Kafkas Univ Vet Fak Derg
26 (3): 391-396, 2020
DOI: 10.9775/kvfd.2019.23329

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

A Molecular Investigation of Carbapenem Resistant Enterobacteriaceae and *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ Genes in Raw Milk [1]

Serhat AL [1,a] Harun HIZLISOY [2,b] Nurhan ERTAŞ ONMAZ [1,c]
Fulden KARADAL [3,d] Mukaddes BAREL [1,e] Yeliz YILDIRIM [1,f] Zafer GÖNÜLALAN [1,g]

[1] *This study was supported by "Erciyes University, Scientific Research Project Coordination Unit" with project number 7485*

[1] Erciyes University, Veterinary Faculty, Food Hygiene and Technology Department, TR-38280 Kayseri - TURKEY
[2] Erciyes University, Veterinary Faculty, Veterinary Public Health, TR-38280 Kayseri - TURKEY
[3] Nigde Omer Halisdemir University, Bor Vocational School, Department of Food Processing, TR-51700 Nigde - TURKEY
[a] ORCID: 0000-0003-2721-9275; [b] ORCID: 0000-0003-3391-0185; [c] ORCID: 0000-0002-4679-6548; [d] ORCID: 0000-0002-5113-5883
[e] ORCID: 0000-0002-1170-8632; [f] ORCID: 0000-0001-8783-3889; [g] ORCID: 0000-0002-3935-6296

Article ID: KVFD-2019-23329 Received: 11.09.2019 Accepted: 10.01.2020 Published Online: 10.01.2020

How to Cite This Article

Al S, Hızlısoy H, Ertaş Onmaz N, Karadal F, Barel M, Yıldırım Y, Gönülalan Z: A molecular investigation of carbapenem resistant Enterobacteriaceae and *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ genes in raw milk. *Kafkas Univ Vet Fak Derg,* 26 (3): 391-396, 2020. DOI: 10.9775/kvfd.2019.23329

Abstract

The success of antibiotic treatment has been negatively affected due to developing and spreading antimicrobial resistance all over the world. The present study was carried out to reveal the presence of carbapenem resistant Enterobacteriaceae and *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ genes responsible for carbapenem resistance in raw milk and to contribute to transmission dynamics and molecular epidemiology of carbapenem resistance, as well as the potential public health risks of milk. In Turkey, there is not sufficient data on the presence and the potential risks posed by carbapenem resistance in animal origin foods. A total of different 427 raw milk samples were collected and subjected to phenotypic microbiological analysis and conventional and Sybergreen real-time PCR targeting *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ genes. In the phenotypic analyses, suspicious isolates were identified by Vitek-2 compact system and antibiotic resistance profiles were revealed. Two *Stenotrophomonas maltophilia* inherently resistant to carbapenems were detected in raw milk samples. Acquired carbapenem resistance and related genes were not found in any of the milk samples. The present study revealed that milk is not epidemiologically involved in the transmission of carbapenem resistance. In order to prevent the environmental distribution of antibiotic resistant microorganisms, control of antibiotics used in human and veterinary medicine should be maintained.

Keywords: *Carbapenem resistant Enterobacteriaceae, Carbapenemases, Raw milk*

Çiğ Sütlerde Karbapenem Dirençli Enterobacteriaceae ve *bla*$_{KPC}$, *bla*$_{NDM}$ ve *bla*$_{OXA-48}$ Gen Varlığının Moleküler Olarak İncelenmesi

Öz

Uygulanan antibiyotik tedavileri, antimikrobiyel dirençliliğin dünya genelinde şekillenmesi ve yayılım göstermesi nedeniyle olumsuz yönde etkilenmektedir. Bu çalışma, potansiyel halk sağlığı risklerinin ortaya konması yanısıra, çiğ sütteki karbapenem dirençli Enterobacteriaceae ve karbapenem dirençliliğinden sorumlu *bla*$_{KPC}$, *bla*$_{NDM}$ ve *bla*$_{OXA-48}$ genlerinin varlığını ortaya koymak ve karbapenem direncinin taşınma dinamikleri ve moleküler epidemiyolojisine katkıda bulunmak amacıyla gerçekleştirilmiştir. Türkiye'de hayvansal gıdalarda karbapenem dirençliliğin varlığı ve sebep olduğu potansiyel riskler hakkında yeterli veri bulunmamaktadır. Toplanan toplam 427 farklı çiğ süt örneği fenotipik mikrobiyolojik analizlere tabi tutuldu ve *bla*$_{KPC}$, *bla*$_{NDM}$ ve *bla*$_{OXA-48}$ genlerini hedef alan konvansiyonel ve Sybergreen real-time PCR işlemleri gerçekleştirildi. Fenotipik analizler sonucunda şüpheli izolatlar VITEK-2 kompakt sistem ile tanımlandı ve antibiyotik dirençlilik profilleri ortaya kondu. Çiğ süt örneklerinde karbapenem grubu antibiyotiklere doğal dirençli iki *Stenotrophomonas maltophilia* tespit edildi. İncelenen hiçbir süt örneğinde kazanılmış karbapenem direnci ve ilişkili genler tespit edilmedi. Bu çalışma, epidemiyolojik olarak sütün karbapenem dirençliliği dağılımında rol almadığını göstermektedir. Antibiyotiğe dirençli mikroorganizmaların çevresel dağılımını önlemek için, insan ve veteriner hekimlikte kullanılan antibiyotiklerin kontrolüne devam edilmelidir.

Anahtar sözcükler: *Karbapenem direneçli Enterobacteriaceae, Karbapenemazlar, Çiğ süt*

INTRODUCTION

Enterobacteriaceae is a family of rod-shaped, Gram negative strains naturally present in the intestinal biota of warm-blooded animals. It contains pathogens that cause severe diseases such as cystitis, pyelonephritis, septicemia, pneumonia, peritonitis and meningitis, and some Enterobacteriacea can cause foodborne disease.

İletişim (Correspondence)
+90 352 3399484/29788, Mobile: +90 543 3258741
serhatal@erciyes.edu.tr

Bacteria belonging to this family can be easily distributed between humans and other warm-blooded organisms via food and water, and genetic elements can be transferred to each other through mobile structures such as plasmids and transposons [1-3]. Due to these properties, Enterobacteriaceae plays a significant role in contributing to multidrug resistance.

Carbapenems form an antibiotic group that is effectively used against the Gram-negative bacilli that develop broad-spectrum β-lactamase antibiotic resistance. Carbapenem resistance make it more difficult to treat nosocomial diseases [4]. Carbapenem resistant Enterobacteriaceae (CRE) is the current common problem reported worldwide [5-7].

The use of carbapenems in farm and pet animals has not been approved due to the clinical importance of these antibiotics and resistance concerns [8]. For this reason, carbapenem resistance can be predicted to be very rare in isolates of animal and related foods and there is limited data on the current state and prevalence of carbapenem-resistant bacteria in animals and their associated environments [9]. However, some studies conducted in different countries have recently shown that carbapenem resistance is observed apart from human isolates. It has been reported that carbapenem-resistant bacteria from various animals were detected in Germany [10], France [11], Belgium [12] and China [13]. For this reason, resistance to antibiotics used effectively in cases of serious infection should be monitored and reported globally.

The present study was conducted to reveal whether CRE could be distributed widely with raw milk which is consumed and processed in the dairy industry. An important public debate continues on the possible benefits of increasing the popularity of raw milk consumption [14]. In this matter, the aim of the study is to determine the role of animal originated foods in the epidemiology of CREs and the role of healthy individuals and animals in reaching the sensitive groups.

MATERIAL and METHODS

Bacterial Strains

Escherichia coli MSC234 (pMSC122); *E. coli* MSC229 (pMSC116), *E. coli* MSC228 (pMSC115) and *Klebsiella pneumoniae* ATCC BAA-1705 cultures were used as a positive control and *E. coli* ATCC 25922 were used as a negative control.

Sample Collection

A total of 427 milk samples from dairy cows were included in the study for a two-year period. Each sample was collected from separate cows from 15 different dairy farms located in Central Anatolia region, Turkey. A total of 40 mL milk samples, 10 mL from each nipple, were taken from the cows into sterile 50 mL falcon tubes and brought to the laboratory under the cold chain. Before sampling, the udder was cleaned with a commercially ready to use iodophor based antiseptic solutions and dried with a paper towel.

Isolation of CRE from the Raw Milk Samples

The Isolation of CRE from raw milk collected in the study was performed by modifying the laboratory protocol proposed by the Centers for Disease Control and Prevention [15]. Briefly, 100 µL homogenized milk samples were put into tubes containing 5 mL sterile Tryptic Soy Broth (Merck, Germany) with a Meropenem disk (10 µg, Oxoid, United Kingdom) and the tubes were incubated at 35±2°C for 24 h. After incubation, 100 µL of the homogenized sample was streaked on the MacConkey Agar (Merck, Germany) and incubated at 35±2°C for 24 h. Suspected colonies with different morphology grown on the MacConkey agar were streaked on Chromagar™ KPC (Chromagar, France) and ChromID® Carba Smart (Biomerieux, France) selective agars and incubated at 35±2°C for 24 h as an initial screening step. Isolates growth in both selective media were evaluated as CRE suspicious.

Determination of Carbapenem Resistance Profiles of Suspected Isolates

Suspicious colonies isolated from selective agars were subjected to the disc diffusion method, Modified Hodge tests (MHT) and Modified Carbapenemase Inactivation method (mCIM) according to CLSI [16,17].

Identification of Carbapenem Resistant Isolates

Phenotypically positive pure isolates were cultivated in blood agar, and identification and antibiotic resistance profiles were analyzed by Vitek® 2 Compact system (Biomerieux, France) following the instructions of the manufacturer. Automated selection of antibiotics was done according to the EUCAST [18].

Determination of bla_{KPC}, bla_{NDM} and bla_{OXA-48} Carbapenemase Genes

Genomic DNA Extraction: The total genomic DNA extraction was performed using the Instagene Genomic DNA Extraction Kit (Bio-Rad, USA) from the suspected colonies according to the manufacturer's protocol. Concentrations of the gDNA samples (µg/µL) were measured by Qubit 3.0 fluorometer (Thermo Fisher, USA) and stored at -20°C until molecular analyzes.

PCR Amplification: The gDNA isolates were subjected to PCR analysis with specific primers in order to amplify the gene regions sought in the suspicious samples. For the amplification of bla_{KPC}, bla_{NDM} and bla_{OXA-48} carbapenemase genes, the primers designed by Poirel et al.[19] were used in the study *(Table 1)*. The Maxima Hot Start Green 2x PCR Master Mix (Thermo Fisher, USA) was used for PCR

analysis according to the manufacturer's instructions. PCR amplification was performed with an initial denaturation of 95°C for 4 min followed by 35 cycles, each consisting of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min. The final extension cycle was performed at 72°C for 10 min (Arktic™ Thermal Cycler; Thermo Fisher, USA). All amplification products were analyzed by agarose gel (1.5%) electrophoresis at 100 V for 45 min. The gels were stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA) and visualized under a UV transilluminator (Vilber Lourmat, France).

SYBR Green Real-Time PCR Amplification: For the real time amplification of bla_{KPC}, bla_{NDM} and bla_{OXA-48} carbapenemase genes, the primers designed by Subirats et al.[20] were used in the study *(Table 1)*. qPCR was performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) on the CFX96 Connect Real-Time System (Bio-Rad, USA) according to manufacturer's instruction. The cycling protocol consisted of 95°C for 3 min, followed by 40 cycles at 95°C for 30 s and 60°C for 45 s. A melting curve was constructed in the range of 60 to 95°C to verify the specificity of the amplified products in all analyses. Each sample was run with duplicate. The positivity and quantitative values in the samples were based on the amplification curves and Ct (dR) (Threshold value cycle) data and the melting curve profiles.

RESULTS

Isolation of CRE from the Raw Milk Samples

In the study, 93 different carbapenem resistant suspected isolates were collected with CDC protocols in conventionally examined raw milk samples. After cultivation on chromogenic agars of isolates obtained by CDC protocol, growth was observed in 52 isolates. In the disc diffusion, two of 52 suspected isolates were found to be meropenem resistant and none of the isolates were found to be positive in the mCIM and MHT tests.

Determination of Carbapenem Resistance Profiles of Suspected Isolates

As a result of phenotypic antimicrobial susceptibility tests, two carbapenem resistant isolates were identified as *Stenotrophomonas maltophilia* with the Vitek-2 Compact system. In the antimicrobial resistance, both isolates were found to be susceptible to Trimethoprim-sulfamethoxazole.

Determination of bla_{KPC}, bla_{NDM} and bla_{OXA-48} Carbapenemase Genes

According to the PCR and qPCR analyses, no bla_{KPC}, bla_{NDM} and bla_{OXA-48} carbapenemase genes were detected in any gDNA obtained from the 52 carbapenem resistant suspected isolates collected from raw milk. Gel electrophoresis results obtained by PCR and Sybergreen qPCR results of bla_{KPC}, bla_{NDM} and bla_{OXA-48} positive controls were shown in *Fig. 1* and *Fig. 2,* respectively.

DISCUSSION

Farm animals are reservoirs for many zoonotic pathogens, also may pose serious hazards to public health if they provide antibiotic resistance to these microorganisms. Particularly, the antibiotic resistance genes can be transferred to other bacteria and mutated to reveal the necessity to focus on animal foods more carefully regarding public health [21].

Nowadays, the control of antibiotic resistance is becoming more difficult. In the absence of a systematic route in the control of bacterial pathogens, the misuse and overuse of antibiotics lead to the development of resistant pathogens. The environmental microbiota has a wide variety of distribution in farms and food industries where animal food is obtained. It is known that mobile genetic elements can be easily shared in these complex circumstances. Animal food is easily contaminated with these pathogens and can reach the consumer as a result of the production of raw

Table 1. *Primer pairs used in this study*

Target Gene	Primer	Sequence (5'-3')	Product (bp)	Reference
bla_{KPC}	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	798	[19]
	KPC-Rm	CTTGTCATCCTTGTTAGGCG		
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	621	
	NDM-R	CGGAATGGCTCATCACGATC		
bla_{OXA-48}	OXA-F	GCGTGGTTAAGGATGAACAC	438	
	OXA-R	CATCAAGTTCAACCCAACCG		
bla_{KPC} allels	Kpc-rtF	CAGCTCATTCAAGGGCTTTC	196	[20]
	Kpc-rtR	GGCGGCGTTATCACTGTATT		
bla_{NDM} allels	Ndm-rtF	GATTGCGACTTATGCCAATG	189	
	Ndm-rtR	TCGATCCCAACGGTGATATT		
bla_{OXA-48} allels	Oxa-rtF	AGGCACGTATGAGCAAGATG	189	
	Oxa-rtR	TGGCTTGTTTGACAATACGC		

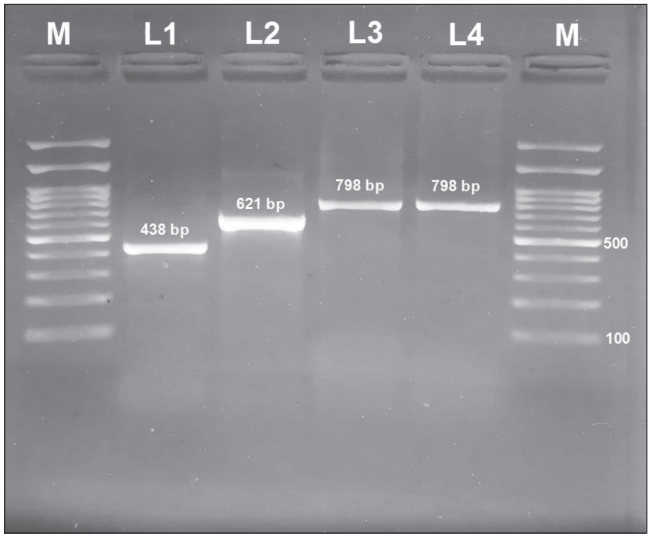

Fig 1. Agarose gel electrophoresis results of PCR assay for the bla_{KPC}, bla_{NDM} and bla_{OXA-48}. M: Marker (Geneaid 100-bp DNA Ladder); L1: Positive control for bla_{OXA-48} (*E. coli* MSC234 [pMSC122]); L2: Positive control for bla_{NDM} (*E. coli* MSC229 [pMSC116]); L3-4: Positive control for bla_{KPC} (*E. coli* MSC228 [pMSC115], *K. pneumoniae* ATCC® BAA-1705, respectively)

the spread of an antimicrobial resistance situation outside of the hospitals. The actual prevalence of carbapenem resistance is not yet known exactly and global scale studies on this subject are ongoing. In a report published in 2015 by the European Centre for Disease Prevention and Control (ECDC), it is stated that OXA-48 enzyme is endemic, NDM shows regional spread and KPC has not been reported in Turkey [27].

In the present study, CRE was not detected in any milk samples collected from different dairy farms in Central Anatolia, Turkey. However, in many recent studies, raw milk and dairy cows were reported to be positive for CRE in different countries. In a study conducted in Lebanon, it was reported that 30.2% CTX-M-15-producing *K. pneumoniae* was detected in raw bovine milk [28]. In another study conducted in India, it was reported that bla_{NDM-5} carbapenemase gene was found in one *E. coli* isolated from milk samples obtained from mastitic cow [29]. In a study organized in China, it was reported that 10 carbapenem resistant *K. pneumonie* were isolated in 65 milk and fecal samples from three dairy farms [30]. In a study

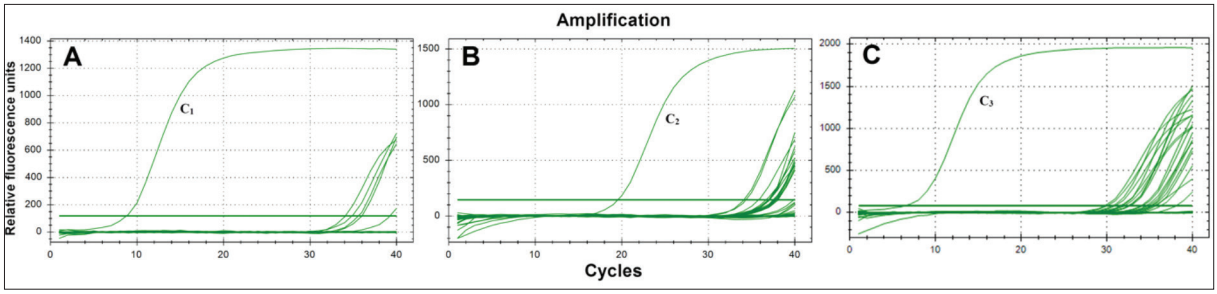

Fig 2. Sybergreen qPCR results of bla_{KPC}, bla_{NDM} and bla_{OXA-48}. **A:** Positive control for bla_{OXA-48} (*E. coli* MSC234 [pMSC122]); **B:** Positive control for bla_{NDM} (*E. coli* MSC229 [pMSC116]); **C:** Positive control for bla_{KPC} (*K. pneumoniae* ATCC® BAA-1705)

materials from non-hygienic farms and food production facilities with insufficient sanitation [22]. Inadequate farm conditions, operational errors such as faulty pasteurization, and personnel related contamination are important in terms of raw milk safety. The use of β-lactams has been limited by the wide spread of resistant *E. coli* strains containing broad-spectrum b-lactamase (ESBL) [23]. In consequence of this situation, the use of carbapenems globally increased due to their resistance to broad-spectrum β-lactamase hydrolysis from bacterial plasmids or chromosomes [24]. Enterobacteriaceae carrying NDM enzyme are resistant to almost all antibiotics with the few exceptions such as colistin. The presence and sharing of this plasmid mediated gene among bacteria originated from animal sources and food chain may pose a serious threat to human health [25].

Carbapenems are frequently used in the treatment of ESBL involved infections and play an important role in the treatment of nosocomial diseases [26]. The development of carbapenem resistance causes serious problems in the use of antibiotics and in the treatment of persistent infections. It is necessary to vary epidemiologic studies to determine

in Algeria, four *E. coli* isolates with identical PFGE profiles were obtained from the raw milk and teats of 34 healthy cows and carbapenem resistance was detected in all of *E. coli* isolates [31]. In a study carried out in Brazil, it is revealed that none of the 117 *E. coli* exhibited carbapenemase activity isolated from raw milk used in cheese production without any heat treatment, as a similar finding to current study [32]. In regard to these data, the main objective of this study was to determine the occurrence of CRE and carbapenemase in raw milk, in order to help clarify risk assessment with regard to potential human transfer through milk consumption. In this regard, it should be stated that there are no concerning levels of CRE in farm animals and related food products in Turkey.

In the findings of the present study, it is revealed that determination based on agar selection is not sufficient. It is observed that chromogenic agars have not exhibited essential sensitivity in the detection as claimed. None of the 52 suspected isolates obtained from selective agar had carbapenem resistance. Also, in the phenotype-based susceptibility methods, there are inherent problems in

differentiation between acquired and intrinsic resistance [33]. The isolates that were found to be cabapenem resistant in the study were detected as *S. maltophilia* by the VITEK compact system. For this reason, it is thought that the most accurate antimicrobial resistance determination can be realized by DNA based molecular methods. *S. maltophilia* is a Gram-negative bacillus showing wide environmental spread and although of low virulence pathogenicity, may cause serious infections in debilitated or immune-compromised individuals and patients. This environmental bacterium is known to have intrinsic resistance to many antibiotics with properties such as efflux pumps and low membrane permeability [34]. In the study, both of *S. maltophilia* inherently resistant to carbapenems were found to be susceptible to Trimethoprim-sulfamethoxazole. It is shown that monitoring of environmental contamination and control measures should be considered in animal food.

The results of the study showed that milk is not involved in the transmission of carbapenem resistance and the epidemiological data obtained are significant. CREs were not detected in any of the 427 milk samples and the resistance of these group antibiotics prohibited in the veterinary field has not developed in the dairy environment. Distribution of environmental microorganisms plays an important role in the continuation of the effectiveness of antibiotics such as carbapenems considered to be last resort antibiotics in the treatment of persistent nosocomial infections. Pathways by which bacteria and antimicrobial resistance instruments spread to humans from farm to fork should be clearly understood.

REFERENCES

1. Partridge SR: Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol Rev*, 35 (5): 820-855, 2011. DOI: 10.1111/j.1574-6976.2011.00277.x

2. Stokes HW, Gillings MR: Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol Rev*, 35, 790-819, 2011. DOI: 10.1111/j.1574-6976.2011.00273.x

3. Toleman MA, Walsh TR: Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev*, 35, 912-935, 2011. DOI: 10.1111/j.1574-6976.2011.00294.x

4. Hawkey PM, Livermore DM: Carbapenem antibiotics for serious infections. *BMJ,* 344:e3236, 2012. DOI: 10.1136/bmj.e3236

5. Ferreira RL, da Silva BCM, Rezende GS, Nakamura-Silva R, Pitondo-Silva A, Campanini EB, Brito MCA, da Silva EML, Freire CCM, da Cunha AF, Pranchevicius MDS: High prevalence of multidrug-resistant *Klebsiella pneumoniae* harboring several virulence and β-lactamase encoding genes in a brazilian intensive care unit. *Front Microbiol*, 9:3198, 2019. DOI: 10.3389/fmicb.2018.03198

6. Lepuschitz S, Schill S, Stoeger A, Pekard-Amenitsch S, Huhulescu S, Inreiter N, Hartl R, Kerschner H, Sorschag S, Springer B, Brisse S, Allerberger F, Mach RL, Ruppitsch W: Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals. *Sci Total Environ*, 662, 227-235, 2019. DOI: 10.1016/j.scitotenv.2019.01.179

7. Yamakawa H, Kosai K, Akamatsu N, Matsuda J, Kaku N, Uno N, Morinaga Y, Hasegawa H, Tsubouchi T, Kaneko Y, Miyazaki T, Izumikawa K, Mukae H[7], Yanagihara K: Molecular and epidemiological analysis of IMP-1 metallo-β-lactamase-producing *Klebsiella pneumoniae* in a tertiary care hospital in Japan. *J Infect Chemother*, 25 (4): 240-246, 2019. DOI: 10.1016/j.jiac.2018.11.012

8. OIE WO: World Organization for Animal Health List of antimicrobial agents of veterinary importance OIE International Commitee, 1-9, 2015.

9. Webb HE, Bugarel M, Den Bakker HC, Nightingale KK, Granier SA, Scott HM, Loneragan GH: Carbapenem-resistant bacteria recovered from faeces of dairy cattle in the high plains region of the USA. *PLoS One*, 11:e0147363, 2016. DOI: 10.1371/journal.pone.0147363

10. Fischer J, Rodrìguez I, Schmoger, Friese A, Roesler U, Helmuth R, Guerra B: *Salmonella enterica* subsp. *enterica* producing VIM-1 carbapenemase isolated from livestock farms. *J Antimicrob Chemother*, 68 (2): 478-480, 2013. DOI: 10.1093/jac/dks393

11. Poirel L, Berçot B, Millemann Y, Bonnin RA, Pannaux G, Nordmann P: Carbapenemase-producing *Acinetobacter* spp. in Cattle, France. *Emerg Infect Dis*, 18 (3): 523-525, 2012. DOI: 10.3201/eid1803.111330

12. Smet A, Boyen F, Pasmans F, Butaye P, Martens A, Nemec A, Deschaght P, Vaneechoutte M, Haesebrouck F: OXA-23-producing *Acinetobacter* species from horses: A public health hazard? *J Antimicrob Chemother*, 67 (12): 3009-3010, 2012. DOI: 10.1093/jac/dks311

13. Wang Y, Wu C, Zhang Q, Qi J, Liu H, Wang Y, He T, Ma L, Lai J, Shen Z, Liu Y, Shen J: Identification of new delhi metallo-β-lactamase 1 in *Acinetobacter iwoffii* of food animal origin. *PLoS One*, 7:e37152, 2012. DOI: 10.1371/journal.pone.0037152

14. Lucey J: Raw milk consumption: Risks and benefits. *Nutr Today*, 50 (4): 189-193, 2015. DOI: 10.1097/NT.0000000000000108

15. CDC: Centers for Disease Control and Prevention. Laboratory protocol for detection of carbapenem-resistant or carbapenemase-producing, *Klebsiella* spp. and *E. coli* from rectal swabs. Centers for Disease Control and Prevention, Atlanta, GA, 2009.

16. CLSI: Performance standards for antimicrobial susceptibility testing. 28th Informational Supplement. Clinical and Laboratory Standards (M100-S28). Wayne, P.A. CLSI Document, Wayne, PA, 2018.

17. CLSI: Performance standards for antimicrobial susceptibility testing. 24th Informational Supplement. Clinical and Laboratory Standards (M100-S24). Wayne, P.A. CLSI Document, Wayne, PA, 2014.

18. EUCAST: The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019.

19. Poirel L, Walsh TR, Cuvillier V, Nordmann P: Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis*, 70 (1): 119-123, 2011. DOI: 10.1016/j.diagmicrobio.2010.12.002

20. Subirats J, Royo E, Balcázar JL, Borrego CM: Real-time PCR assays for the detection and quantification of carbapenemase genes *(bla_{KPC}, bla_{NDM},* and *bla_{OXA-48})* in environmental samples. *Environ Sci Pollut Res*, 24, 6710-6714, 2017. DOI: 10.1007/s11356-017-8426-6

21. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP: Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect Dis*, 13 (9): 785-796, 2013. DOI: 10.1016/S1473-3099(13)70190-7

22. Van Balen J, Kelley C, Nava-Hoet RC, Bateman S, Hillier A, Dyce J, Wittum TE, Hoet AE: Presence, distribution, and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in a small animal teaching hospital: A year-long active surveillance targeting dogs and their environment. *Vector Borne Zoonotic Dis*, 13 (5): 299-311, 2013.

23. Pitout JD, Laupland KB: Extended-spectrum β-lactamase-producing Enterobacteriaceae: An emerging public-health concern. *Lancet Infect Dis*, 8 (3): 159-166, 2008. DOI: 10.1016/S1473-3099(08)70041-0

24. Martin SI, Kaye KM: Beta-lactam antibiotics: Newer formulations and newer agents. *Infect Dis Clin North Am*, 18 (3): 603-619, 2004. DOI: 10.1016/j.idc.2004.04.006

25. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan

S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N.: Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: A molecular, biological, and epidemiological study. *Lancet Infect Dis*, 10 (9): 597-602, 2010. DOI: 10.1016/S1473-3099(10)70143-2

26. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA: Carbapenems: Past, present, and future. *Antimicrob Agents Chemother*, 55 (11): 4943-4960, 2011. DOI: 10.1128/aac.00296-11

27. Albiger B, Glasner C, Struelens MJ, Grundmann H, Monnet DL, European Survey of Carbapenemase-Producing Enterobacteriaceae (EuSCAPE) Working Group: Carbapenemase-producing Enterobacteriaceae in Europe: Assessment by national experts from 38 countries, May 2015. *Euro Surveill*, 20 (45): 30062, 2015. DOI: 10.2807/1560-7917.ES.2015.20.45.30062

28. Diab M, Hamze M, Bonnet R, Saras E, Madec JY, Haenni M: OXA-48 and CTX-M-15 extended-spectrum beta-lactamases in raw milk in lebanon: Epidemic spread of dominant *Klebsiella pneumoniae* clones. *J Med Microbiol*, 66 (11): 1688-1691, 2017. DOI: 10.1099/jmm.0.000620

29. Ghatak S, Singha A, Sen A, Guha C, Ahuja A, Bhattacharjee U, Das S, Pradhan NR, Puro K, Jana C, Dey TK, Prashantkumar KL, Das A, Shakuntala I, Biswas U, Jana PS: Detection of new delhi metallo-beta-lactamase and extended-spectrum beta-lactamase genes in *Escherichia coli* isolated from mastitic milk samples. *Transbound Emerg Dis*, 60 (5): 385-389, 2013. DOI: 10.1111/tbed.12119

30. He T, Wang Y, Sun L, Pang M, Zhang L, Wang R: Occurrence and characterization of bla_{NDM-5} positive *Klebsiella pneumoniae* isolates from dairy cows in Jiangsu, China. *J Antimicrob Chemother*, 72 (1): 90-94, 2017. DOI: 10.1093/jac/dkw357

31. Yaici L, Haenni M, Saras E, Boudehouche W, Touati A, Madec JY: bla_{NDM-5}-carrying *IncX3* plasmid in *Escherichia coli* ST1284 isolated from raw milk collected in a dairy farm in Algeria. *J Antimicrob Chemother*, 71 (9): 2671-2672, 2016. DOI: 10.1093/jac/dkw160

32. Parussolo L, Sfaciotte RAP, Dalmina KA, Melo FD, Costa UM, Ferraz SM: Detection of virulence genes and antimicrobial resistance profiles of *Escherichia coli* isolates from raw milk and artisanal cheese in Southern Brazil. *Semin Cienc Agrar*, 40 (1): 163-178, 2019. DOI: 10.5433/1679-0359.2019v40n1p163

33. Codjoe FS, Donkor ES: Carbapenem resistance. A review. *Med Sci*, 6:1, 2018. DOI: 10.3390/medsci6010001

34. Sánchez MB: Antibiotic resistance in the opportunistic pathogen *Stenotrophomonas maltophilia*. *Front Microbiol*, 6:658, 2015. DOI: 10.3389/fmicb.2015.00658