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# Detection Of Virulence Genes In Klebsiella Pneumoniae Isolates From Respiratory Infected Equines Regarding Their Resistance To Antibiotics

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***Abstract:***

***Background****: Opportunistic pathogens have become elevating relevant as the causative agents of clinical illness in horses. Generally, Klebsiella spp. are gram-negative bacteria that are considered serious public health problems causing urinary tract infections, bloodstream infections, pneumonia infections, and soft tissue infections. This study was performed to evaluate the existence of Klebsiella pneumoniae (K. pneumoniae) in diseased horses suffered from respiratory symptoms as well as determining their virulence and resistance against various antimicrobials.*

***Materials and Methods****: A total of 112 samples including nasal (64), lavage (40), and aborted feti (8) were obtained. The samples were streaked on the suitable culture media. The suspected isolates were confirmed via classical and Vitek procedures. The K. pneumoniae isolates were tested for their susceptibility to various antibiotics. Uniplex and diplex polymerase chain reactions were carried on K. pneumoniae isolates to confirm their virulence determinants; mucoviscosity-associated gene (magA), mucoid phenotype regulator (rmpA), iron uptake (kfuBC) and attachment (fimH) genes.*

***Results****: Twenty seven K. pneumoniae isolates were obtained from examined samples (24.11%); nasal (16), lavage (7), and aborted feti (4). The isolates' identification was secured via biochemical tests and Vitek-2 compact system. Seven isolates exhibited hemolytic activity on horse blood agar (25.93%) while all isolates were non hemolytic on sheep blood agar. On the other hand, seven of the tested K. pneumoniae isolates (25.92%), five (18.52%) and four (14.81%) isolates exhibited lecithinase, gelatinase and caseinase activity respectively. K. pneumoniae isolates were 100% resistant against ampicillin, followed by cefotaxime and ceftazidime (81.84%), while highly sensitive to imipenem (92.59%) and amikacin (85.19%). Concerning the molecular identification of tested virulence genes; eight isolates carried magA gene and five isolates carried rmpA gene. Furthermore, it was found that eleven and six isolates load fimH and kfuBC genes respectively.*

***Conclusion:*** *The results indicated the emerging concern of increased isolation of multidrug resistant K. pneumoniae from livestock as horses. Also, the inclination of K. pneumoniae strains to potentiate surface attachment and biofilm formation leading to an elevated resistance output versus various drugs and disinfectants. The obtained results directed the attention to K. pneumoniae as emerging pathogen belonged to Klebsiella species and give chance to future needing to develop novel combating techniques.*

***Key Word:*** *Antibiogram; fimH; kfuBC; Horses; Klebsiella pneumoniae (K. pneumoniae); virulence.*

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## I. Introduction

Klebsiella species are belonged to the Enterobacteriaceae family. The bacterium is Gram-negative and opportunistic pathogen associated with nosocomial infections. Klebsiella pneumoniae (K. pneumoniae) is the species prime often isolated from ill farm animals as horses, that can colonize oropharynx, skin, or gastrointestinal tract then interspersed from respiratory, gastrointestinal, and renal mucosa to other soft tissues [1]. K. pneumoniae cause various illnesses comprising pneumonia, bacteraemia, suppurative infections, urinary tract infections, and may abortion in animals. The clinical scope of infections can be partly referred to the existence or expression of the virulence agents in addition to the antimicrobial resistance pattern [2]. The hydrolytic enzymes and hemolysins are essential extracellular agents produced frequently by pathogenic bacterial strains [3]. The virulence determinants that have been well depicted to date in K. pneumoniae involve

capsule, siderophores and fimbriae. These are essential in adherence, colonization, biofilm formation, invasion and pathogenesis of infection [4, 5].

It is known that mucoviscosity-associated gene A (magA) is delimited to the capsular serotype K1, whereas capsule-associated gene A (K2A) is delimited to serotype K2. Both magA and K2A are assumed substantial in pathogenesis of hyper virulent Klebsiella pneumoniae (hvKP) infections [6]. Regulator of mucoid phenotype A (rmpA) gene is either chromosomal or plasmid mediated regulator of capsular polysaccharide production. The gene promotes capsule synthesis in hvKP [7]. FimH is the gene responsible for expression of fimbriae and interposes bacterial adhesion either to epithelial cells or biofilm formation at surfaces [8]. The iron transport system is regulated via Klebsiella ferric iron uptake (Kfu) gene. It is important for iron acquisition, usually found in invasive strains and linked with capsule synthesis, hypermucoviscosity [9]. All the above mentioned virulence genes individually or in combination implemented in different degrees of K. pneumoniae pathogenicity; adherence, invasion, severity, spread, and course of infection. Fundamentally, the indistinctive use of antibiotics has implemented in the emergence of the resistance to antibiotic drugs. Recently, the Multidrug Resistant (MDR) Klebsiella spp. has been developing [10]. This study was designated to investigate the prevalence, virulence profile as well as the antibiotic resistance pattern of Klebsiella pneumoniae isolated from ill horses present in stables and stations in Egypt.

## II. Material And Methods

**Ethical approval**

All these clinical samples were obtained from third parties and, therefore, not undergo to reporting obligations of the Ethics and Animal Welfare Commission. During the study period; two successive autumn and winter seasons, (2021 -2022 and 2022- 2023), a number of 112 samples obtained from respiratory ill foals and horses of different ages. The samples obtained from nasal (64), lavage (40), and aborted feti (8). The samples were preserved in ice box till transportation to laboratory. All isolates were stored in glycerol stocks at −80 °.

**K. pneumoniae isolation and identification**

To isolate K. pneumoniae, the samples were inoculated onto MacConkey agar (Himedia, India), for 24 hours at 37 °C and the lactose-fermenting viscous colonies were re-inoculated on eosin methylene blue (EMB) agar (Himedia, India) for an additional 24 h at 37 °C. Each presumptive colony was subjected to further identification using biochemical test as well as Vitek2 identification system.

**Determination of virulence properties**

Hemolytic activity

Isolates were examined for the output of β-hemolysin on sheep and horse blood agar plates [11].

Lecithinase activity

The suspected colony was streaked on 10% egg yolk agar plate and incubated aerobically at 37°C for 24 h, according to Nandy et al. [12]. Lecithinase production and activity were indicated by formation and means of opaque zones respectively.

Caseinase activity

The ability of K. pneumoniae to produce caseinase was identified following the method of Gudmudsdo [13]. The isolates were streaked onto milk agar plates and incubated for 24 h at 37 °C. The caseinase activity was determined by formation of transparent zone.

Gelatinase activity

The gelatinase test was performed using a loop needle of pure colony, then inserted into the nutrient gelatin media in the middle and incubated at 37 °C for ±72 hours., indicates that the microorganism was considered to produce gelatinase exoenzymes, if there was melting of gelatin after refrigeration occurred [14].

**Antimicrobial susceptibility assay**

K. pneumoniae isolates response to eleven antimicrobials was estimated by a disk diffusion method as per the guidelines of the Clinical and Laboratory Standards Institute [15]. Amikacin (30 mg), amoxicillin (25 mg), ampicillin (10 mg), ampicillin-sulbactam (each 10 mg), ciprofloxacin (5 mg), cefotaxime (30 mg), ceftazidime (30 mg), chloramphenicol (30 µg), gentamicin (30 mg), imipenem (10 mg), trimethoprim-sulfamethoxazole (1.25 and 23.75 mg). A reference strain of K. pneumoniae (ATCC 700603) susceptible to all tested antimicrobials was used as a control.

**Polymerase Chain Reaction**

Two hundred microliters were obtained from the twenty seven K. pneumoniae's overnight cultures, mixed with 800 µ of distilled water and boiled for 10 minutes. The obtaining solution was centrifuged and the supernatant used as the DNA template. DNA extraction from samples was done using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's guidelines. The Amplification was performed using an applied biosystem 2720 thermal cycler. Following PCR performance, the reaction products were subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light. The uniplex amplification of the virulence genes was done to find mucoviscosity-associated gene (magA) and mucoid phenotype regulator (rmpA). On the other hand, a diplex PCR was done to detect the fimH and kfuBC genes which responsible for attachment and iron uptake respectively. The used primers and cycling conditions were shown in table (1). Of note that K. pneumoniae ATCC 1290 was used as a positive control, while E. coli ATCC 11303 was used as a negative one.

**Table no 1:** The used primers and cycling conditions for detection of K. pneumoniae isolates' virulence genes

| Gene | Primer | Amplicon size (bp) | Cycling conditions | | | Reference |
|---|---|---|---|---|---|---|
| | | | Denaturation | Annealing | Extension | |
| Maga | F:CGC CGC AAA TAC GAG AAG TG<br>R:GCA ATC GAA GTG AAG AGT GC | 540 | 94°C for 45 sec | 52 °C for 45 sec | 72 °C, 45 sec then 72°C for 5 min | [16] |
| Rmpa | F:ACTGGGCTACC TCTGCTTCA3′<br>R:CTTGCATGAGC CATCTTTCA3′ | 535 | 94°C for 1 min | 58 °C for 1 min | 72 °C, 1 min then 72°C for 10 min | [17] |
| Fimh | F:TGCTGCTGGGC TGGTCGATG<br>R:GGGAGGGTGAC GGTGACATC | 550 | 94°C for 30 s | 55 °C for 30 s | 72 °C, 1 min then 72°C for 10 min | |
| Kfubc | B: GAAGTGACGCTG TTTCTGGC<br>C: TTTCGTGTGGCCA GTGACTC | 797 | | | | [18] |

**Statistical analysis**

SPSS software (version 16.0) was used for analyzing obtained data and a P-value lower than 0.05 was considered statistically significant.

## III. Result

Twenty seven isolates were suspected as K. pneumoniae based on the colonial appearance on cultured media (24.14%). The microorganism can ferment lactose so appeared as rose-pink, mucoid, slimy and medium in size on MacConkey agar plates; figure 1. While on EMB agar the colonies showed purple colonies. The microscopical examination revealed Gram negative non motile bacilli. The suspected K. pneumoniae isolates were indole negative, citrate positive, urease positive and the isolates were more confirmed as K. pneumoniae by Vitek2 system.

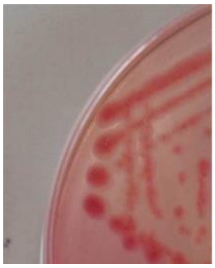

**Figure 1: Colonial appearance of K. pneumoniae isolates on MacConkey agar plate.**

Hemolytic activity

Seven K. pneumoniae isolates showed hemolytic activity horse blood agar (25.93%) while all isolates were non-hemolytic on sheep blood plates.

Hydrolytic activity

Seven K. pneumoniae isolates produced lecithinase (25.93%), five produced gelatinase (18.52%) and four produced caseinase (14.81%).

Antibiotic susceptibility test

The highest resistance pattern of All K. pneumoniae isolates were 100% resistant against ampicillin, followed by cefotaxime and ceftazidime (81.84%), then amoxicillin, trimethoprim-sulfamethoxazole and ampicillin-sulbactam as (70.37 %), (70.37%) and (55.55%) respectively. On the other side, medium resistances were observed against chloramphenicol (48.15%), gentamicin and ciprofloxacin as (44.44%) for both. Finally 25 isolates were sensitive to imipenem (92.59%) and 23 were sensitive to amikacin (85.19%).

Polymerase chain reaction

Eight out of the twenty seven K. pneumoniae isolates (29.63%) revealed the amplification of 540-bp which represent the existence of magA gene indicating they belonged to capsular serotype K1 (figure 2). Another uniplex PCR showed amplification of 535 bp (rmpA gene) in five isolates (18.52%) (figure 3). On the other side, the diplex PCR showed the presence of fimH gene (550-bp) responsible for attachment and biofilm formation in eleven isolates (40.74%) rather than iron uptake gene kfuBC (797-bp) in six isolates (22.22%) (figure 4).

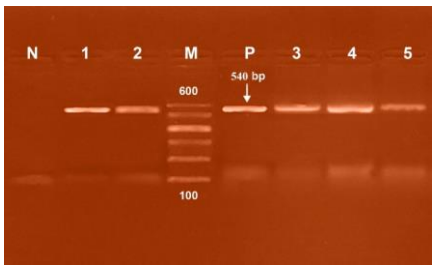


**Fig. 2:** PCR amplification of capsular serotype K1 (magA) gene in of K. pneumoniae isolates. Lane 1–5: positive amplification of PCR product (540 bp). Lane P: control positive. Lane N: control negative. Lane M: 100 bp DNA ladder.

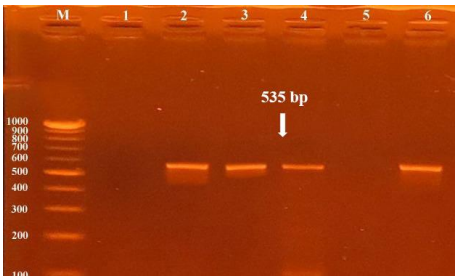


**Fig. 3:** PCR amplification of rmpA gene in of K. pneumoniae isolates. Lanes 3,4 and 6: positive amplification of PCR product (535 bp). Lane 1: control negative. Lane 2: control positive. Lane M: 100 bp DNA ladder.

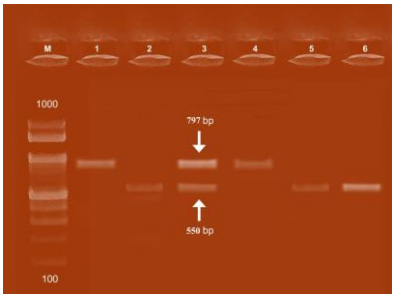


**Fig. 4:** Diplex PCR amplification of two virulence genes in K. pneumoniae isolates. Lanes 1 and 4: positive amplification of PCR product (797 bp) represent kfuBC gene. Lanes 2, 5 and 6: positive amplification of PCR product (550 bp) represent fimH gene. Lane 3: control positive. Lane M: 100 bp DNA ladder.

## IV. Discussion

Recently, K. pneumoniae is in growing link to environmental, community-onset, hospital-acquired infections such as bacteremia, meningitis, pneumonia, and urinary tract infections [19]. Additionally, K. pneumoniae especially MDR becomes an emergence pathogen isolated from various clinically ill animals as well as food chains [20].

Our results demonstrated that 27 isolates were suspected as K. pneumoniae recovered from 112 examined horses samples (24.11%); nasal (16), lavage (7), and aborted feti (4).

Several studies reported K. pneumoniae as a causative agent of respiratory illness in horses; K. pneumoniae was isolated in 40 out of 46 horses suffered from lower respiratory manifestations (86.96%) [21]. K. pneumoniae were isolated from seven foals died from severe respiratory distress, associated with fever [22]. Rahman et al. [23] reported K. pneumoniae (12.1%) among non-racing horses suffered from bronchopneumonia. Also multiple K. pneumoniae isolates were recovered from bronchial aspirates of a mare with pneumonia [24].

On the other side, K. pneumoniae commonly reported as a cause of abortion and reproductive stresses in horses; Akter et al. [25] molecularly detected K. pneumoniae among aborted equine cases in Australia by 29.92%. Also, K. pneumoniae was isolated from Arabian mares suffered from endometritis [26]. Timoney et al. [27] reported K. pneumoniae as a causative pathogen of abortion in mares. Additionally, Loncaric et al. [28] isolated K. pneumoniae (57.14%) from equine fistula samples in Austria.

Generally, K. pneumoniae isolates were differentiated from K. oxytoca via the negativity of indole reaction [29] and confirmed via Vitek-2 identification system.

Our study demonstrated the hemolytic and hydrolytic activities of K. pneumoniae as 25.93%, 25.93%, 18.52% and 14.81% for hemolysin on horse blood agar, lecithinase, gelatinase, caseinase production respectively. The results were near the previous data; K. pneumoniae strains produced hemolysin (33.4%), lecithinase (5.3%), gelatinase (8.9%), caseinase (5.3%), in contrast, 22.8% K. pneumoniae strains caused hemolysis on sheep blood agar [30].

Concerning the antibiotic susceptibility of K. pneumoniae against tested 11 antibiotics revealed that most of the isolates were resistant to two or more antibiotics belonged to different classes. The highest resistances were observed against ampicillin (100%), cefotaxime and ceftazidime (81.84%), then amoxicillin, trimethoprim-sulfamethoxazole (70.37 %). On the other hand the obtained isolates were highly susceptible to imipenem (92.59%) and amikacin (85.19%). Several reports mentioned the analogue antibiotic susceptibility and resistance pattern of K. pneumoniae [28, 31, 32].

Our results revealed the presence of several virulence genes; magA (29.63%), rmpA (18.52%), fimH (40.74%) and kfuBC (22.22%). Regarding the existence of virulence genes among 13 K. pneumoniae isolates obtained from horses suffering from respiratory illness; rmpA(76.9%) and kfu (46.2%). On the contrary, magA was negative in all strains [33]. Also, fimH gene was detected in 61% of K. pneumoniae isolated from dogs [34]. The occurrence of virulence genes involving fimH (100%), rmpA (100%) kfu (68.75%) was noticed among the K. pneumoniae isolates using PCR assay [35]. Clinical ESBL K. pneumoniae isolates found to harbored fimH (89.1%), kfu (27.8%), rmpA (5.1%), and magA (0.2%) [36].

## V. Conclusion

The growing existence of resistant K. pneumoniae against abundant antimicrobial agents among livestock is a developing concern. Also, the inclination of K. pneumoniae strains to form biofilms leading to an elevated resistance output versus various drugs and disinfectants. The obtained results directed the attention to K. pneumoniae as emerging pathogen belonged to Klebsiella species and give chance to future needing to develop novel combating techniques.

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