Molecular epidemiology and extended-spectrum β-lactamases production of *Klebsiella pneumoniae* isolated from three dairy herds[1]

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ABSTRACT.- Nóbrega D.B., Guiduce M.V.S., Guimarães F.F., Riboli D.F., Cunha M.L.R.S., Langoni H., Pantoja J.C.F. & Lucheis S.B. 2013. **Molecular epidemiology and extended-spectrum β-lactamases production of *Klebsiella pneumoniae* isolated from three dairy herds.** *Pesquisa Veterinária Brasileira 33(7):855-859.* Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus de Botucatu, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-900, Brazil. E-mail: diegoborin@yahoo.com

The objectives of this study were to isolate *Klebsiella pneumoniae* from different sources in three dairy cattle herds, to use the pulsed-field gel electrophoresis (PFGE) to measure genotypic similarities between isolates within a dairy herd, to verify the production of extended-spectrum β-lactamases (ESBLs) by the double-disk synergy test (DDST), and to use the PCR to detect the main ESBLs subgroups genes. Three dairy farms were selected based on previous mastitis outbreaks caused by *K. pneumoniae.* Milk samples were collected from lactating cows and from the bulk tank. Swabs were performed in different locations, including milking parlors, waiting room, soil, animal's hind limbs and rectum. *K. pneumoniae* was isolated from 27 cases of intramammary infections (IMI) and from 41 swabs. For farm A isolates from IMI and bulk tank were considered of the same PGFE subtype. One isolate from a bulk tank, three from IMI cases and four from environmental samples were positive in the DDST test. All eight DDST positive isolates harbored the bla_{shv} gene, one harbored the bla_{tem} gene, and three harbored the bla_{ctx-m} gene, including the bulk tank isolate. Our study confirms that ESBL producing bacteria is present in different locations in dairy farms, and may be responsible for IMI. The detection of ESBLs on dairy herds could be a major concern for both public and animal health.

INDEX TERMS: Dairy cattle herds, extended-spectrum β-lactamases (ESBLs), *Klebsiella pneumoniae*, pulsed-field gel electrophoresis (PFGE).

RESUMO.- [**Epidemiologia molecular e produção de β-lactamases de espectro estendido de *Klebsiella pneumoniae* isoladas de três propriedades leiteiras.**] Os objetivos deste estudo foram isolar *Klebsiella pneumoniae* de diferentes localidades em três propriedades leiteiras, utilizar a eletroforese em campo pulsátil para averiguar similaridades genotípicas entre os isolados de uma mesma propriedade, verificar a produção de beta-lactamases de espectro estendido (ESBLs) pela prova da disco-difusão dupla associada (DDST) e utilizar a PCR para detecção dos principais subgrupos genéticos de ESBLs. Três propriedades leiteiras foram selecionadas baseando-se em surtos prévios de mastites causadas por *K. pneumoniae.* Amostras de leite foram coletadas de vacas em lactação e do tanque de expansão. Swabs foram realizados em diferentes localidades, incluindo salas de lactação, salas de espera, solo, reto e membros posteriores de animais. *K. pneumoniae* foi isolada de 27 casos de infecções intramamária (IMI) e de 41 swabs. Para a propriedade A os isolados de IMI e do tanque de expansão foram considerados do mesmo subtipo

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molecular. Um isolado do tanque de expansão, três de casos de IMI e quatro de amostras ambientais foram considerados positivos no teste da DDST. Todos os oito isolados DDST positivos portavam o gene bla_{shv}, um portava o gene bla_{tem}, e três portavam o gene bla_{ctx-m}, incluindo um isolado de tanque de expansão. Nosso estudo confirma que bactérias produtoras de ESBLs estão presentes em diferentes localidades em propriedades leiteiras, e podem ser responsáveis por quadros de IMI. A detecção de ESBLs em propriedades leiteiras pode representar uma grande preocupação para saúde pública e para a saúde animal.

TERMOS DE INDEXAÇÃO: Beta-lactamases de espectro estendido (ESBLs), eletroforese em campo pulsátil, *Klebsiella pneumoniae*, propriedades leiteiras.

INTRODUCTION

Agents such as *Klebsiella pneumoniae* and *Escherichia coli* are commonly found in environments where dairy cattle is kept, hence, they are considered environmental mastitis agents (Hogan & Smith 2003). *K. pneumoniae* is present in soil, faeces, water, among other locations within a dairy herd. The pathogen shows a high genetic diversity (Munoz et al. 2007, Paulin-Curlee et al. 2007), with different strains been isolated from several sources in dairy herds (Paulin--Curlee et al. 2008, Verbist et al. 2011).

Coliforms can cause chronic mastitis that is unresponsive to treatment (Hogan et al. 1989). Treatment of such cases is often ineffective due to great resistance to drugs commonly used to treat mastitis (Rajala-Schultz et al. 2004). It's noteworthy that β-lactam compounds such as penicillin continues to be one of the most frequently used drugs in veterinary medicine (Pitkala et al. 2007). For Gram-negative bacteria, the production of β-lactamase enzymes is the main resistance mechanism to β-lactam antibiotics. Although the development of third-generation cephalosporins resulted in improvement of treatments against β-lactamase-producing bacterial infections, a new class of enzymes that is capable of hydrolyzing even these new antimicrobials is reported (Bradford 2001). Such enzymes are known as extended-spectrum β-lactamases (ESBLs) and are derived from common β-lactamases.

The genes that encode ESBLs are usually found in plasmids, and those encoding ESBLs of types CTX-M (gene bla_{ctx-m}), TEM (bla_{tem}), PER (bla_{per}), VEB (bla_{ver}) and SHV (bla_{shv}) are the main groups (Paterson et al. 2003, Jemima and Verghese 2008).

This study addressed an important topic, as currently there are few studies involving molecular epidemiology and ESBLs in dairy herds. The objectives of this study were to isolate *K. pneumoniae* from different sources in three dairy herds, assess genotypic similarities among isolates within herds using the pulsed-field gel electrophoresis (PFGE), verify the production of ESBLs by the double-disk synergy test (DDST), and detect the main ESBLs subgroups genes. Our hypotheses were that isolates from same farm and source shared high genotypic similarities and ESBLs were present in the studied dairy farms.

MATERIALS AND METHODS

Sample collection

Three dairy cattle farms (A, B and C) located in São Paulo State were selected based on the occurrence of previous mastitis outbreaks caused by *Klebsiella pneumoniae*. All farms performed the pos-dipping routine with iodine solutions and adopted the feeding after milking strategy (up to 40 minutes of waiting time). Studied animals were raised in the respective farms (lactating cows acquired from other farms did not participate in the study). All the animals were of Holstein Friesian breed. Information regarding each farm specifically is listed next.

Farm A. Farm A had 59 lactating cows, raised in a grass pasture system. The average milk production was 16.06 liters per animal, with bulk tank milk SCC of 528.00 cells/mL. The majority of animals consisted of first lactating cows (60.6%) and in the late lactation days (45.4% of the cows with 240-360 Days in Milk - DiM). The pre-milking routine consisted of washing the udder quarters with water, followed by drying with towel papers.

Farm B. Farm B had 290 lactating cows with an average milk production of 25.99 liters per animal and 253.00 cells/mL of bulk tank milk SCC. The majority of animals was first lactating cows (42.1%) located in the range of 120-240 lactation days (47.8%). The farm used a freestall system (sand bedding) with four barns, and the pre-milking routine consisted of washing the udder quarters with water and a commercial disinfectant solution based on chlorhexidine.

Farm C. Farm C had 970 lactating cows, raised in a freestall system (eight barns) with sand bedding. The average milk production was 35.05 liters per animal and had 380.00 somatic cells/mL in the bulk tank milk. The majority of animals was second lactating cows (42.1%) located in the range of 120-240 lactation days (38.7%). The pre-milking routine consisted of washing the udder quarters with water and a commercial disinfectant solution based on chlorhexidine.

Except for Farm C, where 30% of the high-somatic cell count animals (three-month average >200x10³ cells/mL) were sampled, all non-missing teats from lactating cows were sampled once in a three month period. The California Mastitis Test (CMT) (Schalm & Noorlander 1957) was used at the quarter level as a screening method to detect IMI. Milk samples were collected from CMT-positive quarters (score ≥ three in a five point scale). For all farms, bulk tank milk samples (200mL) were collected after homogenization.

Different locations were sampled, including milking parlors, waiting room, freestall beds (farms B and C), environment (farm A, soil samples), animals hind limbs, and rectum. All samples were collected using cotton pad swabs. From the milking parlor and waiting room, two samples (swabs) were collected from the floor (entrance and exit). From farms B and C, eight sand beds were sampled. Each swab was taken by scrubbing five points per square meter of surface, for at least 30 s, and immediately stored in three mL of saline solution under refrigeration. For each farm, 12 animals were randomly selected, six of which had swabs collected from hind limbs, and six from rectums. In both cases, the swabs were scrubbed at the regions for at least 15 s.

Microbiological procedures

Milk. Microbiological procedures were performed following standardized guidelines (National Mastitis Council 1999).

Swabs. Each swab was stored in three mL of saline solution. After homogenization, one mL was used to prepare dilutions (10^{-1}, 10^{-2} and 10^{-3}) on saline. One hundred μL of each dilution (including the undiluted) were streaked onto MacConkey agar, Eosin Methylene Blue Agar (Levine, Oxoid, Basingstoke, United

Kingdom) and MacConkey agar containing ampicillin (10mg/L) (Munoz et al. 2006). Plates were incubated for 24 h at 37°C.

PFGE. The PFGE protocol was performed according to Durmaz et al. (2009) in all isolated *K. pneumoniae*. Clusters were defined as strains with at least, 80% of similarities. PFGE subtypes were defined as strains having at least 95% of similarity.

ESBL Detection. All *K. pneumoniae* isolates were screened for ESBLs production by the DDST as described elsewhere (CLSI, 2008). The following drugs were tested: aztreonam (30 µg), ceftriaxone (30 µg), cefpodoxime (10 µg), ceftazidime (30 µg), cefoxitin (30 µg), and cefotaxime (30 µg) (Oxoid, Basingstoke, United Kingdom).

PCR reactions were conducted in order to identify the main ESBLs subgroups in all isolated *K. pneumoniae*. Primers and re-

Table 1. Oligonucleotide sequences, amplicon size (bp), direction and reference of the primers used to detect the extended-spectrum β-lactamases genes

Gene	Direction	Primer sequence (5' - 3')	Bp*	Reference
bla_{tem}	forward	CATTTTCGTGTCGCCCTTATTC	799bp	Dallenne et al.
bla_{tem}	reverse	CGTTCATCCATAGTTGCCTGAC		(2010)
bla_{ctx-m}	forward	ATGTGCAGYACCAGTAARGT	593bp	Pagani et al.
bla_{ctx-m}	reverse	TGGGTRAARTARGTSACCAGA		(2002)
bla_{shv}	forward	ATGCGTTATATTCGCCTGTGTA	862bp	This study
bla_{shv}	reverse	TTAGCGTTGCCAGTGCTCGATCAG		

*bp = base pairs.

Table 2. Absolute frequency (N) and source of positives samples for the isolation of *Klebsiella pneumoniae* according to the dairy farm, number of isolates producing extended--spectrum β-lactamases (ESBL), genes detected at the ESBLs positives isolates, and pulsed-field gel electrophoresis (PFGE) clusters and subtypes per source location

Farm	Source[d]	N	ESBL	Gene	Clusters[b]	Subtypes[c]
A	IMI	1	0		A1	A1a (1)
	Soil	7	1	bla_{shv}	NA	NA
	Hind limbs	4	0		NA	NA
	Milking parlors	2	1	bla_{shv}	NA	NA
	Rectum	0	0		NA	NA
	Bulk Tank	1	0		A1	A1a (1)
	Bed	NA[a]	0		NA	NA
	Waiting room	0	0		NA	NA
B	IMI	3	2	$bla_{shv;}\ bla_{ctxm}$	B2	B2a (1); B2b (1)
	Soil	NA[a]	0		NA	NA
	Hind limbs	3	1	bla_{shv}	B3	B3a (1)
	Milking parlors	0	0		NA	NA
	Rectum	2	0		B3	B3b (1)
	Bulk Tank	1	1	$bla_{shv;}\ bla_{ctxm}$	B2	B2c (1)
	Bed	6	1	bla_{shv}	B1	B1a (2); B1b (1)
	Waiting room	2	0		NA	NA
C	IMI	23	1	$bla_{shv;}\ bla_{tem}$	NA	NA
	Soil	NA[a]	0		NA	NA
	Hind limbs	4	0		NA	NA
	Milking parlors	0	0		NA	NA
	Rectum	3	0		NA	NA
	Bulk Tank	1	0		NA	NA
	Bed	5	0		NA	NA
	Waiting room	0	0		NA	NA

[a] Not applicable due to the farm's raising system; [b] Defined as strains sharing, at least, 80% of similarities at the PFGE; [c] Defined as strains sharing, at least, 95% of similarities at the PFGE. The subtypes are followed by the number of strains belonging to that category, i.e. cluster A1 had one isolate from an udder and one isolate from a bulk tank. These strains were form the same subtype (A1a); [d] IMI = intramammary infection. * NA = not applicable.

action conditions are presented in Table 1. A positive ESBL producing *K. pneumoniae* (ATCC 700603 harboring a bla_{shv} gene), a *K. pneumoniae* harboring both bla_{ctx-m} and bla_{tem} genes stored in our laboratory, a *K. pneumoniae* ATCC 13883 and an *E. coli* 25922 strain were used as reference controls.

RESULTS AND DISCUSSION

Eighty one milk samples were collected from farm A. Seven milk samples (8.6%) were contaminated and excluded from analysis. Two hundred thirty-one milk samples were collected from farm B, of which 25 were missing. One milk sample (0.4%) was contaminated and excluded from analysis. Three hundred ninety-six milk samples were collected from farm C, of which 41 were missing.

Klebsiella pneumoniae was isolated from 27 cases of IMI and 41 swabs from different locations (Table 2). The dendrograms for farm A and B are shown in Figures 1 and 2. The number of non-typeable isolates found by farm was: farm A: one isolate (an animal's hind limb); farm B: two isolates (one from a hind limb and one from a rectum); and farm C: one isolate from an IMI.

Results of the present study contibute to the understanding of *K. pneumoniae* molecular epidemiology. Clusters of environmental samples were observed for farm B. Three beds shared the same genotype while another genotype was similar between one hind limb and a rectum sample. In contrast to results of previous studies (Verbist et al. 2011), none of these genotypes were found causing IMI. Epidemiological studies involving isolates from bedding, rectum and hind limbs would be necessary in order to study the true nature of the relationship between the different genotypes. Different beds shared the same genotype, and this was not found for rectum or hind limbs samples. We could not confirm fecal contamination of bedding in our study because not all animals were sampled (swabs), and only one isolate per sample was typed. Nonetheless, we could
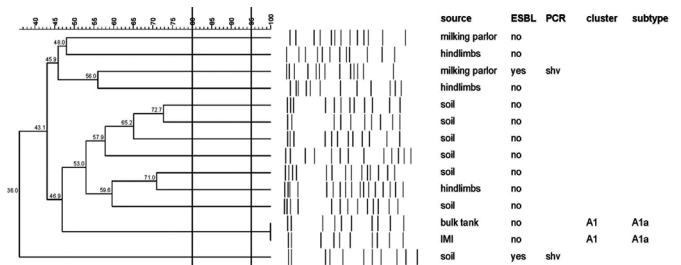

Fig.1. Pulsed field gel electrophoresis (PFGE) pattern of 14 *Klebsiella pneumoniae* isolates from different sources from farm A (IMI = intramammary infection) digested with the *XbaI* restriction enzyme. The dendrogram was constructed with the BioNumerics software 6.0 (Applied Maths NV, Sint-Martens--Latem, Belgium) choosing the Dice coefficient setting both tolerance and optimization at 1%. The horizontal scale on the left side (100 to 40) indicates the level of similarity in percent among fingerprints. The vertical bars indicate the levels of 80% and 95% of similarities, which indicates the minimal levels for defining clusters and subtypes respectively. Production of extended spectrum β-lactamases (ESBL) detected by the double-disk synergy test (DDST) and the detection of the ESBL main genes (shv = bla_{shv}) are also shown.

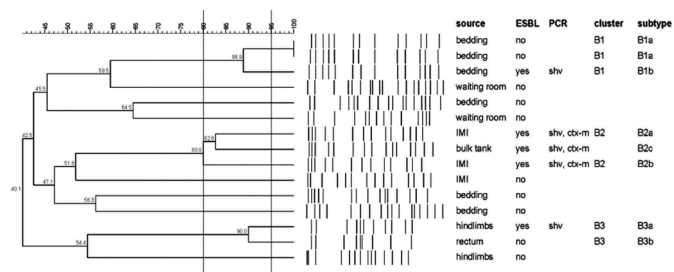| source	ESBL	PCR	cluster	subtype
bedding	no		B1	B1a
bedding	no		B1	B1a
bedding	yes	shv	B1	B1b
waiting room	no			
bedding	no			
waiting room	no			
IMI	yes	shv, ctx-m	B2	B2a
bulk tank	yes	shv, ctx-m		B2c
IMI	yes	shv, ctx-m	B2	B2b
IMI	no			
bedding	no			
bedding	no			
hindlimbs	yes	shv	B3	B3a
rectum	no		B3	B3b
hindlimbs	no			

Fig.2. Pulsed field gel electrophoresis (PFGE) pattern of 15 *Klebsiella pneumoniae* isolates from different sources from farm B (IMI = intramammary infection) digested with the *XbaI* restriction enzyme. The dendrogram was constructed with the BioNumerics software 6.0 (Applied Maths NV, Sint-Martens--Latem, Belgium) choosing the Dice coefficient setting both tolerance and optimization at 1%. The horizontal scale on the left side (100 to 45) indicates the level of similarity in percent among fingerprints. The vertical bars indicate the levels of 80% and 95% of similarities, which indicates the minimal levels for defining clusters and subtypes respectively. Production of extended spectrum β-lactamases (ESBL) detected by the double-disk synergy test (DDST) and the detection of the ESBL main genes (shv = bla_{shv}; (tem = bla_{tem}; (ctx=m = bla_{ctx-m}) are also shown.

not reject the hypothesis of a common source of the bacteria that were present in bedding material. One hypothesis is that unused bedding may introduce a similar genotype (Munoz et al. 2007). Wood-based bedding is considered an important source of *Klebsiella* spp. in dairy herds (Sampimon et al. 2006) but in the present study herds used sand as bedding.

For farms A and B, clusters involving bacteria from bulk tank milk and from IMI were observed. Although coliforms from different sources could be present in bulk tank milk, IMI could be a significant source of contamination. A positive relationship between SCC and coliform count in bulk tank milk has been consistently demonstrated (Jayarao et al. 2004, Pantoja et al. 2009, Pantoja et al. 2011). A single cluster containing isolates from IMI cases and raw milk suggests that mastitis agents, even environmental ones, are able to reach the final product. For farm C, 23 cases of IMI caused by *K. pneumoniae* were isolated from 20 different animals, and no cluster was detected in this herd. Even isolates from multiple milk samples of the same animal showed distinct genotypes.

Five isolates from farm B produced ESBLs detected by the DDST (strains B1b - bed, B2a and B2b - IMI, B2c - bulk tank and B3a - hind limb) (Table 2). For the three strains from cluster B2, both bla_{ctx-m} and bla_{shv} genes were detected (Fig.2). For the two other strains, only the bla_{shv} gene was present. The DDST-positive isolate from farm C also harbored two genes, bla_{tem} and bla_{shv} whereas the two DDST--positive isolates from farm A harbored the bla_{shv} gene. Two isolates from farm B were of the same subtype. For both farm A and B, clusters were detected with isolates from IMI and the bulk tank (Table 2).

Recently, the use of antimicrobials in food-producing animals and its relation with the emergence of resistant bacteria in the food chain has become of great concern. In the present study, eight isolates produced ESBLs (detected by the DDST), including one from the bulk milk tank. Among ESBL-producing microorganisms, *Klebsiella* is the genus that produces the greatest variety of such enzymes (Paterson et al. 2003). *K. pneumoniae* can develop resistance and acquire other genes that contribute to the survival of the pathogen in different environments. Administration of drugs at dry-off, or ineffective treatment of mastitis cases can exert pressure for the selection and maintenance of antimicrobial resistance genes. Additionally, drug resistant pathogens do not necessarily result from unsuccessful intramammary treatment. Coliforms are considered to be environmental agents that have extensive contact with other bacterial populations within the environment, which facilitates the exchange of genetic material such as plasmids. Systemic treatments result in the excretion of antimicrobial residue through animals' feces, urine and milk. Antimicrobial residue contributes to pathogen selection in the environment and, by transferring genetic material, new bacteria can acquire resistance genes. Multi-drug resistant pathogens can, in adequate conditions, infect the mammary gland. Additionally, microorganisms producing resistance enzymes can be found naturally in soil, which facilitates the acquisition of resistance genes by coliforms (Hammad et al. 2008).

There is very little evidence supporting an increase in antimicrobial resistance due to mastitis treatments (Erskine et al. 2002). Bengtsson et al. (2009), showed that *Escherichia coli* from the gastrointestinal tract of young cattle were resistant to antimicrobials not used routinely for treatment of mastitis in Sweden (such as ampicillin and tetracycline). Pathogens with the same resistant profile were isolated from IMI cases, showing that treatment for other diseases in another animal category could also be responsible for IMI cases caused by drug resistant bacteria.

ESBL bacteria were isolated from the environment in dairy herds (Watson et al. 2012). Our study confirms that this type of bacteria is present in different locations within a dairy farm. Under favorable conditions, drug resistant pathogens may reach the mammary gland and be responsible for IMI. As observed in this study, these pathogens may reach the bulk tank and be potential threats to human health. Treatments with β-lactams in cows with IMI caused by ESBL-producing bacteria are not likely to succeed, leading to economic losses (Erskine et al. 2003).

Bla_{tem} gene was observed in only one isolate whereas bla_{shv} in eight. More than 50% of enterobacteria isolated from human patients produce at least some type of TEM enzyme (particularly those from the TEM-1 group), and not all variants are considered to be ESBLs (not showing activity in face of cephalosporins). However, TEM-type ESBLs (mainly derived from TEM-1) are capable of hydrolyzing cephalosporins, but incapable of hydrolyzing carbapenems. Similarly, there are approximately 100 variants of SHV--type enzymes, many of which are not considered ESBLs (Woodford 2010). Although subclasses of these enzymes can be differentiated, the DDST showed the production of ESBLs. It can be hypothesized that these classes of enzymes are responsible for the phenotypic profile observed, or

other classes of enzymes are being produced by the bacteria. TEM and SHV enzymes have already been detected in ESBL bacteria causing IMI in dairy herds (Hammad et al. 2008, Locatelli et al. 2009).

The importance of TEM and SHV enzymes has been considerably diminished due to the rapid growth of the third group of ESBLs, the CTX-M enzymes (Woodford 2010). To our knowledge, our study detected for the first time a *K. pneumoniae* isolated from the bulk milk tank harboring the bla$_{ctx-m}$ gene. Also, we showed that these bacteria can be responsible for IMI on dairy herds, as two strains from the same herd harboring the gene shared the same subtype. Besides, the spread of these genes may result in serious consequences to the veterinary therapeutics.

β-lactams continue to be the base of veterinary therapeutics, and the ESBLs were present in the three dairy herds in different locations. Hence, we observed that this could be worrisome in the long-term.

CONCLUSIONS

A great diversity of *Klebsiella pneumoniae* was found within the studied dairy cattle herds. Even isolates from multiple milk samples of the same cow showed distinct genotypes.

ESBLs are present in dairy herds in bacteria isolated from different sources and may reach the bulk tank and be potential threats to human health.

The detection of ESBLs in dairy cattle herds could be a major concern for both public and animal health, given that the presence of these enzymes can result in resistance to β-lactams, which are currently one of the most used antimicrobials in veterinary medicine.

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