

Molecular Detection of Virulence Genes Associated with Pathogenicity of *Klebsiella* spp. Isolated from the Respiratory Tract of Apparently Healthy as well as Sick Goats

Aher, T.,* Roy, A. and Kumar, P.

Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand- 388001. (Gujarat) India.

* Corresponding author: Email: drtusharher@yahoo.in; Cellular phone: 09922724213

ABSTRACT

In the present study, 102 nasal swabs and 96 tissue samples were collected from apparently healthy as well as sick goats. All of these samples were screened for the presence of microbial pathogens by cultural isolation and PCR. Out of 144 isolates obtained, 8 isolates (5.6%) were confirmed as *Klebsiella* spp., on the basis of mucoid colony production and biochemical characteristics. Isolates were screened for the presence of *magA* (mucoviscosity associated gene), *uge* (uridine diphosphate galacturonate 4-epimerase gene) and *kfu* (iron uptake system gene) gene by PCR using specific primers, which yielded specific PCR products of 1280 bp, 534 bp and 797 bp, respectively. The *magA*, *uge* and *kfu* gene were amplified in one, five and two isolates out of eight isolates of *Klebsiella* spp. The presence of these genes confirmed the pathogenic potential of *Klebsiella* spp. isolates and their possible association with clinical manifestations in respiratory tract infections of goats.

Key Words: *Klebsiella* spp., Respiratory tract, Biochemical characteristics, Virulence genes, PCR.

INTRODUCTION

Klebsiella species are gram negative, medium sized rods (0.4-0.6 x 2-3 µm). They are facultative anaerobic, fermentative, catalase positive, oxidase negative lactose fermenters, reduce nitrate to nitrite and are motile by peritrichous flagella. They are capable of growth on non-enriched media such as nutrient agar and produce pale pink mucoid colonies on MacConkey agar (1). *Klebsiella pneumoniae* is a common opportunistic pathogen of community-acquired and nosocomial infections (2). They cause a wide spectrum of infections, such as coliform mastitis in cattle, cervicitis and metritis in the mare, urinary tract infections in dogs and pneumonia and suppurative infections in foals including meningitis, and purulent abscesses at various sites, especially in the liver. In particular, it is also associated with a distinctive clinical syndrome, which is characterized by community-acquired *K. pneumoniae* bacteremia with primary liver abscesses, metastatic meningitis and endophthalmitis (3). Furthermore, a

novel chromosomal gene, *magA* (mucoviscosity-associated gene A), causes hypermucoviscosity, as defined by positive results in the string test of mucoviscosity (4).

K. pneumoniae typically expresses a smooth lipopolysaccharide (LPS with O antigen) and capsule polysaccharide (K antigen) on its surface, both contributing to the pathogenesis of diseases caused by this species (5, 6, 7). They produce virulence factors such as adhesin for attachment to host cells, capsules that are antiphagocytic, siderophores that aid the bacterium in its competition with the host for iron and various endotoxins (1). These species produce mucoid colonies on primary isolation which is indicative of the presence of a large capsule surrounding the individual cells (8).

In this study, the isolates were screened for the presence of *magA* (mucoviscosity associated gene), *uge* (uridine diphosphate galacturonate 4-epimerase gene) and *kfu* (iron uptake system gene). These factors contribute to virulence and are responsible for colonization, invasion and pathoge-

nicity. The *magA* gene is part of the capsular polysaccharide gene cluster of *K. pneumoniae* serotype K1 and contributes to the bacteria's virulence (2). The *uge* gene codes for uridine diphosphate galacturonate 4-epimerase, express both smooth lipopolysaccharide (LPS) with O antigen molecules and capsule polysaccharide (K antigen) on the surface, therefore, is essential for *K. pneumoniae* virulence (9). The *kfu* gene which codes for an iron uptake system is a putative pathogenic gene, significantly associated with the virulent hypermucoviscosity phenotype and purulent tissue infections (10). In addition to these, it produces several other extracellular virulence factors which affect the host cell metabolism.

In the present study, the animals were screened for respiratory tract infections caused by *Klebsiella* spp. by cultural and molecular techniques. To study their possible role in pathogenesis, the isolates were also examined for the presence of various virulence associated genes by PCR as well as their biochemical characteristics.

MATERIALS AND METHODS

Sample collection

A total of 102 nasal swab and 96 tissue samples from apparently healthy and sick goats were collected from established

farms and a local meat market and transported to the laboratory on ice. All the samples were processed and screened for possible microbial pathogens.

Cultural isolation of the organisms

Bacterial isolation was carried out following standard techniques (1) by inoculating tissue samples and nasal swabs primarily on blood agar plates (HiMedia, India) and incubated for 24-48 hrs at 37°C. After incubation, the nature of growth and cultural characters of colonies were studied. Preliminary morphological identification was done based on Gram's staining. Cultural characteristics of the isolates were further studied on MacConkey agar (HiMedia, India).

Biochemical identification of *Klebsiella* spp.

Primarily the isolates were characterized by KOH, catalase, oxidase and O-F tests (1). Following primary biochemical tests, the isolates were characterized by various secondary biochemical tests (11). Secondary biochemical tests includes indole test, methyl-red (MR) test, Voges-Proskauer (VP) test, citrate utilization test, urease test, string test for mucoviscosity, hydrogen sulphide production on TSI agar and carbohydrate fermentation test.

Table 1: Primers used for amplification of virulence genes of *Klebsiella* spp.

Primers	Sequences (5'-3')	Target Gene	Size of amplified product (bp)	Reference
magA F magA R	GGT GCT CTT TAC ATC ATT GC GCA ATG GCC ATT TGC GTT AG	<i>magA</i>	1280	(2)
uge F uge R	TCT TCA CGC CTT CCT TCA CT GAT CAT CCG GTC TCC CTG TA	<i>uge</i>	534	(9)
kfu F kfu R	GAA GTG ACG CTG TTT CTG GC TTT CGT GTG GCC AGT GAC TC	<i>kfu</i>	797	(10)

magA, mucoviscosity associated gene; *uge*, Uridine diphosphate galacturonate 4-epimerase gene; *kfu*, Iron uptake system gene.

Table 2: Cycling condition for PCR

Primers (forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
<i>magA</i> F <i>magA</i> R	94°C 1min	94°C 30sec	59°C 45sec	74°C 2min	72°C 6min
<i>Uge</i> F <i>Uge</i> R	94°C 5min	94°C 60sec	54°C 45sec	72°C 60sec	72°C 7min
<i>kfu</i> F <i>kfu</i> R	94°C 5min	94°C 60sec	54°C 45sec	72°C 60sec	72°C 7min
Repeated for 35cycles					

Table 3: Specific Identification of *Klebsiella* spp. isolates-

Test	Characteristics
MacConkey Agar	Lactose fermenting colonies
Indole test	-ve
Methyl Red test	-ve
Voges-Proskauer test	+ve
Citrate utilization test	+ve
Urease test	+ve
Triple Sugar Iron agar slant	Yellow/Yellow/H ₂ S-
String test for mucoviscosity	+ve
Gelatine liquefaction test	-ve

DNA Extraction

The template DNA from the colony was prepared (12) with minor modifications. Briefly, from the culture plate, bacterial colonies were picked and suspended in 100 µl milli-Q water (Manufacturer- MILLIPORE, Method- REVERSE OSMOSIS). The samples were boiled for 15 min, cell debris was removed by centrifugation at 5000 rpm and the supernatant was collected and used as a template DNA.

Polymerase chain reaction (PCR) for amplification of virulence associated genes:

PCR was carried out with template DNA (3 µl), forward and reverse primers (1 µl), 12.5 µl of master mix (2x) (Fermentas, India) and 7.5 µl DNase Free Water (Fermentas, India) in a total volume of 25 µl. The DNA was amplified using the specific cycling conditions (Table 2). PCR products were separated in 1.5% agarose gel (HiMedia, India) for 90 min at 80 V, stained with ethidium bromide (1%) (Sigma, India) added at 0.5 µl/100ml and detected by UV transillumination (wavelength 312 nm). Amplified genes were identified

Table 4: Molecular Characterization of *Klebsiella* isolates-

Name of gene	No. of isolates positive	In percentage
<i>magA</i> (1250 bp)	1	12.5%
<i>uge</i> (534 bp)	5	62.5%
<i>kfu</i> (797 bp)	2	25%

on the basis of fragment size shown in Table 1 and the cyclic conditions for PCR are explained in table 2.

RESULTS AND DISCUSSION

Confluent growth on blood agar medium was inoculated onto MacConkey agar, where lactose fermenting mucoid colonies producing pink discoloration of the medium was further tested. These isolates were positive for urease production and citrate utilization and negative on gelatine stab and acidic reaction on triple sugar iron agar slant. The isolates were negative for H₂S production, on TSI agar slant. After primary biochemical identification, the isolates were found positive for KOH, catalase and negative for oxidase reaction and showed a fermentative reaction in the O-F test (Table 3).

In the present study, the prevalence of *Klebsiella* spp. was found to be 5.6%. Higher prevalence of *Klebsiella* spp. has been reported (13, 14 and 15). A low prevalence of *Klebsiella* spp. has also been reported (16) at the rate of 1.3%.

In the present study, *magA*, *uge* and *kfu* genes were detected by PCR using specific primer sequences which yielded product sizes of 1280 bp, 534 bp and 797 bp, respectively (Table 4). Out of total 8 isolates, 1 isolate (12.5%) was positive for *magA* gene (Figure 1), 5 isolates (62.5%) were positive for *uge* gene (Figure 2) and 2 isolates (25%) were positive for *kfu* gene (Figure 3).

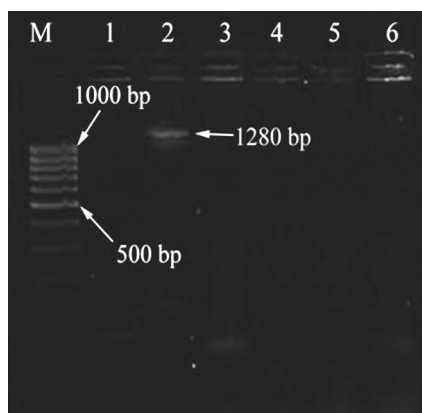
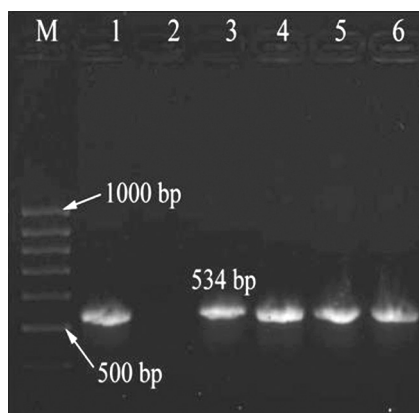
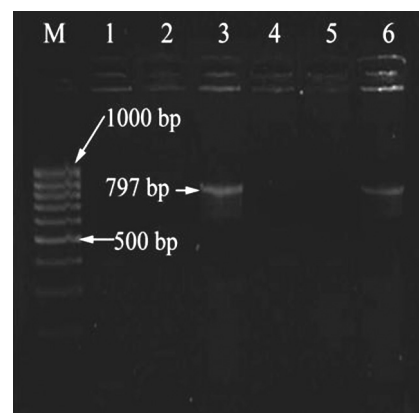
**Figure 1****Figure 2****Figure 3**



Figure 4

The results of the present study are in agreement with reports of (2, 9, 10 and 4) who designed these oligos complementary to each end of the sequence of the *magA*, *uge* and *kfu* genes which specifically amplified the product size of 1250 bp, 534 bp and 797 bp, respectively. The prevalence of virulence associated genes viz., *magA*, *uge* and *kfu* genes were found at the rate of 12.5%, 62.5% and 25%, respectively, which is in contrast with the reports of (4) who detected prevalence at the rate of 29%, 96% and 35%, respectively. In the present study, string test of hypermucoviscosity (Fig. 4) was positive in all isolates but only one isolate was positive for *magA* gene amplification. Thus hypermucoviscosity was not confined to *magA* positive isolates. These results were also in agreement with one report (17). Detection of these genes may indicate the virulence potential of the isolates.

Consequently, although the prevalence rate of the organism in the respiratory tract infections is low, the presence of the virulence associated genes provides them considerable pathogenic potential in the cases where the host is immunocompromised. The molecular detection of these genes provides an avenue for early diagnosis of the infection from susceptible hosts. In view of all the findings, further investigation is needed to define the role of other host and pathogen factors which may assist in the progression of disease at the physiological and molecular levels.

ACKNOWLEDGEMENT

The authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, Anand (Gujarat) for providing the facilities to pursue this work.

REFERENCES

1. Quinn, P. J., Carter, M. E., Markey, B. K. and Cartey, G. E.: Clinical Veterinary Microbiology. Section-2. Bacteriology, Mosby-Year Book Europe Limited, Lynton House, London, England. 1994.
2. Fang, C. T., Chuang, Y. P., Shun, C. T., Chang, S. C. and Wang, J. T.: A novel virulence gene in *K. pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J. Exp. Med.* 199:697-705, 2004.
3. Ko, W. C., Paterson, D. L., and Sagnimeni, A. J.: Community-acquired *Klebsiella pneumoniae* bacteremia: global differences in clinical patterns. *Emerg. Infect. Dis.* 8:160-166, 2002.
4. Yu, W., Ko, W., Cheng, K., Lee, H., Ke, D., Lee, C., Fung, C., and Chuang, Y.: Association between *rmpA* and *magA* Genes and Clinical Syndromes Caused by *Klebsiella pneumoniae* in Taiwan. *Clin. Infect. Dis.* 42:1351-1358, 2006.
5. Kenne, L., and Linberg, B.: Bacterial polysaccharides, In G. O. Aspinall (ed.), *The polysaccharides*. Academic Press Inc., New York, pp. 287-363. 1983.
6. Kelly, R. F., Severn, W. B., Richards, J. C., Perry, M. B., MacLean, L. L., Tomas, J. M., Merino, S., and Whitfield, C.: Structural variation in the O specific polysaccharides of *Klebsiella pneumoniae* serotype O1 and O8 lipopolysaccharide: evidence for clonal diversity in *rfb* genes. *Mol. Microbiol.* 10:615-625, 1993.
7. Izquierdo, L., Merino, S., Regue, M., Rodriguez, F. and Tomas, J. M.: Synthesis of a *Klebsiella pneumoniae* O antigen heteropolysaccharide (O12) requires an ABC 2 transporter. *J. Bacteriol.* 185:1634-1641, 2003.
8. Podschun, R. and Ullmann, U.: *Klebsiella* spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. *Clin. Microbiol. Rev.* 11(4):589-603, 1998.
9. Regue, M., Hita, B., Pique, N., Izquierdo, L., Merino, S., Fresno, S., Benedi, V. J., and Tomas J. M.: A Gene, *uge*, Is Essential for *K. pneumoniae* Virulence. *Infect. Immun.* 54-61, 2004.
10. Ma, L. C., Fang, C. T., Lee, C. Z., Shun, C. T. and Wang, J. T.: Genomic heterogeneity in *K. pneumoniae* strains is associated with primary pyogenic liver abscess and metastatic infection. *J. Infect. Dis.* 192:117-128, 2005.
11. Barrow, G. I. and Feltham, R. K. A.: *Cowan and Steel's Manual for the Identification of Medical Bacteria*. 3rd ed. Cambridge University Press, Cambridge, 1993.
12. Smyth, R. W., Kahlmeter, G., Liljequist, O. B. and Hoffman, B.: Methods for identifying methicillin resistance in *Staphylococcus aureus*. *J. Hosp. Infect.* 48:103-107, 2001.
13. Ramchandran, S. and Sharma, G. L.: Observations on the incidence and histopathology of pneumonia of sheep and goats in India. *Ind. Vet. J.* 46:16-29, 1969.
14. Sharma, R. K., Boro, B. R. and Borachi, P. C.: Incidence of Caprine Pneumonia and associated bacterial isolates. *Ind. J. Anim. Sci.* 61:54-55, 1991.
15. Srinivasan, P., Jyue, M. and Kumar, R. A.: Bacteriological Studies of ovine pneumonia in an organized farm. *Ind. Vet. J.* 80:311-313, 2003.
16. Yimer, N. and Asseged, B.: Aerobic bacterial flora of the respiratory tract of healthy sheep slaughtered in Dessie municipal abattoir, north-eastern Ethiopia. *Revue Méd. Vét.* 158:473-478, 2007.
17. Turton, J. F., Engleder, H., Gabriel, S. N., Turton, S. E., Kaufmann, M. E. and Pitt, T. L.: Genetically similar isolates of *Klebsiella pneumoniae* serotype K1 causing liver abscesses in three continents. *J. Med. Microbiol.* 56:593-597, 2007.