Phenotypic, antimicrobial susceptibility profile and virulence factors of *Klebsiella pneumoniae* isolated from buffalo and cow mastitic milk

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Studies on the prevalence and virulence genes of *Klebsiella* mastitis pathogens in a buffalo population are undocumented. Also, the association of *rmpA*, *kfu*, *uge*, *magA*, Aerobactin, K1 and K2 virulent factors with *K. pneumoniae* buffalo, and cow mastitis is unreported. The virulence of *K. pneumoniae* was evaluated through both phenotypic and molecular assays. *In vivo* virulence was assessed by the Vero cell cytotoxicity, suckling mouse assay and mice lethality test. Antimicrobial susceptibility was tested by disk diffusion method. The 45 *K. pneumoniae* isolates from buffalo ($n=10/232$) and cow ($n=35/293$) milk were isolated (45/525; 8.6%) and screened via PCR for seven virulence genes encoding uridine diphosphate galactose 4 epimerase encoding gene responsible for capsule and smooth lipopolysaccharide synthesis (*uge*), siderophores (*kfu* and aerobactin), protectins or invasins (*rmpA* and *magA*), and the capsule and hypermucoviscosity (K1 and K2). The most common virulence genes were *rmpA*, *kfu*, *uge*, and *magA* (77.8% each). Aerobactin and K1 genes were found at medium rates of 66.7% each and K2 (55.6%). The Vero cell cytotoxicity and LD (50) in mice were found in 100% of isolates. A multidrug resistance pattern was observed for 40% of the antimicrobials. The distribution of virulence profiles indicate a role of *rmpA*, *kfu*, *uge*, *magA*, Aerobactin, and K1 and K2 in pathogenicity of *K. pneumoniae* in udder infections and invasiveness, and constitutes a threat for vulnerable animals, even more if they are in combination with antibiotic resistance.

Keywords: *K. pneumoniae*, Buffalo and cow mastitis, Virulence genes, Antibiotic susceptibility

Introduction

Escherichia coli, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Streptococcus* sp. are the major organisms found to cause environmental mastitis.¹ Among the Gram-negative bacterial causes of mastitis, *Klebsiella* spp. cause the most severe cases.^{2–4}

The search for the pathogenic mechanism of *Klebsiella* infections has identified a number of virulence factors in *K. pneumoniae* associated with primary pyogenic liver abscess which include capsular polysaccharides especially K1 or K2, lipopolysaccharides, hypermucoviscosity, adhesins, and iron acquisition systems.^{5–11} However, very little is known about the presence and roles of virulence-associated genes products involved in the pathogenesis of *K. pneumoniae* associated with mastitis.

Subclinical diagnosis of *Klebsiella* infections commonly is through milk cultures performed at herd surveys. Several isolates that were identified as being *K. pneumoniae* based on phenotypic, failed to differentiate *Raoultella* spp., some *E. cloacae* isolates and a single *Providencia* isolate from *Klebsiella* spp.^{13–15}

Consequently, we evaluated *Klebsiella* by both phenotypic and molecular assays through the use of 16S rRNA for species identification and virulence genes for *Klebsiella* identification. Studies on the prevalence and correlated virulence genes of *Klebsiella* mastitis pathogens in a buffalo population are undocumented. Also, the association of the some virulence determinants: K1, K2, *magA* (mucoviscosity-associated gene specific to K1 capsule serotype), *uge* (uridine diphosphate galactose 4 epimerase encoding gene responsible for capsule and smooth lipopolysaccharide synthesis) and *kfu* (iron uptake system gene), *rmpA* gene (regulator of the mucoid phenotype), and aerobactin

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in buffalo and cow mastitis are unreported. Therefore, in the present study, we screened buffalo and cows affected by clinical or subclinical mastitis for *Klebsiella* species via microbiological, biochemical characteristics and molecular techniques. In addition, antimicrobial resistance of *Klebsiella* isolates was studied for their antimicrobial resistance pattern.

Materials and Methods

Sample collection

The study took place in private dairy farms surrounding Cairo, Egypt. A total of 525 milk samples were aseptically collected from apparently healthy (buffalo, $n=112$ and cows, $n=125$), subclinical (buffalo, $n=23$ and cows, $n=10$), and clinical mastitic animals (buffalo, $n=97$ and cows, $n=158$). The animals had not been treated with an antibiotic for at least 30 days prior to collection. Udders and teats of the randomly selected, lactating animals were cleaned, dried, and disinfected with 70% ethanol before sample collection. Three streams of foremilk was expressed from each quarter and then discarded. During sample collection, 10–15 ml of milk from each quarter was manually expressed into separate sterile 25 ml universal tubes. After gently suspending each sample, the milk was poured into a separate, unused container. As previously indicated,¹⁶ a composite milk sample representing one udder was created; this sample was comprised of all four quarters in one collection vial. A subsample of 15 ml of milk, taken from the composite milk sample, was transferred to sterile universal bottles. The milk samples were quickly transported to the laboratory under chilled conditions and stored at 4°C until bacteriologically analyzed, and had its antimicrobial susceptibility, pathogenicity, and molecular typing determined.

Milk samples were processed using routine methods for the isolation and identification of mastitis pathogens.¹² Briefly, approximately 0.01 ml of each milk sample was swabbed onto trypticase soy agar with 5% sheep blood and 0.1% esculin. Growth was evaluated after 24 and 48 hours of incubation at 37°C. For samples from buffalo and cows with CM, a 1:1 mixture with Todd-Hewitt broth was prepared, and after 4 hours of enrichment at 37°C, additional TSA-BE plates were streaked and incubated in the same manner as the rest of the milk samples. Coliform isolates were identified based on colony morphology, subcultured onto MacConkey and incubated overnight at 37°C. Pink-yellow, mucoid, lactose-positive colonies were considered to be *Klebsiella* spp. and identified to the species level based on motility and biochemistry (citrate utilization and indole production).¹² The isolates were classified phenotypically as mucoid or non-mucoid. Colonies were touched with a loop; the loop was then

lifted vertically from the surface of the agar plate. Mucoid phenotype was defined as being present when a string-like growth was observed to attach to the loop as it was lifted from the plate.¹⁷

Antimicrobial susceptibility

Once they had been isolated and identified, pure cultures of *Klebsiella* were tested for an antibacterial susceptibility panel of 28 antibiotics belonging to 11 drug classes (Table 3) with the disc diffusion assay on Mueller-Hinton agar. Testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute.¹⁸ Antimicrobials were selected for testing based on the licensing for mastitis treatment in cattle, use in human medicine and potential resistant-determinant phenotypes.^{19,20}

Capsule presence by light microscopy

Capsule formation was assessed by India ink method. An unstained region around central bacterial cells indicated the presence of a capsule.¹⁷

Assessment of Congo red uptake

The method of Qadir *et al.*²¹ was used. Congo red (0.003%, w/v) was incorporated into nutrient agar before autoclaving. Plates streaked with test strains were incubated at 37°C for 18 hours. Colonies were examined for the presence (red, crb +ve) or absence (white, crb -ve) of Congo red binding.

Cytotoxicity of *Klebsiella* species

The procedure of Raja *et al.*²² was adopted. Cell Free Culture Supernatants of the nine *Klebsiella* isolates were prepared. Each bacterial strain was grown in Brain Heart Infusion broth. All of the culture supernatants were adjusted to pH 7.3–7.5. One hundred microliters of 5×10^4 tissue culture cells per milliliter in Eagle's minimal essential medium containing kanamycin and 10% fetal calf serum was added to the wells of 96-well microtiter plates and incubated in air containing 5% CO₂ at 37° for 2 days. Aliquots of 25 µl of the cell-free culture supernatant of *Klebsiella* diluted in a two-fold series with Dulbecco's phosphate-buffered saline (pH 7.4) were then added to 100 µl of the monolayer cultures in a well, and the microtiter plates were incubated at 37°C in CO₂ incubator for 48 hours. The results of the cytotoxicity assay was expressed as a reciprocal of the highest final dilution of culture supernatant that caused rounding of more than 90% of cultured cells in the wells.

Animal inoculation

This work was performed in accordance with the recommendations in the updated Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.²³ All procedures were approved by the Cairo University Ethical Committee

in compliance with the United Kingdom (UK) Animals (scientific procedures) Act of 1986 and all required approvals were obtained prior to the experiments.

Mice lethality test

Female BALB/cByl 6-week-old mice were used for inoculation. *K. pneumoniae* inocula consisting of 10^2 – 10^6 mid-logarithmic growth phase colony-forming units were diluted in 100 µl normal saline and injected intraperitoneally.²⁴ Survival of the inoculated mice was recorded and the LD₅₀ was calculated using the SigmaPlot (version 7.0) program from SPSS Inc. (Chicago, IL, USA).

Suckling mouse assay

The *K. pneumoniae* strains to be tested for production of heat stable enterotoxin were screened by the suckling mouse assay according to Giannella.²⁵ Strains were grown in casamino acid yeast extract and 0–1 ml was injected intragastrically in mice 2–4 days old. After three hours the mice were killed by decapitation and the ratio from gut weight divided by body weight was determined. Values greater than 0–0.83 were considered positive; values less than 0–0.75 were considered negative; intermediate results were considered doubtful.

Molecular typing

Molecular methods were used for strain typing and to confirm the species identity of isolates that had been classified as *Klebsiella* spp. based on phenotypic characteristics. Crude DNA extracts from *Klebsiella* isolates were obtained by 10-minute boil preparation and used as templates for m-PCR.²⁶ Strain typing was performed using m-PCR. The primers, sequences, conditions, and predicted sizes of the amplified products are outlined in Table 1. New *Klebsiella*-like colonies were boiled in 400 µl of 1 × Tris-EDTA buffer (pH 8.0) for 10 minutes and centrifuged at 14 000 rev/min for 10 minutes to remove denatured proteins and bacterial membranes. *Klebsiella* spp. was distinguished on the basis of the 16S rRNA revealed by PCR. Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Electrophoresis of amplified products was carried out using 1.5% agarose gels, with 20 5-mm-wide wells, run in 0.5 × Tris-borate-EDTA buffer for 1.5 hours in a horizontal electrophoresis system at ~95 V. Gels were stained with ethidium bromide and visualized through UV transillumination with the Molecular Imager Gel Doc XR system and Quantity One software, version 4.4.1 (Bio-Rad, Hercules, CA, USA).

Table 1 Oligonucleotide primers sequences and size of the PCR-targeted products for the *Klebsiella* species

Primer	Function	Forward	Reverse	bp	cycling conditions	Reference
<i>K1</i>	On the basis of mouse lethality,	5'-GGTGCTCTTTA	5'-GCAATGGCCAT	1283	95°C for	6
<i>K2</i>	capsular serotype is the most virulent when injected intraperitoneally	CATCATTGC-3'	TTGCGTTAG-3'		5 minutes	
		5'-GACCCGATATTCAT	5'-CCTGAAGTAAAATCG	641	Amplification (35 cycles of)	27
		ACTTGACAGAG-3'	TAAATAGATGGC-3'		94°C for	
					30 seconds,	
<i>16S rRNA</i>	Used for species identification	5'-ATTGAAGAGGT	5'-TTCACCTCTGAATTT	130	58°C for	28
		TGCAAACGAT-3'	CTTGTGTTTC-3'		90 seconds,	
<i>rmpA</i>	Extracapsular polysaccharide synthesis regulator (regulator of the mucoid phenotype A)	5'-ACTGGGCTACCTCT	5'-CTTGCATGAGCCA	536	72°C for	29
		GCTTCA-3'	TCTTCA-3'		90 seconds	
					Final extension	
					72°C for	
					10 minutes	
<i>uge</i>	Related to sugar nucleotide epimerases (for uridine diphosphate galacturonate 4-epimerase)	5'-TCTTCACGCCTTCC	5'-GATCATCCGGTCT	534	94°C for	17
		TTCACT-3'	CCCTGTA-3'		1 minutes	
					Amplification (35 cycles of)	
					94°C for	
					1 minute,	
<i>kfu</i>	Responsible for an iron uptake system	5'-GAAGTGACGCTGT	5'-TTTCGTGTGGCCA	797	55°C for	30
		TTCTGGC-3'	GTGACTC-3'		45 seconds,	
					72°C for	
<i>magA</i>	Causes hypermucoviscosity (mucoviscosity-associated gene A)	5'-GGTGCTCTTTACA	5'-GCAATGGCCATT	1280	2 minutes	27
		TCATTGC-3'	TGCGTTAG-3		Final extension	
					72°C for	
					7 minutes	
<i>Aerob-actin</i>	A hydroxamate-type siderophore iron-uptake system	5'-GCATAGGCGGATA	5'-CACAGGGCAATTG	556		26
		CGAACAT-3'	CTTACCT-3'			

Table 2 Prevalence of *Klebsiella* species in buffalo and cow raw milk

Animals	<i>n</i> of examined samples	<i>K. pneumoniae</i>		Total	
		<i>n</i>	%	<i>n</i>	%
Buffalo raw milk					
Apparently healthy animal	112	6	5.4	6	5.4
Subclinical mastitic animal	23	...	...	...	...
Clinical mastitic animal	97	4	4.12	4	4.12
Total	232	10	4.3	10	4.3
Cow raw milk					
Apparently healthy animal	125	12	9.6	12	9.6
Subclinical mastitic animal	10	...	...	...	...
Clinical mastitic animal	158	23	14.6	23	14.6
Total	293	35	11.9	35	11.9

Table 3 Antimicrobial susceptibility patterns of nine *Klebsiella* isolates

Antimicrobial agents	Susceptibility					
	Susceptible		Intermediate		Resistance	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Ampicillin (10 µg)	0	0	0	0	9	100
Carbenicillin (100 µg)	9	100	0	0	0	0
Cephalexime (30 µg)	9	100	0	0	0	0
Chloramphenicol (30 µg)	0	0	2	17.4	8	82.6
Colistin Sulphate (10 µg)	0	0	2	17.4	8	82.6
Erythromycin (15 µg)	0	0	2	21.7	8	87.3
Flumequine (30 µg)	9	100	0	0	0	0
Gentamicin (10 µg)	9	100	0	0	0	0
Kanamycin (30 µg)	9	100	0	0	0	0
Neomycin (30 µg)	8	86.9	2	13.0	0	0
Nitrofurantoin (300 µg)	9	91.3	1	8.7	0	0
Oxytetracycline (30 µg)	7	69.6	3	30.4	0	0
Penicillin G (10 IU)	0	0	3	21.7	7	78.3
Streptomycin (10 µg)	0	0	2	17.4	8	82.6
Sulphamethoxazole/Trimethoprim (1.25 µg + 23.75 µg)	6	60.9	2	21.7	2	17.4

Results

Prevalence and antibiotic profile

The results revealed the prevalence for *K. pneumoniae* from cow was 11.9%, while in buffalo, it was significantly lower (4.3%) (Table 2).

The obtained results recorded in Table 3 revealed that, all strains of *Klebsiella* species were susceptible to carbenicillin, cephalexime, flumequine, gentamicin and kanamycin (100%). Most strains were susceptible to neomycin (86.96 %) and nitrofurantoin (91.30 %). On the other hand, most *K. pneumoniae* were resistant to ampicillin (100%), chloramphenicol, colistin sulphate, erythromycin and streptomycin (88.9%), and penicillin G (77.8%).

Relationship between capsular serotype, Vero cell cytotoxicity, and lethality in mice

The results in the present study indicated that all *Klebsiella* isolates were capsulated. When the mastitic strains were examined, 100% (9/9) cytotoxicity and lethality were observed with strains of K1 and K2 serotypes.

Relationship between mucoid phenotype, serotypes, and infection

The mucoid phenotype was observed in 100% (9/9) of strains from mastitic animals (Table 4); 100% (9/9) of

the isolates of serotypes K1 and K2 were found to be mucoid.

Association between phenotypic evidence of mucoidity, and presence of *rmpA* gene

Phenotypic evidence of mucoidity was highly correlated with the presence of the *rmpA* gene. Of the nine mucoid isolates, 77.8% (7/9) were *rmpA* gene-positive, and 22.2% (2/9) were *rmpA* gene-negative (Table 4).

Table 4 Distribution of the virulence genes within the isolated *K. pneumoniae* strains

Animals	Virulence genes						
	K1	K2	<i>rmpA</i>	<i>kfu</i>	<i>uge</i>	<i>magA</i>	Aerobactin
Buffalo	D	ND	ND	D	D	ND	D
	D	ND	ND	ND	ND	ND	D
	D	D	D	ND	ND	D	D
	D	D	D	D	D	D	D
Cow	D	D	D	D	D	D	ND
	D	ND	D	D	D	D	ND
	ND	ND	D	D	D	D	D
	ND	D	ND	D	D	D	ND
	ND	D	D	D	D	D	D

Note: D, detected; ND, not detected.

Figure 1 Agarose gel electrophoresis showing positive amplification of product 130 bp fragment of 16srRNA gene of *Klebsiella pneumoniae* performed with specific primer. Lane 1: 100–1500 bp DNA ladder; lane 2: negative control *E. coli* ATCC 25922; lane 3: positive control *K. pneumoniae* ATCC 13883; lanes 4–6, 8, and 9: *K. pneumoniae* from cow milk; lane 7: negative control *S. Typhimurium* 14028; lanes 10–13: *K. pneumoniae* from buffalo milk.

Relationship between aerobactin production and type of infection

The presence of the *rmpA* gene and phenotypic evidence of aerobactin production were closely correlated. 57% of *rmpA* gene-positive isolates were aerobactin producers (4/7); aerobactin was produced by 100% of isolates that were *rmpA* gene-negative (2/2) (Table 4).

Molecular identification and virulotyping

The results observed in Fig. 1 show the positive amplification of the 130 bp fragment of primer specific for the 16s rRNA gene in all of the examined *K. pneumoniae* (9/9; 100%). The isolated *K. pneumoniae* strains revealed that they were encoded by *K1* (6/9; 66.7%), *K2* (5/9; 55.6%), *rmpA* (7/9; 77.8%) genes (Fig. 2), *uge* (7/9; 77.8%), *aerobactin* (6/9; 66.7%), *kfu* (7/9; 77.8%), and *magA* (7/9; 77.8%) genes (Fig. 3).

Discussion

Mastitis is one of the most economically problematic diseases of dairy cattle, particularly for the backyard farmers worldwide.³¹ The prevalence of mastitis varies between countries and geographical regions due to national differences in legislation, veterinary, and laboratory services and farmers' management practices. Usually, the highest prevalence of mastitis is found in countries with a poorly developed dairy sector and lack of udder hygiene. *Klebsiella* mastitis is a major concern in the USA.^{4,24,32,33} By contrast, *Klebsiella* mastitis outbreaks are only occasionally reported in Europe,^{34,35} although a high rate has been reported in a study from the Alps in France (92%).³⁶

Our low prevalence rates are closely related to the findings in Canada³⁷ and significantly lower from several other global surveys on the prevalence of *Klebsiella* intra-mammary infections in Europe, Asia and South America^{38–41} which have reported values ranging between 33.5% and 45.0 % and in Africa^{42–49} which have reported a prevalence of 15–16%. The absence of *Klebsiella* spp. isolates in cases of subclinical mastitis highlights the insignificant role of this enterobacteria in subclinical mastitis contrary to other reports.^{42–46,49}

The existence of an oro-fecal transmission cycle has been suggested for *K. pneumoniae* in dairy herds, with fecal shedding resulting in the contamination of feed and water and the subsequent re-ingestion of the organism, resulting in renewed fecal shedding.^{15,50–52} Fecal shedding of *Klebsiella* spp. contributes to pathogen loads in the environment, including in milking machines, recycled manure, bedding material, alleyways, and holding pens.^{4,15,34,35,51,53–55} Studies have illustrated a correlation between bovine teat colonization by *Klebsiella* and the use of sawdust bedding.^{56–58}

The antimicrobial susceptibility of udder pathogens varies greatly between studies. The disc diffusion technique is the most widely used method for determining the susceptibility of animal pathogens, especially in clinical work when it is necessary to determine the correct treatment. Therefore any comparison with studies that use other methods of susceptibility testing is not acceptable.⁴⁰ The prolonged use of antibiotics in the treatment of mastitis

Figure 2 Results of the m-PCR amplification of products 16s *rRNA*, *rmpA*, *k2*, and *k1* genes at 130, 536, 641, and 1283 bp in all of the nine tested *K. pneumoniae* (lanes 4–13). Lane 1: 100–1500 bp DNA ladder; lane 2: negative control *E. coli* ATCC 25922; lane 3: positive control *K. pneumoniae* ATCC 13883; lanes 4–6, 8, and 9: *K. pneumoniae* from cow milk; lane 7: negative control *S. Typhimurium* 14028; lanes 10–13: *K. pneumoniae* from buffalo milk.

and as growth promoters has led to the additional problem of emergence antibiotic resistant strains, hence the constant concern about the resistant strains entering the food chain.⁶³ *Klebsiella*'s economic impact may be more devastating as many cows die or end up being culled.⁴ *Klebsiella* is usually referred to as particularly aggressive and is prone to cause severe clinical mastitis, which responds poorly to treatment and as a consequence, infections tend to be severe and long lasting with a fatal outcome.^{3,24,60,64} Antibiotic-resistant *Klebsiella* on fresh vegetables and in sawdust pose a potential health problem with

respect to bovine mastitis.⁵⁷ One possible contributing factor is the documented overuse of antibiotics in both agriculture and medical practice.^{65,66} A possible explanation for the resistance phenomenon could be that ampicillin, chloramphenicol, colistin sulphate, erythromycin and streptomycin, and penicillin G have been the class of antimicrobial most widely used for treatment of several infections for many years.

The environment can contain a large variety of *K. pneumoniae* strains with pathogenic potential,^{24,52,54,59,60} and mastitis cases within a dairy herd are usually caused by many different strains which

Figure 3 Results of the m-PCR amplification of products *uge*, *aerobactin*, *kfu*, and *magA* genes at 534, 556, 797, and 1280 bp in all of the nine tested *Klebsiella pneumoniae*. Lane 1: 100–1500 bp DNA ladder; lane 2: negative control *E. coli* ATCC 25922; lane 3: positive control *K. pneumoniae* ATCC 13883; lanes 4–6, 8, and 9: *K. pneumoniae* from cow milk; lane 7: negative control *S. Typhimurium* 14028; lanes 10–13: *K. pneumoniae* from buffalo milk.

confirms our recording for K1 and K2.^{33,51,61,62} *Klebsiella* produces 77 serologically distinct types of capsules¹¹ and their degree of virulence may be related to the mannose content of the capsular polysaccharide.⁶⁷ Mucoid strains of K1 or K2 serotype were more virulent to mice than non-mucoid strains of the same serotype.¹⁷ Although one of the few consistencies is that organisms that produce either the K1 or K2 capsular serotypes are more often associated with human disease,^{11,28} both capsular serotypes were observed to be associated with the K. pneumoniae strains causing mastitis in our investigation, an indication that the source of *Klebsiella* infection was human.^{11,68,69}

Capsular serotypes K1 and K2 that carry genes *magA*⁷⁰ and *rmpA*^{71,72} make the bacteria more invasive and more resistant to phagocytosis.^{73,74} A previous study has shown that the mucoid phenotype may be due to a gene designated *rmpA*^{71,72} which frequently coexists with aerobactin production.¹⁷ The strong association found in this study between mucoid phenotype A (*rmpA*-positive isolates) and aerobactin production suggests that the two virulence characteristics might be genetically coupled on a large virulence plasmid.^{17,29,70,71,75} This paper also shows that *magA*, *uge*, *kfu*, *rmpA*, and Aerobactin rather than the K1 and K2 capsule *per se* are important virulence genes in invasive K. pneumoniae strains causing mastitis.

Conclusion

The isolation of K. pneumoniae from milk of apparently healthy animals supports the notion that the presence of *Klebsiella* on teat ends may lead to opportunistic intramammary infections.⁷⁶ Therefore, to improve uptake of *Klebsiella* mastitis, control measures must be implemented: (1) the application of molecular or DNA-based methods in mastitis diagnostics; (2) insight into socioeconomic factors that motivate farmers to control *Klebsiella* mastitis; (3) hygiene of alleyways and holding pens is an important component of *Klebsiella* control on dairy farms; (4) mastitis controls will need to be developed to meet the specific requirements of an individual country or segment of the dairy industry; (5) identification of the virulence genes in K. pneumoniae strains causing mastitis will be helpful in tracing the origins of this infectious disease and vaccine production; and (6) pre-milking preparation procedures is of utmost importance. Cows with soiled udders arrive in the milking parlor, they are likely to still have *Klebsiella* on teat ends after pre-milking udder preparation. Not even the best pre-dipping disinfectant can cope with excess organic matter on heavily soiled udders.

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