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DETECTION OF HYPERMUCOVISCOUS *Klebsiella pneumoniae* IN CAMEL (*Camelus dromedarius*) DURING AN OUTBREAK OF ACUTE RESPIRATORY TRACT INFECTION

S.K. Sharma, A.K. Kataria, B.N. Shringi, P. Nathawat, T. Bhati and N. Mohammed

Department of Veterinary Microbiology & Biotechnology, College of Veterinary & Animal Science,
Rajasthan University of Veterinary and Animal Sciences, Bikaner, India

ABSTRACT

The present study was undertaken with an objective to identify hypermucoviscous (HMV) *Klebsiella pneumoniae* isolates in camels (*Camelus dromedarius*) suffering from acute respiratory tract infection in an outbreak form. Of the 96 nasal swabs from affected and 67 from healthy camels, 47 and 18 isolates of *K. pneumoniae*, respectively were obtained. Only 25 (53.19%) isolates of *K. pneumoniae* from acute respiratory tract infected camels and none from healthy camels showed existence of virulent HMV phenotype. Seven (14.89%) of the HMV phenotypes were also positive for virulent K5 serotype. All studied isolates did not show presence of K1 (*magA* gene), K2 and *rmpA* genes.

Key words: Acute respiratory tract infection, camel, hypermucoviscous phenotypes, *Klebsiella pneumoniae*

Klebsiella pneumoniae is a capsulated Gram-negative opportunistic pathogen associated with various systemic and hospital acquired infections in human and animals (Podschun and Ullmann, 1998).

In India, *K. pneumoniae* has been reported from lungs of pneumonic camels affected with acute respiratory infections (locally known as "Khurak"), which is characterised by alveolar serofibrinous exudation, capillary hyperaemia and microbial bronchopneumonia (Arora and Kalra, 1973). This organism has also been reported from acute destructive bronchopneumonia and community-acquired bacterial pneumonia with increased tendency to develop abscess, cavitation and empyema in camels (Zubair *et al,* 2004; Kane *et al,* 2005; Abubakar *et al,* 2010).

The organism *K. pneumoniae* has multifactorial virulence mechanism, which includes capsular polysaccharides (CPS), lipopolysaccharide (LPS), iron-scavenging systems (siderophores), adhesins and hypermucoviscosity (Podschun and Ullmann, 1998; Kawai, 2006). A total 78 different types of capsular (K) antigens were identified (Glucks, 2007) in *K. pneumoniae* of which K1 through K6 have been found to be most virulent (Gierczynski *et al,* 2007; Shu *et al,* 2009; Sobirk *et al,* 2010). These capsular antigens play an important role in defending the organisms against phagocytosis and bactericidal effects of serum factors (Highsmith and Jarvis, 1985).

Some *K. pneumoniae* exhibit large amounts of mucopolysaccharide web of capsular and extracapsular polysaccharides to produce more virulent hypermucoviscous (HMV) phenotypes (Wiskur *et al,* 2008).The HMV phenotype has significant role in virulence since these are resistant to phagocytosis and from killing by complement system. These are commonly associated with community acquired pneumonia, bacteremia and distinct invasive syndromes such as primary liver abscesses, meningitis, and endophthalmitis regardless of the capsule serotype (Yu *et al,* 2008; Lin *et al,* 2010; Vila *et al,* 2011). In HMV phenotypes capsular polysaccharide biosynthesis is mainly governed by the flanking regions of *magA* (mucoviscosity-associated gene A) gene which is strictly associated with K1 capsular serotype (Fang *et al,* 2005; Struve *et al,* 2005) and extracapsular polysaccharide synthesis is associated with plasmid mediated *rmpA* (regulator of the mucoid phenotype A) gene (Nassif *et al,* 1989). Both genes have been reported to increase the mucoviscosity and virulence, resulting in severe septicaemia and death (Yoshida *et al,* 2000; Chuang *et al,* 2006).

Since capsular serotypes K1 (*magA* gene), K2 and K5, HMV phenotypes and *rmpA* gene

SEND REPRINT REQUEST TO S.K. SHARMA email: drsharmask01@hotmail.com

are important for determining the virulence of the organism, the present study was designed to characterise the *K. pneumoniae* isolates obtained from healthy and acute respiratory diseased camels for presence of HMV phenotype, K1 (*magA* gene), K2, K5 serotypes and *rmpA* gene

Materials and Methods

Sample collection, isolation and identification of K. pneumoniae isolates

A total of 163 nasal discharge swab samples, 96 from acute respiratory diseased camels and 67 from apparently healthy camels were collected which were then subjected to cultivation on Simmon's citrate agar with 1% inositol (SCIA) (Van Kregten *et al*, 1984), MacConkey agar (MCA) and Eosin methylene blue (EMB) agar followed by various other biochemical tests for phenotypic identification (Cowan and Steel, 1975; Balows *et al*, 1992). The phenotypically identified isolates were further confirmed genotypically on the basis of 16S-23S rDNA internal transcribed spacer (ITS) region as per the method described by Liu *et al* (2008).

The method of Chen and Kuo (1993) was used to isolate genomic DNA and the sets of primers for PCR used for species level confirmation of the *K. pneumoniae* isolates is mentioned in Table 1.

Virulent Hypermucoviscous (HMV) phenotype detection (String test)

The 16S-23S rDNA ITS confirmed *K. pneumoniae* isolates were streaked on brain heart infusion (BHI) agar plates to obtain isolated colonies of bacteria and incubated overnight at 37°C. A standard inoculation loop was used to stretch a mucoviscous string vertically from a single colony. The formation of a mucoid string >5 mm was regarded as a virulent HMV phenotype (Wiskur *et al*, 2008).

Serotype K1 (magA gene), K2, K5 and rmpA gene detection

Conventional PCR for serotype specific targets such as K1 (*magA* gene), K2, K5 and *rmpA* gene detection was carried out by using the specific primers (Table 1). The genomic DNA (Chen and Kuo, 1993) was used as template for serotype K1 (*magA* gene), K2, K5 detection and the plasmid DNA (Sambrook and Russel, 2001) for *rmpA* gene confirmation. The PCR reactions were carried out as described earlier by Turton *et al* (2008) and Nadasy *et al* (2007) using Promega (USA) gene amplification kit. Thermocycler (Applied Biosystems) conditions were: 95°C for 5 min followed by 40 cycle of 95°C for 1 min, 50°C for 5 min, 72°C for 2 min and final extension at 72°C for 7 min. The PCR products were separated in an 8% native PAGE (Sambrook and Russel, 2001). The gel was analysed under UV light (UVP gel documenting system) and photographs were obtained.

Results and Discussion

From 163 samples processed, 65 (39.87%) isolates were identified as *K. pneumoniae* by using species-specific primers based on 16S–23S rDNA ITS region (Fig 1) of which 47 isolates (48.95%) belonged to acute respiratory diseased camels and 18 isolates (26.86%) to apparently healthy camels. Abubakar *et al* (2010) also recorded more frequency of isolation from pneumonic than from healthy camels. Similarly Al-Tarazi (2001) reported *K. pneumoniae* to be associated with interstitial and chronic pleuropneumonia in camels.

In the study of the 65 *K. pneumoniae* isolates only 25 (38.46%) isolates from respiratory tract infected camels showed existence of virulent hypermucoviscous (HMV) phenotype (Fig 2) while none of the *K. pneumoniae* isolates from healthy camel exhibited HMV phenotype. Yu *et al* (2006)

Table 1. Details of primer sets used for PCR reactions for different target sequences of *K. pneumoniae*.

S. No.	Target	Sequence (5'–3')	Product size (bp)	Reference
1	*K. pneumoniae* 16S–23S ITS*	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130	Liu *et al* (2008)
2	K1 serotype (magA gene)	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1283	Fang *et al* (2004)
3	K2 serotype	GACCCGATATTCATACTTGACAGAG CCTGAAGTAAAATCGTAAATAGATGGC	641	Turton *et al* (2008)
4	K5 serotype	TGGTAGTGATGCTCGCGA CCTGAACCCACCCCAATC	280	Turton *et al* (2008)
5	rmpA gene	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	516	Nadasy *et al* (2007)

* Internal transcribed spacer.

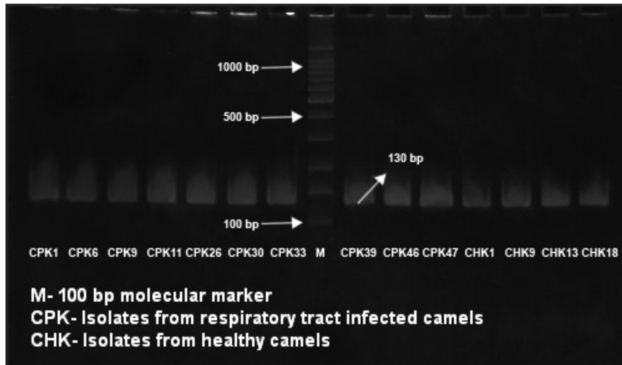

Fig 1. 16S-23S rDNA ITS (internal transcribed spacer) region based genotyping of *Klebsiella pneumoniae*.

Fig 2. Virulent Hypermucoviscous (HMV) phenotype of *Klebsiella pneumoniae* (Positive string test).

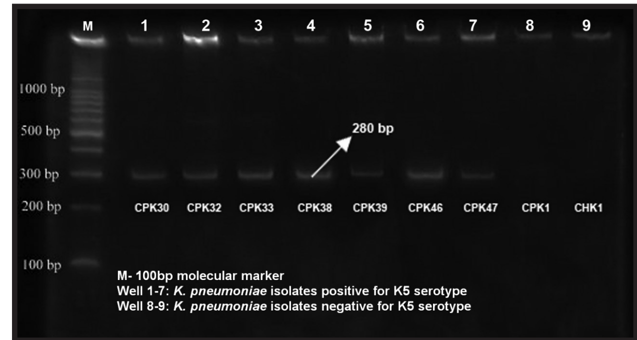

Fig 3. Detection of K5 serotype of *Klebsiella pneumoniae* with specific primers.

also reported 38% prevalence of hypermucoviscous (HMV) phenotype in human clinical isolates of *K. pneumoniae*. The absence of hypermucoviscous (HMV) phenotype in isolates from healthy camels is supported by the findings of Whitehouse *et al* (2010) who also did not find HMV phenotype in apparently healthy vervets. However, a variable occurrence of HMV phenotype has been reported by different workers in various species of animals (Twenhafel *et al,* 2008; Hartman *et al,* 2009; Jang *et al,* 2010)

The detection of *K. pneumoniae* HMV phenotype in various infections of human (Yu *et al,* 2006; Lin *et al,* 2010), coastal marine mammals (Jang *et al,* 2010), monkeys, non-human primates (Twenhafel *et al,* 2008; Burke *et al,* 2009; Hartman *et al,* 2009) and in camels suffering from acute respiratory infections includes in present study highlights the pathogenic nature of HMV phenotypes of *K. pneumoniae*. The recovery of these virulent pathogens from animals envisages the importance of screening domestic, wild and marine mammals for these infectious agents with potential human health implications (Cowan *et al,* 2001).

In the present study, all *K. pneumoniae* isolates were subjected to PCR based identification of K1 (*magA* gene), K2, K5 and *rmpA* genes wherein only seven (14.89%) isolates from acute respiratory tract infected camels were found belonging to K5 virulent serotypes (Fig 3). All other isolates from infected and healthy camels were not detected with K1, K2 and K5 serotype nor with *rmpA* gene. However, Arora and Kalra (1973) found K11 serotype in *K. pneumoniae* isolates obtained from camel pneumonia in India. Similar to observations in the present investigation Turton *et al* (2008) reported 14.28% occurrence of K5 serotypes of *K. pneumoniae* from clinical samples from horses whereas lower frequency of 6.12% K5 serotype was reported by Lin *et al* (2010) in community-acquired pneumonia in humans. Similarly, Turton *et al* (2010) also confirmed the presence of K5 serotype in clinical isolates of *K. pneumoniae* in horses.

Absence of K1 serotype (*magA* gene) has also been reported by Pinsky *et al* (2009) in isolates from human clinical syndrome characterised by liver abscesses, bacteraemia and metastatic lesions. Anstey *et al* (2010) reported variations in the geographical distribution of *K. pneumoniae* serotypes. Likewise, Hartman *et al* (2009) also verified the existence of multiple genotypes and high degree of genetic diversity in isolates of *K. pneumoniae*. In a previous study, Glucks (2007) reported that serotypes K1 to K6 are commonly found in respiratory tract infections of which K1 and K5 are predominant in pneumonia and K2 and K6 are predominant in urinary tract infections of animals while Platt *et al* (1976) and Henton *et al* (1994) found that K1, K2 and K5 serotypes are most commonly associated with metritis of mares while K7 serotype is frequently found in normal preputial flora of stallions but did not cause disease in mares. Fang *et al* (2007) conducted a retrospective study involving 177 cases of *K. pneumoniae* pyogenic liver abscess in human beings and found six genotypes

(K1, K2, K54, K5, K20 and K57) contributing for 92% of the 177 cases.

The absence of *rmpA* gene in the present study is partially supported by earlier observations of Hartman *et al* (2009) who found that 76.27% clinical isolates of primates were negative for *rmpA* gene and Turton *et al* (2010) who observed the absence of *rmpA* gene in 52.32% clinical isolates obtained from horses. However, in regards to isolates from healthy camels our observations are similar to those of Nadasy *et al* (2007) and Whitehouse *et al* (2010) who reported that *K. pneumoniae* isolates from healthy individuals were negative for *rmpA* gene. The absence of *rmpA* gene in isolates from diseased camels is in contrast to observations of Yeh *et al* (2007) who recorded presence of *rmpA* gene in all invasive strains of *K. pneumoniae* they studied in human beings.

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