Short communication: Bovine mastitis caused by a multidrug-resistant, *mcr-1*-positive (colistin-resistant), extended-spectrum β -lactamase-producing *Escherichia coli* clone on a Greek dairy farm

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ABSTRACT

We performed a survey aimed at analyzing milk samples collected from cows with mastitis for the presence of extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli*. Single-quarter mastitic milk samples obtained from 400 cows in 23 Greek dairy herds with a history of *E. coli* mastitis were processed for the selective isolation of ESBL-producing *E. coli*. The antimicrobial susceptibility of the ESBL-producing isolates was analyzed using agar disk diffusion, and minimum inhibitory concentrations of colistin were determined by broth microdilution. We used PCR followed by DNA sequencing to characterize the β -lactamases and *mcr-1* (colistin resistance) genes, and for phylotyping and multilocus sequence typing. We found a total of 89/400 (22.25%) *E. coli* isolates from 12/23 (52%) farms. Six isolates originating from 6 cows on a single farm were ESBL producers and were resistant to cefquinome, amoxicillin-clavulanic acid, aztreonam, ampicillin, and colistin. Five of these isolates were resistant to trimethoprim-sulfamethoxazole, and 5 to streptomycin. The 6 ESBL producers were *mcr-1*-positive and carried *bla*_{TEM-1} genes; 3 also carried *bla*_{CTX-M} genes, and 3 carried *bla*_{SHV} genes. All of the ESBL producers belonged to phylogroup A, multilocus sequence type ST666 (*n* = 5), or a single locus variant of ST666 (*n* = 1). To our knowledge, this is the first report of endemic bovine mastitis caused by *mcr-1*-positive, ESBL-producing *E. coli*. These results highlight the value of active surveillance of antimicrobial resistance not commonly tested

by diagnostic laboratories for the early detection of novel resistant strains.

Key words: *Escherichia coli*, extended-spectrum β -lactamase (ESBL), *mcr-1*, colistin resistance, bovine mastitis, antimicrobial resistance

Short Communication

The rapid emergence of antimicrobial resistance in bacteria of clinical significance represents a serious threat to human health. The World Health Organization encourages the implementation of active surveillance of resistance patterns not usually tested by diagnostic laboratories to enable early detection of novel resistant strains (World Health Organization, 2014). Resistant bacteria relevant to human health may emerge in livestock (Mather et al., 2013; Spoor et al., 2013); hence the need for active surveillance in food-producing animals as well.

Escherichia coli causes a broad range of infections in humans and is among the most common environmental mastitis-causing species in dairy cattle (Shpigel et al., 2008). In *E. coli*, horizontal transfer of genes coding for extended-spectrum β -lactamases (ESBL) is a common mechanism of dissemination of resistance to a broad range of β -lactams. Moreover, ESBL-producing *E. coli* strains have a higher propensity to express multidrug resistance than non-ESBL-producing strains (Karkaba et al., 2017), further complicating infection management.

Colistin (also known as polymyxin E) and other polymyxins are considered last-resort antimicrobials for the treatment of severe human infections with *Pseudomonas aeruginosa* and carbapenem-non-susceptible ESBL-producing *Enterobacteriaceae* and *Acinetobacter* spp.

Received July 23, 2019.

Accepted September 26, 2019.

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(Nation and Li, 2009). Acquired resistance of *E. coli* to colistin has been assumed to be due to chromosomal mutations, and therefore not readily horizontally transferable. However, this assumption has been recently challenged by the identification of the plasmid-mediated *mcr-1* gene conferring colistin resistance in *E. coli* (Liu et al., 2016), followed by reports of human infections with *mcr-1*-positive strains. Such strains have also been found in foods of animal origin (Skov and Monnet, 2016; Liu and Liu, 2018), and there are indications of *mcr-1* gene flow from animals to humans (Hernández et al., 2017). Here, we report for the first time the occurrence of IMI with multidrug-resistant, *mcr-1*-positive, ESBL-producing *E. coli* in dairy cattle. The bacterium was isolated from milk samples collected from 6 cows during a bacteriological survey performed on Greek dairy farms. The survey was carried out between June 2015 and December 2016 on 23 dairy farms with a history of *E. coli* mastitis, after consultations with 6 local veterinarians. Of the farms, 16 were located in the Macedonia region in northern Greece (mean herd size: 88; range: 54–135), and 7 in the Thessaly region in central Greece (146; 100–225). Milk samples from quarters showing abnormal milk were collected opportunistically by veterinarians at any stage during the survey period. Samples were collected aseptically before treatment was administered, and they were refrigerated and shipped within 12 h to the microbiology laboratory at the Faculty of Veterinary Medicine of Aristotle University, Thessaloniki. Additional farm- and cow-level data were not collected.

Within 2 h of arrival, 50 μ L of milk was spread onto the surface of selective-differential Tryptone Bile X-glucuronide agar plates (TBX; Oxoid, Basingstoke, UK) and incubated at 44°C for 18 to 24 h, as recommended by the manufacturer for selection of *E. coli*. To maximize the identification of true *E. coli* IMI, only samples yielding a minimum of 25 β -glucuronidase-positive (blue) colonies (i.e., 5 colonies for every 10 μ L of inoculum) were considered *E. coli*-positive. Samples yielding 2 or more dissimilar colony types were not analyzed further. One colony from each *E. coli*-positive plate was subcultured and identified using the API-20E kit (bioMérieux, Marcy l'Etoile, France) and by means of a PCR for amplification of the *E. coli* *usp(A)* gene (Chen and Griffiths, 1998). Isolates producing metabolic profiles and bands on gels consistent with *E. coli* were screened for the presence of Ambler class A ESBL production using the double-disk synergy test. Isolates displaying synergy between third-generation cephalosporins and amoxicillin-clavulanic acid were classified as ESBL producers (Timofte et al., 2014). We assessed the in vitro susceptibility of the ESBL producers us-

ing agar disk diffusion according to the Clinical and Laboratory Standards Institute standard (CLSI, 2015). The following disks were used: nalidixic acid (30 μ g), ciprofloxacin (5 μ g), gentamicin (10 μ g), trimethoprim and sulfamethoxazole (1.75/23.75 μ g), streptomycin (10 μ g), cefquinome (30 μ g), amoxicillin-clavulanate (20/10 μ g), aztreonam (30 μ g), ampicillin (10 μ g), piperacillin (100 μ g), and imipenem (10 μ g) (Oxoid Ltd.). We determined the MIC of colistin by broth microdilution using the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2016). Briefly, 2-fold colistin dilutions ranging between 0.03 and 256 mg/L were prepared in Mueller-Hinton broth (Oxoid), and colistin-susceptible and colistin-resistant *E. coli* reference strains (ATCC 25922 and NCTC13846) were used for quality control. Results were interpreted with a resistance breakpoint of 2 μ g/mL.

We extracted DNA from the ESBL-producing isolates using kits (NucleoSpin Microbial DNA Kit; Macherey-Nagel, Düren, Germany), and used PCR-based approaches to analyze their phylogenetic relationships and antimicrobial resistance. Briefly, phylotyping was performed using the Clermont method, which assigns strains to a phylogroup based on the presence or absence of 4 specific genes (Clermont et al., 2013). Multilocus sequence typing at 7 housekeeping genes was implemented as described by Wirth et al. (2006). The sequence types (ST) were determined online by searching the gene sequences in a publicly available *E. coli* database (Enterobase; https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search).

We assessed the presence of genes encoding the ESBL *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM}, and the plasmid-mediated Ambler class C enzymes *bla*_{FOX}, *bla*_{DHA}, *bla*_{CIT}, *bla*_{ACC}, and *bla*_{EBC}, using PCR sequencing as previously described (Pérez-Pérez and Hanson, 2002; Fang et al., 2008). For analysis of the *mcr-1* gene, we obtained nucleotide sequences of the gene from the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>) and aligned them using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) and BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Based on this alignment, we determined a set of forward and reverse primers expected to yield amplicons of 521 bp. The PCR products of all resistance genes were bidirectionally sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) using dRohamine-labeled terminators. Raw sequences were manually edited using MEGA7 software (www.megasoftware.net) and compared with those available in the NCBI database using BLAST (Altschul et al., 1990). The primers for the amplification of the resistance genes are reported in Table 1.

Table 1. Primers, expected amplicon sizes, and annealing temperatures used for the amplification of extended-spectrum- β -lactamase and *mcr-1* genes

Sequence	Expected amplicon size (kb)	Annealing temperature (°C)	Gene designation	Reference
CGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	62	<i>bla</i> _{TEM}	Fang et al., 2008
ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593	62	<i>bla</i> _{CTX-M}	Fang et al., 2008
CTTTATCGGCCCTCACTCAA AGGTGCTCATCATGGGAAAG	237	62	<i>bla</i> _{SHV}	Fang et al., 2008
GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520	72	<i>bla</i> _{MOX}	Pérez-Pérez and Hanson, 2002
TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	72	<i>bla</i> _{CTT}	Pérez-Pérez and Hanson, 2002
AACTTTACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTTGC	405	72	<i>bla</i> _{DHA}	Pérez-Pérez and Hanson, 2002
AACAGCCTCAGCAGCCGGTGA TTCGCCGCAATCATCCCTAGC	346	72	<i>bla</i> _{ACC}	Pérez-Pérez and Hanson, 2002
TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	302	72	<i>bla</i> _{EBC}	Pérez-Pérez and Hanson, 2002
AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	109	72	<i>bla</i> _{FOX}	Pérez-Pérez and Hanson, 2002
GCAACCAAGCCTGATATGCG CGCTTAAATACGCAGGCC	521	55	<i>mcr-1</i>	Designed in this study

We analyzed a total of 400 milk samples from an equal number of cows; the number of samples per farm varied from 9 to 28. Eighty-nine samples from 12 farms yielded more than 25 blue colonies on TBX agar. The farms were classified as *E. coli*-positive farms, and each farm had 4 to 12 *E. coli*-positive samples. Seventeen samples were contaminated, and 9 yielded 1 to 13 blue colonies and were not analyzed further. The remaining 285 samples yielded no blue colonies on TBX agar. We found no correlation between the number of samples collected and the number of *E. coli*-positive samples from the positive farms (not shown).

Six of 89 (6.7%) *E. coli* isolates displayed the ESBL phenotype. The 6 ESBL producers were collected on 2 sampling occasions from 6 mastitic cows on a single farm in the Macedonia region. The first sampling took place in October 2015 and included 5 mastitic cows, 2 of which were infected with ESBL producers (isolates VNF₀₁ and VNF₀₂; Table 2). The second sampling took place in March 2016 and included another 5 mastitic cows, 4 of which were infected with ESBL producers (VNF₀₃ to VNF₀₆). The other 4 samples collected on that farm were *E. coli*-negative. All ESBL-producing isolates displayed resistance to at least 1 antimicrobial from 3 or more pharmacological groups and were classified as multidrug-resistant. All isolates were resistant to cefquinome, amoxicillin-clavulanic acid, aztreonam, ampicillin, piperacillin, and colistin, and susceptible to nalidixic acid, ciprofloxacin, gentamicin, and imipenem. Five isolates were resistant to trimethoprim-

sulfamethoxazole, and 5 to streptomycin (Table 2). The 6 ESBL producers yielded amplicons for the *yjaA* and *arpA* genes, and therefore belonged to phylogroup A. The isolates were monoclonal: 5 of 6 belonged to sequence type ST666, and the sixth was a single locus variant of ST666, but its allelic profile did not match any sequence type in the *E. coli* database. All of the ESBL producers carried the *mcr-1* gene, and 5 carried the *bla*_{TEM-1} gene. The *mcr-1* sequences were identical between isolates. Three isolates also carried *bla*_{CTX-M} genes, and 3 were *bla*_{SHV}-positive. We found 99 to 98% sequence similarity between the isolates at these 2 loci. We detected no plasmid-mediated Ambler class C genes (Table 2). The ESBL gene sequences identified in this survey have been deposited in the GenBank under accession numbers MF566034 to MF566050.

Infections with ESBL-producing *E. coli* are relevant for both human and bovine medicine because of the widespread use of β -lactams in both clinical settings. Such ESBL-producing *E. coli* have been reported from cases of bovine mastitis for more than a decade (Brías et al., 2003; Haftu et al., 2012), and the identification of 1 ESBL-positive farm in our survey is consistent with previous data suggesting a low prevalence of such strains in Europe (Locatelli et al., 2010; Dahmen et al., 2013). To our knowledge, this is the first report of bovine mastitis caused by an *mcr-1*-positive ESBL-producing *E. coli*.

Colistin has been marketed for decades in Europe for the oral treatment of infections in pigs, poultry, cattle,

sheep, goats, and rabbits; in aquaculture; and for the topical treatment of bovine mastitis (European Medicines Agency, 2013). Colistin resistance is unlikely to be of clinical importance in the case of bovine mastitis, because the use of this drug (or other polymyxins) for the treatment of mastitis is not common. However, our finding carries public health implications. First, ESBL-producing *E. coli* lineages found in food-producing animals have also been found in human infections (Carattoli, 2008; Lifshitz et al., 2018). Moreover, the widespread use of β -lactams in dairy cattle may coselect ESBL-producing colistin-resistant strains, increasing the abundance and mobilization of the *mcr-1* gene between strains, or even between bacterial species, potentially increasing human exposure to the gene through various pathways (European Medicines Agency, 2013).

The 6 ESBL-producing isolates belonged to phylogroup A. Phylogroups have been widely used as predictors of *E. coli* pathogenicity in humans and, to a lesser extent, cattle. Studies focusing on human medicine often consider phylogroup A to be an “environmental” group (Johnson et al., 2001), but 1 study assigned approximately 13% of human bacteremia isolates to this phylogroup (Bukh et al., 2009). Interestingly, although there is no consensus on what constitutes a “pathogenic” *E. coli* strain in cattle, phylogroup A is one of the most common groups reported in bovine mastitis worldwide (Suojala et al., 2011; Blum and Leitner, 2013; Zhang et al., 2018). Because phylotyping lacks discriminatory power for epidemiological source tracking, we also applied multilocus sequence typing.

Five isolates belonged to ST666, and the sixth was a single locus variant, indicating that the infections were monoclonal. We did not find information in the literature about the pathogenicity of ST666. In the database consulted, ST666 is reported as a singleton sequence type (i.e., it is not grouped within any clonal complex), and was reported only once in humans, 3 times in cattle, once in shellfish, and once in the environment (database consulted on July 1, 2019).

The results of the molecular typing add complexity to our understanding of the transmission routes of *E. coli* and other environmental mastitis-causing bacteria. In a recent review, Klaas and Zadoks (2018) described 2 common modes of transmission of environmental mastitis pathogens: point-source, in which cows within farms are infected with a genetically homogeneous bacterial lineage transmitted from a single localized source; and multiple environmental sources, in which cows within farms are infected with genetically heterogeneous organisms originating from various sources. Our results did not completely conform to either of these models. A close look at the data in Table 2 indicates that the 2 isolates obtained in October 2015 (VNF₀₁ and VNF₀₂) were very similar, with only 4 nucleotide substitutions observed in the *bla*_{CTX-M} sequence (3 of which were synonymous; data not shown), and the 3 isolates obtained in March 2016 were more heterogeneous. Hence, even though the monoclonal descent of the isolates was consistent with the point-source transmission model, the variable resistomes and antibiograms indicated different recent evolutionary histories and perhaps multiple

Table 2. Colistin MIC, antibiogram, and occurrence of resistance genes in 6 extended-spectrum- β -lactamase-producing *Escherichia coli* strains isolated from bovine mastitis cases¹

Isolate designation	Colistin MIC	Antimicrobial resistance	Antimicrobial susceptibility	Reference NCBI accession numbers for nucleotide sequences and % similarity
VNF ₀₁	4 mg/L	STX, SMN, CEQ, AMC, ATM, AMP, PIR	NAL, CIP, GEN, IMI	<i>bla</i> _{TEM-1} (MH742416.1; 100%) <i>bla</i> _{CTX-M} (MH190876.1; 100%) <i>mcr-1</i> (CP031550.1; 100%)
VNF ₀₂	4 mg/L	STX, SMN, CEQ, AMC, ATM, AMP, PIR	NAL, CIP, GEN, IMI	<i>bla</i> _{TEM-1} (MH742416.1; 100%) <i>bla</i> _{CTX-M} (MH190876.1; 98%) <i>mcr-1</i> (CP031550.1; 100%)
VNF ₀₃	4 mg/L	STX, SMN, CEQ, AMC, ATM, AMP, PIR	NAL, CIP, GEN, IMI	<i>bla</i> _{TEM-1} (MH742416.1; 100%) <i>bla</i> _{CTX-M} (MH190876.1; 100%) <i>mcr-1</i> (CP031550.1; 100%)
VNF ₀₄	4 mg/L	STX, SMN, CEQ, AMC, ATM, AMP, PIR	NAL, CIP, GEN, IMI	<i>bla</i> _{TEM-1} (MH742416.1; 100%) <i>bla</i> _{SHV} (MF993523.1; 99%) <i>mcr-1</i> (CP031550.1; 100%)
VNF ₀₅	4 mg/L	STX, SMN, CEQ, AMC, ATM, AMP, PIR	NAL, CIP, GEN, IMI	<i>bla</i> _{TEM-1} (MH742416.1; 100%) <i>bla</i> _{SHV} (MF993523.1; 100%) <i>mcr-1</i> (CP031550.1; 100%)
VNF ₀₆	8 mg/L	CEQ, AMC, ATM, AMP, PIR	NAL, CIP, GEN, IMI, SXT, SMN	<i>bla</i> _{SHV} (MF993523.1; 100%) <i>mcr-1</i> (CP031550.1; 100%)

¹AMC = amoxicillin-clavulanic acid; AMP = ampicillin; ATM = aztreonam; CEQ = cefquinome; CIP = ciprofloxacin; GEN = gentamicin; IMI = imipenem; NAL = nalidixic acid; PIR = piperacillin; SMN = streptomycin; STX = trimethoprim and sulfamethoxazole.

sources. Mobile resistome is readily transferrable within and between strains (Hu et al., 2016), and our results may reflect horizontal gene transfer and diversification of the ST666 during or before the survey. Future studies combining intensive longitudinal sampling with whole-genome sequencing would enable a better understanding of the significance of subtle genetic differences deriving from within-farm evolution of mastitis-causing *E. coli* and other *Enterobacteriaceae*.

This is the first report of the circulation of a multidrug-resistant, ESBL-producing, colistin-resistant (*mcr-1*-positive) *E. coli* clone causing IMI in dairy cattle. The prevalence of such strains is not known, and further studies are needed to assess the effects of *mcr-1*-positive *E. coli* circulating in dairy cattle. The results also highlight the usefulness of active surveillance of resistance not usually tested by diagnostic laboratories, for the early detection of novel resistant strains.

ACKNOWLEDGMENTS

This work was financed by national funds through the research committee of Aristotle University of Thessaloniki. The authors report no conflict of interest.

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