while the isolation was noticed in 13.5% of samples collected from other normal animals (Gebru *et al*., 2018).

Antibiotics are being used extensively to inhibit or to treat microbial infections in human and veterinary practices. Therefore, a great concern has been growing pertaining to antimicrobial resistance in recent years included in what is termed as One Health concept (Matar *et al*., 2020).

Our data revealed that all tested *K. pneumoniae* isolates were sensitive to both imipenem and levofloxacin (100%), and have also showed considerably high susceptibility against ciprofloxacin (93.3%) and ceftriaxon (73%). On the other hands, it was observed that all recovered isolates were resistant to amoxicillin, ampicillin and gentamycin.

The choice of quinolone group of antibiotics in effective treatment of diseases caused by *K. pneumoniae* has been observed earlier (Coskun *et al*., 2020). The resistance of *K. pneumoniae* to amoxicillin, ampicillin or gentamycin has been reported in several previous reports (El-Mahmood *et al*., 2009; Moawad *et al*., 2011; Sharma *et al*., 2013; Ahmed and Musa, 2015; Borgio *et al*., 2021; Fouad *et al*., 2022).

In our study, all the five tested isolates showed presence of *bla*TEM and *aad*B genes and interestingly this existence was expressed in all isolates clinically by resistance to amoxicillin, ampicillin or gentamycin in susceptibility test as mentioned before. The high presence of these resistance genes which are responsible for antibiotic resistance among clinical *K. pneumoniae* isolates have been documented earlier (Stolle *et al*., 2013; Lev *et al*., 2018; Sivaraman *et al*., 2020; Ejikeugwu *et al*., 2021; Wareth and Neubauer, 2021).

K. pneumonia is characterized by numerous virulence factors which are playing important roles in the microbial pathogenesis. The *mag*A is considered virulence factor encoded for an outer membrane protein, and help in the resistance of phagocytosis. The *rmp*A is another virulence factor that facilitates the expression of the hypermucoviscous phenotypes (Fang *et al*., 2004).

In our study, we have found two out of five tested *K. pneumoniae* harbor *mag*A gene (40%) while no isolate carry *rmp*A gene. The variation of these two genes' existence among *K. pneumonia* clinical isolates has been reported and the absence incidence are documented (Pinsky *et al*. (2009), Sharma *et al*. (2013), Wen-Liang *et al*. (2015)). On the other side, high detection of *mag*A and *rmp*A (77.8%) was reported by Osman *et al*. (2014) *rmp*A (45.7%) and Tan *et al*. (2019). Hartman *et al*. (2009), Turton *et al* (2010) and Lev *et al*. (2018) have demonstrated a moderate (25%) ratio of *rmpA* gene detection.

CONCLUSION

In conclusion, *K. pneumoniae* may become a significant pathogen among farmed animals such as camels with the emergence of multidrug resistance that may constitute public health concern. A special vigilance is required to avoid camels becoming a possible reservoir for *K. pneumonia*.

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Ethical statement

As per CPCSEA guidelines, ethical approval was not needed as the study included clinical and postmortem samples.

Statement of conflict of interest

The authors have declared no conflict of interest.

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