Virulence Determinants and Plasmid-Mediated Colistin Resistance *mcr* Genes in Gram-Negative Bacteria Isolated From Bovine Milk

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A major increase of bacterial resistance to colistin, a last-resort treatment for severe infections, was observed globally. Using colistin in livestock rearing is believed to be the ground of mobilized colistin resistance (*mcr*) gene circulation and is of crucial concern to public health. This study aimed to determine the frequency and virulence characteristics of colistin-resistant Gram-negative bacteria from the milk of mastitic cows and raw unpasteurized milk in Egypt. One hundred and seventeen strains belonging to Enterobacteriaceae (n = 90), *Pseudomonas aeruginosa* (n = 10), and *Aeromonas hydrophila* (n = 17) were screened for colistin resistance by antimicrobial susceptibility testing. The genetic characteristics of colistin-resistant strains were investigated for *mcr*-1–9 genes, phylogenetic groups, and virulence genes. Moreover, we evaluated four commonly used biocides in dairy farms for teat disinfection toward colistin-resistant strains. Multidrug-resistant (MDR) and extensive drug-resistant (XDR) phenotypes were detected in 82.91% (97/117) and 3.42% (4/117) of the isolates, respectively. Of the 117 tested isolates, 61 (52.14%) were colistin resistant (MIC >2 mg/L), distributed as 24/70 (34.29%) from clinical mastitis, 10/11 (90.91%) from subclinical mastitis, and 27/36 (75%) from raw milk. Of these 61 colistin-resistant isolates, 47 (19 from clinical mastitis, 8 from subclinical mastitis, and 20 from raw milk) harbored plasmid-borne *mcr* genes. The *mcr*-1 gene was identified in 31.91%, *mcr*-2 in 29.79%, *mcr*-3 in 34.04%, and each of *mcr*-4 and *mcr*-7 in 2.13% of the colistin-resistant isolates. Among these isolates, 42.55% (20/47) were *E. coli*, 21.28% (10/47) *A. hydrophila*, 19.12% (9/47) *K. pneumoniae*, and 17.02% (8/47) *P. aeruginosa*. This is the first report of *mcr*-3 and *mcr*-7 in *P. aeruginosa*. Conjugation experiments using the broth-mating technique showed successful transfer of colistin resistance to *E. coli* J53-recipient strain. Different combinations of virulence genes...
were observed among colistin-resistant isolates with almost all isolates harboring genes. Hydrogen peroxide has the best efficiency against all bacterial isolates even at a low concentration (10%). In conclusion, the dissemination of mobile colistin resistance mcr gene and its variants between MDR- and XDR-virulent Gram-negative isolates from dairy cattle confirms the spread of mcr genes at all levels; animals, humans, and environmental, and heralds the penetration of the last-resort antimicrobial against MDR bacteria. Consequently, a decision to ban colistin in food animals is urgently required to fight XDR and MDR bacteria.

**Keywords:** colistin, Gram-negative bacteria, mastitis, milk, mcr, virulence factors, multidrug-resistance

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**INTRODUCTION**

*Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* are the most common Gram-negative environmental pathogens in dairy farms. They could infect the mammary glands of dairy cows through the teat end and colonize the mammary tissue, causing mastitis (Scatamburlo et al., 2015; Gao et al., 2017). Although *Aeromonas* species are autochthonous to aquatic environments, *Aeromonas hydrophila* has been reported as a common contaminant in a diverse variety of foods such as milk and milk products (Sharma and Kumar, 2011). Contamination of milk with pathogenic Gram-negative bacteria is often derived directly from the udder excretion of infected animals or the farm environment, thus putting the consumers who consume unpasteurized milk at a high risk for developing foodborne diarrheal diseases (Garcia et al., 2019).

In Egypt, there are no regulations that control the use of antibiotics in animal husbandry for growth promotion or the prevention and treatment of bacterial diseases (WHO, 2013). The misuse of antibiotics in dairy production is a matter of concern for the effective antibiotic therapy of infected cows, food safety, and occupational exposure, and a potential threat to public health due to the selection of multidrug-resistant (MDR) bacteria and entry of antibiotic residues into bulk tank milk (Oliveira and Ruegg, 2014; Locatelli et al., 2019). The emergence of MDR and extensive drug-resistant (XDR) Gram-negative bacteria in livestock production increased the interest in the use of colistin (polymyxin E) as a last-resort antibiotic for the treatment of these pathogens (Kempf et al., 2016). Although colistin was prohibited in developed countries, it is still used in animal husbandry in Egyptian dairy farms (Lima Barbieri et al., 2017; Dandachi et al., 2019). Hence, the extensive use of colistin increases colistin-resistant Gram-negative bacteria. Also, it imposes a major public health concern (Lima Barbieri et al., 2017) due to the dissemination of colistin resistance genes (mcr) through mobilized plasmids among animal strains and subsequently transmitted to humans through the food chain or direct contact (McEachran et al., 2015; Garcia et al., 2018; Hmede and Kassem, 2019). Nine plasmid-borne mcr-family genes (mcr-1–mcr-9) have been reported in over 40 countries from different continents across the globe; most of them were detected in several enterobacteria, including *E. coli* and *K. pneumoniae* (Luo et al., 2020). The mcr-1 gene has been detected in most continents, while other genes have only been found in a few countries (Ling et al., 2020). The mcr-1 gene has been reported previously in colistin-resistant *E. coli* isolated from raw milk (Coton et al., 2012; Hassen et al., 2019), mastitis bovine milk (Filioussis et al., 2019; Liu G. et al., 2020), and cheese (Coton et al., 2012; Hammad et al., 2019). A higher frequency of mcr-1 compared with other mcr genes has been reported in colistin-resistant *E. coli* (98.9%), *K. pneumoniae* (100%), and *P. aeruginosa* (100%) recovered from animal and human sources (Javed et al., 2020). The implementation of pre- and post-milking teat disinfection is a critical effective means of reducing the incidence of clinical and subclinical mastitis as well as new intramammary infections caused by Gram-negative environmental pathogens in dairy farms such as *E. coli, K. pneumoniae,* and *P. aeruginosa* (Tiwari, 2013; Kamal and Bayoumi, 2015; Böhm et al., 2017; Rowe et al., 2018). Choosing the ideal disinfectant with an accurate concentration is vital to control resistant bacteria in dairy farms. The resistant bacteria can survive at concentrations many folds below the minimum inhibitory concentration (MIC) of the biocidal agents (Hughes and Andersson, 2012). Notably, Gram-negative bacteria have a risk for promoting antibiotic resistance after exposure to sublethal concentrations of some disinfectants such as chlorhexidine (Kampf, 2018).

Most putative virulence determinants contributing to the pathogenicity of *E. coli, K. pneumoniae, Aeromonas* species, and *P. aeruginosa* are chromosomally encoded. However, previous studies have indicated the occurrence of some virulence factors on plasmids of *E. coli* (Rodriguez-Siek et al., 2005; Johnson et al., 2006; Mellata et al., 2010; Cyoia et al., 2015), *K. pneumoniae* (Dolejska et al., 2013; Chen et al., 2020), *Aeromonas* species (Brown et al., 1997), and *P. aeruginosa* (Moraes-Espinosa et al., 2012). *E. coli* strains possess virulence genes that play a significant role in the survival and pathogenesis of the strain in the host through bacterial adhesion (*ipfA*), hemolysis (*hlyF*), iron acquisition (*iroN* and *ireA*), and increased serum survival and resistance to phagocytosis (*iss* and *traT*) (Serowska et al., 2019; Adzitey et al., 2020). *K. pneumoniae* hypervirulent strains possess different genes that determine virulence and severity of infection in the host. These virulence genes include *entB* (enterobactin biosynthesis gene), *allS* (associated with allantoin metabolism), *ybtS* (yersiniabactin biosynthesis), and *mrkD* and *fimH* (fimbrial adhesin that mediates binding to the extracellular matrix to
form the biofilm) (Rosen et al., 2008; Wasfi et al., 2016; Ye et al., 2016; Russo and Marr, 2019). The most important A. hydrophila virulence factors include hemolysin A (hlyA), aerolysin (aer), cytotoxic heat-stable enterotoxin (ast), cytotoxic enterotoxin (act), and lipases (lip) (Furmanek-Blaszk, 2014; Rather et al., 2014). P. aeruginosa produces extracellular products that contribute to its pathogenicity, such as protein exotoxin A (toxA), proteases (lasB), type III secretion system exoenzymes (exoU and exoS) (Casilag et al., 2016; Fadhil et al., 2016), and genes responsible for pyocyanin production (phzM) (Novroozi et al., 2012). Genes encoding virulence and MDR/XDR are often found with mcr genes on plasmids in environmental isolates (Anyanwu et al., 2020). There are limited reports that studied the occurrence of plasmid-encoded virulence and colistin resistance genes (mcr) among colistin-resistant Gram-negative bacteria isolated from cow’s milk in our geographic region. Hence, the current study aimed to: (1) determine the frequency of colistin-resistant Gram-negative bacteria from the milk of mastitic cows as well as raw unpasteurized milk; (2) screen for mcr resistance determinants in bacterial isolates and their virulence characteristics; (3) evaluate four frequently used biocides in dairy farms for teat disinfection toward colistin-resistant isolates.

MATERIALS AND METHODS

Gram-Negative Bacterial Strains
A total of 117 Gram-negative bacterial strains belonging to Enterobacteriaceae (n = 90), P. aeruginosa (n = 10), and A. hydrophila (n = 17) were included in this study. The bacterial isolates were recovered previously from milk samples of dairy cows showing mastitis as well as bulk tank raw milk during 2018–2020. Gram-negative isolates were selected based on the provided data from the Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt. Enterobacteriaceae strains included non-O157 E. coli (n = 30), O157 E. coli (n = 12), K. pneumoniae (n = 18), Enterobacter species (n = 10), and Citrobacter species (n = 20). The isolated bacteria were sourced from different farms. All the isolates were independent and were not clonally duplicated from the same source. The presumptive bacterial isolates were confirmed adopting the standard microbiological procedures (Quinn et al., 1994). In brief, MacConkey’s, eosin methylene blue (Oxoid, Cambridge, UK) and HiCrome klebsiella-selective agar media (HiMedia, Mumbai, India) were used for the cultivation of Enterobacteriaceae strains. Aeromonas species were grown on Rimler-Shotts medium (HiMedia, India) with a novobiocin (Oxoid, UK) supplement, while P. aeruginosa strains were cultivated on Pseudomonas cetridime agar (Oxoid, UK). Serotyping of E. coli was applied using diagnostic polyvalent and monovalent O and H antisera (Denka Seiken Co., Tokyo, Japan). Genomic DNA was extracted from fresh bacterial cultures using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Polymerase chain reaction (PCR)-based confirmation of Gram-negative bacteria was applied using genus- and species-specific primer sets depicted in Supplementary Table S1.

Antimicrobial Susceptibility Testing
The antimicrobial susceptibilities of Gram-negative bacterial strains (n = 117) against a panel of 24 commonly used antimicrobial agents were determined using the Kirby-Bauer disk diffusion assay following the Clinical and Laboratory Standards Institute guidelines and interpretative criteria (CLSI, 2019). The tested antimicrobial discs (Oxoid, Cambridge, UK) were ampicillin (10 µg), amoxicillin-clavulanic acid (30 µg), piperacillin-tazobactam (40 µg), cefazolin (30 µg), cephalothin (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), ceftazidine (30 µg), cefepime (30 µg), imipenem (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), trimethoprimsulphamethoxazole (25 µg), chloramphenicol (30 µg), tetracycline (30 µg), aztreonam (30 µg), tigecycline (15 µg), fosfomycin (50 µg), and colistin (25 µg). The minimum inhibitory concentration (MIC) of colistin (Sigma-Aldrich, Seelze, Germany) was determined against Gram-negative bacterial strains by broth microdilution technique following the relevant CLSI document (CLSI, 2019).

The multiple antibiotic resistance (MAR) indices were evaluated as documented elsewhere (Tambekar et al., 2006), while the MDR and XDR phenotypes were reported according to Magiorakos et al. (2012). E. coli ATCC 25922, P. aeruginosa ATCC 27853, and A. hydrophila ATCC 7966 were used as quality control strains.

Detection of Plasmid-Mediated Colistin Resistance Genes
Plasmid DNAs were extracted from colistin-resistant Gram-negative bacterial strains (E. coli, K. pneumoniae, P. aeruginosa, and A. hydrophila) using Plasmid DNA Miniprep Kits (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. PCR amplifications of colistin resistance genes (mcr-1 to mcr-9) from phenotypic colistin-resistant Gram-negative bacteria isolates were performed using oligonucleotide primer sequences and their annealing temperatures listed in Supplementary Table S1. Conventional PCRs were performed using a programmable 2720 thermal cycler (Applied Biosystem, Waltham, MA, USA) in a total reaction volume of 25 µl containing 12.5 µL of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (20 pmol) (Sigma-Aldrich, Co., St. Louis, MO, USA), 5 µl of template DNA, and 5.5 µl of nuclease-free water. The amplified products were visualized after 30 min of electrophoresis on a 2% agarose gel containing ethidium bromide.

DNA Sequencing and Phylogenetic Analysis of mcr Genes
DNA sequencing of the PCR products was applied to validate their genetic identity and to verify any mutations in the mcr genes. The amplified DNA fragments were purified with the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France). Sanger sequencing was performed in both directions using Bigdyce Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Inc. Waltham, MA, USA) in an applied Bioystems 3130
genetic analyzer (Hitachi, Tokyo, Japan). The nucleotide sequences were compared with those available in the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Nucleotide and deduced protein sequences were analyzed using the MEGA7 program (Kumar et al., 2016). To investigate the genetic relatedness and the evolutionary distance among Gram-negative strains harboring the mcr genes, a phylogenetic tree was constructed using the neighbor-joining (maximum composite likelihood) method. DNA sequences generated in the study were deposited into the GenBank database with accession numbers MW811398-MW811434 and MZ648218-MZ648227.

Conjugation Assay
The transmissibility of colistin-resistant mcr genes between donors (mcr-positive isolates) and the recipient bacteria (E. coli J53; Na azide-resistant) was evaluated by the conjugation assays using the broth mating technique (Wang et al., 2003). An equal ratio (1:1) of donor and recipient cells (0.5 ml of each) in a logarithmic phase were added to 4 ml of sterile Luria-Bertani (LB) broth (Sigma-Aldrich, USA) then incubated overnight at 37°C without shaking. The mating mixture was serially diluted then the transconjugants were cultured on LB agar (Sigma-Aldrich, USA) supplemented with colistin (2 mg/L) or Na azide (100 mg/L). MICs for the donors, recipients, and transconjugants were determined by the broth microdilution method following the CLSI guidelines (CLSI, 2019). Potential transconjugants were examined for the existence of mcr genes using PCR assays. The conjugation efficiency was calculated as the number of transconjugants per donor as previously documented (Wang et al., 2003).

PCR Amplification of Virulence-Associated Genes
PCR detection of virulence-related genes of E. coli (hlyF, ireA, iroN, iss, lpfA, and traT), K. pneumoniae (entB, allS, mrkD, fimH, ybtS, and irp1), P. aeruginosa (exoU, exoS, lasB, toxA, and phzM), and A. hydrophila (hlyA, aer, lip, ast, and act) was performed in conventional PCR assays using the oligonucleotide primer sequences presented in Supplementary Table S1.

Disinfectant Susceptibility Testing
Gram-negative bacterial strains (three of each species exhibiting colistin resistance) were evaluated against four commonly used biocides in dairy farms for teat disinfection, namely chlorhexidine gluconate (0.5% in alcohol 70%; Pfizer, Manhattan, NY, USA), hydrogen peroxide (H₂O₂; 6%, and A. hydrophila sequences presented in conventional PCR assays using the oligonucleotide primer sequences presented in Supplementary Table S1.

Bioinformatics and Data Analysis
Statistical analyses were performed using the software SAS (SAS Institute Inc., 2012). Results of conjugation efficiency of Gram-negative bacteria were graphed by a boxplot through GraphPad Software (version 8.0.1, GraphPad Software Inc., La Jolla, CA, USA). Resistance phenotypes and frequencies of virulence genes among isolates were compared using the Chi-squared (χ²) test. P < 0.05 was considered significant. A heatmap with hierarchical clustering was generated to visualize the overall distribution of mcr variants and antimicrobial resistance phenotypes in colistin-resistant isolates using the “heatmap” package in R software (version 3.4.2) (Kolde, 2019). To determine the shared mcr variants among colistin-resistant isolates from different sources, a Venn diagram was generated using the Venny 1.0 tool, https://bioinfgp.cnb.csc.es/tools/venny/index.html. Spearman correlations were used to provide an estimate for the association among various variables (resistance phenotypes, colistin resistance, and virulence genes). The correlation coefficients and their p-values were visualized using a correlation plot. The correlation analyses and visualization were done using R packages rcorr (https://hbiostat.org/R/Rmisc/) and corrplot (https://github.com/taiyun/corrplot). Genes or phenotypes that were present or absent in all analyzed subjects were not considered in these analyses.

RESULTS
Resistance Phenotypes of Gram-Negative Bacteria Isolated from Mastitis and Raw Milk
Overall, the MDR phenotype was determined for 70 Enterobacteriaceae isolates including 37/42 (88.1%) E. coli (27/30 were non-O₁₅₇ and 10/12 were O₁₅₇ E. coli), 18/18 K. pneumoniae (100%), 10/10 Enterobacter species (100%), and 5/20 Citrobacter species (25%). All P. aeruginosa and A. hydrophila isolates were MDR with MAR indices ranged from 0.33 to 0.79. Three P. aeruginosa and one E. coli (O111:H4) exhibited an XDR phenotype as being resistant to at least one antibiotic of all tested antimicrobial classes but remained susceptible to two classes (carbapenems and monobactams in P. aeruginosa, carbapenems and glycolycyclines in E. coli).

E. coli isolates showed high resistance to ampicillin, cephalothin, cefazolin, ceftazidime (100% each), cefoxitin (97.62%), cefotaxime (95.23%), and amoxicillin-clavulanic acid (90.47%). In addition, over 50% of isolates were resistant to piperacillin-tazobactam (71.43%), colistin, and fosfomycin (57.14%, each).

All K. pneumoniae isolates were resistant to ampicillin, amoxicillin-clavulanic acid, fosfomycin, cefotaxin, cefazolin, and cephalothin. Meanwhile, 94.4% of the isolates were resistant to both ceftazidime and piperacillin-tazobactam, 88.89% were resistant to tigecycline, 77.78% to ceftriaxone, cefotaxime, and colistin, and 55.56% were resistant to sulfamethoxazole-trimethoprim.
A. hydrophila isolates were sensitive to amikacin, imipenem, ciprofloxacin, and levofloxacin (100% each). However, the highest resistance was for the tetracycline and cephalosporins groups (ceftriaxone, cefotaxime, cefazidime, cefoxitin, and cephalothin (100% each)) followed by colistin (88.24%), nalidixic acid (76.47%), sulfamethoxazole-trimethoprim (70.59%), and cefazolin (58.8%).

P. aeruginosa isolates exhibited resistance to sulfamethoxazole-trimethoprim, amoxicillin-clavulanic acid, fosfomycin, cefoxitin, cefalothin, and cephalothin (100% each); tetracycline, chloramphenicol, and ampicillin (90% each); colistin and nalidixic acid (80% each); and ceftazidime, ciprovloxacin, and levofloxacin (60% each). However, the most active antimicrobial agents against P. aeruginosa isolates were imipenem (100%), aztreonam (90%), piperacillin-tazobactam, and ceftriaxone (80% each).

Cephalosporins (cefotaxime, cefazidime, cefoxitin, cefazolin, and cephalothin), ampicillin, and amoxicillin-clavulanic acid were the least active antimicrobials against both Enterobacter and Citrobacter species as well as cefepine, sulfamethoxazole-trimethoprim, and tetracycline in Enterobacter species (100% resistance rate). Citrobacter species were completely sensitive to both sulfamethoxazole-trimethoprim and tetracycline. Meanwhile, both species were highly sensitive to imipenem, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin, colistin, piperacillin-tazobactam, and tigecycline (100% each).

**Trends in Bacterial Resistance and MAR Indices**
The overall resistance rates of Gram-negative isolates from clinical mastitis, subclinical mastitis, and raw milk to each tested antimicrobial were analyzed using $\chi^2$ test. As revealed in Table 1, a significant ($p < 0.05$) difference in resistance rates of isolates from clinical, subclinical mastitis, and raw milk was observed against ampicillin, cefazolin, ceftriaxone, nalidixic acid, tobramycin, fosfomycin, colistin, and tetracycline. All isolates from different sources were highly resistant to the first and second generations of cephalosporins especially cephalothin (100%). Furthermore, the MAR index of each antimicrobial was calculated to understand the level of antibiotic use. High MAR index (0.04) of ampicillin, amoxicillin-clavulanic acid, and the first and second generations of cephalosporins was observed. This implies the excessive use of these antimicrobials in the veterinary sector and human medicine. Meanwhile, imipenem achieved a lower MAR index.

**Frequency of Colistin Resistance mcr Genes in MDR and XDR Gram-Negative Bacteria**
In total, 61/117 tested isolates (52.14%), 24/70 (34.29%) from clinical mastitis, 10/11 (90.91%) from subclinical mastitis, and 27/36 (75%) from raw milk were colistin resistant, showing colistin MICs >2 mg/L. Among them, 19 (27.14%), 8 (72.73%), and 20 (55.56%) isolates from clinical, subclinical mastitis, and raw milk, respectively, were positive for plasmid-borne mcr genes.

From the PCR and DNA sequencing results, the mcr-1 gene was identified in 31.91% (13 MDR and two XDR isolates), mcr-2 in 29.79% (13 MDR and one XDR), mcr-3 in 34.04% (15 MDR and one XDR), and each of mcr-4 and mcr-7 in 2.13% of the colistin-resistant Gram-negative isolates (Table 2). Of these isolates, 42.55% (20/47) were E. coli, 21.28% (10/47) A. hydrophila, 19.12% (9/47) K. pneumoniae, and 17.02% (8/47) P. aeruginosa (Figure 1).

A Venn diagram (Supplementary Figure S1) was generated to determine the shared mcr variants between colistin-resistant isolates from clinical mastitis, subclinical mastitis, and raw milk. E. coli mcr-4 and both mcr-7 and mcr-2 of P. aeruginosa were unique for clinical mastitis; whereas K. pneumonia mcr-3 and A. hydrophila mcr-1 to mcr-3 variants were exclusive for raw milk. Only E. coli mcr-3 and K. pneumonia mcr-2 were common among all sources.

**Phylogeny of Colistin Resistance mcr Variants**
As presented in Figure 2, the phylogenetic tree was constructed based on nucleotide sequences for all identified colistin resistance mcr variants (mcr-1 to mcr-4 and mcr-7) in 47 Gram-negative bacteria isolates of various species. The phylogenetic analysis revealed five potential clusters indicating sequence heterogeneity among the mcr variants. Despite the overall diversity of bacterial isolates, which were originating from different on-farm locations/sources, the phylogenetic tree demonstrated the presence of the same clonal lineages of each mcr variant separately. Nonetheless, two mcr variants (mcr-4, accession number MW811433, and mcr-7, accession number MW811434) were clonally related; whereas the latter shared the same lineage with another mcr-2 variant (accession number MW811412).

**Transferability of mcr Genes**
Transconjugation assays revealed that representative Gram-negative bacteria isolates of various species harboring mcr-1, mcr-2, mcr-3, mcr-4, and mcr-7 were capable of transferring their genes to the E. coli J53-recipient strain. The conjugation efficiencies of these isolates ranged from 2.01 ± 0.816 × 10^{-6} to 9.50 ± 2.663 × 10^{-3} CFU/donor (Table 3 and Figure 3). As expected, the recipient J53 strain did not grow on colistin and Na azide-incorporated media. The colistin MICs for transconjugants ranged from 4 to 64 µg/ml, demonstrating an increase of three to sevenfold relative to E. coli J53-recipient strain (MIC = 0.5 µg/ml) (Table 3).

**Virulence Characteristics of Colistin-Resistant Gram-Negative Bacteria**
The profiles of virulence-associated genes detected in 47 colistin-resistant isolates are depicted in Table 2. Different combinations of virulence genes were observed among colistin-resistant isolates with almost all isolates harboring genes ranging from one to six. LpfA was the most frequently detected gene in the tested E. coli isolates (20/20; 100%) followed by hlyF (17/20; 85%), iroN (16/20; 80%), iss (15/20; 75%), ireA (13/20; 65%), and traT (12/20; 60%). The entB gene was detected in all nine colistin-resistant K. pneumoniae isolates examined; both mrkD
Resistance rates of 117 Gram-negative bacteria from milk samples against the tested antimicrobial agents and the overall multiple antibiotic resistance (MAR) index.

| AMA    | Clinical mastitis | Subclinical mastitis | Raw milk | MAR indexa | p-valuea |
|--------|-------------------|----------------------|----------|------------|----------|
|        | E. coli (n = 25)  | K. pneumoniae (n = 8) | Enterobacter species (n = 10) | Citrobacter species (n = 20) | P. aeruginosa (n = 7) | E. coli (n = 5) | K. pneumoniae (n = 3) | P. aeruginosa (n = 3) | E. coli (n = 12) | K. pneumoniae (n = 7) | A. hydrophila (n = 17) |
| AM     | 25 (100.00)       | 8 (100.00)           | 10 (100.00) | 20 (100.00) | 6 (85.71) | 12 (100.00) | 7 (100.00) | 9 (52.94) | 0.04 | 0.0004 |
| AMC    | 25 (100.00)       | 8 (100.00)           | 10 (100.00) | 20 (100.00) | 7 (100.00) | 5 (100.00) | 3 (100.00) | 7 (100.00) | 8 (47.06) | 0.04 | 0.000007 |
| CEF    | 25 (100.00)       | 8 (100.00)           | 10 (100.00) | 20 (100.00) | 7 (100.00) | 3 (100.00) | 3 (100.00) | 7 (100.00) | 17 (100.00) | 0.04 | NA |
| CFZ    | 25 (100.00)       | 8 (100.00)           | 10 (100.00) | 20 (100.00) | 7 (100.00) | 5 (100.00) | 3 (100.00) | 7 (100.00) | 10 (58.82) | 0.04 | 0.0002 |
| FOX    | 24 (96.00)        | 8 (100.00)           | 10 (100.00) | 20 (100.00) | 7 (100.00) | 12 (100.00) | 7 (100.00) | 17 (100.00) | 0.04 | 0.7128 |
| CAZ    | 25 (100.00)       | 8 (100.00)           | 10 (100.00) | 20 (100.00) | 4 (57.14) | 3 (100.00) | 5 (100.00) | 12 (100.00) | 0.04 | 0.6833 |
| CTX    | 23 (92.00)        | 5 (62.50)            | 10 (100.00) | 20 (100.00) | 1 (14.29) | 3 (100.00) | 1 (33.33) | 6 (85.71) | 0.04 | 0.1211 |
| CRO    | 6 (24.00)         | 6 (75.00)            | 5 (50.00)  | 15 (75.00)  | 2 (28.57) | 2 (40.00) | 3 (100.00) | 6 (85.71) | 0.02 | 0.00088 |
| FEB    | 9 (36.00)         | 2 (25.00)            | 10 (100.00) | 0 (0.00)    | 1 (14.29) | 1 (33.33) | 5 (41.67) | 4 (23.53) | 0.01 | 0.8673 |
| IPM    | 0 (00.00)         | 1(12.50)             | 0 (0.00)   | 0 (0.00)    | 0 (0.00) | 0 (0.00) | 0 (0.00) | 0 (0.00) | 0.00 | 0.7128 |
| NA     | 5 (20.00)         | 0 (00.00)            | 5 (25.00)  | 5 (71.43)   | 5 (71.43) | 1 (20.00) | 3 (100.00) | 6 (85.71) | 0.01 | 0.00466 |
| CIP    | 4 (16.00)         | 0 (00.00)            | 0 (00.00)  | 4 (57.14)   | 4 (57.14) | 0 (00.00) | 2 (66.67) | 4 (33.33) | 0.06 | 0.7614 |
| LVX    | 4 (16.00)         | 0 (00.00)            | 0 (00.00)  | 4 (57.14)   | 4 (57.14) | 2 (40.00) | 2 (66.67) | 4 (33.33) | 0.05 | 0.799 |
| TOB    | 1 (4.00)          | 0 (00.00)            | 0 (00.00)  | 0 (00.00)   | 3 (42.86) | 2 (40.00) | 2 (66.67) | 1 (33.33) | 0.05 | 0.00005 |
| AK     | 0 (00.00)         | 0 (00.00)            | 0 (00.00)  | 3 (42.86)   | 0 (00.00) | 0 (00.00) | 1 (33.33) | 0 (0.00) | 0.00 | 0.2763 |
| CN     | 3 (12.00)         | 0 (00.00)            | 0 (00.00)  | 3 (42.86)   | 0 (00.00) | 0 (00.00) | 1 (33.33) | 3 (25.00) | 0.05 | 0.2512 |
| SXT    | 10 (40.00)        | 6 (75.00)            | 10 (100.00) | 7 (100.00)  | 3 (42.86) | 0 (00.00) | 1 (33.33) | 4 (33.33) | 0.02 | 0.623 |
| TGC    | 11 (44.00)        | 8 (100.00)           | 0 (00.00)  | 0 (00.00)   | 3 (42.86) | 1 (20.00) | 3 (100.00) | 2 (16.67) | 0.01 | 0.632 |
| ATM    | 5 (20.00)         | 1 (12.50)            | 0 (00.00)  | 0 (00.00)   | 0 (00.00) | 0 (00.00) | 0 (00.00) | 0 (00.00) | 0.005 | 0.068 |
| TPZ    | 21 (84.00)        | 8 (100.00)           | 0 (00.00)  | 0 (00.00)   | 3 (100.00) | 3 (100.00) | 3 (100.00) | 5 (41.67) | 0.02 | 0.429 |
| CHL    | 5 (20.00)         | 2 (25.00)            | 0 (00.00)  | 0 (00.00)   | 6 (85.71) | 0 (00.00) | 3 (100.00) | 5 (41.67) | 0.01 | 0.1378 |
| FOF    | 15 (60.00)        | 8 (100.00)           | 0 (00.00)  | 7 (100.00)  | 7 (100.00) | 7 (100.00) | 3 (25.00) | 2 (11.76) | 0.02 | 0.0001 |

(Continued)
and fimH were identified in 88.8%, ybtS and irp1 in 77.7%, and alls in 44.4% of K. pneumonia isolates. The frequencies of toxA, exoS, lasB, exoU, and phzM genes in eight colistin-resistant P. aeruginosa isolates were 100%, 87.5%, 87.5%, 75%, and 25%, respectively. The aer gene is the most prevalent (70%) in A. hydrophila isolates followed by lip (30%) and act (20%). Both ast and hlyA genes were detected in one A. hydrophila isolate (10%). There are significant differences in the frequencies of virulence genes among E. coli isolates (p = 0.04), A. hydrophila (p = 0.01), and P. aeruginosa (p = 0.005).

Correlations Between the Antimicrobial Resistance and Virulence Traits

The correlation plots among pairs of antimicrobial resistance phenotypes, colistin resistance genes (mcr), and virulence genes in E. coli, K. pneumoniae, P. aeruginosa, and A. hydrophila are displayed in Supplementary Figure S2 and Supplementary Tables S2A–H. Significant positive correlations were pronounced between fluoroquinolone resistance and the existence of mcr-1 gene in E. coli (r = 0.58–0.67; p < 0.001). On the other hand, a strong positive correlation was significantly detected between a fimH virulence gene and the resistance to certain antimicrobials; ceftazidime, cefotaxime, ceftriaxone, and piperacillin-tazobactam in K. pneumonia isolates (r = 1; p < 0.001). Correlations between virulence traits and antimicrobial resistance were obvious in P. aeruginosa and A. hydrophila isolates. Concerning P. aeruginosa, non-significant positive correlations were found between the analyzed virulence genes and antimicrobial agents/genes including the last-resort ones. The correlations between exoU and phzM virulence genes and gentamicin (r = 0.58; p = 0.1), exoS and fluoroquinolones (r = 0.65; p = 0.07), exoU and tigecycline (r = 0.58; p = 0.01), phzM and mcr2 (r = 0.65; p = 0.07), and phzM and tigecycline (r = 0.58; p = 0.1) were mostly distinct. For A. hydrophila, the hlyA gene showed significant positive correlations with tobramycin (r = 0.67; p = 0.03) or piperacillin-tazobactam (r = 1; p < 0.001). Of interest, significant strong positive correlations were detected between the last resort antimicrobials; mcr-2 and tigecycline (r = 0.76; p = 0.01) and mcr-2 and fosfomycin (r = 0.67; p = 0.03).

Efficacy of Disinfectants on Colistin-Resistant Gram-Negative Bacteria

According to the agar well-diffusion results, certain disinfectants had a wide efficacy against the test bacteria. However, some disinfectants were ineffective even at high concentrations (Supplementary Table S3). E. coli and K. pneumonia isolates were resistant to chlorhexidine gluconate at concentrations lower than 50% (MIC = 40%), whereas P. aeruginosa and A. hydrophila were sensitive to such disinfectant even at low concentrations (MIC = 10%). E. coli and K. pneumoniae were sensitive to iodine at high concentrations (MIC = 80%), while P. aeruginosa and A. hydrophila exhibited resistance at all tested concentrations. On the other hand, hydrogen peroxide had the best inhibitory effect against all tested bacterial isolates even at a low concentration (10%), except for E. coli, which was sensitive to H2O2 at a concentration of 50%. All tested bacteria were resistant to ethanol at all concentrations.
### TABLE 2 | Resistance phenotypes, virulence genes profiles, and plasmid-mediated mcr genes of 47 Gram-negative bacteria recovered from milk samples.

| Isolate No. | Species          | Source          | Antimicrobial resistance patterns | MAR index | MIC (mg/L) | mcr gene | Accession No. | Virulence gene families |
|-------------|------------------|-----------------|----------------------------------|------------|------------|----------|---------------|------------------------|
| 1           | *E. coli*        | Clinical mastitis | CFZ, FOX, AM, AMC, TPZ, CEF, CAZ, FOF, CTX, TET, CHL, TGC, CST | 0.54       | 128        | mcr-2    | MW811407     | hlyF, rea, iss, lpfA    |
| 2           | *E. coli*        | Raw milk        | CFZ, FOX, AM, AMC, TPZ, CEF, CAZ, FO, CTX, CST | 0.42       | 8         | mcr-3    | MW811419     | hlyF, rea, iroN, lpfA   |
| 3           | *K. pneumoniae*  | Subclinical mastitis | CFZ, FOX, AM, AMC, TET, TPZ, CEF, CAZ, TGC, CST, FO, CTX, NA | 0.5        | 32        | mcr-3    | MW811420     | hlyF, iroN, lpfA        |
| 4           | *K. pneumoniae*  | Clinical mastitis | CFZ, FOX, AM, AMC, TET, TPZ, CEF, CAZ, TGC, CST, FO, CTX, NA | 0.54       | 64        | mcr-3    | MW811421     | iroN, iss, lpfA         |
| 5           | *K. pneumoniae*  | Clinical mastitis | CFZ, CRO, FOX, SXT, AM, AMC, TPZ, CEF, CAZ, TGC, CST, TET, ATM, FO, FEP, CTX | 0.67       | 128       | mcr-2    | MW811408     | hlyF, rea, iroN, lpfA   |
| 6           | *K. pneumoniae*  | Clinical mastitis | CFZ, CRO, FOX, SXT, AM, AMC, TPZ, CEF, CAZ, TGC, CST, TET, ATM, FO, FEP, CTX | 0.66       | 128       | mcr-2    | MW811405     | hlyF, rea, iroN, lpfA   |
| 7           | *K. pneumoniae*  | Clinical mastitis | CFZ, FOX, AM, AMC, CEF, CAZ, CTX, FO, TET, CST, FO, CST | 0.46       | 16        | mcr-4    | MW811433     | hlyF, rea, iroN, lpfA   |
| 8           | *K. pneumoniae*  | Subclinical mastitis | CFZ, FOX, AM, AMC, CEF, CAZ, CTX, FO, TET, FO, FEP, CST | 0.42       | 16        | mcr-3    | MW811427     | hlyF, rea, iroN, lpfA   |
| 9           | *K. pneumoniae*  | Clinical mastitis | CFZ, FOX, TPZ, CEF, ORO, CAZ, CTX, FEP, NA, CIP, LVX, SXT, ATM, AM, AMC, CHL, TE, FO, CST | 0.79       | 16        | mcr-1    | MW811398     | hlyF, rea, iroN, iss, lpfA, traT |
| 10          | *K. pneumoniae*  | Clinical mastitis | CFZ, TPZ, CEF, CAZ, CTX, FEP, FO, FOX, SXT, AM, ATM, AMC, TET, CST, FO, CST | 0.63       | 16        | mcr-2    | MW811409     | hlyF, rea, iroN, iss, lpfA, traT |
| 11          | *K. pneumoniae*  | Clinical mastitis | CFZ, CAZ, CRO, FEP, FO, FOX, SXT, NA, CIP, LVX, SXT, ATM, AM, TPZ, AMC, CHL, CST, FO, CST | 0.74       | 64        | mcr-3    | MW811423     | hlyF, rea, iroN, iss, lpfA |
| 12          | *K. pneumoniae*  | Clinical mastitis | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST | 0.5        | 8         | mcr-2    | MW811410     | hlyF, rea, iroN, iss, lpfA |
| 13          | *K. pneumoniae*  | Subclinical mastitis | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST, FO, CST | 0.46       | 128       | mcr-3    | MW811424     | iroN, iss, lpfA, traT   |
| 14          | *K. pneumoniae*  | Subclinical mastitis | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST, FO, CST | 0.38       | 16        | mcr-2    | MW811411     | hlyF, rea, iroN, iss, lpfA, traT |
| 15          | *K. pneumoniae*  | Clinical mastitis | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST, FO, CST | 0.42       | 16        | mcr-2    | MW811412     | hlyF, rea, iroN, iss, lpfA |
| 16          | *K. pneumoniae*  | Clinical mastitis | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST, FO, CST | 0.42       | 32        | mcr-3    | MW811425     | hlyF, rea, iroN, iss, lpfA, traT |
| 17          | *K. pneumoniae*  | Clinical mastitis | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST, FO, CST | 0.54       | >128       | mcr-2    | MW811413     | hlyF, rea, iroN, iss, lpfA, traT |
| 18          | *K. pneumoniae*  | Raw milk         | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST, TET | 0.5         | 64        | mcr-2    | MW811414     | iroN, iss, lpfA, traT   |
| 19          | *K. pneumoniae*  | Raw milk         | CFZ, CRO, FO, FOX, CAZ, TGC, AM, TPZ, AMC, CST, TET | 0.5         | >128       | mcr-1    | MW811399     | hlyF, rea, iroN, iss, lpfA, traT |
| 20          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.33       | 128       | mcr-3    | MW811426     | hlyF, rea, iroN, iss, lpfA |
| 21          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.63       | 8         | mcr-2    | MW811415     | entB, als, mKd, fimH, ybtS, ip1 |
| 22          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.63       | 128       | mcr-2    | MW811416     | entB, mKd, fimH, ybtS, ip1 |
| 23          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.29       | 4         | mcr-1    | MW811400     | entB, mKd, ybtS, ip1 |
| 24          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.5         | 128       | mcr-1    | MW811401     | entB, als, mKd, fimH, ip1 |
| 25          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.58       | 64        | mcr-3    | MW811428     | entB, als, mKd, fimH, ip1 |
| 26          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.54       | 64        | mcr-4    | MW811402     | entB, als, mKd, fimH, ip1 |
| 27          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.54       | 8         | mcr-3    | MW811429     | entB, als, mKd, fimH, ybtS, ip1 |
| 28          | *K. pneumoniae*  | Raw milk         | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.5         | 16        | mcr-1    | MW811403     | entB, fimH, ybtS, ip1 |
| 29          | *K. pneumoniae*  | Subclinical mastitis | CFZ, FOX, AM, AMC, CHL, FO, CST | 0.5         | 32        | mcr-2    | MW811417     | entB, mKd, fimH, ybtS, ip1 |
| 30          | *P. aeruginosa*  | Clinical mastitis | CFZ, FOX, NA, CIP, LVX, SXT, TGC, AM, AMC, CHL, FO, CST, TET | 0.63       | >128       | mcr-7    | MW811434     | toxA, exoS, lasB, exoU |
| 31          | *P. aeruginosa*  | Clinical mastitis | AK, CN, TOB, CEF, CFZ, FOX, CTX, CRO, NA, CIP, LVX, SXT, AM, AMC, CHL, FO, CST, TET | 0.79       | 128       | mcr-2    | MW811418     | toxA, exoS, lasB, exoU, phaM |

(Continued)
TABLE 2 | Continued

| Isolate No. | Species          | Source       | Antimicrobial resistance patterns | MAR index | MIC (mg/L) | mcr gene | Accession No. | Virulence gene |
|-------------|------------------|--------------|-----------------------------------|-----------|-----------|----------|---------------|----------------|
| 32          | P. aeruginosa    | Subclinical mastitis | TOB, CEF, FOX, CAZ, CFZ, NA, AM, AMC, SXT, CHL, FOF, TET, CST | 0.54      | 32        | mcr-1    | MW811404      | toxA, exoS, lasB |
| 33          | P. aeruginosa    | Clinical mastitis | AK, CN, TOB, CEF, FOX, CAZ, CFZ, NA, CIP, LVX, AM, AMC, CHL, FOF, TET, CST | 0.71      | 64        | mcr-3    | MW811430      | toxA, exoS, exoU |
| 34          | P. aeruginosa    | Subclinical mastitis | CEF, FOX, CFZ, NA, CIP, LVX, SXT, AM, AMC, CHL, FOF, TET, CST | 0.54      | 64        | mcr-3    | MW811431      | toxA, exoS, lasB |
| 35          | P. aeruginosa    | Clinical mastitis | TOB, CEF, FOX, CFZ, SXT, AM, AMC, CHL, FOF, TET, CST | 0.45      | 32        | mcr-1    | MW811405      | toxA, lasB, exoU |
| 36          | P. aeruginosa    | Clinical mastitis | AK, CN, CEF, FOX, CFZ, CRO, FEP, NA, CIP, LVX, SXT, TGC, AM, AMC, CHL, FOF, TET, CST | 0.75      | 16        | mcr-3    | MW811432      | toxA, exoS, lasB, exoU, phzM |
| 37          | P. aeruginosa    | Subclinical mastitis | AK, CN, CEF, FOX, CFZ, CAX, CTX, FEP, NA, CIP, LVX, SXT, TGC, AM, AMC, CHL, FOF, TET, CST | 0.79      | >128      | mcr-1    | MW811406      | toxA, exoS, lasB, exoU |
| 38          | A. hydrophila    | Raw milk      | CAZ, CTX, CEF, CRO, FOX, NA, SXT, AM, AMC, CHL, CST, TET | 0.5        | 128       | mcr-1    | MZ648218      | aer |
| 39          | A. hydrophila    | Raw milk      | CFZ, CEF, FOX, CAZ, CTX, NAO, SXT, ATM, AM, CST, TET | 0.5        | 16        | mcr-1    | MZ648219      | act |
| 40          | A. hydrophila    | Raw milk      | TOB, CEF, FOX, CAZ, CTX, CRO, TGC, AM, AMC, AM, AMC, FOF, CHL, CST, TET | 0.54      | 32        | mcr-2    | MZ648224      | aer, lip |
| 41          | A. hydrophila    | Raw milk      | TOB, CEF, CFZ, FOX, CAZ, CTX, CRO, CRO, FEP, NA, SXT, AM, AMC, CHL, CST, TET | 0.63      | 64        | mcr-1    | MZ648220      | aer, lip |
| 42          | A. hydrophila    | Raw milk      | CEF, FOX, CAZ, CTX, CRO, NA, SXT, CST, TET | 0.38      | 64        | mcr-3    | MZ648226      | lip |
| 43          | A. hydrophila    | Raw milk      | CN, CEF, FOX, CAZ, CTX, CRO, NA, SXT, AT, AM, CST, TET | 0.5        | 128       | mcr-3    | MZ648227      | aer |
| 44          | A. hydrophila    | Raw milk      | CN, TOB, CEF, FOX, CAZ, CTX, NA, SXT, ATM, AM, AMC, CHL, CST, TET | 0.63      | 128       | mcr-1    | MZ648221      | hlyA, aer |
| 45          | A. hydrophila    | Raw milk      | CEF, FOX, CAZ, CFZ, CTX, CRO, NA, SXT, ATM, AM, AMC, CHL, CST, TET | 0.5        | 64        | mcr-1    | MZ648222      | act |
| 46          | A. hydrophila    | Raw milk      | CEF, FOX, CAZ, CTX, CRO, FEP, NA, SXT, TGC, CST, TET | 0.46      | 32        | mcr-2    | MZ648225      | aer |
| 47          | A. hydrophila    | Raw milk      | CEF, FOX, CAZ, CTX, CRO, FEP, NA, SXT, TGC, CST, TET | 0.5 <128   | mcr-1      | MZ648223      | aer, ast |

AM, ampicillin; AMC, amoxicillin-clavulanic acid; TPZ, piperacillin-tazobactam; CFZ, cefazolin; CEF, cephalothin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEB, cefepime; PM, piperacillin; CN, gentamicin; TOB, tobramycin; AK, amikacin; NA, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; TGC, tigecycline; CST, colistin; TET, tetracycline; TGC, tigecycline; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; ATM, aztreonam; FOX, fosfomycin. All isolates are multidrug resistant (MDR) except the bold ones are extensive drug resistant (XDR).

**DISCUSSION**

The recent global dissemination of mcr genes has raised the alarm of colistin resistance as a serious and urgent threat to public health (WHO, 2019). Consequently, large numbers of studies were carried out to explore the distribution of mcr genes in different reservoirs. Herein, we studied colistin-resistant Gram-negative bacteria isolated from bovine milk concerning the mcr resistance determinants and virulence characteristics. Unfortunately, 52% of the tested isolates were colistin resistant. The high proportion of colistin-resistant bacteria and the diversity of mcr variants detected here indicates a lot of independent acquisitions of mcr genes by colistin-sensitive bacteria and confirm the hypothesis that animals and food chain may play a key role in mcr transmission (Luo et al., 2020). This is considering an alarming ratio since previous studies have detected colistin resistance in 37%, 40%, and 41% of bacterial isolates from pigs, poultry, and human clinical samples, respectively (Savin et al., 2020; Lay et al., 2021; Qadi et al., 2021). Of note, we identified these isolates in milk samples, which included various bacterial species. These findings could be attributed to the frequent usage of colistin in dairy farms that in turn could be responsible for the development of colistin resistance. This is consistent with a previous report of a Canadian dairy farm, which uses colistin to increase the milk yield although its usage is not officially licensed in veterinary medicine (Saini et al., 2012; Webb et al., 2017).

Colistin-resistant E. coli isolates exhibited resistance to cephalothin, cefazolin, cefazidime, ampicillin, amoxicillin-clavulanic acid, cefoxitin, cefotaxime, piperacillin-tazobactam, and fosfomycin. Concurrently, nearly similar patterns were obtained recently for E. coli against several antibiotics in India, Croatia, and Egypt (Zdolc et al., 2016; Singh et al., 2018; Ramadan et al., 2021; Soliman et al., 2021). For *K. pneumoniae* isolates, the antibiotic resistance pattern was closely like the pattern of Brazilian MDR *K. pneumoniae* isolates (Ferreira et al., 2019). They showed resistance to cefazolin, cephalothin, cefoxitin, cefazidime, cefotaxime, ceftriaxone, sulfamethoxazole-trimethoprim, and tigecycline. On the contrary, our colistin-resistant *P. aeruginosa* isolates showed resistance to most groups of antibiotics; however, previous colistin-resistant *P. aeruginosa* isolates from Iran showed only resistance to ticarcillin, ciproflaxacin, aztreonam, cefazidime, gentamicin, imipenem, and piperacillin/tazobactam (Goli et al., 2016). Our *A. hydrophila* isolates have an antimicrobial resistance pattern much akin to the antibiotic resistance profiles of pathogenic aeromonads isolated from ornamental fish with resistance to gentamicin, cephalothin, cefoxitin, cefazidime, cefotaxime, ceftriaxone, nalidixic acid, sulfamethoxazole-
FIGURE 1 | A heatmap supported by a dendrogram for colistin-resistant Gram-negative isolates (n = 47) from bovine mastitis and raw milk showing their antimicrobial resistance phenotypes and the distribution of mcr genes. Mcr variant, source, and species are color-coded feature categories. AM, ampicillin; AMC, amoxicillin-clavulanic acid; TPZ, piperacillin-tazobactam; CFZ, cefazolin; CEF, cephalothin; FOX, cefoxitin; CTX, cefotaxime; CRO, ceftizoxime; FEB, cefepime; IPM, imipenem; CN, gentamicin; TOB, tobramycin; AK, amikacin; NA, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; GST, colistin; TET, tetracycline; TGC, tigecycline; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; ATM, aztreonam; FOF, fosfomycin.

FIGURE 2 | A phylogenetic tree shows the genetic relationship between mcr gene sequences of colistin-resistant Gram-negative bacteria recovered from clinical, subclinical mastitis, and raw milk. The tree was constructed using a neighbor-joining method with a bootstrap value for 1,000 replicates.
TABLE 3 | Conjugation efficiencies of some mcr-1-, mcr-2-, mcr-3-, mcr-4-, and mcr-7-positive isolates and colistin MIC values for transconjugants.

| Bacterial species | Code No. | mcr gene | Transferability | Transconjugant MIC (µg/ml)* | Recipient CFU/ml | Donor CFU/ml | Transconjugant CFU/ml | Conjugation efficiency |
|-------------------|----------|----------|-----------------|-------------------------------|------------------|-------------|-----------------------|------------------------|
| E. coli           | 9        | mcr-1    | +               | 16                           | 2.62 × 10^8      | (3.14 ± 0.548) × 10^6 | (2.31 ± 0.142) × 10^3 | (7.36 ± 2.101) × 10^-3 |
| K. pneumoniae     | 26       | mcr-1    | +               | 32                           | (4.63 ± 0.856) × 10^6 | (1.36 ± 0.211) × 10^3 | (2.94 ± 0.621) × 10^-4 |
| P. aeruginosa     | 37       | mcr-1    | +               | 64                           | (7.12 ± 0.891) × 10^7 | (5.70 ± 1.224) × 10^2 | (8.01 ± 2.663) × 10^-6 |
| A. hydrophila     | 41       | mcr-1    | +               | 64                           | (5.53 ± 1.024) × 10^4 | (3.24 ± 0.882) × 10^2 | (5.86 ± 1.174) × 10^-3 |
| E. coli           | 10       | mcr-2    | +               | 16                           | (1.69 ± 0.442) × 10^6 | (7.01 ± 1.663) × 10^2 | (4.15 ± 1.111) × 10^-3 |
| K. pneumoniae     | 21       | mcr-2    | +               | 8                            | (5.46 ± 1.105) × 10^6 | (4.06 ± 1.022) × 10^2 | (7.47 ± 2.337) × 10^-4 |
| P. aeruginosa     | 31       | mcr-2    | +               | 32                           | (3.91 ± 0.482) × 10^6 | (4.11 ± 1.121) × 10^2 | (1.05 ± 0.184) × 10^-4 |
| A. hydrophila     | 40       | mcr-2    | +               | 16                           | (7.23 ± 1.335) × 10^5 | (2.64 ± 0.451) × 10^2 | (3.65 ± 0.326) × 10^-4 |
| E. coli           | 3        | mcr-3    | +               | 8                            | (3.61 ± 0.710) × 10^6 | (3.43 ± 0.352) × 10^2 | (9.50 ± 2.663) × 10^-3 |
| K. pneumoniae     | 27       | mcr-3    | +               | 4                            | (6.40 ± 0.633) × 10^6 | (4.26 ± 0.222) × 10^2 | (6.66 ± 1.812) × 10^-3 |
| P. aeruginosa     | 34       | mcr-3    | +               | 32                           | (3.12 ± 0.223) × 10^7 | (6.47 ± 1.362) × 10^2 | (2.07 ± 0.634) × 10^-4 |
| A. hydrophila     | 42       | mcr-3    | +               | 32                           | (6.46 ± 0.384) × 10^6 | (2.23 ± 0.331) × 10^2 | (3.45 ± 1.125) × 10^-5 |
| E. coli           | 7        | mcr-4    | +               | 16                           | (4.15 ± 0.361) × 10^6 | (1.81 ± 0.114) × 10^2 | (4.36 ± 1.632) × 10^-4 |
| P. aeruginosa     | 30       | mcr-7    | +               | 64                           | (5.46 ± 0.966) × 10^7 | (1.30 ± 0.314) × 10^2 | (2.01 ± 0.816) × 10^-6 |

*Colistin MIC value = 0.5 µg/ml.
Conjugation efficiency = number of transconjugants/number of donor cells.
CFU, colony-forming unit.
CFU values were retrieved from duplicate measurements from two independent experiments.

FIGURE 3 | Transconjugation frequencies of 14 mcr-harboring Gram-negative isolates from bovine milk. The data are presented as mean values ± standard deviations. Code numbers of isolates are shown in Table 2.
trimethoprim, aztreonam, and chloramphenicol (Saengsittisak et al., 2020). Colistin-resistant isolates found in this study exhibited extreme emergence and spread of antimicrobial resistance and high virulence as well.

It is almost established that *E. coli* is deemed to be the topmost bacteria for the *mcr* genes by carrying *mcr-1* up to *mcr-5* with the chance for coexistence of more than one *mcr* gene (Yin et al., 2017; Hammerl et al., 2018). Similarly, colistin-resistant *E. coli* isolates in our study were found to carry *mcr-1*, *mcr-2*, *mcr-3*, or *mcr-4* genes; 20% out of which were from raw milk and carried *mcr-1*, *mcr-2*, or *mcr-3* genes. It is the first detection of *mcr*-carrying isolates in raw milk samples. A previous study has identified *mcr-1* gene in one *E. coli* isolate from a cow suffering from subclinical mastitis in Egypt (Khalifa et al., 2016). Although the *mcr-1* gene was detected frequently from different samples (Khalifa et al., 2016; Lima Barbieri et al., 2017; Ramadan et al., 2021), it is foremost found here in the raw milk samples. Marvelously, one *E. coli* isolate carrying *mcr-1* gene obtained from hard cheese made from raw milk was reported in Egypt very lately (Ombarak et al., 2021), which in turn lightens the from hard cheese made from raw milk was reported in Egypt. It is almost agreed that the importance of raw milk quality control checks in Egypt, focusing on farm workers as well as the animal itself.

The prevalence of *mcr-3* gene in our isolated *E. coli* was the dominant one, followed by the *mcr-2* gene. Only one *E. coli* isolate was *mcr-4* positive. However, in this study, the *mcr-1*, *mcr-2*, and *mcr-3* genes were found in *E. coli* isolated from raw milk as well as clinical and subclinical mastitis samples; however, the only *mcr-4*-positive *E. coli* was isolated from a clinical mastitis sample. The variants *mcr-2*, *mcr-3*, and *mcr-4* were identified formerly in *E. coli* isolates from Belgium, China, and Italy, respectively, and were isolated from calves and pigs (Xavier et al., 2016; Carattoli et al., 2017; Yin et al., 2017). All these data confirmed that *E. coli* is the chief bacteria carrying *mcr* genes from different samples and in different geographical areas.

Notably, all our colistin-resistant *A. hydrophila* were isolated from raw milk samples, and the predominant gene in them was *mcr-1* followed by *mcr-2* and *mcr-3*. In contrast, colistin-resistant *A. hydrophila* were found to harbor *mcr-7* gene in cooked and raw meats and in environmental samples (Liu Y. et al., 2020). No previous reports until now showed the isolation of colistin-resistant *A. hydrophila* from raw milk samples. Our findings warrant that the source of raw milk contamination is most probably the farm workers rather than the animal source, particularly, that *mcr-3* gene was previously identified in isolates from human rectal swabs (Shen et al., 2018).

Of the identified colistin-resistant isolates, 19.12% were *K. pneumoniae* and were carrying *mcr-1*, *mcr-2*, or *mcr-3* genes. The three variants were identified in the isolates from raw milk samples, while *mcr-1* was found in subclinical mastitis isolates and *mcr-2* in both isolates from clinical and subclinical mastitis. It is almost agreed that the *mcr-1* gene is one of the few and clear examples of animal origin of a resistance trait that could hit the entire human health system (Poirel and Nordmann, 2016). Appealingly, three *mcr-1*-positive *K. pneumoniae* isolates were isolated from raw milk samples ensuring the role of humans in contamination and the transmission from animals to humans.

On the other hand, no colistin-resistant *P. aeruginosa* were found in the raw milk samples. They were isolated only from clinical and subclinical mastitis and were carrying *mcr-1*, *mcr-2*, *mcr-3*, or *mcr-7* genes. Although, we detected 37.5% and 12.5% of the colistin-resistant *P. aeruginosa* isolates carrying *mcr-1* and *mcr-2* genes; respectively, it was found lately in Egypt that colistin-resistant *P. aeruginosa* were negative for *mcr-2* gene and 50% were positive for *mcr-1* (Abd El-Baky et al., 2020). We also found that the *mcr-3* variant was prevalent with 37.5% among our colistin-resistant *P. aeruginosa* isolates. Even, to our knowledge, there is no report detected on *mcr-3* in *P. aeruginosa* isolates before. Moreover, this is the first-time reporting *mcr-7* from Egyptian isolates, and it was identified in *P. aeruginosa* from clinical mastitic milk. The first plasmid-mediated colistin resistance gene *mcr-7* was detected in *K. pneumoniae* from chickens in China (Yang et al., 2018). Recently, the *mcr-7* gene was detected in a water sample from an environment of an alligator (dos Santos et al., 2020). The detection of *mcr-7* in our isolate from clinical mastitis confirms the dissemination of *mcr* gene and its variants at all levels; humans, animals, and environmental. This was confirmed here while application of the conjugation assay as the plasmid harboring *mcr* genes (*mcr-1* to *mcr-4*, and *mcr-7*) successfully transferred colistin resistance to the recipient strain through a broth-mating assay, representing their capability to mobilize the *mcr*-genes between isolates (Migura-Garcia et al., 2020).

The virulome of our colistin-resistant isolates was screened in each species. Many of the identified virulence genes in our study were required for bacterial survival and had a role in causing mastitis (Tartor and El-Naenaey, 2016; Gao et al., 2019; Guerra et al., 2020). This in turn raises the alarm that most of the colistin-resistant Gram-negative isolates are highly pathogenic. Here, the establishment of an interplay between antimicrobial resistance and virulence traits corresponded to each analyzed Gram-negative bacteria, revealing positive correlations between the existence of certain virulence genes and resistance to antimicrobial agents/genes. A previous study suggested that the mobile genetic elements of proficient pathogens favor the coselection of both resistance and virulence-associated traits (Hennequin and Robin, 2016).

The virulome, as well as the resistome patterns of our colistin-resistant isolates, confirmed the elevated pathogenicity and the widespread antibiotic resistance that correlated to these isolates. The phylogenetic analysis of the nucleotide sequences of our colistin resistance *mcr* variants showed three distinguishable clusters. Interestingly, each cluster has the same *mcr* variant, and this is in accordance with what was reported before about *mcr-1*, *mcr-2*, and *mcr-3* (Zhang et al., 2018). The presence of each variant of the *mcr* gene in the same clonal lineage despite the different sources of isolation confirms the high spread of colistin resistance from one source to another. Each of the *mcr-4* and *mcr-7* variants was shown in a unique cluster. This was unsurprising since our study showed the first report of *mcr-7* in *P. aeruginosa*. Similarly, *mcr-4* was previously found in a unique phylogenetic group (Zhang et al., 2019). Our findings proved that each *mcr* gene has a distinct evolution and spread path producing a characteristic phenotypic colistin resistance.
All the above information has raised the alert for controlling the spread of colistin-resistant isolates. Thus, we decided to evaluate four commonly used biocides in the dairy farms against colistin-resistant isolates. In accordance with Lanjri et al. (2017), all isolates were sensitive to chlorhexidine with different concentrations. This is attributed to its cationic nature that helps in electrostatic interaction with the anionic group lipopolysaccharide in the cytoplasmic membrane of Gram-negative bacteria, resulting in modification of the membrane permeability and causing membrane disruption and cell death due to coagulation of the cytoplasm (Estrela et al., 2003). Another tested biocide was iodine; it showed an inhibitory effect on *E. coli* and *K. pneumoniae* at high concentrations and no inhibition effect on *P. aeruginosa* and *A. hydrophila* at all tested concentrations. Iodine is proven to be considered a broad-spectrum, rapidly acting bactericidal, and effective against all mastitis-causing bacteria (Gleeson et al., 2009). It acts through its penetration into the proteins, nucleotides, and fatty acids of microorganisms causing cell death (Gupta et al., 2018), which makes the development of resistance unlikely. However, in the field, the presence of some residues in the environment like the organic matter of the teat skin or in the challenge suspension may have been reacting with the disinfectant and thus less germicidal activity may have been produced (Enger et al., 2015). These conditions permit some bacteria to be exposed too much to lower concentrations of the active ingredients consequently and thus increasing their resistance to the used disinfectants (Wales and Davies, 2015). These results were in agreement with what was reported earlier that a dip containing the highest free iodine provided a greater significantly superior reduction of pathogens compared with a lower concentration of iodine dip (Foret et al., 2005). Furthermore, all isolates showed a sensitivity to H$_2$O$_2$, and this had been confirmed previously. H$_2$O$_2$ produces oxidants as singlet oxygen, superoxide radicals, and hydroxyl radicals with high penetration power and not specific in the points of contact by attacking bacteria at any essential cell components, leading to a decrease in the probability of bacteria to create resistance with these compounds (Nilima, 2011; Benavides, 2014). This was in contrast with Enger et al. (2015) who reported that H$_2$O$_2$ was the least efficacious tested teat dip, and these differences may be due to the evolution of resistance by the gradual use of the disinfectant (Wales and Davies, 2015). However, different concentrations of ethanol showed no antimicrobial activity against all tested bacteria, and this is in agreement with what was proved before about ethanol effectiveness (Al-Dabbagh et al., 2015). This ethanol tolerance mainly appears to result in large part from adaptive and evolutionary changes in cell membrane composition (Ingram, 1990).

**REFERENCES**

Abd El-Baky, R. M., Masoud, S. M., Mohamed, D. S., Waly, N. G., Shafik, E. A., Mohareb, D. A., et al. (2020). Prevalence and Some Possible Mechanisms of Colistin Resistance Among Multidrug-Resistant and Extensively Drug-Resistant *Pseudomonas aeruginosa*. *Infet. Drug Resist.*, 13, 323–332. doi: 10.2147/IDR.S238811

Adzitey, F., Asante, J., Kumalo, H. M., Khan, R. E., Somboro, A. M., and Amoako, D. G. (2020). Genomic Investigation Intothe Virulome, Pathogenicity, Stress Response Factors, Clonal Lineages, and Phylogenetic Relationship of *Escherichia coli* Strains Isolated from Meat Sources in Ghana. *Genes* 11 (12), 1504. doi: 10.3390/genes11121504

Al-Dabbagh, S. Y. A., Ali, H. H., Khalil, I. L., and Hamada, M. (2015). A Study of Some Antibiotics; Disinfectants and Antiseptics Efficacy Against Some Species of Pathogenic Bacteria. *Assiut Vet. Med. J.* 61 (147), 210–217.

Anyanwu, M. U., Jaja, I. F., and Nwobi, O. C. (2020). Occurrence and Characteristics of Mobile Colistin Resistance (*mcr*) Gene-Containing Isolates *P. aeruginosa* and *A. hydrophila*. *Infect. Drug Resist.* 13, 323–332. doi: 10.2147/IDR.S238811

Conversely, all isolates were sensitive to chlorhexidine with different concentrations. This is attributed to its cationic nature that helps in electrostatic interaction with the anionic group lipopolysaccharide in the cytoplasmic membrane of Gram-negative bacteria, resulting in modification of the membrane permeability and causing membrane disruption and cell death due to coagulation of the cytoplasm (Estrela et al., 2003). Another tested biocide was iodine; it showed an inhibitory effect on *E. coli* and *K. pneumoniae* at high concentrations and no inhibition effect on *P. aeruginosa* and *A. hydrophila* at all tested concentrations. Iodine is proven to be considered a broad-spectrum, rapidly acting bactericidal, and effective against all mastitis-causing bacteria (Gleeson et al., 2009). It acts through its penetration into the proteins, nucleotides, and fatty acids of microorganisms causing cell death (Gupta et al., 2018), which makes the development of resistance unlikely. However, in the field, the presence of some residues in the environment like the organic matter of the teat skin or in the challenge suspension may have been reacting with the disinfectant and thus less germicidal activity may have been produced (Enger et al., 2015). These conditions permit some bacteria to be exposed too much to lower concentrations of the active ingredients consequently and thus increasing their resistance to the used disinfectants (Wales and Davies, 2015). These results were in agreement with what was reported earlier that a dip containing the highest free iodine provided a greater significantly superior reduction of pathogens compared with a lower concentration of iodine dip (Foret et al., 2005). Furthermore, all isolates showed a sensitivity to H$_2$O$_2$, and this had been confirmed previously. H$_2$O$_2$ produces oxidants as singlet oxygen, superoxide radicals, and hydroxyl radicals with high penetration power and not specific in the points of contact by attacking bacteria at any essential cell components, leading to a decrease in the probability of bacteria to create resistance with these compounds (Nilima, 2011; Benavides, 2014). This was in contrast with Enger et al. (2015) who reported that H$_2$O$_2$ was the least efficacious tested teat dip, and these differences may be due to the evolution of resistance by the gradual use of the disinfectant (Wales and Davies, 2015). However, different concentrations of ethanol showed no antimicrobial activity against all tested bacteria, and this is in agreement with what was proved before about ethanol effectiveness (Al-Dabbagh et al., 2015). This ethanol tolerance mainly appears to result in large part from adaptive and evolutionary changes in cell membrane composition (Ingram, 1990).

**CONCLUSION**

Ultimately, the spread of mobile *mcr* genes between MDR and XDR virulent Gram-negative isolates from dairy cattle confirms the dissemination of the *mcr* gene and its variants at all levels: human, animal, and environmental. Consequently, a decision to stop using colistin in food animals is a principal demand to defy XDR and MDR emergence. Additionally, establishing a perfect quality control check system in Egypt, focusing on both farm workers and the animal itself is urgent.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

YT and NA contributed equally in the conception and design of the study and participated with RG, HE, EK, and AA in the application of classical microbiological techniques. YT and NA carried out the PCR assays, sequencing approaches, and participated with HR in sequence analysis. HR performed the bioinformatics and statistical analyses of the data. RG, HE, SE, EK, AA, SA, and HR conceived the study and participated in the design. YT, RG, NA, HE, SE, and AA wrote the initial draft of the manuscript. All authors revised the manuscript critically for important intellectual content and gave the final approval of the version to be published.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.761417/full#supplementary-material

**Supplementary Figure S1** | A Venn diagram showing the intersection between the *mcr* variants of colistin-resistant isolates from clinical, subclinical mastitis, and raw milk.

**Supplementary Figure S2** | | Correlation plot between antimicrobial resistance phenotypes, colistin resistance genes (*mcr*) and virulence genes in *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. hydrophila*. In each plot, each cell represents the *P* value (correlation coefficient) for each pair of features. The color and circle size indicate the value of correlation (red = negative and blue = positive correlation). The more intense the color, the higher the correlation value. Stars referring to significant correlation determined at a cutoff = 0.05. Insignificant correlations are left without stars.

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Positive (Colistin-Resistant), Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* Clone on A Greek Dairy Farm. *J. Dairy Sci.* 103 (1), 852–858.

Forét, C. J., Corbellini, C., Young, S., and Janowicz, P. (2005). Efficacy of Two Iodine Teat Dips Based on Reduction of Naturally Occurring New Listermannary Infections. *J. Dairy Sci.* 88 (1), 426–432. doi: 10.3168/jds.s0022-0302(05)72704-1

Furmank-Blaszak, B. (2014). Phenotypic and Molecular Characteristics of an *Aeromonas hydrophila* Strain Isolated From the River Nile. *Microb. Res.* 169 (7–8), 557–562. doi: 10.1016/j.microres.2013.11.001

Gao, J., Barkema, H. W., Zhang, L., Liu, G., Deng, Z., Cai, L., et al. (2017). Incidence of Clinical Mastitis and Distribution of Pathogens on Large Chinese Dairy Farms. *J. Dairy Sci.* 100 (6), 4797–4806. doi: 10.3168/jds.2016-12334

Gao, J., Li, S., Zhang, J., Zhou, Y., Xu, S., Barkema, H. W., et al. (2019). Prevalence of Potential Virulence Genes in *Klebsiella* spp. Isolated From Cows With Clinical Mastitis on Large Chinese Dairy Farms. *Foodborne Pathog. Dis.* 16 (12), 856–863. doi: 10.1089/fpd.2019.2657

Garica, V., Garcia-Menlo, I., Mora, A., Flament-Simon, S. C., Diaz-Jimenez, D., Blanco, J. E., et al. (2018). Co-Occurrence of Mcr-1, Mcr-4 and Mcr-5 Genes in Multidrug-Resistant ST10 Enterotoxigenic and Shiga Toxin-Producing *Escherichia coli* in Spain, (2006-2017). *J. Antimicrob. Agents* 52 (1), 104–108. doi: 10.1093/jiai/lijantimicag.2018.03.022

Garcia, S. N., Osbourn, B. L., and Cullor, J. S. (2019). A One Health Perspective on Dairy Production and Dairy Food Safety. *One Heal.* 7, 100086. doi: 10.1016/j.oneh.2019.100086

Gleeson, D., O’Brien, B., Flynn, J., O’Callaghan, E., and Galli, F. (2009). Effect of Pre-Milk Teat Preparation Procedures on the Microbial Count on Teats Prior to Clusters Application. *Ir. Vet. J.* 62 (7), 461–467. doi: 10.1186/2046-0481-62-7-461

Goli, H. R., Naehei, M. R., Rezaei, M. A., Hasani, A., Kafi, H. S., and Aghazadeh, M. (2016). Emergence of Colistin Resistant *Pseudomonas aeruginosa* at Tabriz Hospitals, Iran. *Iran J. Microbiol.* 8 (1), 62–69.

Guerra, S. T., Orsi, H., Joaquim, S. F., Guimarães, F. F., Lopes, B. C., Dalanezi, F. M., et al. (2020). Short Communication: Investigation of Extra-Intestinal Pathogenic *Escherichia coli* Virulence Genes, Bacterial Motility, and Multidrug Resistance Pattern of Strains Isolated From Dairy Cows With Different Severity Scores of Clinical Mastitis. *J. Dairy Sci.* 103 (4), 3606–3614. doi: 10.3168/jds.2019-17477

Gupta, P., Bhatia, M., Gupta, P., and Omar, B. (2018). Emerging Biocide Resistance Among Multidrug-Resistant Bacteria: Myth or Reality? A Pilot Study. *J. Pharm. Biomed. Sci.* 10 (2), 96–101. doi: 10.4103/JPBS.JPBS_24_18

Hammad, A. M., Hoffmann, M., Gonzalez-Escalona, N., Abbas, N. H., Yao, K., Koenig, S., et al. (2019). Genomic Features of Colistin Resistant *Escherichia coli* ST09 Strain Harboring Mcr-1 on IncHII Plasmid From Raw Milk Cheese in Egypt. *Infect. Genet. Evol.* 73, 126–131. doi: 10.1016/j.meegid.2019.04.021

Hammerl, J. A., Borowiak, M., Schmoger, S., Shamoun, D., Grobbel, M., Malorny, B., et al. (2018). Mcr-5 and a Novel Mcr-5-2 Variant in *Escherichia coli* Isolates From Food and Food-Producing Animals, Germany 2010 to 2017. *J. Antimicrob. Chemother.* 73 (5), 1433–1435. doi: 10.1093/jac/dky020

Hassen, B., Saloua, B., Abbassi, M. S., Ruiz-Ripa, L., Mama, O. M., Hassen, A., et al. (2019). Mcr-1 Encoding Colistin Resistance in CTX-M-1/CTXM-15 Producing *Escherichia coli* Isolates of Bovine and Caprine Origins in Tunisia. First Report of CTX-M-15 ST394/ D. E. coli from Goats. *Comp. Immunol. Microbiol. Infect. Dis.* 67, 101366. doi: 10.1016/j.cimid.2019.101366

Hennequin, C., and Robin, F. (2016). Correlation Between Antimicrobial Resistance and Virulence in *Klebsiella pneumoniae*. *Eur. J. Clin. Microbiol.* Infect. Dis. 35 (3), 333–341. doi: 10.1007/s10027-015-2559-7

Hmede, Z., and Kassem, I. I. (2019). First Report of the Plasmid-Borne Colistin Resistance Gene (Mcr-1) in Proteus Mirabilis Isolated From a Toddler in Non-Clinical Settings. *IDCases* 18, e00651. doi: 10.1016/j.idcases.2019.e00651

Hughes, D., and Andersson, D. I. (2012). Selection of Resistance at Lethal and Non-Lethal Antibiotic Concentrations. *Crit. Rev. Microbiol.* 38 (4), 305–319. doi: 10.3109/00922705.2012.669734

Javed, H., Saleem, S., Zafar, A., Ghafari, A., Shazad, A. B., Fiaz, H., et al. (2020). Emergence of Plasmid-Mediated Mcr Genes From Gram-Negative Bacteria At The Human-Animal Interface. *Gut Pathog.* 12 (1), 1–9. doi: 10.1186/s13099-020-00392-3
Savin, M., Bierbaum, G., Blau, K., Parcina, M., Sib, E., Smalla, K., et al. (2020). Colistin-Resistant Enterobacteriaceae Isolated From Process Waters and Wastewater From German Poultry and Pig Slaughterhouses. Front. Microbiol. 11, 575391. doi: 10.3389/FMICB.2020.575391

Scamburlo, T. M., Yamazi, A. K., Cavicchioli, V. Q., Pieri, F. A., and Nero, L. A. (2015). Spoilage Potential of Pseudomonas Species Isolated From Goat Milk. J. Dairy Sci. 98 (2), 759–764. doi: 10.3168/JDS.2014-8747

Sharma, I., and Kumar, A. (2011). Occurrence of Enterotoxigenic Aeromonas Species in Foods of Animal Origin in North East India. Eur. Rev. Med. Pharmacol. Sci. 15 (8), 883–887.

Shen, Y., Xu, C., Sun, Q., Schwarz, S., Ou, Y., Yang, L., et al. (2018). Prevalence and Genetic Analysis of Mcr-3-Positive Aeromonas Species From Humans, Retail Meat, and Environmental Water Samples. Antimicrob. Agents Chemother. 62 (9), e04094–e04098. doi: 10.1128/AAC.04094-18

Singh, A., Chhabra, D., Sikrodia, R., Shukla, S., Sharda, R., and Audarya, S. (2018). Isolation of E. coli From Bovine Mastitis and Their Antibiotic Sensitivity Pattern. Int. J. Curr. Microbiol. Appl. Sci. 7 (10), 11–18. doi: 10.20546/ijcmas.2018.710.002

Soliman, A. M., Ramadan, H., Zarad, H., Sugawara, Y., Yu, L., Sugai, M., et al. (2021). Co-Production of Tet(X7) Conferring High-Level Tigecycline Resistance, Fosfomycin FosA4 and Colistin Mcr-1.1 in Escherichia coli Strains From Chickens in Egypt. Antimicrob. Agents Chemother. 65 (6), e02084–e02020. doi: 10.1128/AAC.02084-20

Tambekar, D., Dhanorkar, D., Gulhane, S., Khandelwal, V., and Dudhane, M. (2006). Antibacterial Susceptibility of Some Urinary Tract Pathogens to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. Afr. J. Biotechnol. 5 (17), 1562–1565.

Tartor, Y. H., and El-Naenaeey, E.-S. Y. (2016). RT-PCR Detection of Exotoxin Genes Expression in Multidrug Resistant Pseudomonas aeruginosa. Cell Mol. Biol. (Noisy-le-grand) 62, 56–62.

Tiwari, J. (2013). Trends In Therapeutic and Prevention Strategies for Management of Bovine Mastitis: An Overview. J. Vaccines Vaccin. 4, 176. doi: 10.4172/2157-7560.1000176

Wales, A. D., and Davies, R. H. (2015). Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. Antibiotics 4 (4), 567–604. doi: 10.3390/antibiotics4040567

Yang, Y. Q., Li, Y. X., Lei, C. W., Zhang, A. Y., and Wang, H. N. (2018). Novel Plasmid-Mediated Colistin Resistance Gene Mcr-2 in Escherichia coli, Belgium. Eurosurveillance (Belgium) 21 (27). doi: 10.2807/1560-7917.ES.2016.21.27.30280

Ye, M., Tu, J., Jiang, J., Bi, Y., You, W., Zhang, Y., et al. (2016). Clinical and Genomic Analysis of Liver Abscess-Causing Klebsiella pneumoniae Identifies New Liver Abscess-Associated Virulence Genes. Front. Cell. Infect. Microbiol. 6, 165. doi: 10.3389/FICMB.2016.00165

Zdoicic, N., Dobranic, V., Butkovic, I., Koturic, A., Filipovic, I., and Medvid, V. (2016). Antimicrobial Susceptibility of Milk Bacteria From Healthy and Antibacterial Susceptibility of Milk Bacteria From Healthy and Drug-Treated Cow Udder. Vet. Arch. 86 (2), 163–172.

World Health Organization (2013). Report on the Consultative Meeting on Antimicrobial Resistance for Countries in the Eastern Mediterranean Region: From Policies to Action Sharm El Sheikh, Egypt. Available at: http://apps.who.int/iris/handle/10665/11

World Health Organization (2019). WHO List of Critically Important Antimicrobials for Human Medicine (WHO CIA List) (World Health Organization). Available at: https://apps.who.int/iris/handle/10665/325036.

Xavier, B. B., Lammens, C., Ruhl, R., Malhotra-Kumar, S., Butaye, P., Goossens, H., et al. (2016). Identification of a Novel Plasmid-Mediated Colistin Resistance Gene, Mcr-2, in Escherichia coli, Belgium. Eurosurveillance (Belgium) 21 (27). doi: 10.2807/1560-7917.ES.2016.21.27.30280

Yang, Y. Q., Li, Y. X., Lei, C. W., Zhang, A. Y., and Wang, H. N. (2018). Novel Plasmid-Mediated Colistin Resistance Gene Mcr-7.1 in Klebsiella pneumoniae. J. Antimicrob. Chemother. 73 (7), 1791–1795. doi: 10.1093/jac/dky111

Zhang, J., Chen, L., Yassin, A. K., Butaye, P., Kelly, P., et al. (2018). Molecular Detection of Colistin Resistance Genes (mcr-1, mcr-2 and mcr-3) in Nasal/oropharyngeal and Anal/cloacal Swabs From Pigs and Poultry. Sci. Rep. 8 (1), 3705. doi: 10.1038/s41598-018-22084-4

Zhang, H., Hou, M., Xu, Y., Sririnas, S., Huang, M., Liu, L., et al. (2019). Action and Mechanism of the Colistin Resistance Enzyme Mcr-4. Commun. Biol. 2 (1), 1–14. doi: 10.1038/s42003-018-0278-1

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