Characterization of Enterococci- and ESBL-Producing Escherichia coli Isolated from Milk of Bovides with Mastitis in Egypt

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Abstract: This study aimed to investigate the prevalence and antimicrobial resistance of enterococci- and ESBL-producing E. coli isolated from milk of bovine mastitis cases in Egypt. Fifty milk samples of dairy animals were collected from localities in the Nile Delta region of Egypt. Isolates were identified using MALDI-TOF MS, and antibiotic susceptibility testing was performed by the broth microdilution method. PCR amplifications were carried out, targeting resistance-associated genes. Seventeen Enterococcus isolates and eight coliform isolates could be cultivated. Vancomycin resistance rate was high in Ent. faecalis. The VITEK 2 system confirmed all E. coli isolates as ESBL-producing. All Ent. faecalis isolates harbored erm(B), tetL and aac-aphD genes. The vanA gene was detected in Ent. faecalis isolate, vanB was found in other Enterococcus, while one isolate of E. casseliflavus exhibited the vanA gene. E. coli isolates exhibited high prevalence of erm(B) and tetL. E. coli isolates were analyzed by DNA microarray analysis. Four isolates were determined by O-serotyping as O8 (n = 1), O86 (n = 2) and O157 (n = 1). H-serotyping resulted in H11, H12, H21 (two isolates each) and one was of bovine mastitis [3]. The most important coliform pathogen is Escherichia (E.) coli that causes mastitis with high incidence in comparison to other coliforms. Especially, strain type O157:H7 is of great importance because of its zoonotic character [4].

Keywords: enterococci; Escherichia coli; resistance gene; DNA microarray; mastitis; Egypt

1. Introduction

Bovine mastitis is the most common and costly disease affecting dairy cattle throughout the world [1]. It leads to a reduction of the amount and quality of milk produced, increasing of veterinary costs and culling of severely infected animals [2].

Coliform bacteria are the most common pathogens that have been isolated from bovine mastitis [3]. The most important coliform pathogen is Escherichia (E.) coli that causes mastitis with high incidence in comparison to other coliforms. Especially, strain type O157:H7 is of great importance because of its zoonotic character [4].

Beside mastitis, E. coli is the most common pathogen responsible for various severe gastrointestinal or urinary tract infections, and even bacteremia in humans, causing thousands of deaths worldwide every year. Emergence of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, especially E. coli, has been increased over the last decades. Possibly, a cause is the usage of cephalosporins as preferred agents to avoid mastitis-associated economic losses in dairy cattle [5]. High prevalence of ESBL-producing
E. coli in animals has been demonstrated in many studies and varies between countries and animal species [6].

Enterococci are one of the environmental causative agents of mastitis. These opportunistic bacteria are part of normal physiological gut flora in humans and animals, but over the last years, they have become one of the main pathogens causing numerous infections in humans, mainly those hospital-acquired, such as bacteremia and infections of the urinary tract, skin, soft tissue, abdomen and pelvis and central nervous system. These infections are caused mainly by Enterococcus (Ent.) faecalis (about 80.0%) and Ent. faecium (10.0–15.0%). The high tolerance of enterococci to disadvantageous conditions allows their long survival in the environment, including in abattoirs [7]. In addition to their importance in causing diseases, enterococci can evolve resistance to antibiotics, and additionally to their intrinsic resistance properties. Enterococci may harbor multidrug resistance determinants for antimicrobial agents such as cephalosporins and aminoglycosides [8]. Generally, bacteria can produce antibiotic resistance with remarkably new mechanisms [9]. Resistance genes are often easily transferred to other species through conjugative transposons and plasmids, showing a broad host profile [10].

Due to the little information known about enterococci and E. coli from mastitis in bovines in Egypt, the aim of this study was to investigate the prevalence and antimicrobial resistance of enterococci and ESBL-producing E. coli isolated from milk of bovine mastitis cases in Egypt. The study completes the first part regarding staphylococci and streptococci from milk [11].

2. Materials and Methods

2.1. Sample Collection and Cultivation

The present study was carried out in 2018 and 2019 on 50 milk samples of dairy animals from 50 different localities in Qalyubia and Monufia governorates in the Nile Delta region of Egypt. All dairy cattle and buffalo were local Egyptian breed and kept by smallholders (1–5 animals). They were hand-milked twice daily.

All animals were subjected to clinical examination. Animals with clinical mastitis were defined when one or more of the following signs were observed: cardinal signs of inflammation in one or more of the udder quarters, signs of systemic reaction such as fever, depression and disturbed appetite, and abnormal physical character of milk such as clot formation, discoloration, alterations in viscosity, aberrant smell or presence of blood. Due to the absence of observable clinical signs in animals, the presumptive diagnosis of subclinical mastitis was done based on laboratory diagnostic tests of milk samples, including the California Mastitis Test (CMT).

Milk samples were taken and stored as previously described [11].

Isolation of bacteria from milk samples was carried out as described by the National Mastitis Council [12]. A loopful of milk sample was streaked on blood agar (Oxoid Deutschland GmbH, Wesel, Germany) supplemented with 5% sheep red blood cells and then sub-cultured on selective media: Mannitol Salt Agar, Edwards Medium and Brilliance ESBL Agar (Oxoid Deutschland GmbH) for identification of ESBL-producing microorganisms. All plates were incubated aerobically at 37 °C for 24 h. The plates were examined for colony morphology, pigmentation and hemolytic characteristics after 24 and 48 h.

2.2. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [13]. Briefly, bacteria from overnight cultures were suspended in 300 µL of bi-distilled water and mixed with 900 µL of ethanol (96% vol/vol; Carl Roth GmbH, Karlsruhe, Germany) for precipitation. After centrifugation for 5 min at 10,000 × g, the supernatant was removed, and the pellet was re-suspended in 50 µL of 70% (vol/vol) formic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Fifty microliters of acetonitrile (Carl Roth GmbH) were added, mixed and centrifuged for
5 min at 10,000× g. One and a half microliters of the supernatant were transferred onto a MTP 384 Target Plate Polished Steel TF (Bruker Daltonik GmbH, Bremen, Germany). After air-drying, the material was overlaid with 2 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie GmbH) in a mix of 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich Chemie GmbH). After air-drying, spectra were acquired with an Ultraflex instrument (Bruker Daltonik GmbH). The instrument was calibrated with the IVD Bacterial Test Standard (Bruker Daltonik GmbH). Analysis was carried out with the Biotype 3.1 software (Bruker Daltonik GmbH). Interpretation of results was performed according to the manufacturer’s recommendation: score of ≥ 2.3 represented reliable species level identification, score 2.0–2.29, probable species level identification, score 1.7–1.9, probable genus level identification, and score ≤ 1.7 was considered an unreliable identification.

2.3. Antibiotic Susceptibility Testing using Broth Microdilution

The antibiotic susceptibility testing of all Enterococcus isolates was performed with the MICRONAUT system for Gram-positive bacteria (MICRONAUT-S MRSA/GP; Merlin, Bornheim, Germany) according to the manufacturer’s recommendations. It allowed the determination of minimum inhibitory concentrations (MICs) of 22 antimicrobial agents, including ampicillin (β-lactam), cefoxitin (β-lactam; cephamycin), ceftaroline (cephalosporin 5th generation), clindamycin (lincosamide), daptomycin (cyclic lipopeptide), erythromycin (macrolide), erythromycin/clindamycin, fosfomycin (epoxide antibiotic), fusidic acid (steroid antibiotic), gentamicin (aminoglycoside), linezolid (oxazolidinone), moxifloxacin (fluoro- nolone 4th generation), mupirocin, oxacillin (β-lactam), penicillin G (β-lactam), rifampicin (ansamycine), streptomycin (streptogramine), tetracycline (tetracycline), trimethoprim/sulphamethoxazole (trimethoxybenzyl pyrimidine/sulfonamide) and vancomycin (glycopeptide).

The MICRONAUT-S FLI MHK plates allowed the determination of MICs for E. coli against 14 antimicrobial agents including AMK (amikacin), AMC (amoxicillin/clavulanic acid), CAZ (ceftazidim), CMP (chloramphenicol), CIP (ciprofloxacin), ERY (erythromycin), GEN (gentamicin), IMP (imipenem), LEV (levofloxacin), PEN (penicillin G), RAM (rifampicin), STR (streptomycin), TET (tetracycline) and T/S (trimethoprim/sulphamethoxazole) in serial dilutions of the antibiotics.

Overnight grown bacteria were suspended in NaCl solution (0.9%) to obtain a turbidity corresponding to a McFarland standard of 0.5 (Dr. Lange, CADAS photometer 30, Berlin, Germany). Three hundred microliters of the suspension were diluted with 11 mL of Mueller–Hinton broth (Oxoid Deutschland GmbH), resulting in a concentration of approximately 10^6–10^7 colony forming units (cfu)/mL. In total, 100 µL of the inoculum were given in each well of the plate. After sealing the plates, they were incubated for 18 to 24 h at 37 °C. Reading of plates was done optically. Interpretation was carried out as recommended by the Clinical and Laboratory Standards Institute [14].

2.4. Antibiotic Susceptibility Testing using the VITEK 2 System

All E. coli isolates suspected as ESBL producers were subsequently confirmed using an automated microdilution system (VITEK 2, bioMérieux Deutschland GmbH, Nürtingen, Germany) according to the instructions of the manufacturer. For this study, the test card AST-N289 (bioMérieux Deutschland GmbH) was used, which included the following antibiotics: piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEB), aztreonam (ATM), imipenem (IMP), meropenem (MEM), amikacin (AMK), gentamicin (GEN), tobramycin (TOP), ciprofloxacin (CIP), moxifloxacin (MXF), tigecycline (TGC), fosfomycin (FOS), colistin (CT) and trimethoprim/sulfamethoxazole (T/S).
2.5. DNA Extraction and Detection of Resistance-Associated Genes

Genomic DNA was extracted from bacterial cultures using the High Pure PCR Template Purification Kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer.

For *E. coli*, PCR amplifications were carried out for colistin resistance genes (*mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5), erythromycin resistance genes (*erm*(A) and *erm*(B)), macrolide resistance genes (*msr*(C)), aminoglycoside resistance genes (*aac*-*aph*(2)) and tetracycline resistance genes (*tet*K, *tet*L and *tet*M). Primer sequences and target genes are given in Table 1.

### Table 1. Primers and their sequences used for the detection of antibiotic resistance-associated genes in *Enterococcus* species and *Escherichia coli* isolates.

| Antibiotic          | Target Gene | Primer Sequences *(5′–3′)* | Expected Amplicon Size (bp) | Reference |
|---------------------|-------------|-----------------------------|----------------------------|-----------|
| Methicillin/oxacillin | *mec*A      | F: TCC AGA TTA CAA CTT CAC CAG G R: CCA CTT CAT ATC TGG TAA CG | 161 | [15] |
|                     | *mec*B      | F: TTA ACA TAT ACA CCC GCT TG R: TAA AGT TCA TTA GGC ACC ACC TCC | 2263 | [16] |
|                     | *mec*C     | AL3: TCA AAT TGA GTT CCC TTA TCA AL4: AAC TIG GTT ATT CAA AGA TGA CGA | 1931 | [16] |
| Penicillin          | *bla*Z      | F: AAG AGA TTT GCC TAT GCT TC R: GCT TGA CCA CTT TTA TCA GC | 517 | [17] |
| Vancomycin          | *van*A     | F: ATG AAT AGA ATA AAA GGT GTA ATA R: CCC CTT TAA GCA TAA TAC GAT CAA | 1030 | [18] |
|                     | *van*B     | F: AAG GTA TCG AAG AAG CCA TG R: CCG ACA AAA TCA TCC TC | 536 | [18] |
|                     | *van*C1    | F: GGA ATC AAG GAA ACC TC R: CTT CCG CCA TCA TAG CT | 822 | [19] |
| Erythromycin        | *erm*(B)   | F: GAA AAG GTA CTC AAC CAA ATA R: AGT AAC TGT TCA AAT ATT TAC | 639 | [20] |
|                     | *erm*(A)   | F: TAT CTT ATC GCT GAG AAG GTA TT R: CTA CAC TGG GCT TAG GAT GAA A | 138 | [15] |
|                     | *erm*(C)   | F: CTT CTT GAT CAC GAT AAT TTC C R: TTC TTT TAG CAA ACC GCT ATTC | 189 | [15] |
| Macrolide           | *msr*(C)   | F: AAG GAA TCC TTC TTC CTC CCG R: GTA AAC AAA ATC GGT CCC G | 342 | [21] |
| Tetracycline        | *tet*K     | F: TCG ATA GGA ACA GCA GTA R: CAG CAG CAT CTA CTC CTT | 169 | [22] |
|                     | *tet*L     | F: TCG TTA GCG TGC TGT CAT R: GTA TCC CAC CAA TGT AGC CG | 267 | [22] |
|                     | *tet*M     | F: GTG GAC AAA GGT ACA ACG AG R: CGG TAA AGT TCG TCA CAC AC | 406 | [22] |
| Aminoglycoside      | *aac*-*aph*(2) | F: CCA AGA GCA ATA AGG GCA TA R: CAC TAT CAT AAC CAC TAC CG | 219 | [23] |
|                     | *aac*-*aph*(D) | F: TAA TCC AAG AGC AAT AAG GGC R: GGC ACA CTA TCA TAA CCA CTA | 227 | [15] |
| Linezolid, chloramphenicol | *optr*A | F: AAG TGG TCA CGG ACC TCA R: ATC AAC TGT TCC CAT TCA | 1400 | [24] |
| Oxazolidinone       | *cfr*      | F: TGA AGT ATA AAG CAG GTG GGG AGT CA R: ACC ATA TAA TIG ACC ACA AGC AGC | 400 | [24] |
| Lincosamide         | *ltn*D    | F: AGC GAG GGA TCA CAT GGT AA R: TCT CTC GCA TAA TAA TAC TAC TCC | 475 | [25] |
|                     | *ltn*A    | F: GGT GCC TGC GGG GTA GAT GTA TAA ACT CG R: GCT CTC TTT GAA ATA CAT GGT ATT TTT CTA GC | 323 | [26] |
| Colistin            | *mcr*-1   | F: AGT CCG TTT GTT GTG GC R: AGA TCA TGG TGT GTC GCT TGG | 320 | [27] |
|                     | *mcr*-2   | F: CAA GTG TGT TGG CAG AG R: TCT AGC CCG ACA AGC AGA ATC CC | 715 | [27] |
|                     | *mcr*-3   | F: AAA TAA AAA TTTGC CGC TTA TG R: AAT GGA GAT CCC GTG TTT | 929 | [27] |
Table 1. Cont.

| Antibiotic Target | Gene | Primer Sequences (5′-3′) | Expected Amplicon Size (bp) | Reference |
|-------------------|------|--------------------------|----------------------------|-----------|
| mcr-4             | F: TCA CTT TCA TCA CGT TG | R: TTG GTC CAT GAC TAC CAA TG | 1116 | [27] |
| mcr-5             | F: ATG CCG TTG TCT GCA TTT ATC | R: TCA TGG TGG TCC TTT TCT G | 1644 | [28] |

Enterococci PCR amplifications (primers, see Table 1) were done for vancomycin resistance genes (vanA, vanB and vanC1), erythromycin resistance genes (erm(B), erm(A) and erm(C)), penicillin resistance gene (blaZ), linezolid resistance genes (optRA and cfr), macrolide resistance gene (msrC), aminoglycoside resistance genes (aac-aphD), tetracycline resistance genes (tetK, tetM, tetL and tetO) and lincosamide resistance genes (InuA and InuD).

PCR products were analyzed by electrophoresis on 2% agarose gel following staining with ethidium bromide and visualizing under UV.

2.6. GenoSerotyping, Detection of Antibiotic Resistance and Virulence-Associated Genes of E. coli Isolates by Microarray Analysis

Serotypes of E. coli isolates were determined using the E. coli SeroGenoTyping AS-1 Kit (Alere Technologies GmbH, Jena, Germany). Five microliters of extracted RNA-free DNA (with a concentration of at least 100 ng/µL) were biotin-labeled by a primer extension amplification using the E. coli SeroGenoTyping AS-1 Kit according to the manufacturer’s instructions. The procedures for multiplex labeling, hybridization and data analysis were carried out as described in a previous study [29].

Antimicrobial resistance (AMR) genotypes and resistance-associated genes were ascertained using the CarbDetect AS-2 Kit and E. coli PanType AS-2 Kit, respectively (Alere Technologies GmbH). The data were automatically summarized by the “result collector”, a software tool provided by Alere Technologies GmbH. The detection of virulence-associated genes was performed using the E. coli PanType AS-2 Kit. Twenty-eight different gene loci connected with resistance to antibiotics and virulence factors associated with adhesion, fimbriae production, secretion systems, SPATE (serine protease auto-transporters), toxins and miscellaneous genes were detected. Analysis was done as described above.

3. Results
3.1. Bacterial Isolation and Identification by MALDI-TOF MS

In this study, 17 Enterococcus isolates (34.0%) were obtained from 50 milk samples of cattle and buffaloes. Identification by MALDI-TOF MS resulted in Ent. faecalis (n = 13; 26.0%), Ent. casseliflavus (n = 2; 4.0%) and Ent. hirae (n = 2; 4.0%). Additionally, 8 coliform isolates (7 E. coli and one Enterobacter cloacae) could be cultivated. Distribution of isolates from cattle and buffalo is given in Table 2.

Table 2. Prevalence of Enterococcus and coliform isolates in milk samples.

| Origin of Milk | Number of Milk Samples | Enterococcus faecalis | Enterococcus casseliflavus | Enterococcus hirae | Escherichia coli | Enterobacter cloacae |
|----------------|------------------------|-----------------------|---------------------------|-------------------|-----------------|---------------------|
|                |                        | n | %  | n | %  | n | %  | n | %  | n | %  | n | %  |
| Clinical mastitis |                        | 22 | 5 | 22.7 | 1 | 4.5 | 1 | 4.5 | 2 | 9.1 | 0 | 0.0 |
| Cattle         |                        | 10 | 3 | 30.0 | 0 | 0.0 | 1 | 10.0 | 1 | 10.0 | 1 | 10.0 |
| Buffalo        |                        | 5 | 3 | 60.0 | 1 | 20.0 | 0 | 0.0 | 2 | 40.0 | 0 | 0.0 |
| Subclinical mastitis |                        | 13 | 2 | 15.4 | 0 | 0.0 | 0 | 0.0 | 2 | 15.4 | 0 | 0.0 |
| Cattle         |                        | 5 | 3 | 60.0 | 1 | 20.0 | 0 | 0.0 | 2 | 40.0 | 0 | 0.0 |
| Buffalo        |                        | 10 | 3 | 30.0 | 0 | 0.0 | 1 | 10.0 | 1 | 10.0 | 1 | 10.0 |
| Total          |                        | 50 | 13 | 26.0 | 2 | 4.0 | 2 | 4.0 | 7 | 14.0 | 1 | 2.0 |
3.2. Antimicrobial Susceptibility Profiles of Enterococcus Isolates

All Enterococcus isolates were examined for their susceptibility to 22 antimicrobial agents. Table 3 shows that all Ent. faecalis isolates were resistant to clindamycin, erythromycin, gentamicin, rifampicin, synercid, trimethoprim/sulfamethoxazole and daptomycin. The resistance rate for linezolide, moxifloxacin, erythromycin and others reached 92.3%. Vancomycin resistance rate was high, too (76.9%). Resistance rates regarding other antibiotics ranged between 38.4% for ampicillin and 84.6% for oxacillin.

Table 3. Antimicrobial resistance in Enterococcus isolates.

| Antibiotic                  | Class                        | Enterococcus faecalis (n = 13) | Other Enterococcus Species (n = 4) |
|-----------------------------|------------------------------|-------------------------------|------------------------------------|
|                             | S   | I  | R  | Resistance Rate (%) | S   | I  | R  | Resistance Rate (%) |
| Ampicillin                  | β-Lactam | 8  | 0  | 5  | 38.4 | 2  | 0  | 2  | 50.0 |
| Cefoxitin                   | β-Lactam; cephamycin         | 1  | 0  | 12 | 92.3 | 0  | 0  | 4  | 100 |
| Cefaroline                  | Cephalosporin 5th generation | 1  | 0  | 12 | 92.3 | 1  | 2  | 1  | 25.0 |
| Clindamycin                 | Lincosamide                  | 0  | 0  | 13 | 100  | 0  | 0  | 4  | 100 |
| Daptomycin                  | Cyclic lipopeptide           | 0  | 0  | 13 | 100  | 0  | 0  | 4  | 100 |
| Erythromycin                | Macrolide                    | 0  | 1  | 12 | 92.3 | 0  | 0  | 4  | 100 |
| Erythromycin/clindamycin    |                             | 0  | 0  | 13 | 100  | 0  | 0  | 4  | 100 |
| Fosfomycin                  | Epoxide antibiotic           | 1  | 0  | 12 | 92.3 | 0  | 0  | 4  | 100 |
| Fusidic acid                | Steroide antibiotic          | 1  | 0  | 12 | 92.3 | 0  | 0  | 4  | 100 |
| Gentamicin                  | Aminoglysidies               | 0  | 0  | 13 | 100  | 0  | 0  | 4  | 100 |
| Gentamicin high level       | Aminoglysidies               | 1  | 0  | 12 | 92.3 | 0  | 1  | 3  | 75.0 |
| Linezolid                   | Oxazolidinone                | 1  | 0  | 12 | 92.3 | 0  | 1  | 3  | 75.0 |
| Moxifloxacin                | Fluorchinolone 4th generation| 1  | 0  | 12 | 92.3 | 0  | 0  | 4  | 100 |
| Mupirocin                   |                             | 1  | 2  | 10 | 76.9 | 0  | 2  | 2  | 50.0 |
| Oxacillin                   | beta-Lactam                  | 2  | 0  | 11 | 84.6 | 0  | 0  | 4  | 100 |
| Penicillin G                | beta-Lactam                  | 1  | 5  | 7  | 53.8 | 1  | 2  | 1  | 25.0 |
| Rifampicin                  | Ansamycine                   | 0  | 0  | 13 | 100  | 0  | 0  | 4  | 100 |
| Synercid                    | Streptogramine               | 0  | 0  | 13 | 100  | 0  | 2  | 2  | 50.0 |
| Teicoplanin                 | Glycopeptide                 | 3  | 0  | 10 | 76.9 | 3  | 0  | 1  | 25  |
| Tigecycline                 | Glycylcycline                | 2  | 0  | 11 | 84.6 | 1  | 0  | 3  | 75.0 |
| Trimethoprim/sulphamethoxazole | Dihdrofolatredctase/Sulfonamide | 0  | 0  | 13 | 100  | 0  | 0  | 4  | 100 |
| Vancomycin                  | Glycopeptide                 | 2  | 1  | 10 | 76.9 | 2  | 1  | 1  | 25.0 |

Ent. casseliflavus and Ent. hirae isolates were resistant to clindamycin, erythromycin, fosfomycin, fusidic acid, daptomycin, rifampicin, trimethoprim/sulfamethoxazole, oxacillin, moxifloxacin and gentamicin. Resistance rate for vancomycin was 25.0%.

3.3. Antimicrobial Susceptibility Profiles of Escherichia coli Isolates

All E. coli isolates were resistant to penicillin, streptomycin, erythromycin, chloramphenicol, rifampicin and trimethoprim/sulfamethoxazole (Table 4). Cefazidim, ciprofloxacin,
gentamicin, levofloxacin and tetracycline followed with a resistance rate of 85.7%. Other antibiotics showed resistance rates of 42.9% for amoxicillin/clavulanic acid and imipenem and of 71.4% for amikacin, respectively.

Table 4. Phenotypic resistance detected by MICRONAUT system and resistance-associated genes found in of *Escherichia coli* isolates.

| Isolate     | Phenotypic Antimicrobial Resistance | Detected Resistance-Associated Genes |
|-------------|------------------------------------|--------------------------------------|
| 19CS0095-1  | PEN, STR, CAZ, CIP, LEV, GEN, AMK, TET, ERY, CMP, RAM, T/S | *erm*(B), *tet*K                      |
| 19CS0065    | PEN, STR, AMC, CAZ, IMP, ERY, CMP, RAM, T/S | *erm*(B)                              |
| 19CS0080-1  | PEN, STR, AMC, CAZ, IMP, CIP, LEV, GEN, TET, ERY, CMP, RAM, T/S | *tet*L, *tet*K                        |
| 19CS0092-1  | PEN, STR, AMC, CAZ, IMP, CIP, LEV, GEN, AMK, TET, ERY, CMP, RAM, T/S | *erm*(B), *msr*C, *tet*L             |
| 19CS0078-1  | PEN, STR, CIP, LEV, GEN, AMK, TET, ERY, CMP, RAM, T/S | *erm*(B), *aac*6-*aph*2, *tet*L       |
| 19CS0069    | PEN, STR, CAZ, CIP, LEV, GEN, AMK, TET, ERY, CMP, AM, T/S | *msr*C                                |
| 19CS0098-1  | PEN, STR, AMP, CAZ, CIP, LEV, GEN, TET, ERY, CMP, RAM, T/S | *erm*(B), *tet*L                      |

AMK (amikacin), AMC (amoxicillin/clavulanic acid), CAZ (ceftazidime), CMP (chloramphene-nicol), CIP (ciprofloxacin), ERY (erythromycin), GEN (gentamicin), IMP (imipenem), LEV (levofloxacin), PEN (penicillin G), RAM (rifampicin), STR (streptomycin), TET (tetracycline), T/S (trimethoprim/sulfamethoxazole).

The VITEK 2 system confirmed all 7 *E. coli* isolates as ESBL-producing. They showed resistance to piperacillin, cefotaxime, ceftazidime, astreonam, cefepime, gentamicin and trimethoprim/sulfamethoxazole, as shown in Table 5. All isolates were susceptible to imipenem and meropenem.

Table 5. Results of antimicrobial resistance test for 7 *E. coli* isolates using the VITEK 2 system.

| Isolate     | PIP | TZP | CTX | CAZ | FEB | ATM | IMP | MEM | AMK | GEN | TOB | CIP | MXF | TGC | FOS | CT | T/S |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 19CS0095-1  | R   | I   | R   | R   | R   | R   | S   | S   | S   | R   | R   | R   | S   | S   | S   | R   | ESBL |
| 19CS0065    | R   | I   | R   | R   | R   | R   | S   | S   | S   | S   | S   | S   | S   | S   | S   | S   | R   | ESBL |
| 19CS0080-1  | R   | I   | R   | R   | R   | R   | S   | S   | S   | S   | S   | S   | S   | S   | S   | S   | R   | ESBL |
| 19CS0092-1  | R   | I   | R   | R   | R   | R   | S   | S   | R   | R   | R   | R   | R   | R   | R   | R   | R   | ESBL |
| 19CS0078-1  | R   | I   | R   | R   | R   | R   | S   | S   | R   | R   | R   | R   | R   | R   | R   | R   | ESBL |
| 19CS0069    | R   | I   | R   | R   | R   | R   | S   | S   | R   | R   | R   | R   | R   | R   | R   | R   | ESBL |
| 19CS0098-1  | R   | I   | R   | R   | R   | R   | S   | S   | R   | R   | R   | R   | R   | R   | R   | R   | ESBL |

PIP—piperacillin, TZP—piperacillin/tazobactam, CTX—cefotaxime, CAZ—ceftazidime, FEB—cefepine, ATM—aztreonam, IMP—imipenem, MEM—meropenem, AMK—amikacin, GEN—gentamicin, TOB—tobramycin, CIP—ciprofloxacin, MXF—moxifloxacin, TGC—tigecycline, FOS—fosfomycin, CT—colistin, T/S—trimethoprim/sulfamethoxazole.
3.4. Detection of Resistance-Associated Genes in Enterococcus Isolates

All Ent. faecalis isolates harbored the \textit{erm}(B) gene, associated with erythromycin resistance, the \textit{tet}L gene, connected with tetracycline resistance, and \textit{aac}-\textit{aph}D, responsible for aminoglycoside resistance (Table 6). Other frequently detected resistance determinants were \textit{bla}Z, associated with penicillin resistance, and \textit{tet}M, associated with tetracycline resistance (84.6%). The \textit{van}A gene was detected in 53.8% of Ent. faecalis isolates, while \textit{van}B and \textit{van}C1 genes were not found. Other detected resistance-associated genes were \textit{msr}C (n = 3), \textit{opt}r\textit{A} (n = 2), \textit{Inu}\textit{A} (n = 2), \textit{Inu}\textit{D} (n = 1) and \textit{erm}(A) (n = 1).

Table 6. Antibiotic resistance-associated genes detected in \textit{Enterococcus} isolates.

|                      | Enterococcus faecalis (n = 13) | Other Enterococcus Species (n = 4) |
|----------------------|---------------------------------|-----------------------------------|
|                      | Positive (n) | %    | Positive (n) | %    |
| Vancomycin resistance genes | vanA | 7 | 53.8 | 1 | 25.0 |
|                         | vanB | 0 | 0.0 | 3 | 75.0 |
|                         | vanC1 | 0 | 0.0 | 1 | 25.0 |
| Erythromycin resistance genes | \textit{erm} (A) | 1 | 7.7 | 0 | 0.0 |
|                         | \textit{erm}(B) | 13 | 100 | 4 | 100 |
|                         | \textit{erm}(C) | 0 | 0.0 | 0 | 0.0 |
| Penicillin resistance gene | \textit{bla}Z | 11 | 84.6 | 1 | 25.0 |
| Linezolide resistance genes | \textit{opt}r\textit{A} | 2 | 15.4 | 0 | 0.0 |
|                         | \textit{cfr} | 0 | 0.0 | 0 | 0.0 |
| Macrolide resistance gene | \textit{msr}C | 3 | 23.1 | 0 | 0.0 |
| Aminoglycoside resistance genes | \textit{aac}-\textit{aph}D | 13 | 100 | 3 | 75.0 |
| Tetracycline resistance genes | \textit{tet}K | 0 | 0.0 | 0 | 0.0 |
|                         | \textit{tet}M | 11 | 84.6 | 1 | 25.0 |
|                         | \textit{tet}L | 13 | 100 | 4 | 100 |
|                         | \textit{tet}O | 0 | 0.0 | 0 | 0.0 |
| Lincosamide resistance genes | \textit{lnu}A | 2 | 15.4 | 0 | 0.0 |
|                         | \textit{lnu}D | 1 | 7.7 | 2 | 50.0 |

Other \textit{Enterococcus} (n = 4) isolates exhibited high prevalence of resistance genes \textit{erm}(B), \textit{tet}L, \textit{aac}-\textit{aph}2 and \textit{van}B, with 100%, 100%, 75.0% and 75.0%, respectively. There were two \textit{Ent. casseliflavus} isolates, one exhibited the \textit{van}A gene and the other one carried the \textit{van}B gene.

3.5. Detection of Resistance-Associated Genes in Coliform Bacteria

\textit{E. coli} isolates exhibited high prevalence of the \textit{erm}(B) gene (71.4%), followed by \textit{tet}L (57.1%), while the \textit{tet}K and \textit{msr}C genes, responsible for macrolide resistance, were found only in 2 isolates (28.6%). Aminoglycoside resistance-associated genes \textit{aac}6-\textit{aph}2 were detected only in one isolate (14.3%). No colistin resistance genes have been detected.

In this study, one \textit{Enterobacter cloacae} isolate was examined for the presence of antibiotic resistance genes. It harbored \textit{bla}Z, \textit{erm}(B), \textit{tet}L, \textit{tet}O and \textit{aac}6-\textit{aph}2 genes.
3.6. GenoSerotyping and Analysis of Escherichia coli Isolates by Microarray Investigation

Table 7 shows the data detected by microarray analysis. All of the 7 isolates were detected to be *E. coli*. Four isolates were determined by O-serotyping as O8 (n = 1), two were O86 and one isolate was O157. Analysis of three isolates failed. H-serotyping resulted in H11, H12, H21 (two isolates each) and one was of H16 type.

Table 7. Results of DNA microarray analysis of *E. coli* isolates including genoserotyping and detection of genes associated with virulence and antibiotic resistance.

|            | 19CS0065 | 19CS0069 | 19CS0078-1 | 19CS0080-1 | 19CS0092-1 | 19CS0095-1 | 19CS0098-1 |
|------------|----------|----------|------------|------------|------------|------------|------------|
| *Escherichia coli* | +        | +        | +          | +          | +          | +          | +          |
| *dnaE*     | +        | +        | +          | +          | +          | +          | +          |
| *gapA*     | -        | +        | -          | +          | +          | +          | +          |
| *dfrA*     | +        | +        | +          | +          | +          | +          | +          |
| *rrs*      | +        | +        | +          | +          | +          | +          | +          |
| O-serotyping | 08       | 086      | -          | -          | 086        | -          | O157       |
| H-serotyping | H11      | H12      | H21        | H11        | H12        | H16        | H21        |
| *lpfA*     | -        | -        | +          | -          | -          | -          | +          |
| *lsb*      | -        | +        | -          | -          | -          | -          | -          |
| *astA*     | -        | +        | -          | +          | +          | +          | +          |
| *cma*      | -        | +        | +          | +          | +          | +          | +          |
| *hemL*     | -        | +        | +          | +          | +          | +          | +          |
| *intI1/2*  | +        | +        | +          | +          | +          | +          | +          |
| *iroN*     | -        | -        | -          | -          | -          | -          | -          |
| *iss*      | -        | -        | -          | -          | -          | -          | -          |
| *blaCTX-M1, M15* | -       | +        | -          | -          | +          | -          | -          |
| *blaTEM*   | -        | +        | +          | +          | +          | +          | +          |
| *Aminoglycosides* | aadA1, aadA4 | aadA1, aphA, strA, strB | aadA1, aphA, strA | aadA1, aadA4, aphA | aadA1, aphA, strA, strB | strA, strB | aadA1, aphA, strA |
| *Chloramphenicol* | cmlA1 | cmlA1, floR, catA1 | cmlA1, floR | cmlA1, floR | cmlA1, floR, catA1 | floR | cmlA1, floR |
| *Macrolides* | -       | mphA     | -          | mphA       | mphA       | mphA       | mphA       |
| *Quinolones* | -       | qnrA1, qnrS | -          | -          | -          | qnrA1, qnrS | qnrA1, qnrS |
| *Tetracycline* | -       | tetA     | -          | -          | -          | tetA       | -          |
| *Sulphonamides* | sul3 | sul1, sul2, sul3 | sul1, sul3 | sul3 | sul3 | sul2 | sul3 |
| *Trimethoprim* | dfrA17 | dfrA12 | dfrA12 | dfrA17 | dfrA1, dfrA14 | dfrA12 | dfrA12, dfrA14 |

a Family, genus and species-specific marker, b Genes encoding virulence factors—fimbriae, c Genes encoding virulence factors—SPATE, d Genes encoding virulence factors—toxins, e Genes encoding virulence factors—miscellaneous, f ESBL genes, g Genes associated with antimicrobial resistance.

Different virulence-associated genes were detected in *E. coli* isolates, including the fimbria-associated gene (*lpfA*). Toxin genes *astA*, *cma*, *cma* and *cma* were detected in 6 isolates, whereas only isolates 19CS0069 and 19CS0092-1 carried all four determinants (Table 7). Others were carriers of one or two of these genes. Miscellaneous virulence-associated genes like *hemL* and *intI1* were detected in all isolates, *intI2* was found in one isolate. Two isolates carried *iroN* and *iss* genes, respectively. No Shiga toxin gene or those responsible for adhesion or secretion systems were detected.

Various antibiotic resistance-associated genes were detected (Table 7). Associated with aminoglycoside resistance, the *aadA1* gene was identified in 6 isolates, *aadA4* in 2, *aphA* in 4, *strA* in 5 and *strB* in 3 isolates. The most frequently detected chloramphenicol resistance...
genes were cmxA1 and floR, found in 6 isolates. Two isolates carried the catA1 gene. The mphA gene responsible for macrolide resistance was detected in 3 isolates. Quinolone resistance-associated genes qnrA1 and qnrS were found in two isolates. The tetA gene connected with tetracycline resistance was found in 3 isolates. Three sulphonamide resistance determinants including sul1, sul2 and sul3 were detected in two, two and 6 isolates respectively, while four different genes associated with trimethoprim resistance were identified, including dfrA12 in 3 isolates and dfrA17, dfrA1 and dfrA14 in 2 isolates each.

Genes characteristic for ESBL-producing E. coli were detected in all isolates to different degrees: blaCTX-M9 in 5 isolates, blaCTX-M1, M15 in 2 isolates and blaTEM in 4 isolates.

4. Discussion

Raw milk consumption could present a potential risk for public health due to the presence of foodborne pathogens and spoilage bacteria from raw milk samples.

Enterococcus species were encountered in studies regarding milk connected with mastitis. In this study, prevalence of Enterococcus species in milk of cattle and buffalo was 34.0%, which is similar to a report of the authors of Reference [30], who found that Enterococcus species were present in 31.0% of cow milk samples in Iraq.

One of the most important environmental pathogens causing mastitis is Ent. faecalis. In this study, the most frequently isolated Enterococcus species was Ent. faecalis, which is in agreement with other authors’ previous work [30–32].

Ent. faecalis was found in 26.0% of isolates, similar to other studies that reported the prevalence of Ent. faecalis in bovine mastitis cases to be 19.5% in Egypt [33] and 20.9% in Czech Republic [34]. Other enterococci, Ent. casseliflavus (4.0%) and Ent. hirae (4.0%), were found in similar percentages, as reported in Reference [35].

Enterococci have developed various intrinsic and acquired resistance mechanisms to antibiotics. They have an intrinsic resistance to β-lactams, cephalosporins, clindamycin and low concentrations of aminoglycosides, while acquired resistance to erythromycin, linezolid, daptomycin, tetracyclines, ciprofloxacin and vancomycin was recognized [36].

Vancomycin-resistant enterococci (VRE) were first reported in 1998 and their incidence has increased rapidly through the world after that. Vancomycin-resistance determinants are van genes which can be transferred to other Gram-positive bacteria [8]. In this study, phenotypic determined vancomycin resistance was found in 76.9% of E. faecalis isolates: 53.8% of them carried the vanA gene, which is in contrast to results of the authors of Reference [37], who found E. faecalis originated from mastitis cases in Turkey resistant to vancomycin at low levels (1.06%).

All Enterococcus isolates were found to be phenotypically resistant to erythromycin. This result can be explained by the presence of the erm(B) gene in all of them, which is in agreement to reports given by other authors [31,38]. They also found that the erm(B) gene was the most prevalent erythromycin resistance gene found in enterococci from both human and animals. Tetracycline resistance in enterococci is often connected with the presence of tet genes. Here, tetL and tetM genes were found frequently, which is nearly in agreement with data reported in Reference [39]. The high percentage of erythromycin and tetracycline resistance found in this and in other studies [38–41] is due to common and prolonged usage of these antibiotics in the dairy industry for prophylaxis and treatment of mastitis-diseased cattle.

One of the most significant bacterium causing mastitis is E. coli. It is isolated in high incidence and it is more dangerous for public health, as a serotype like O157:H7 is enteropathogenic and can cause gastroenteritis, food intoxication, hemorrhagic colitis and hemolytic uremic syndrome. Furthermore, E. coli is one of the pathogens that is related to severe clinical mastitis, it is considered as a fatal mastitis pathogen, in some cases it has led to animal death [42]. In this study, in 14.0% of milk samples, E. coli was isolated, which is comparable to other Egyptian studies on mastitis-diseased cattle in Egypt with 13.3% and 18.7%, respectively [43,44]. Microarray analysis of E. coli isolates revealed that one was serotyped as O157. O157 serotype was reported in some studies in Egypt as a
mastitis-causing pathogen [42,43,45] as well as one of the most harmful STEC that can cause severe human infections. Other isolates belonged to serotype O86 within the EPEC group. Isolates of this serogroup were also described as connected with bovine mastitis [46].

ESBL-encoding genes have been categorized into three main types: blaCTX-M, blaSHV and blaTEM. The blaCTX-M isolates have been further categorized into five sub-groups (blaCTX-M-1, blaCTX-M-2, blaCTX-M-8, blaCTX-M-9, blaCTX-M-25) and more than 150 variants have been documented (http://www.lahey.org/studies). In our study, all E. coli isolates were ESBL-producing, as confirmed by the VITEK 2 system, and this finding was explained by the presence of one or two types of ESBL genes. It agrees with results obtained by several authors for E. coli isolates from bovine mastitis cases [5,47,48]. Previous studies suggest that the blaCTX-M type, predominantly blaCTX-M-15, was the most prevalent ESBL type worldwide [49]. This result was alarming because E. coli which produce ESBLs of blaCTX-M-15 type are important causal agents of healthcare-oriented as well as community-based infections in humans [50] and have recently also been increasingly reported from food-producing animals [51]. The cause for frequent occurrence of ESBL-producing bacteria could be the use of β-lactams and even fourth generation cephalosporins in veterinary medicine [52]. Another reason is possibly co-selection by multiple resistance mechanisms through the usage of various antibiotics due to the fact that resistance genes for aminoglycosides, tetracyclines and trimethoprim-sulfamethoxazole are frequently placed on single conjugative plasmids, as is often also the case with blaESBL genes [53]. Generally, ESBL genes are located on plasmids that could spread easily among commensal and pathogenic bacteria within herds and the environment. All ESBL-producing E. coli were multidrug-resistant and showed resistance to cephalosporins, β-lactam and non-β-lactam antibiotics, such as ampicillin, aminoglycosides, macrolides, tetracyclines and fluoroquinolones. Many studies confirmed that ESBL-producing E. coli were multidrug-resistant independent from the source, like cattle [54,55], poultry [56], pigs [51] and humans [57].

Erythromycin resistance can be connected with the presence of different genes like erm(B), msrC or mphA, which were found in E. coli isolates of the milk samples. Similar data have been reported [58]. Other resistance determinants regarding erythromycin resistance played no role.

Phenotypic chloramphenicol resistance was detected in all E. coli isolates and DNA microarray analysis resulted in the detection of genes cmlA1, floR and catA1 responsible for it. Previously, the floR gene was detected in Egyptian E. coli isolates from neonatal calves [59] as well as floR and cmlA in E. coli from bovine mastitis cases [60].

Resistance to trimethoprim/sulphamethoxazole was detected in all 7 E. coli isolates by both the microdilution method and the VITEK 2 system, which resulted from the presence of sulphonamide resistance genes sul1, sul2 and sul3. The isolates carried at least one or two of these genes, which was already reported previously [61], while carriage of genes dfrA1, dfrA12, dfrA14 and dfrA17 responsible for resistance to trimethoprim were described for Egyptian isolates from calves and mastitis cases [59,60].

All E. coli isolates carried at least one aminoglycoside resistance determinant like aadA1, aadA4, aac6-aph2, aphA, strA or strB genes. Similar results have been found previously in E. coli isolates from bovine mastitis cases in Egypt [61] and in Iran [62]. Plasmid-mediated quinolone resistance genes qnrA1 and qnrS were present in only a few isolates, a fact which was reported in the past, too [59,60]. Nevertheless, the detection of these genes is very important due to the possibility of spread of these resistance determinants between bacteria through plasmid mobility [62].

High detection rates of tet genes described here were also found in other investigations previously [63,64], in which mainly tetA was present in E. coli isolates from bovine mastitis cases. Here, the cause of the high presence rate of tetracycline resistance genes is the widespread and uncontrolled usage of this antibiotic for treatment and prevention of infections in livestock in Egypt.

In general, increasing Enterococcus and E. coli resistance to antibiotics results in excessive use of antimicrobials in different genetic resistance mechanisms, vertically by
inheriting genes to new generations or horizontally by exchanging genetic materials among bacteria.

5. Conclusions

This study clearly showed that milk from small farmers in Egypt, which is unpasteurized and used as food, is traded or will be processed further, was contaminated with bacteria which are potentially hazardous for human health. To make matters worse, many of these bacteria became multidrug-resistant, which makes therapy of resulting diseases hard.

As a consequence, training of farmers is desirable regarding a better hygiene system andpasteurization of produced milk without exception. A surveillance system by governmental institutions should be introduced.

Concerning antibiotic resistance, a reduction of uncontrolled administering of antibiotics should be a primary aim. Only in cases of diseases after serious diagnosis should antimicrobials be used, not as prophylaxis or even as growth promoters. The usage of last-line antibiotics like vancomycin is not allowed in veterinary medicine.

Author Contributions: W.A., H.H., H.T. and A.A.A.E.-T. participated in the conception and design of the study; W.A., H.H., F.I.E.H. and S.M. performed farm and laboratory work; W.A., H.H., H.N., H.T. and S.M. analyzed the data, wrote the manuscript and contributed to manuscript discussion. All authors read and approved the final manuscript.

Funding: There was no external funding for this study.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank the Egyptian Cultural and Educational Office of the Arab Republic of Egypt in Germany and the Egyptian Ministry of Higher Education for financial support. The authors would like to thank Byrgit Hofmann, Peggy Methner, Elke Müller and Annett Reissig for their excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interests.

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