Scientific Papers. Series D. Animal Science. Vol. LXVII, No. 2, 2024
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

ISOLATION OF CARBAPENEM-RESISTANT *Klebsiella pneumoniae* FROM MASTITIC COWS AND THEIR ENVIRONMENTS

Mevlüt ATALAY[1], Uçkun Sait UÇAN[2]

[1]Konya Food Control Laboratory, Ministry of Agriculture and Forestry, 42090, Konya, Turkey
[2]Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University, 42003, Konya, Turkey

Corresponding author email: mevatalay@gmail.com.tr

Abstract

Klebsiella species cause infections occurring in different tissues of various hosts. In terms of bovine health it is a well-known opportunistic pathogen playing a role in the pathogenesis of mastitis. In fact, such bacteria can spread widely in bovine farm environments mostly through dairy facilities and breeding areas causing eventually mastitis. Characterizing aetiological agents thoroughly can assist to understand pathogenesis of the opportunistic infections. In this study, a total of 1206 dairy cows from 6 farms were first screened by California Mastitis Test (CMT). Samples found positive by CMT, samples from clinical mastitic udders and also swab samples obtained from both the same animals' rectal and nasal orifices and their surrounding environments were all cultured aerobically and a complete identification of the isolates was achieved by phenotyping and genotyping. Some bovine Klebsiella strains from the culture bank of the Department were also included as organ isolates in the study. Lastly, antibiotic resistance of the strains was detected. There is no difference between numbers of coliforms from the farms using either robotic milking or classical milking systems ($p > 0.05$). The highest prevalence of Klebsiella mastitis in the farms examined in this study was 8.75%. It was common to see colistin resistance in the Klebsiella isolates from all farms anyway. The lowest 12% and highest 50% resistances for colistin were seen in rectal and organ originated strains, respectively. Unexpectedly, carbapenem (Imipenem) resistance was detected and was the highest 50% in isolates from environments. The lower occurrence of carbapenem resistance 18.2% was measured in Klebsiella spp. isolated from mastitic milk samples. Carbapenem resistancy was further verified molecularly.

Key words*: Carbapenem, dairy cow health, environmental contamination, opportunistic infections.*

INTRODUCTION

Klebsiella species are Gram-negative, rod-shaped, encapsulated, lactose positive (with an exception of the species *Klebsiella pneumoniae* subsp. *rhinoscleromatis*), non-motile, H_2S negative, facultatively anaerobic bacteriae (Atalay, 2023; Cheng et al., 2021) *Klebsiella pneumoniae* (*K. pneumoniae*) causes high morbidity rates and significant economic losses in cases of mastitis (Oliver, Gonzalez, Hogan, Jayarao, & Owens, 2004). Bovine mastitis leads to major economic losses and some profound, negative effects on animal welfare. Thus, it is important to successfully manage such infections including especially those that at the beginning or subclinical stage of the inflamemation. *K. pneumoniae* is generally considered as one of the opportunistic pathogens causing not only environment-derived bovine mastitis but also upper respiratory tract infection of dairy cattle. It is an emerging zoonotic and foodborne pathogen with a presence in many countries worldwide (Darniati et al., 2021). *K. pneumoniae* is now considered as one of the major pathogens of international concern due to the dramatic increase in the occurrence of its hypervirulent as well as carbapenem-resistant strains (Chang et al., 2021). The bacterium's widespread presence in areas heavily used by humans, such as dairy farms increases the risk of infection (Jayarao et al., 2006). Compared to other pathogenic microorganisms, *K. pneumoniae* can lead to a faster and more severe occurrence of mastitis by means of its strong host specific growth feature (Schukken et al., 2012). This often increases an urgent need for appropriate and rapid treatment (Vikova et al., 2017). However, many *K. pneumoniae* strains show resistance to different antibiotics, which limits treatment choices, and more complex treatment protocols are required (Paczosa & Mecsas, 2016; Ruegg, 2017). More researches on *Klebsiella* were rather focused on human health until recently (Chang et al., 2021;

Schukken et al., 2012). However, it can be argued that virulence genes in the human isolates show similarities to those of animal-derived strains. This suggests that the adoption of any approaches to follow up sources of infections epidemiologically may be necessary (Yang et al., 2019). More investigation is still needed on genotypic and phenotypic features that may affect the good management of bovine mastitis. Specifically, for *K. pneumoniae* to establish an udder infection, the bacteria must overcome various mechanical and chemical barriers and bypass the humoral and cellular defense system of the host (Piperaki et al., 2017). Antibiotic resistance profiles of animal originated strains are also required to be updated at all time to treat effectively patients of animal origin.

Researches on bovine mastitis caused by *Klebsiella* spp. conducted in this country so far produced little data and showed some occurrence rates. An early of these studies conducted on 1277 dairy cows from some state farms located in Bursa, Eskişehir and Ankara reported no *Klebsiella* isolation. Another study later carried out in a farm from Ankara also reported no isolation (Arda & Istanbulluoglu, 1980; Ulusoy, 1985). Among 21 other studies on bovine mastitis in Turkey covering the period from 1979 to 2022, the highest rate for *Klebsiella* spp. isolation was observed 34.3% in Aydın region (Erdoğdu, 2019). In another study from the same city 20 years later, a total of 141 milk samples taken from cows with clinical mastitis were examined and *K. pneumoniae* was detected in 5% of 141 milk samples examined (Kaya et al., 1999).

In other studies in Turkey, the isolation rates were between 0% and 17.9% depending on the year and geography of the studies as reviewed elsewhere (Atalay, 2023).

As for the studies in Konya and its district; *Klebsiella* isolation was not stated in previous reports from cow milk with mastitis (Ateş et al., 1991; Bozkır, 1985; Dinç et al., 1991; Tekeli et al., 1985). The first report ever on *Klebsiella* spp. in Konya region was made from sheep mastitic milk (Erer et al., 1990). In the following years, *Klebsiella* spp. has been beginning to be isolated from bovine mastitic milk as evidenced by a few studies (Nizamlıoğlu et al., 1992). Therefore, we aimed to detect up-to-date prevalence and profile of carbapenem-resistance in *Klebsiella* spp. isolates from the cases of bovine mastitis, Konya by this study.

MATERIALS AND METHODS

Sampling, Isolation and Identification

The study was conducted on 1206 lactating cows from 6 intensively managed farms in Konya. All the cows were examined clinically first and then those that were not showing any visible signs of mastitis were screened using California Mastitis Test (CMT). Subclinical mastitis was diagnosed based on the screening test. The CMT positive milk samples (n = 124) as well as milk samples from udders showing any signs of clinical mastitis (n = 67) were collected to the 15 mL sterile tubes and transferred to the lab in isothermal boxes 4°C for bacterial culture. The microbiological examinations were carried out at the Microbiology laboratory of the Veterinary Faculty of Selçuk. A loopful of milk sample inoculating onto Tryptic Soy Agar (TSA) enriched with 5% sheep blood (Merck 105459/Almanya) and were incubated for 24-48 hours at 37°C under aerobic conditions. Suspicious coliform isolates based on colony morphology and gram staining characteristics were then transferred to McConkey Agar (Lab M Limited/UK) and incubated at 37°C overnight. Identification of the isolates was performed using Lassen Triple Tube Method, IMVIC and additional tests (Hogan et al., 1999; Lassen, 1975; Quinn et al., 2002). Swab samples taken from nasal, rectal orifices and from environmental surfaces were taken into tubes containing Todd Hewit Broth (Neogen-NCM0061/USA) and inoculated onto the same agar as above and followed the same procedures for isolation and identification. *K. pneumoniae* ATCC 700603 strain was used as positive control in molecular tests.

Antimicrobial sensitivity

The susceptibility of the isolated strains to antimicrobials was evaluated according to the Kirby-Bauer Disk Diffusion Method (Bauer, 1996) and the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (PA, 2010). Bacterial suspensions were adjusted to 0.5 McFarland Standard and planted on Mueller Hinton Agar. Detection of Extended Spectrum β-Lactamase (ESBL) production was performed

using the double disk synergy method (PA, 2010).

DNA Extraction

Boiling and freeze-thawing protocol, a simple, efficient, reproducible and inexpensive technique was used to extract DNA from each colony (Hasibuan et al., 2018). Isolated DNA contents were stored at -20°C until use.

Molecular typing

Following morphological and biochemical characterizations, identification of suspected colonies was further performed by *gyr*A gene PCR amplification specific for *K. pneumoniae* subsp., and *peh*X specific for *K. oxytoca*. Primers used in PCR analysis are presented in Table 1.

Table 1. Primers used in the study

Amplified Gene or Sequence	Oligonucleotide sequence (5'-3')	Product Length (bp)	References
gyrA[1]	F-CGCGTACTATACGCCATGAACGTA, R-ACCGTTGATCACTTCGGTCAGG	441	(Brisse & van Duijkeren, 2005)
K. pneumoniae[2]	F-ATTTGAAGAGGTTGCAAACGAT, R- TTCACTCTGAAGTTTTCTTGTGTTC	130	(Liu et al., 2008)
pehX[3]	F-ATACGGAGTATGCCTTTACGGTG, R-TAGCCTTTATCAAGCGGATACTGG	343	(Younis et al., 2017)
KPC	F- TCGCTAAACTCGAACAGG, R- TTACTGCCCGTTGACGCCCAATC	785	(Monteiro et al., 2009)
OXA	F- TGTTTTTGGTGGCATCGAT, R- GTAAMRATGCTTGGTTCGC	177	(Monteiro et al., 2012)
VIM	F-GATGGTGTTTGGTCGCATA R- CGAATGCGCAGCACCAG	382	(Poirel et al., 2007)

[1]*gyrA* = is for *Klebsiella* detection at genus level; [2]*K. pneumoniae* = Specific for *K. pneumonia* detection; [3]*pehX* = Specific for *K. oxytoca* detection.

PCR and Multiplex-PCR Protocols

To determine isolates at genus and species level molecularly, PCR was performed in 50 µL volume contained 5 µl target DNA, 10 pmol of each primer, 10 µl 5 × Master Mix (Solisbiodyne, Estonya), and 33,6 µl of ultra-pure distilled water. Thirty-five cycles with a profile of 95°C for 15 min (denaturation), 94°C for 30 sec (second denaturation), 58°C for 90 sec (annealing), and 72°C for 90 sec (extension) were run on a Techne PCR thermal cycler (BIORAD T100). Cycling was preceded by a final extension at 72°C for 10 min. Amplified PCR products were analyzed by electrophoresis in ethidium bromide-stained 1.5% agarose (w/v) gels and visualized with a UV-transilluminator.

Multiplex PCR Detection of Carbapenemase Genes

Primers designed to amplify the 3 genes encoding carbapenemases (*blaKPC, blaOXA and blaVIM*) were used (Ellington et al., 2007). The PCR mixture contained 5 µL DNA, 10 µL 5× Master Mix (Solisbiodyne, Estonia), 10 pmol of each primer, 34 µl ultrapure water was used.

RESULTS AND DISCUSSIONS

Of 1206 cows, 51 were clinically infected and bacteriologically positive. 20 of 51 clinical mastitis cases were culture-positive for *Klebsiella*. These results were confirmed molecularly and are shown in Figure 1.

CMT as a screening test indicates that 124/1206 (10.28%) cows and 180/489 (36.8%) quarters examined (except for 7 breast lobes - blind) were observed positive for subclinical mastitis. Out of 124 CMT-positive cows 18/124 (14.52%) cows and from 72 quarters 18/72 (25%) quarters were positive for *Klebsiella* isolation (Table 2).

Table 2. Prevalences of mastitis in the farms, Konya

%	Prevalence* in Farms					
Mastitis caused by	A	B	C	D	E	G
n	200	250	342	135	137	142
K. pneumoniae	1	3.2	2.04	1.48	5.83	0
K. oxytoca	0	1.6	0	0	0.73	0
Other *Klebsiella*	0	0	0.88	0	2.19	0
Total	1	4.8	2.92	1.48	8.75	0

The CMT produced statistically significant results on type of the farms ($p > 0.05$). Aerobic bacteria grow more in milk samples from manure littered bans. There is no difference between the numbers of coliforms from the farms using either robotic milking or classical milking systems ($p > 0.05$). This was also the case for the of aerobic bacteria numbers for the same farms ($p > 0.05$).

In farms that robotic milking systems was in use the negativity value for mastitis infection by *Klebsiella* was statistically quite low ($p < 0.05$). More *Klebsiella* spp. isolations occurred in the manure litter groups.

In farms equipped with milking systems, a correlation analysis was made between the some infectious/physiological data (intramammary subclinical and clinical mastitis status of dairy cows, lactation numbers of milkers, milking day after birth and milk yield) and isolations of *Klebsiella* spp. Between the isolation of *Klebsiella* spp. and day of milking a moderate negative correlation (r = -237) was observed with a P value of 0.016. Thus, as the milking day increases the *Klebsiella* spp. detection rate decreases.

Of the strains, 11 of the 16 ESBL positive isolates (64.28%) were found in farm B. No ESBL enzyme detection was seen in farms A and D. Ten of the strains with ESBL positivity were obtained from clinically mastitic cows. Molecular results regarding carbapenem resistance of isolates obtained from various sources are shown in Figure 2 and Table 3. The antimicrobial resistance profiles of the isolates, categorized by their sources of isolation, are presented in Table 4.

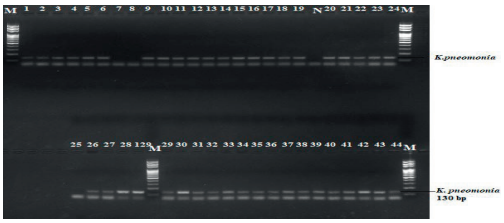

Figure 1. *K. pneumoniae* species-specific 16S-23S gene gel image of isolates, M: 100 bp DNA ladder

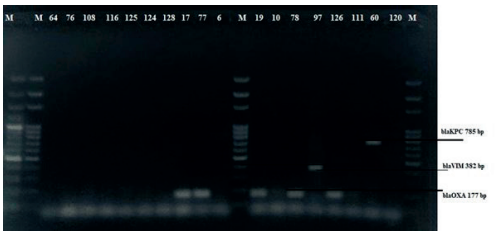

Figure 2. Gel image of carbapenem genes of isolates, M: 100 bp DNA ladder

Table 3. Distribution of carbapenem positive samples determined molecularly

Sources	*blaKPC*	*blaVIM*	*blaOXA*
Nasal (n = 45)	12*/17**	8*/17**	4*/17**
%	70.59	47.06	23.52
Rectal (n = 25)	5*/8**	5*/8**	2*/8**
%	62.5	62.5	25
Milk (n = 44)	1*/14**	4*/14**	10*/14**
%	7.14	28.57	71.43
Organ (n = 6)	1*/3**	1*/3**	1*/3**
%	33.33	33.33	33.33
Environmental (n = 8)	0*/2**	0*/2**	2*/2**
%	0	0	100
Total	19	18	19

*Test Result
**Total Number of Samples

Table 4. Percent resistances of the isolates to antimicrobials based on isolation sources

Antimicrobial	Milk	Nasal	Rectal	Environmental	Organ
AMC	29.5	44.4	48	16.7	75
C	36.4	13.3	8	16.7	87.5
PY	79.5	88.9	88	83.3	87.5
T	54.5	42.2	48	83.3	87.5
IPM	18.2	20.0	12	50.0	0
SXT	34.1	13.3	12	33.3	87.5
E	100	100	100	100	100
CN	13.6	8.9	16	0	87.5
AM	100	100	100	100	100
CTX	38.6	20.0	16	16.7	25
N	45.5	55.6	36	16.7	62.5
FLM	15.9	6.7	20	33.3	87.5
Colistin	22.7	31.1	12	33.3	50

AM10: Ampicillin (10 mg), PY100: Carbenicillin (100 mg), CTX30: Cephataxime (30 mg), C30: Chloramphenicol (30 mg), E15: Erythromycin (15 mg), CN10: Gentamicin (10 mg), N30 Neomycin (30 mg), T30:Oxytetracycline (30 mg), SXT25: Sulphamethoxazole/ Tripethoprim (1.25/23.75 mg), AMC30: Amoxicillin- Clavulanate (30 mg), IPM10: Imipenem (10 mg), FLM: Flumequin (30 mg)

Housing conditions, bedding material, milking systems, and udder hygiene are all critical environmental factors that directly affect udder health. The environment is sometimes itself the factor that allows some amount of pathogens to enter the udder tissue and thus increases the risk of infection (Srivastava & Kumaresan, 2015). *K. pneumoniae* is considered an opportunistic pathogen that poses a risk to humans and animals as evidenced by other studies so far (Fu et al., 2022; Mirzaie & Ranjbar, 2021). However, epidemiological studies of bovine mastitis caused by *Klebsiella* spp. are still insufficient in many part of the World (Fu et al., 2022). A complete history of bovine *Klebsiella* mastitis prevalence in Turkey is described elsewhere (Atalay, 2023). From 1979 on, occurrence has been reported between 0-17.9% depending on the city and year. In Konya, the detection of *Klebsiella* spp. as causative of bovine mastitis was first reported in 1992. No declining trend in the occurrences of *Klebsiella* mastitis in cattle can be observed in the Country anyway (Atalay, 2023)

All of the farms in this study were free from bovine Tuberculosis and Brucellosis, use automated milking systems and contained 100 lactating cows/each at least. One (Farm E) of the farms sampled that was bedded with manure and with free stall, open roof, shed-typed barn gives the highest prevalence of *Klebsiella* spp. mastitis ($p<0.05$). Percentages of mastitis (subclinical and clinical) cases in total according to farms A, B, C, D, E, and G were as follows; 15% (12.5%; 2.5%), 21.2% (14.8%; 6.4%), 9.06% (5.26%; 3.80%), 13.33% (8.14%; 5.18%), 16.05% (12.14%; 3.65%), and 14.78% (11.26%; 3.52%), respectively. However, Farm G was unique in the characteristics that no *Klebsiella* mastitis was detected at all although mastitis (either of type) is present (14.78%) in this farm with similar ratios to other farms. This might be because of that Farm G has more strict entry and exit allowances. On the other hand, by performing statistical analysis of the CMT results based on the litter type of the farms; differences were not statistically significant ($p > 0.05$).

In parallel with previous reports, as the day on lactation period increases, the detection rate of *Klebsiella* spp. decreases ($p < 0.05$). This supports the fact that coliforms causes mastitis in the early stages of bovine lactation since *Klebsiella* is one of the genera included in Coliform bacteriae (Constable et al., 2016). This early stage of vulnerability to *Klebsiella* spp. may be due to an increased susceptibility of the host or some other host specific reasons such as changed levels of lactoferrin and citrates (Burvenich et al., 2003). However, *K. pneumoniae* is proposed to be superior to most *E. coli* strains in bypassing the barriers posed by lactoferrin and infiltrating the mammary gland. On the other hand, similar to a *E. coli* infection, *K. pneumoniae* infection usually begins with subclinical mastitis at the end of the dry period, which can develop into a clinical form with the onset of lactation (Bradley & Green, 2000).

In Turkey, ESBL has been noted to be detected in one *K. oxytoca* isolate and four *E. coli* isolates obtained from mastitic cows (Babacan, 2022). At the present study, ESBL-producing strains were detected in 12.5% of all *Klebsiella* spp. isolates obtained from cattle. The corresponding figure for milk samples in total was 25%. The rates of ESBL enzyme in the *Klebsiella* obtained from lactating cows with clinical or subclinical mastitis were very close to each other (25.9%, 23.53%).

It was reported more than 10 years ago that coliform bacteria (*E. coli, Klebsiella* spp.) were 100% sensitive to carbapenems (Büyükcangaz et al., 2012). In the present study, 9 of 16 ESBL-positive isolates were resistant to Imipenem (IPM). IPM resistance was 17.9% when all of the isolates were taken into account. Two of the resistant strains obtained from the environment originated from the walls of animal shelters. These environmental isolates were from only one farm. The farm where we detected IPM resistance from the environmental samples was the farm that the highest rate of 55% IPM resistance and the highest rate of 69% ESBL enzyme occurred together. This suggests that it seems to pose a risk in terms of transmission to and between animals. Some of the primary protection practices, namely udder washing, drying, and teat dipping must be practiced in dairy farms. Testing cows by CMT is also crucial to detect early subclinically infected cows. A new introduction into the herd should not be allowed in any circumstances, either. Protection of udder health by the administration of proper vaccines is recommended as the first

barrier to specific pathogens, too. Therefore, all these primary protection measures make the preventive approach in question indispensable to the dairy industry.

The development of antimicrobial resistance in veterinary medicine is driven by the wide utilization and misuse of antibiotics (Bedawy et al., 2024). The presence of the natural existence of β-lactamase in *K. pneumoniae* can explain the resistance to β-lactams and 3rd and 4th generation cephalosporins (Marr & Russo, 2019). Additionally, extended-spectrum β-lactamases (ESBLs) in this bacterial species is a well-known phenomenon as one of the plasmid-mediated enzymes (Bedawy et al., 2024; Koovapra et al., 2016). When extended-spectrum beta-lactamase activity was examined in *Klebsiella* strains, it was determined that bacteria with this enzyme spread in the environment and this feature could pass on to other bacteria quickly. This spread can occur between similar or different bacterial species (Caniça et al., 2015; DuPont & Steffen, 2017). It has been pointed out that animal foods play an important role in the spread of such bacteria among humans (Wu et al., 2013). In Farm B in this study, the antibiotics used must be resistant to the β-lactamase enzyme since ESBL had the highest rate of positivity with 69%. Therefore, antibiotics that would be used in combination with β-lactamase inhibitors such as sulbactam and clavulonic acid may increase their effectiveness. Detection of β-lactamase positivity before starting treatment is critical for identifying resistant isolates.

Carbapenems are recommended for high risk community acquired and nosocomial infections (Cole et al., 2022; Logan & Weinstein, 2017). Carbapenemase positive bacteria have been proposed to sometimes exhibit only a slight increase of MIC values for carbapenems. Genotypic rather than phenotypic methods to detect resistance to carbapenems is more convenient (Cornaglia et al., 2007). The present study shows that carbapenem resistance is evidenced genotypically in all of 6 dairy farms sampled and frequency was between 2-34%.

In the present study, the identification of isolates positive for carbapenem resistance genes from the same animal's either rectal or nasal samples along with a finding that a high rate of such resistance was seen in a particular farm together reveals that a potential risk of contamination between the cows is present.

Plasmid-mediated transfer of codes for enzymes may enable the transfer of resistance between bacterial populations. In our study, the carbapenem resistance gene was found in approximately one-third of the *Klebsiella* isolates from milk-originated samples (31.81%, n = 44). Although no animals in Konya are known to be treated with carbapenem-based antimicrobials, this study suggests that *K. pneumoniae* carrying genes for resistance to carbapenems and third-generation cephalosporins present in dairy cows can have critical potential for pathogenicity for humans. The fact that *K. pneumoniae* carries the same virulence genes in both animals and humans indicates a possible zoonotic potential of this microorganism. It may mean that the infection that can be mutually transmitted between animals and humans cannot be ignored.

The European Antimicrobial Resistance Surveillance Network (EARS-Net) performs surveillance of antimicrobial susceptibility of eight bacterial pathogens in humans including *K. pneumoniae*. To strengthen health surveillance throughout the whole of Europe, EARS-Net and the European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (EARS-Vet) have been decided to collaborate and started to work together. As an outcome of this collaboration an alert on how carbapenem-resistant *K. pneumoniae* is transmitted between animals and humans was set (Mader et al., 2021). We recommend establishing a surveillance program covering both bovine and human isolates within the framework of *One Health*, in Turkey.

In this study, colistin resistance was determined as 22.7%, 31.1%, 33.3%, 12% in milk, nasal, environmental and rectal samples, respectively. This type of resistance was not found in nasal, rectal and milk samples from two farms (A and C). We believe that this has been interpreted as the colistin resistance may occur based on farm management practicals (those in which colistin was in use).

CONCLUSIONS

The detection of carbapenem resistance genes in both animal and environmental samples poses a

significant risk to animal and public health, suggesting potential horizontal gene transfer. These findings support the implementation of comprehensive surveillance programs covering both animal and human health sectors, in line with the One Health approach, to effectively manage and reduce the spread of resistant pathogens.

To treat specific infections caused by multiresistant Gram-negative bacteria few drugs are recommendable. Carbapenems are of great importance and are used to treat these kinds of infections. Traditionally, those caused by extended-spectrum beta-lactamase (ESBL) producing bacteriae that are members of Order *Enterobacterales* and multi-drug-resistant organisms, such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. are prescribed.

Carbapenem-resistant organisms (CRO) have recently become a significant threat to human health and healthcare systems. In human medicine, controlling CROs is seen as a priority issue, necessitating a multifaceted approach that includes aggressive infection control strategies, enhanced surveillance, and more effective antimicrobial stewardship measures.

The use of carbapenems in veterinary medicine should be out of choice since it is off-label. Thus it has been common to consider carbapenem as a reserved antibiotic only for cases with limited therapeutic alternatives. Carbapenem is not routinely prescribed for veterinary treatment purposes in Turkey.

ACKNOWLEDGEMENTS

This study has been supported by the Selçuk University Scientific Research Projects Coordinator in Konya/Turkey (project number 20212034).

REFERENCES

Arda, M., & Istanbulluoglu, E. (1980). Mastitislere neden olan aerob, anaerob, mikoplasma ve mantarlarin izolasyonu, identifikasyonu, bunlara karsi etkili olan antibiyotik ve fungusitlerin saptanmasi. *Dergisi - Ankara Universitesi, Veteriner Fakultesi. Journal of the Faculty of Veterinary Medicine*.

Atalay, M. (2023). Sığır Mastitisi Ve Çevresel Kaynaklı Klebsiella Suşlarının Virulans Faktörlerinin Belirlenmesi. *Sağlık Bilimleri Enstitüsü.*

Ateş, M., Erganiş, O., Çorlu, M., & Serpek, B. (1991). Konya yöresindeki mastitisli ineklerden elde edilen süt örneklerinin mikrobiyel florası ve LDH aktivitesi. *Turk. J. Vet. Anim. Sci., 16*, 19-29.

Babacan, O. (2022). *First detection of carbapenem resistance in Enterobacteriaceae from animal isolates in Turkey.* Ankara Üniversitesi Veteriner Fakültesi Dergisi.

Bauer, A. (1996). Antibiotic susceptibility testing by a standardized single disc method. *Am. J. of Clinc. Path., 45*, 149-158.

Bedawy, Y. M., Homouda, S. N., Ahmed, H. A., & Abd-El Tawab, A. A. (2024). Genotyping and antibiotic resistance profile of *Klebsiella pneumoniae* and *Corynebacterium bovis* isolates recovered from clinical and subclinical mastitis milk samples. *Journal of Advanced Veterinary Research, 14*(3), 349-355.

Bozkır, M. (1985). Konya ve Yöresindeki Süt İneklerinde Klinik ve Subklinik Mastitis Olgularından Aerob Patojenik Etken İzolasyonu ve İdentifikasyonu ile Bunlara etkili Antibiyotiklerin Tesbiti. *Etlik Veteriner Mikrobiyoloji Dergisi, 5*(8-9), 104-138.

Bradley, A., & Green, M. (2000). A study of the incidence and significance of intramammary enterobacterial infections acquired during the dry period. *Journal of dairy science, 83*(9), 1957-1965.

Brisse, S., & van Duijkeren, E. (2005). Identification and antimicrobial susceptibility of 100 *Klebsiella* animal clinical isolates. *Veterinary microbiology, 105*(3-4), 307-312.

Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., & Duchateau, L. (2003). Severity of *E. coli* mastitis is mainly determined by cow factors. *Veterinary research, 34*(5), 521-564.

Büyükcangaz, E., Burak, M., & Ahmed, M. K. A. A. (2012). Subklinik mastitisli sığır sütlerinin mikrobiyolojik analizi ve izolatların antimikrobiyal direnç profili. *Uludağ Üniversitesi Veteriner Fakültesi Dergisi, 31*(2), 35-44.

Caniça, M., Manageiro, V., Jones-Dias, D., Clemente, L., Gomes-Neves, E., Poeta, P., … & Ferreira, E. (2015). Current perspectives on the dynamics of antibiotic resistance in different reservoirs. *Research in microbiology, 166*(7), 594-600.

Chang, D., Sharma, L., Dela Cruz, C. S., & Zhang, D. (2021). Clinical epidemiology, risk factors, and control strategies of *Klebsiella pneumoniae* infection. *Frontiers in microbiology, 12*, 750662.

Cheng, J., Zhou, M., Nobrega, D. B., Cao, Z., Yang, J., Zhu, C., …. & Gao, J. (2021). Virulence profiles of *Klebsiella pneumoniae* isolated from 2 large dairy farms in China. *Journal of dairy science, 104*(8), 9027-9036.

Cole, S., Perez-Bonilla, D., Hallowell, A., & Redding, L. (2022). Carbapenem prescribing at a veterinary teaching hospital before an outbreak of carbapenem-resistant *Escherichia coli*. *Journal of Small Animal Practice, 63*(6), 442-446.

Constable, P. D., Hinchcliff, K. W., Done, S. H., & Grünberg, W. (2016). *Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs and goats*. Amsterdam, ND: Elsevier Health Sciences.

Cornaglia, G., Akova, M., Amicosante, G., Cantón, R., Cauda, R., Docquier, J.D., … & Galleni, M. (2007). Metallo-β-lactamases as emerging resistance

determinants in Gram-negative pathogens: open issues. *International journal of antimicrobial agents, 29*(4), 380-388.

Darniati, D., Setiyaningsih, S., Agungpriyono, D. R., & Handharyani, E. (2021). First evidence of *Klebsiella pneumoniae* infection in Aceh cattle: Pathomorphology and antigenic distribution in the lungs. *Veterinary World, 14*(4), 1007.

Dinç, D. A., Erganiş, O., Güler, M., & Uçan, U. S. (1991). İneklerin subklinik mastitislerinde Baytril'in etkisi. *Hayv Araş Derg, 1*, 12-15.

DuPont, H. L., & Steffen, R. (2017). Use of antimicrobial agents for treatment and prevention of travellers' diarrhoea in the face of enhanced risk of transient fecal carriage of multi-drug resistant enterobacteriaceae: setting the stage for consensus recommendations. *Journal of travel medicine, 24*(suppl_1), S57-S62.

Ellington, M. J., Kistler, J., Livermore, D. M., & Woodford, N. (2007). Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. *Journal of Antimicrobial Chemotherapy, 59*(2), 321-322.

Erdoğdu, M. (2019). Mastitisli sütlerden klebsiella: SPP izolasyonu ve antibiyotik dirençliliğinin belirlenmesi. *Sağlık Bilimleri Enstitüsü.*

Erer, H., Ateş, M., Kaya, O., Kıran, M. M., & Berkin, Ş. (1990). Koyun Mastitisleri Üzerinde Patolojik ve Bakteriyolojik İncelemeler. *Etlik Veteriner Mikrobiyoloji Dergisi, 6*(6), 79-97.

Fu, S., Wen, C., Wang, Z., Qiu, Y., Zhang, Y., Zuo, J., … & Chen, W. (2022). Molecular Epidemiology and Antimicrobial Resistance of Outbreaks of *Klebsiella pneumoniae* Clinical Mastitis in Chinese Dairy Farms. *Microbiology Spectrum, 10*(6), e02997-02922.

Hasibuan, M., Suryanto, D., & Kusumawati, R. L. (2018). Phenotypic and molecular detection of BLACTX-M gene extended-spectrum beta-lactamases in escherichia coli and Klebsiella pneumoniae of north sumatera isolates. *Paper presented at the IOP Conference Series: Earth and Environmental Science*.

Hogan, J., Gonzalez, R., Harmon, R., Nickerson, S., Oliver, S., Pankey, J., & Smith, K. L. (1999). Laboratory handbook on bovine mastitis. *National Mastitis Council, Madison, WI, 78*(7), 485-488.

Jayarao, B. M., Donaldson, S. C., Straley, B. A., Sawant, A. A., Hegde, N. V., & Brown, J. (2006). A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. *Journal of dairy science, 89*(7), 2451-2458.

Kaya, O., Kırkan, Ş., Gülal, M., & Ünal, B. (1999). Aydın yöresinde ineklerde klinik mastitise neden olan mikroorganizmaların saptanması ve bunların antibiyotiklere duyarlılıklarının incelenmesi. *Pendik Veteriner Mikrobiyoloji Dergisi, 30*(1), 25-29.

Koovapra, S., Bandyopadhyay, S., Das, G., Bhattacharyya, D., Banerjee, J., Mahanti, A., … & Mukherjee, R. (2016). Molecular signature of extended spectrum β-lactamase producing Klebsiella pneumoniae isolated from bovine milk in eastern and north-eastern India. *Infection, Genetics and Evolution, 44*, 395-402.

Lassen, J. (1975). Rapid identification of Gram-negative rods using a three-tube method combined with a dichotomic key. *Acta Pathologica Microbiologica Scandinavica Section B Microbiology, 83*(6), 525-533.

Liu, Y., Liu, C., Zheng, W., Zhang, X., Yu, J., Gao, Q., … & Huang, X. (2008). PCR detection of *Klebsiella pneumoniae* in infant formula based on 16S–23S internal transcribed spacer. *International journal of food microbiology, 125*(3), 230-235.

Logan, L. K., & Weinstein, R. A. (2017). The epidemiology of carbapenem-resistant Enterobacteriaceae: the impact and evolution of a global menace. *The Journal of infectious diseases, 215*(suppl_1), S28-S36.

Mader, R., Damborg, P., Amat, J.-P., Bengtsson, B., Bourély, C., Broens, E. M., . . . Fitzgerald, W. (2021). Building the European antimicrobial resistance surveillance network in veterinary medicine (EARS-Vet). *Eurosurveillance, 26*(4), 2001359.

Marr, C. M., & Russo, T. A. (2019). Hypervirulent *Klebsiella pneumoniae*: a new public health threat. *Expert review of anti-infective therapy, 17*(2), 71-73.

Mirzaie, A., & Ranjbar, R. (2021). Antibiotic resistance, virulence-associated genes analysis and molecular typing of *Klebsiella pneumoniae* strains recovered from clinical samples. *AMB Express, 11*, 1-11.

Monteiro, J., Santos, A. F., Asensi, M. D., Peirano, G., & Gales, A. C. (2009). First report of KPC-2-producing *Klebsiella pneumoniae* strains in Brazil. *Antimicrobial agents and chemotherapy, 53*(1), 333.

Monteiro, J., Widen, R. H., Pignatari, A. C., Kubasek, C., & Silbert, S. (2012). Rapid detection of carbapenemase genes by multiplex real-time PCR. *Journal of Antimicrobial Chemotherapy, 67*(4), 906-909.

Nizamlıoğlu, M., Kalaycıoğlu, L., Dinç, D. A., Erganiş, O., & Özeren, F. (1992). İneklerde Subklinik Mastitisin Erken Teşhisi Amacıyla Sütte N-Asetil B-D Glukozaminidaz (NAG ase) Enzim Aktivitesinin Tayini. *Selçuk Üniversitesi Fakülte Dergisi*, *8*(2), 60-63.

Oliver, S., Gonzalez, R., Hogan, J., Jayarao, B., & Owens, W. (2004). Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. *Verona: National Mastitis Council*, 47.

PA, W. (2010). Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing: 20th informational supplement. *CLSI document M100-S20*.

Paczosa, M. K., & Mecsas, J. (2016). Klebsiella pneumoniae: going on the offense with a strong defense. *Microbiology and molecular biology reviews, 80*(3), 629-661.

Piperaki, E.-T., Syrogiannopoulos, G. A., Tzouvelekis, L. S., & Daikos, G. L. (2017). *Klebsiella pneumoniae*: virulence, biofilm and antimicrobial resistance. *The Pediatric infectious disease journal, 36*(10), 1002-1005.

Poirel, L., Pitout, J. D., & Nordmann, P. (2007). Carbapenemases: molecular diversity and clinical consequences. *Future microbiology*, *2*(5), 501-512.

Quinn, P., Markey, B. K., Carter, M., Donnelly, W., & Leonard, F. (2002). *Veterinary microbiology and microbial disease*. Hoboken, USA: Wiley Blackwell Publishing House.

Ruegg, P. L. (2017). A 100-Year Review: Mastitis detection, management, and prevention. *J. Dairy Sci., 100*(12), 10381-10397.

Schukken, Y., Chuff, M., Moroni, P., Gurjar, A., Santisteban, C., Welcome, F., & Zadoks, R. (2012). The "other" gram-negative bacteria in mastitis: *Klebsiella*, *Serratia*, and more. *Veterinary Clinics: Food Animal Practice, 28*(2), 239-256.

Srivastava, A., & Kumaresan, A. (2015). *Mastitis in dairy animals: an update*. Satish Serial Publishing House.

Tekeli, T., Baysal, T., & Gökçay, Y. (1985). Investigations on the treatment of the subclinical mastitis by penicillin-streptomycin in dry cows. *Eurasian Journal of Veterinary Sciences, 1*(1), 71-79.

Ulusoy, E. (1985). Mastitisli İnek Sütlerinden İzole Edilen Mikroorganizmalarin İdentifikasyonlari Ve Antibiyotiklere Duyarliliklari Üzerinde Bir Araştirma. *Ankara Üniversitesi Veteriner Fakültesi Dergisi, 32*(02).

Vikova, H., Babak, V., Vrtkova, I., Cervinkova, D., Marosevic, D., Moravkova, M., & Jaglic, Z. (2017). Epidemiology of intramammary infections with *Staphylococcus aureus* and mastitis streptococci in a dairy cattle herd with a history of recurrent clinical mastitis. *Polish Journal of Veterinary Sciences, 20*(1).

Wu, G., Day, M. J., Mafura, M. T., Nunez-Garcia, J., Fenner, J. J., Sharma, M., … & Kadlec, K. (2013). Comparative analysis of ESBL-positive *Escherichia coli* isolates from animals and humans from the UK, The Netherlands and Germany. *PLoS One, 8*(9), e75392.

Yang, F., Deng, B., Liao, W., Wang, P., Chen, P., & Wei, J. (2019). High rate of multiresistant *Klebsiella pneumoniae* from human and animal origin. *Infection and Drug Resistance*, 2729-2737.

Younis, A., Elbialy, A., Abo Remila, E., & Ammar, A. (2017). Molecular detection of genus *Klebsiella* and genotypic identification of *Klebsiella pneumoniae* and *Klebsiella oxytoca* by duplex polymerase chain reaction in poultry. *Glob. Vet., 18*(3), 234-241.