Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 76 (2): 95-103 Jan 2023
DOI: 10.5455/ajvs.98143

Phenotypic and Genotypic Characterization of *Klebsiella pneumoniae* isolated from Clinical Mastitis in Buffaloes

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Key words:
Buffaloes, mastitis, .PCR, Resistance, Virulence

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Article History
Received: 25 Oct 2022
Accepted: 31 Dec 2022

ABSTRACT

Mastitis is the most common serious and economical disease affecting the dairy industry. The current study aimed to determine the molecular characterization of *K. pneumoniae* isolated from buffaloes with clinical mastitis as well as determine the antimicrobial pattern and virulence associated genes. Out of examination one hundred and fifty dairy buffaloes at Beheria governorate, 57 (38%) showed clinical mastitis signs. All samples are submitted to bacteriological examination and confirmed by biochemical tests. The result showed that *K.pneumoniae* was identified in only five (8.7%). Regarding to the antibiogram profile, *K. pneumoniae* isolates exhibited high resistance pattern against Carbenicillin, Cefotaxime, Flumequine, Gentamicin, Kanamycin (100%) followed by Neomycin, Nitrofurantoin, Penicillin (80%) and Oxytetracycline, Sulfamethoxazole / trimethoprim (60%). Meanwhile, higher susceptibility to Ampicillin (100%), followed by Chloramphenicol, Colistin Sulphate, Erythromycin, and Streptomycin (80%). Molecular identification using the mPCR approach applied efficiently to detect the *K. pneumoniae* virulence genes (*mrk*A, *mrk*D, *iut*A) at 475, 226, 300bp respectively. In addition, the successfully detection of *bla*TEM and *bla*SHV for β lactams resistance genes at 516 and 392 bp. In conclusion, the phenotypic and genotypic resistance pattern of *K. pneumoniae* isolates indicates the portability of transmission of resistance genes through milk and food chain with public health concern.

1-INTRODUCTION

Mastitis define as inflammation in the mammary glands and constitute the frequent serious global disease causing economic losses through milk yield losses, high culling rate, veterinary costs, and occasionally mortality in complicated or no treated cases (Aghamohammadi et al., 2018). Moreover, it is the major cause for the antibiotics use in veterinary practices and their prevalence varies according to difference in geographic location, veterinary and laboratory facilities (Osman et al., 2014). Two major groups; contagious and environmental pathogens are involved in intrammamry infection (Chehabi et al., 2019). Coliforms bacteria are Gram-negative pathogens have been elaborate as major environmental cause of bovine mastitis in dairy herds (Batool, et al., 2012). *Klebsiella pneumoniae* (*K. pneumoniae*) is recognized as an environmental mastitis pathogen threat the dairy industry worldwide with low treatment recovery (Munoz et al., 2006; Schukken et al., 2012; Fuenzalida and Ruegg, 2019a), and the relapse of clinical episodes has occasionally occur (Oliveira et al., 2013). *K. pneumonia* is found as contaminant for the dairy farms environment causing intrammamary infection (Zadoks et al., 2011).

According to (Magesh, et al., 2013; Shon et al., 2014; Cheng and Han, 2020) several virulence determinants are involved in pathogenesis and infectivity mechanism of *K. pneumoniae* such as capsule, lipopolysaccharide, fimbrial and nonfimbrial adhesions, siderophores and the biofilm production that help in the colonization and infection establishment. Furthermore, (Holt et al., 2015; Yang et al., 2019) described the existence of Fe3+ transport-associated *fec* genes, *lac* operon, and genes correlated to metals metabolism has a substantial role in the *K. pneumoniae* infection and pathogenicity.

Interestingly, capsule of *K. pneumoniae* hinder the recognition by the immune system through antiphagocytosis, prevention inflammatory reaction, neutralization of antimicrobial, and prevent the cell maturation (Paczosa and Mecsas, 2016). Additionally, type 1 (*fim*) and type 3 (*mrk)* fimbriae in *K. pneumoniae* may permit virulence through adhering to mucosal or epithelial surfaces and stimulate biofilm formation (Schroll et al., 2010).

The continuous pressure to minimize the antimicrobial use in food-producing animals, especially due to the emerging threat of antimicrobial resistance should put in consider during the antibiotics treatment (Van Boeckel et al., 2019). For example, the emerging increase in antimicrobial resistance in *K. pneumoniae* mainly due to the abuse of antibiotics that contribute severe health risks and a public health complaint (Singh et al., 2021).Therefore, *K. pneumoniae* may have a role in the dissemination of antimicrobial resistance in the environmental dairy farms. The prospect of zoonosis, together with the establishment of animal reservoirs that sustain the infection pattern and antimicrobial resistance is now a growing source of concern (Messenger et al., 2014).

The antimicrobial resistance profile against multiple antibiotics groups was attributed to the presence of resistance genes such as β-lactams (*bla*CTX-M, *bla*SHV, and *bla*TEM) had been documented in numerous research among *K. pneumoniae* isolates from bovine origin (Locatelli et al., 2010; Timofte et al., 2014; Holt et al., 2015; Yang et al., 2019). Therefore, there is need for further investigation to gain more information about the antimicrobial resistance profile of *K. pneumoniae* isolates from different sources in dairy herds to identify the potential reservoirs for the and antimicrobial resistance dissemination. Therefore, the goal of the current study was to determine the prevalence of *K. pneumonia* from clinical mastitis in buffaloes as well as determine the antimicrobial pattern and virulence and antibiotics resistance associated genes.

2-MATERIALS &METHODS

2.1. Study area: Beheira Governorate is a coastal governorate situated in the northern part of Egypt in the Nile Delta. It is considered an important strategical place, west of the Rosetta branch of the Nile. According to the 2015 population estimates, the majority of residents in this governorate lived in rural areas, with an urbanization rate of only 19.5%. Out of the total 5,804,262 people residing in the governorate, 4,674,346 people lived in rural areas and only 1,129,916 in urban areas. Beheira is famous for some important industries such as cotton, chemicals, carpets, electricity, and fishing. The poverty rate is more than 60% with recent high investment in many animals breeding industries projects along the new reclaimed areas in this governorate (wikipedia, 2012).

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions of virulence and antibiotics resistance genes of *K.pneumonia*

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Colom *et al*., 2003
blaSHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
mrkD	CCACCAACTATTCCCTCGAA ATGGAACCCACATCGACATT	226	94˚C 5 min.	94˚C 30 sec.	52˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Melo *et al*., 2014
iutA	GGCTGGACATGGGAACTGG	300	94˚C 5 min.	94˚C 30 sec.	63˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Yaguchi *et al*., 2007
*fim*A	CGGACGGTACGCTGTATTTT GCTTCGGCGTTGTCTTTATC	436	94˚C 5 min	94˚C 30 sec	55˚C 40 sec	72˚C 45 sec	72˚C 10 min	Alcántar-Curiel et al., 2013
*mrk*A	CGGTAAAGTTACCGACGTAT CTTGT ACTG GCTGTTAACCACACCGGTGGT AAC	475	94˚C 5 min.	94˚C 30 sec	55˚C 40 sec	72˚C 45 se	72˚C 10 mi	

2.2. Animals and samples: One hundred and fifty buffaloes in a private farm in Beheira governorate from the period extend from November 2021 till March 2022 during several visits were clinically examined for mastitis and seven –fifty buffaloes showed signs of clinical mastitis which assessed according to (**Islam et al., 2012**). The acute mastitis sings were assessed according to (fever, inflamed udder &edema, abnormal milk secretion as clotted and flacks milk). After that, milk samples were collected aseptically from clinically affected quarters in sterile tubes and transported in a cool icebox to the laboratory for further bacteriological examination.

2.3. Phenotypic characterization of *K. pneumoniae:* The samples were subculture onto MacConkey and EMB agar medium and then incubated aerobically at 37 C for 24-48h. The characteristic pure colonies appear as pink mucoid colonies and confirmation was carried out based on the biochemical tests include; IMViC tests and microscopically appearance as described by (Ewing, 1986; Quinin et al., 2002).

2.4.Antimicrobial suscptability test of *K .pneumoniae* isolated from buffoles:

Five *K.pneumonia*e isolates were subjected to the antimicrobial susceptibility test by disk diffusion method against 15 antibiotics discs of different antimicrobials classes; (Oxoid Ltd., Basingstoke, UK): 100 IU Penicillin, 20/10 µg, amoxicillin /clavulanic acid, 30µg Cefotaxime, 10 µg Norfloxacin 30 µg Chloramphenicol, 30 µg Tetracycline, 10 µg Gentamicin, 100 µg Spectinomycin , 30 µg Streptomycin, 30µg Amikacin and 1.25/23.75 µg Sulfamethoxazole/ trimethoprim. The prepared bacterial suspension was adjusted to the turbidity of a 0.5 McFarland standard (1.5 × 108 colony forming units (CFU) ml−1). The reading of the results was recorded as resistance, sensitive, and intermediate according to (CLSI, 2018).

2.5. Molecular detection of *K. pneumoniae* virulence and antibiotics resistance genes.

DNA extraction was carried out by commercial kits (QIAamp DNA Mini kit, Qiagen, Germany, GmbH) with manufacturer's recommendations. Briefly, 200 µl of the bacterial suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. Then, 200 µl of 100% ethanol was added to the lysate followed by washing and centrifugation according the manufacturer's recommendations. Finally, 100 µl of elution buffer was added to yield the nucleic acid. Oligonucleotide Primer: were supplied from Metabion (Germany) are listed in table (1). For multiplex PCR, Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products: were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

3. RESULTS & DISCUSSION:

Mastitis is the common costly disease causing adverse influence on animal health and milk quality in the dairy industry (Patil et al., 2021). Although, it is not usually fatal illness but have severe economic effect on both dairy farmers and milking industry. In addition, it is common prevalent in both buffaloes and cattle with nearly 25% reduction in milk production with human risk transmission of pathogenic bacteria and toxins that may not affected by heat treatment (Ashraf et al., 2020). In the current study, out of examination one hundred and fifty dairy buffaloes at Beheria governorate, 57 (38%) showed clinical mastitis signs, 20 of them with single quarter infection, 15 with two quarters, 18 have three quarters, 4 with 4 quarters affected. After bacteriological culturing *K. pneumonia*e was identified in only five (8.7%) through the traditional method and biochemical tests. This was compared with (Javed et al., 2022) reported prevalence of clinical mastitis in buffaloes in Pakistan (24.69%). In a previous study in Egypt, (El-Khodery & Osman, 2008) detected *K. pneumonia*e in 16 (28.6%) out of 56 buffaloes with clinical mastitis. The lower prevalence rate of *K. pneumonia*e in our study was in contact with (Gröhn et al., 2004) as well as in a similar Egyptian study conducted by (Ammer et al., 2021) recorded prevalence rate of *K. pneumoniae* 4% (2/50) from bovine mastitis. In addition, wet animal bedding had supported the growth of coliform bacteria, particularly *K. pneumonia* (Oz et al. 1985). The difference in prevalence in mastitis and *K. pneumoniae* may be attributed to the difference in number of the examined animals, location area, milking type and mangemental practices.

Regarding to the antibiogram profile of the tested *K. pneumonia*e isolates against different antimicrobials groups, the results revealed high resistance pattern against Carbenicillin, Cefotaxim, Flumequine, Gentamicin, Kanamycin (100%) as well as Neomycin, Nitrofurantoin, Penicillin (80%) and Oxytetracycline,
Sulfamethoxazole and trimethoprimtrimethoprim (60%). Meanwhile, exhibited more sensitivity to Ampicillin (100%), Chloramphenicol, Colistin Sulphate, Erythromycin, and Streptomycin (80%) as showed in table (2). These results was compared with (Ammar et al., 2021) reported that *K. pneumoniae* displayed high resistance rates to ampicillin and ampicillin/clavulanate (100% each) with multiple antibiotic resistance followed by azithromycin, cefepime, and trimethoprim/sulphamethoxazole (91.7%) while, high sensitivity were exhibited to chloramphenicol (83.3%). Moreover, (Enferad and Mahdavi, 2020) recorded that all *K. pneumoniae* resistant to ampicillin, ceftriaxone, gentamicin and nitrofurantoin with (100%) (75%), to (70%) (70%) with high susceptibility to amikacin and chloramphenicol (47.5%). In addition, (Badri et al., 2018) recorded higher resistance pattern against ampicillin, ciprofloxacin, gentamycin, amikacin and cefepime with 94%, 89.2%, 46%, 82,5% and 92%, respectively. In a related study, strong susceptibility to ofloxacin, gentamicin, amikacin, pefloxacin and ciprofloxacin and high resistance to carbenicillin, piperacillin, ampicillin, co-trimoxazole, cefotaxime, chloramphenicol and tetracycline was noticed among K. *pneumoniae* (Sikarwar and Batra, 2011). On the other hand, a comparative study carried out in Egypt showed that *Klebsiella* species recorded high susceptibility to carbenicillin, cephotaxime, flumequine, gentamicin and kanamycin (100%), neomycin (86.96 %) and nitrofurantoin (91.30 %), and strong resistant to ampicillin (100%), chloramphennicol, colistin sulphate, erythromycin and streptomycin (88.9%), and penicillin G (77.8%)(Osman et al., 2014). In a recent study in China (Wu et al., 2022) demonstrated that *K. pneumoniae* from dairy cows, exhibit high sensitivity to meropenem and colistin, but full resistant was noticed to ampicillin (100%), sulfisoxazole (94.56%), cephalothin (47.28%), and streptomycin (30.13%). The difference between studies in the degree and rate of resistance can be explain the difference in the methods used to estimate the antibiogram pattern such as disk diffusion methods and other which considered as non-acceptable for adequate comparison and evaluation against different antimicrobials agents (Schwarz et al., 2010).

Molecular identification using the mPCR approach to detect the *K. pneumoniae* virulence genes (*mrk*A, *mrk*D, *iut*A, *fim*A) was successfully applied at 475, 226, 300, 436bp respectively as showed in figure (1,&2). This was similar to (Anjali and Kashyap, 2017) who developed a multiplex PCR as an efficient tool for identifying the common mastitis pathogens in a rapid and single reaction with high accuracy. Conclusively, previous studies (Ahe et al., 2012; Lee et al., 2014; Osman et al., 2014; Gan et al., 2020) mentioned the significance of PCR and mPCR methods to identify the virulence gene of *K. pneumoniae* by specific primer sets. Similarly, (Wu et al., 2022) recognized the prevalence rate of *(fim*H, *mrk*D, and *iut*A) in more than 85% from tested 68 *K. pneumonia*e isolates. Furthermore, a comparative study (Tsuka et al., 2022) screened several virulence-associated genes for *K. pneumoniae* from bovine mastitis origin including *(iuc*A, *ent*B, *fe*pA, *ybt*S, *psn*, and *kfu*) that contributed substantial role in the pathogenicity with common prevalence of both *ent*B and *fep*A genes and absence of the *iuc*A gene.

Of note, the rapid and global increase in the resistance pattern of multidrug *Klebsiella* sp. is a big serious problem (Gundogan et al., 2011). Therefore, both antibiotic treatment and mass vaccination had become low or limited value against bovine mastitis control (Munoz et al., 2007). Cumulatively, the multidrug resistance bacteria and their treatment with antimicrobial agents as well as zoonotic importance are considered as important concerns worldwide (Unakal and Kaliwal, 2010; Mohanty et al., 2013). The emerging of drug-resistant strains has led to further increases in the use of antibiotics, which will not only lead to environmental influence but also human public health concern (Han et al., 2022).

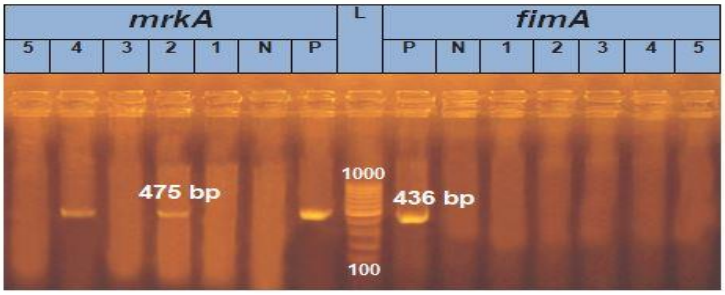

Fig. (1): Amplification of *mrk*A *and fim*A genes of *K. pneumoniae* at 475 and 436 bp respectively, by multiplex PCR. Lane L: 100 bp DNA ladder; Lane 2, 4 positive samples for *mrk*A at 475 bp; and no detection of *fim*A gene at 436 bp in all isolates ; Lane N: Control Negative; Lane P: Control Positive

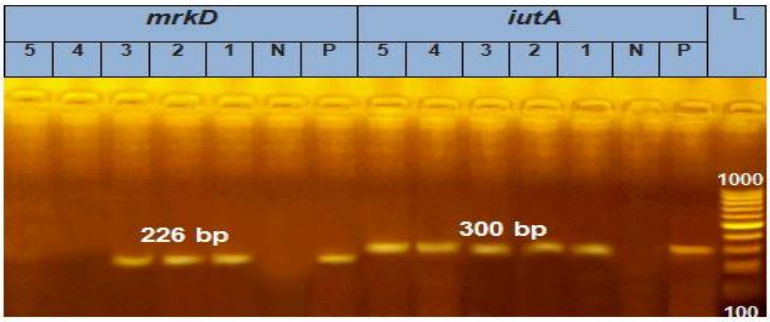

Fig. (2): Amplification of *mrk*D and *iut*A genes of *K. pneumoniae* at 226 and 300 bp by multiplex PCR. Lane L: 100 bp DNA ladder; Lane 1–3 positive samples for *mrk*D gene. While Lane 1-5 positive for the *iut*A gene at 300bp, Lane N: Control Negative; Lane P: Control Positive.

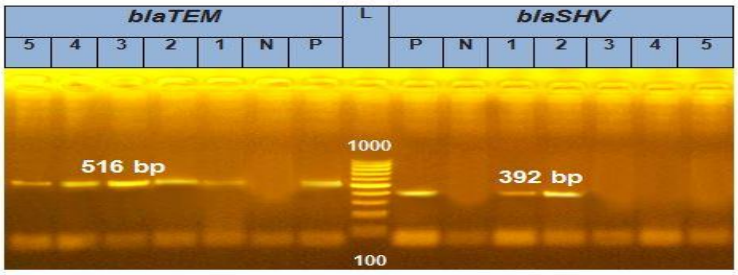

Fig. (3): Amplification of *bla*TEM and *bla*SHV genes of *K. pneumoniae* at 516 and 392 bp by multiplex PCR. Lane L: 100 bp DNA ladder; Lane 1–5 positive samples for *bla*TEM gene and Lane 1,2 positive for *bla*SHV gene ; Lane N: Control Negative; Lane P: Control Positive.

Table (2): Antimicrobial susceptibility pattern of the *K. pneumonia* from clinical mastitis in buffaloes

antibiotics	**groups**	**Resistance**	**Intermediate**	**Sensitive**
Ampicillin	Beta-lactam	0	0	100
Carbenicillin	Beta-lactam	100	0	0
Cefotaxime	Beta-lactam	100	0	0
Chloramphenicol	phenicol	0	20	80
Colistin Sulphate	polypeptide	0	20	80
Erythromycin	Macrolides	0	20	80
Flumequine	Fluoroquinolones	100	0	0
Gentamicin	aminoglycosides	100	0	0
Kanamycin	aminoglycosides	100	0	0
Neomycin	aminoglycosides	80	20	0
Nitrofurantoin	Fluoroquinolones	80	20	0
Oxytetracycline	tetracyclines	60	40	0
Penicillin	Beta-lactam	80	20	0
Streptomycin	aminoglycosides	0	20	80
Sulfamethoxazole and tr imethoprim	Sulfonamide	60	20	20

In our study, the amplification of the *bla*TEM and *bla*SHV resistance genes was detected in five (100%) and three (60%) of the tested isolates as showed in figure (3). In a similar studies, (Rosenblueth et al., 2004; Garza-Ramos et al., 2007) reported a multidrug-resistance phenotypes and extended-spectrum β-lactamase for *K. variicola* during an intra-hospital outbreak at a pediatric hospital in Mexico. In a similar finding, (Chong et al., 2018) reported the widespread of the extended spectrum β-lactamase producing *K. pneumoniae* that represented a critical alarm for treatment against multidrug-resistant bacteria. Furthermore, (Wu et al., 2022) recognized the prevalence rate of *bla*TEM, *bla*SHV in more than 50% from 68 *K. pneumoniae* isolates and thus may allow the spread of environmental multidrug-resistant *K. pneumoniae* through the food chain causing public health hazard. Additionally, *K. pneumoniae* reported as a major carrier of resistance genes from environmental sources to clinically important bacteria and some can carry acquired antimicrobial resistance genes or plasmid to transfer between environmental, human, and animal (Wyres and Holt, 2018). More worryingly, several surveys have been documented that the abuse of antibiotics, had led to the emerging of several multidrug resistant bacterial strains in human, animals, birds and fish that constitute a public health hazard (Algammal et al., 2020a &b). Particularly, multidrug-resistant *K. pneumoniae* strains may associated with epidemics and outbreaks (Gu et al., 2018; Alanezi et al., 2022; Arteaga-Livias et al., 2022).

CONCLUSION

K. pneumoniae is an opportunistic coliform bacteria involved in clinical mastitis in buffaloes in our study. The high phenotypic resistance pattern for the multiple antibiotics groups was observed in our study and correlated to the existence of the resistance genes such as *bla*TEM and *bla*SHV. In addition, the virulence genes were substantial for the pathogenicity and infectivity of *K. pneumoniae.* Further studies are needed to determine the actual role of other virulence and antibiotics resistance genes among *K. pneumoniae* to achieve an adequate control measures. As well, the linked correlation between the phenotypic, genotypic and virulence activity among *K. pneumoniae* from mastitis origin.

Ethics approval: Ethics approval: All studies have been conducted as per the guidelines of the Institutional Animal Ethics Committee, Department of Animal Medicine and Infectious Diseases, University of Sadat City, Egypt. However, farmers in Giza governorate rear the buffaloes species used in this study. Therefore, we have obtained permission from the IACUC, Faculty of Veterinary Medicine, University of Sadat City, Egypt (approval nu/ VUSC- 013-1- 22).

Acknowledgements:
The authors would like to thank the staff members of Animal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, University of Sadat City as well as Animal Health Research Institute, Agricultural Research Center for the help for finishing the manuscript.

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