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Lineages, Virulence Gene Associated and Integrons among Extended Spectrum β-Lactamase (ESBL) and CMY-2 Producing Enterobacteriaceae from Bovine Mastitis, in Tunisia

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Abstract: Extended Spectrum Beta-Lactamase (ESBL) Enterobacteriaceae are becoming widespread enzymes in food-producing animals worldwide. Escherichia coli and Klebsiella pneumoniae are two of the most significant pathogens causing mastitis. Our study focused on the characterization of the genetic support of ESBL/pAmpC and antibiotic resistance mechanisms in cefotaxime-resistant (CTX) and susceptible (CTX) Enterobacteriaceae isolates, recovered from bovine mastitis in Tunisia, as well as the analyses of their clonal lineage and virulence-associated genes. The study was carried out on 17 ESBL/pAmpC E. coli and K. pneumoniae and 50 CTX E. coli. Detection of resistance genes and clonal diversity was performed by PCR amplification and sequencing. The following β-lactamase genes were detected: blaCTX-M-15 (n = 6), blaCTX-M-15 + blaOXA-1 (2), blaCTX-M-15 + blaOXA-1 + blaTEM-1b (2), blaCTX-M-15 + blaTEM-1b (4), blaCMY-2 (3). The MLST showed the following STs: ST405 (n = 4 strains); ST58 (n = 3); ST155 (n = 3); ST471 (n = 2); and ST101 (n = 2). ST399 (n = 1) and ST617 (n = 1) were identified in p(AmpC) E. coli producer strains. The phylogroups A and B1 were the most detected ones, followed by the pathogenic phylogroup B2 that harbored the shigatoxin genes stx1/stx2, associated with the cnf, fimA, and aer virulence factors. The qnrA/qnrB, aac(6′)-Ib-cr genes and integrons class 1 with different gene cassettes were detected amongst these CTXβ-isolated strains. The presence of different genetic lineages, associated with resistance and virulence genes in pathogenic bacteria in dairy farms, may complicate antibiotic therapies and pose a potential risk to public health.

Keywords: ESBL; clonal lineages; p(AmpC); mastitis; pathogenic bacteria; Tunisia; virulence; integrons

1. Introduction

Bovine Mastitis (BM) is one of the most common and costly diseases that affects dairy cattle worldwide [1]. It leads to loss of milk production, mandatory culling, and costly veterinary services [2]. Enterobacteriaceae species such as Escherichia coli and klebsiella pneumoniae are considered as significant etiological agents of bovine mastitis [3].

Mastitis due to E. coli varies significantly from mild to very severe and even fatal infection [4,5], which is associated with clinical signs [6]. Besides, E. coli has a zoonotic character and is involved in severe gastrointestinal or urinary tract infections in humans. Although their prevalence is low, Klebsiella infections in cattle appear to be particularly problematic due to their relatively long period of infection, leading to significant losses of milk production and increased mortality of affected cows [7]. Most E. coli infections associated with clinical mastitis are typically commensal; however, pathogenic variants have also been reported [8] and shigatoxigenic E. coli (STEC) are amongst the pathogenic variants inducing clinical mastitis [8]. Several studies elucidated the virulence determinants and reported shigatoxin-encoding genes (stx1, stx2) as being the most important virulence determinants in E. coli isolated from bovine mastitis [9]. The effect of the virulence factors (VF)s of E. coli on the severity of clinical mastitis in dairy cattle remains unclear [10].
Mastitis was reported as the most common consequence of antimicrobial administration in livestock [11]. Although, these drugs may also be used in a prophylactic manner, to prevent bacterial infection or promote livestock growth, this trend can adversely affect the World Health Organization (WHO) ‘One Health’ policy because of the ease with which Gram-negative bacteria (GNB) harbor multidrug resistance genes and can zoonotically move from animals to humans and vice versa [12]. The overuse of the extended spectrum cephalosporins causes the emergence of extended spectrum beta-lactamases (ESBLs) and/or plasmid-mediated AmpC (pAmpC) producing Enterobacteriaceae [13]. Based on the diversity of ESBL enzymes among E. coli/K. pneumoniae isolates from bovine mastitis, CTX-M-1 and CTX-M-14-producing E. coli are broadly disseminated in bovine mastitis cases in Europe [14]. On the contrary, CTX-M-15 was the most prevalent ESBL type found in E. coli mastitis in Asia [13,15]. Furthermore, Enterobacteriaceae producing p(AmpC) beta-lactamases are more alarming than those possessing ESBLs because they are poorly inhibited by the commercially available beta-lactam inhibitors (i.e., clavulanate, sulbactam, and tazobactam) [16]. In particular, the CMY-2 types represent the most commonly encountered p(AmpC) beta-lactamases identified in E. coli and other Enterobacteriaceae of human origin [17]. However, this beta-lactamase variant was identified among commensally E. coli from healthy and diseased animals in Tunisia [18,19].

There are few reports describing the genetic features of E. coli and K. pneumoniae strains of bovine mastitis origin; the observed data being very heterogeneous and the genetic diversity remaining unclear.

This study aims to characterize the genetic support of ESBL/pAmpC Enterobacteriaceae isolates and the study of antibiotic resistance mechanisms in cefotaxime-susceptible (CTXS) E. coli isolates recovered from bovine mastitis in Tunisia, as well as the analysis of their clonal lineage and their virulence-associated genes.

2. Results
2.1. Antibiotic Resistance Rates for ESBL/pAmpC Enterobacteriaceae Isolates

The occurrences of antibiotic resistance of 14 ESBL Enterobacteriaceae (ESBL-EB) (10 E. coli, 4 K. pneumoniae isolates) and 3 cefotaxime-resistant E. coli isolates are presented in Table 1.

| Strains | Farm | Genes Encoding Beta-Lactamases | blaCTX-M Genetic Environment | Phenotypes of Resistance to Non-beta-Lactams | Other Resistance Genes Detected Outside Integrons | Gene Cassette Arrays in Class 1 Integron | Phylogroup and Virulence Genes | ST |
|---------|------|--------------------------------|-----------------------------|----------------------------------------------|-----------------------------------------------|----------------------------------------|---------------------------------|----|
| E. C 1  | 1    | blaCTX-M-15 + blaOXA-1         | ISEcp1-orf477               | TET NAL SUL SXT                              | tetB, sul3, sul2, aac(6')-Ib-cr               | Int1, dfrA17                           | A                               | 405 |
| E. C 2  | 5    | blaCTX-M-15 + blaOXA-1         | ISEcp1-orf477               | TET SXT SUL                                   | tetB, sul2                                   | Int1, dfrA17                           | B2, cnf1, stx2, aer               | 405 |
| E. C 3  | 8    | blaCTX-M-15 + blaOXA-1 +      | ISEcp1-orf477               | TET NAL                                       | tetB, aac(6')-Ib-cr                          | --                                    | B2, aer, fimA, stx2               | 58  |
| E. C 4  | 2    | blaCTX-M-15 + blaOXA-1 +      | IS26/ISEcp1-orf477          | TET SUL NAL SXT                               | tetB, sul3, sul2, aac(6')-Ib-cr               | Int1, dfrA17                           | D, aer, stx2                     | 58  |
| E. C 5  | 10   | blaCTX-M-15 + blaOXA-1 +      | ISEcp1-orf477               | TET STR                                        | tetB, strA                                    | --                                    | B1                               | 405 |
| E. C 6  | 6    | blaCTX-M-15 + blaOXA-1 +      | ISEcp1-orf477               | TET                                           | tetB                                         | --                                    | A                               | 405 |
| E. C 7  | 18   | blaCTX-M-15 + blaOXA-1 +      | ISEcp1-orf477               | TET                                           | qnrA, sul2                                    | --                                    | A                               | 155 |
Table 1. Cont.

| Strains | Farm | Genes Encoding Beta-Lactamases | blaCTX-M Genetic Environment | Phenotypes of Resistance to Non β-Lactams | Other Resistance Genes Detected Outside Integrons | Gene Cassette Arrays in Class 1 Integron | Phylogroup and Virulence Genes | ST |
|---------|------|--------------------------------|------------------------------|------------------------------------------|-----------------------------------------------|------------------------------------------|---------------------------------|----|
| E. C 8  | 20   | blaCTX-M-15                     | IS26/ISEcp1-orf477           | CIP NAL SUL                              | qnrA, sul3                                    | ND                                       | A                              | 10 |
| E. C 9  | 23   | blaCTX-M-15                     | ISEcp1-orf477                | NAL SUL TET                              | tetB, sul3, sul2, strA                        | ND                                       | A                              | 155|
| E. C 10 | 9    | blaCTX-M-15                     | ISEcp1-orf477                | TET SUL NAL SXT STR                      | tetA, sul2                                   | ND                                       | A                              | 58 |
| E. C 11 *| 11  | blaCMY-2                        | ISEcp1-orf477                | TET SUL SXT                              | tetA                                         | Int1, adaA1, dfrA1                       | B1                             | 155|
| E. C 12 *| 14  | blaCMY-2                        | ISEcp1-orf477                | TET SUL SXT                              | tetB, qnrA, sul2                             | –                                        | –                              | 399|
| E. C 13 *| 16  | blaCMY-2                        | ISEcp1-orf477                | TET SUL CIP                              | tetB, qnrA, sul1, sul2, adaA5                 | –                                        | B1                             | 617|
| Kp1     | 1    | blaCTX-M-15 + blaTEM-1b         | IS26/ISEcp1-orf477           | TET SUL NAL SXT                          | tetA, sul1, sul2, qnrB                       | –                                        | –                              | 471|
| Kp2     | 2    | blaCTX-M-15 + blaTEM-1b         | ISEcp1-orf477                | CIP NAL SUL SXT                          | qnrB, sul2                                  | Int1, dfrA17                            | –                              | 471|
| Kp3     | 3    | blaCTX-M-15 + blaTEM-1b         | ISEcp1-orf477                | TET SUL SXT STR                          | tetA, sul1, sul2                            | Int1, dfrA17 + adaA5                    | –                              | 101|
| Kp4     | 4    | blaCTX-M-15 + blaTEM-1b         | IS26/ISEcp1-orf477           | TET SUL CIP                              | tetA, aac(6’)-Ib-cr                         | –                                        | –                              | 101|

EC: E. coli; Kp: K. pneumoniae; NAL: nalidixic acid; CIP: ciprofloxacin; SUL: sulfonamide; SXT: trimethoprim/sulfamethoxazole; TET: tetracycline; CHL: chloramphenicol; STR: streptomycin; TOB: tobramycin; ST: sequence type; ND: integron not detected in trimethoprim/sulfamethoxazole resistance isolates; –: integron is not sought in strains susceptible to trimethoprim/sulfamethoxazole. *: E. coli p(AmpC) producing isolates.

The analysis of the antibiotic susceptibility test for all tested isolates showed high rates of resistance for tetracycline (82.35%), sulfonamides (76.5%), and nalidixic acid (64.5%). Moderate to low rates of resistance were observed for trimethoprim/sulfamethoxazole (47%), streptomycin (23.5%), ciprofloxacin (29.4%), and chloramphenicol (6%); no resistant isolate being detected for imipenem or ertapenem. Ten isolates showed multi-drug resistance phenotypes, including resistance to at least three families of antimicrobial agents. According to double disc synergy test (DDST), three E. coli CTXR strains were AmpC producers and presented resistance to cefoxitin and amoxicillin-clavulanic acid.

2.2. Genetic Support of ESBL/p(AmpC) Enterobacteriaceae Isolates

As shown in Table 1, the amplification and sequencing of β-lactamase genes of 14 (ESBL-EB) and 3 (AmpC) producers of E. coli isolates revealed that all the tested ESBL E. coli or K. pneumoniae strains carried blaCTX-M-15 or co-associated to blaOXA-1 + blaTEM-1b (n = 2 strains), blaOXA-1 (n = 2), and blaTEM-1 (n = 4); blaCMY-2 being detected in the three p(AmpC) phenotype E. coli strains. The regions surrounding both blaCTX-M-15 and blaCMY-2 genes were analyzed by PCR. The orf477 sequence was detected downstream of blaCTX-M-15 and blaCMY-2 in all ESBL and p(AmpC) producer strains. Nevertheless, IS26 sequence was identified upstream of blaCTX-M-15 and blaCMY-2 gene in 12 isolates. Interestingly, the IS26 flanked a partially truncated ISEcp1 element in five blaCTX-M-15 positive E. coli isolates (Figure 1). The sequences of the amplicons of ESBL/p(AmpC) gene detected in this study were presented in FASTA form as a Supplementary file.
yses revealed that our isolates belong to p

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\]

and \(E. coli\) \(aac(6' - \text{Integrons class 1 structures in ESBL Enterobacteriaceae}\).

\(\text{This collection. Three different arrangements were detected in variable regions of class 1 }\beta\\text{Figure 1.}\)

\(\text{Figure 2.}\) Antimicrobial agents of ESBL-EB and p(AmpC)-Producer \(E. coli\) Strains

Table 1 presents antibiotic resistance phenotypes and genotypes of different (ESBL-Eb) and p(AmpC) \(E. coli\) strains. Class 1 integrons were detected in 7 out of 14 (ESBL-EB) and 3 p(AmpC)-producer \(E. coli\) isolates (41.17%); class 2 integrons were not identified in this collection. Three different arrangements were detected in variable regions of class 1 integrons: (1) \(\text{dfra17}\) confers resistance to trimethoprim in five strains; (2) \(\text{dfra17 + aadA1}\) were involved in streptomycin and trimethoprim resistance, respectively, in one strain; (3) \(\text{dfra17 + aadA5}\) (1 strain). Figure 2 illustrated the structures of integrons class 1 in ESBL/p (AmpC) \(\text{Enterobacteriaceae}\) strains and CTX\(^S\) \(E. coli\) isolates.

- 4 strains CTXM-15 positives (\(E.C.1, E.C.2, E.C.4, Kp2\))
- 1 strain CMY-2 positive (\(E.C.12\))
- 1 strain CMY-2 positive (\(E.C.11\))
- 7 \(E. coli\) isolates CTX\(^3\)
- 3 \(E. coli\) isolates CTX\(^5\)
- 1 strain CTX-M-15 positive \(Kp3\)
- 4 \(E. coli\) isolates CTX\(^8\)

The sequences of the amplicons of the variable region of integrons class 1 for each gene cassette detected in this study are presented in FASTA form as a Supplementary file.

Different resistance genes to non-\(\beta\)-lactam antibiotics situated outside the integrons were observed in all tested strains: the \(\text{tet(A)}\) and \(\text{tet(B)}\) genes were detected in five and nine tetracycline resistance (ESBL-Eb) and p(AmpC) \(E. coli\) strains, respectively. Quinolones resistance was conferred by \(\text{aad(6')} - \text{Ib-cr}\) gene in three \(E. coli\) and 1 \(K. pneumoniae\) strains,
by qnrA in three E. coli including one CMY-2 producer strain, and by qnrB gene in two K. pneumoniae strains. Different variants of sul genes were observed in 11 sulfonamide resistance strains: sul2 + sul3 (n = 3), sul1 + sul2 (n = 2), sul2 (n = 5), and sul3 (n = 1). The streptomycin resistance was conferred by strA gene in two strains.

2.4. Molecular Typing, Virulence Factors and Phylotyping of ESBL–EB and p(AmpC)-Producer E. coli Strains

The analyses of multilocus-sequence typing (MLST) showed seven sequence types (STs); the most frequent being ST405 (n = 4 strains); ST58 (n = 3); ST155 (n = 3); ST471 (n = 2); and ST101 (n = 2). The remaining two STs, ST399/n = 1 and ST617/n = 1, were identified in p(AmpC) E. coli producer strains. The sequences of the ampiclon of the seven housekeeping genes of E. coli and K. pneumoniae for each sequence type detected in this study are presented in FASTA form as a Supplementary file.

The phylogeny analyses revealed that our isolates belong to phylogroups A (six isolates), B1(four isolates), D (one isolate), and B2 (two isolates). For virulence determinant factors, only two strains assigned to the phylogroup B2, harbored two associations out of three virulence factors (cnf1, stx2, aer) and (aer, fimA, stx2) among the genes investigated. In addition, the ESBL E. coli producer isolates assigned to the phylogroup D harbored only the aer and stx2 virulence factors. The virulence factors stxl and stx2 were detected in three E. coli strains and were characterized as shigatoxigenic E. coli (STEC).

2.5. Antibiotic Resistance Rates for CTXS E. coli Isolates

Table 2 presents antibiotic resistance phenotypes of 50 E. coli isolates susceptible to cefotaxime. Amongst this collection, 28 E. coli isolates were resistant to at least three antibiotic families. The highest antimicrobial resistance phenotype of 50 E. coli was observed for ampicillin, ticarcillin, and tetracycline (78%, 66%, and 78%, respectively). For sulfonamides, streptomycin, and trimethoprim-sulfamethoxazole, the percentage of resistance was 52%, 40%, and 40%, respectively. Whilst low resistance has been detected to nalidixic acid, ciprofloxacin (4%), cefoxitin (10%), and chloramphenicol (6%), no isolate was resistant to carbapenem or aminoglycoside families (Figure S1).

| Strains | Antibiotic Resistance Phenotypes | Resistance Genes | Class 1 Integron | Gene Cassette Arrays in Class 1 Integron | Phylogroup and Virulence Genes |
|---------|----------------------------------|------------------|-----------------|---------------------------------------|------------------------------|
| E. C 168 | TET AMP SXT TIC SUL FOX STR CHL | TEM-1b, tetA, catA | +               | dfrA12 + aadA2                       | B1                           |
| E. C 3  | TET AMP SUL NAL CIP TIC SXT     | TEM-1b, tetA, qnrA, sul2 | ND              | —                                     | B1                           |
| E. C 78 | TET AMP SXT TIC SUL STR CHL     | TEM-1b, tetA, catA, strA | +               | dfrA 1 + aadA1                       | B2, sx2, cnf1                |
| E. C 115| TET AMP SXT TIC SUL STR         | TEM-1b, tetA     | +               | dfrA1 + aadA1                        | B1                           |
| E. C 81 | TET AMP SXT TIC SUL STR         | TEM-1b, tetA, strA, dfrA1a | ND              | —                                     | B2, fimA, sx2                |
| E. C 82 | TET AMP SXT TIC SUL STR         | TEM-1b, tetA     | +               | dfrA12 + aadA2                       | B2, sx2, cnf1                |
| E. C 85 | TET AMP SXT TIC SUL STR         | TEM-1b, tetB, strA | ND              | —                                     | A                           |
| E. C 91 | TET AMP SXT TIC SUL STR         | TEM-1b, tetA     | +               | dfrA12 + aadA2                       | B1                           |
| E. C 134| TET AMP SXT TIC SUL STR         | TEM-1b           | +               | dfrA1 + aadA1                        | B2, acr, fimA, cnf1          |
Table 2. Cont.

| Strains | Antibiotic Resistance Phenotypes | Resistance Genes | Class 1 Integron | Gene Cassette Arrays in Class 1 Integron | Phylogroup and Virulence Genes |
|---------|----------------------------------|------------------|------------------|-----------------------------------------|-------------------------------|
| E. C 162 | TET AMP SXT TIC SUL STR          | TEM-1b, tetB     | +                | dfrA1 + aadA1                           | B2, cnf1, stx2 |
| E. C 164 | TET AMP SXT TIC SUL STR          | TEM-1b, tetA     | +                | dfrA1 + aadA1                           | B2, cnf1 |
| E. C 167 | TET AMP SXT TIC SUL STR          | TEM-1b, tetA     | +                | dfrA1 + aadA1                           | B1 |
| E. C 184 | TET AMP SXT TIC SUL STR          | TEM-1b, tetA     | +                | dfrA17 + aadA5                          | B1 |
| E. C 186 | TET AMP SXT TIC SUL STR          | TEM-1b, tetA     | +                | dfrA17 + aadA5                          | B1 |
| E. C 196 | TET AMP SXT TIC SUL STR          | TEM-1b, tetB     | +                | dfrA17 + aadA5                          | B1 |
| E. C 116 | TET SXT SUL NAL CIP STR          | tetA, qnrA       | +                | dfrA1 + aadA1                           | B1 |
| E. C 123 | TET AMP CHL NAL CIP SXT          | TEM-1b, tetA, catA, qnrA, dfrA1 a | ND | –                                      | B2, cnf1 |
| E. C 10  | TET AMP SXT TIC SUL STR          | TEM-1b, tetA, sul2, dfrA12 a | ND | –                                      | B2, acr, cnf1 |
| E. C 28  | TET AMP SXT TIC SUL STR          | TEM-1b, tetA, sul2, dfrA1 a | ND | –                                      | A |
| E. C 29  | TET AMP SXT TIC SUL STR          | TEM-1b, tetA, dfrA1 a | ND | –                                      | B1 |
| E. C 40  | TET AMP SXT TIC SUL STR          | TEM-1b, tetA, dfrA1 a | ND | –                                      | B1 |
| E. C 171 | TET AMP SXT TIC SUL STR          | TEM-1b, tetA, dfrA1 a | ND | –                                      | B2, stx2, fimA |
| E. C 175 | TET AMP SXT TIC SUL STR          | TEM-1b, tetA, dfrA1 a | ND | –                                      | B1 |
| E. C 30  | TET AMP SXT TIC SUL STR          | TEM-1b, tetA     | ND | –                                      | B2, stx2, acr, fimA |
| E. C 17  | AMP TIC NAL CIP SXT              | TEM-1b, qnrA     | –                | –                                      | A |
| E. C 18  | AMP TIC NAL CIP SXT              | TEM-1b, qnrA     | –                | –                                      | A |
| E. C 195 | AMP TIC TET STR SUL STR          | TEM-1b, tetB, strA | –                | –                                      | A |
| E. C 137 | AMP SUL STR                      | TEM-1b, sul2, strA | –                | –                                      | A |
| E. C 14  | TET AMP TIC SUL STR              | TEM-1b, tetA     | –                | –                                      | A |
| E. C 25  | TET AMP TIC SUL STR              | TEM-1b, tetA     | –                | –                                      | A |
| E. C 39  | TET AMP TIC SUL STR              | TEM-1b, tetB     | –                | –                                      | B1 |
| E. C 70  | TET AMP TIC SUL STR              | TEM-1b, tetB     | –                | –                                      | B1 |
| E. C 31  | TET AMP TIC SUL STR              | TEM-1b, tetB     | –                | –                                      | B2, stx2, acr, cnf1 |
| E. C 32  | TET AMP TIC SUL STR              | TEM-1b, tetB     | –                | –                                      | A |
| E. C 135 | TET SUL STR                      | tetB, sul2, strA | –                | –                                      | B1 |
| E. C 183 | FOX AMP                          | TEM-1b           | –                | –                                      | A |
| E. C 23  | AMP TIC                          | TEM-1b           | –                | –                                      | A |
| E. C 179 | AMP STR                          | TEM-1b, strA     | –                | –                                      | A |
| E. C 190 | TET STR                          | tetB, strA       | –                | –                                      | A |
| E. C 119 | AMP                              | TEM-1b           | –                | –                                      | A |
| E. C 146 | FOX                              | –                | –                | –                                      | A |
| E. C 147 | FOX                              | –                | –                | –                                      | B1 |
| E. C 163 | AMP                              | TEM-1b           | –                | –                                      | A |
| E. C 194 | TET                              | –                | –                | –                                      | A |

EC: E. coli; NAL: AMP: ampicillin; TIC: ticarcillin; FOX: cefoxitin; NAL: nalidixic acid; CIP: ciprofloxacin; SUL: sulfonamide; SXT: trimethoprim/sulfamethoxazole; TET: tetracycline; CHL: chloramphenicol; STR: streptomycin; TOB: tobramycin. ND: Integron not detected in trimethoprim/sulfamethoxazole resistance isolates; **: integron is not sought in strains susceptible to trimethoprim/sulfamethoxazole; * gene detected outside of integron; * same phenotype and genotype in six strains; +: Integron detected in trimethoprim/sulfamethoxazole resistance isolates.

In addition, similar different association of phenotype resistance were observed in our E. coli isolate collections from different farms and samples: [TET][AMP][SXT][TIC][SUL][STR] (n = 12); [TET][AMP][SXT][TIC][SUL] (n = 7); [AMP][TIC][NAL][CIP] (n = 2) and [TET][AMP][TIC] (n = 6).
2.6. Genotypic Features of CTXS E. coli Isolates

The resistance genes detected among our 50 CTX\textsuperscript{S} E. coli isolates are shown in Table 2. The PCR amplification and sequencing of different \( \beta \)-lactamases resistance genes revealed that all ampicillin-resistant E. coli strains harbor \( \text{bla}_{\text{TEM-1b}} \) encoding TEM \( \beta \)-lactamase enzyme. Tetracycline resistance is conferred by \( \text{tet}(A) \) and \( \text{tet}(B) \) in 23 and 16 isolates, respectively. The \( \text{gmrA} \) gene was detected in five quinolone-resistant E. coli strains. Furthermore, the 24 isolates resistant to trimethoprim-sulfamethoxazol presented different types and combinations of \( \text{dfrA} \) and \( \text{aadA} \) genes (confering resistance to trimetoprim and streptomycin). Some of them were identified inside the structure of class 1 integron in 14 strains, with the following gene cassette arrangements (number of strains): \( \text{dfrA1 + aadA1} \) (7), \( \text{dfrA12 + aadA2} \) (4), and \( \text{dfrA17 + addA5} \) (3) (Table 2, Figure 2). Other different resistance genes were identified outside integrons [antibiotic/gene (number of isolates)]: sulfonamide/\( \text{sul2} \) (5); streptomycin/\( \text{strA} / \text{strB} \) (1); chloramphenicol/\( \text{catA} \) (3); trimetoprim resistance being conferred by \( \text{dfrA1} \) (7) and \( \text{dfrA12} \) (1).

Phylogenetic group analyses by PCR amplification showed the prevalence of commensal phylogroup A (\( n = 21 \)) followed by B1 (\( n = 18 \)), while the virulent phylogroup B2 was detected in 11 E. coli strains. At least two to three virulence factors (\( \text{aer + cnf1, timA + stx2, stx2 + cnf1, stx2 + aer + cnf1} \)) were detected in only the pathogenic phylogroup B2 E. coli isolates and six of them were characterized as shigatoxigenic E. coli (STEC) (Table 2).

3. Discussion

To the best of our knowledge, there is no published information regarding clonal lineages, virulence factors, and detection of p(AmpC) producing E. coli from milk of clinical mastitis in Tunisia. Only the study of Saidani et al. [20] reported some data on the antimicrobial resistance genes in ESBL Enterobacteriaceae recovered from bovine mastitis, in Tunisia; such type of data are very scarce in other African countries [21,22].

According to the report of Klibi et al. [23], ESBL-producing E. coli and K. pneumonia included in this study harbored \( \text{bla}_{\text{CTXM}} \) gene. For this, our study focused on the molecular characterization of the genetic support of ESBL/p(AmpC) Enterobacteriaceae isolates, the analyses of their phylogeny and clonal lineage, as well as the virulence factors revealed in 300 clinical mastitis milk samples collected from Tunisian farms. Furthermore, our study was also interested in characterizing the mechanism of antibiotic resistance, identifying the virulence factors and establishing the phylogeny of 50 cefotaxime susceptible E. coli isolates recovered from the same samples.

The sequencing and the analyses of CTXM gene of ESBL Enterobacteriaceae isolates producers showed the dominance of the variant \( \text{bla}_{\text{CTX-M-15}} \) \( \beta \)-lactamase in all ESBL strains, although CTX-M-15 has been frequently related to human ESBL-producing Enterobacteriaceae worldwide [24]. Recently, in Tunisia, it was reported in ESBL E. coli and K. pneumonia strains from mastitis bovine milk [20]; in E. coli isolates recovered from diarrheal chickens [19]; and in flocks of healthy poultry, sheep, goat, and calf [25].

Our findings are in accordance with previous studies that report ESBL-producing E. coli isolates from bovine mastitis cases in Egypt, Turkey, China, United Kingdom, and Germany [6,13,22,26,27]. Nevertheless, other studies have described that \( \text{bla}_{\text{CTX-M-1}} \) and \( \text{bla}_{\text{CTX-M-14}} \) genes were the most detected from cows with mastitis in France [28] and \( \text{bla}_{\text{CTX-M-2}} \) type in Japan and Germany [27,29]. The emergence of \( \text{bla}_{\text{CTX-M-15}} \) type in Enterobacteriaceae isolates from cows was scary because ESBL E. coli and K. pneumonia producers, especially this CTX-M type, are important causal agents of healthcare-oriented as well as community-based infections in humans [30]. The reason for such a frequent presence of ESBL-producing bacteria in cows with mastitis could be related to the use of \( \beta \)-lactam, especially cephalosporin \( \beta \)-lactam class in veterinary medicine [22]. On the other hand, the cause could generally be related to the location of ESBL genes on plasmids that may spread easily among commensal and pathogenic bacteria within herds and the environment. Furthermore, \( \text{bla}_{\text{CTX-M-15}} \) was associated to \( \text{bla}_{\text{OXA-1}} \) in four ESBL E. coli-
producing strains. This association has been recently described in diarrheic poultry [19] and emerged in clinical ESBL E. coli strains in Tunisia [31,32].

It is important to highlight that the three CTXβ E. coli strains are p(AmpC) producers encoded by blaCMY-2. To the best of our knowledge, this appeared to be the first description of blaCMY-2 involved in bovine mastitis in Tunisia. This β-lactamase type was recently identified in E. coli isolated from diarrheic poultry [19]. However, its presence was rare in cows with bovine mastitis and only few reports identified blaCMY from dairy cows with clinical mastitis in Lebanon [33], China [34], Taiwan [35], and Switzerland [36].

Antimicrobial susceptibility testing showed that 10 ESBL/p(AmpC) Enterobacteriaceae producers and 28 of CTXβ E. coli isolates are multidrug resistant to at least three antibiotics families and present resistance to β-lactam (amoxicillin, Ticarcillin), tetracyclines, quinolones, sulfonamides, and trimethoprim-sulphamethoxazole and aminoglycosides. Different reports confirmed that ESBL-producing E. coli and K. pneumoniae isolates are multiresistant, independently from their origin such as cattle [20,22], poultry [11,19] or human [31,37]. This finding is in concordance with the veterinary treatment used in Tunisia and based on penicillin, cloxacillin, streptomycin, enrofloxacin, marbofloxacin, sulfonamide, trimethoprim, or colistin, which are the most used antibiotics to treat bovine mastitis alone or in combination [20]. In the present study, tetracycline and sulfonamide resistances of ESBL/p(AmpC) Enterobacteriaceae and CTXβ E. coli isolates are coded by tet and sul genes, respectively. These results were in agreement with previous reports from Tunisia, Egypt, and Lebanon [20,22,33]. Moreover, it seems that tet(A) gene is the most dominant in our collection, identified from bovine mastitis cases in Tunisia. This is in line with the recent report, which described the highest proportion of tet(A) in cattle with mastitis in Egypt [22].

In this study, a high frequency of quinolone resistance (64.5% for nalidixic acid) was detected in ESBL/p(AmpC) Enterobacteriaceae strains. This frequency is higher than that reported by the only study interested in ESBL-EB strains from bovine mastitis in Tunisia [20]. Similar findings documented a high quinolone resistance rate in Egypt (85.7%) and in China (73.3%) [22,34]. Such correlation analysis between plasmid-mediated quinolone resistance (PMQR) and ESBL genes in ESBL-EB isolates showed that aac(6′)-Ib-cr, qnrA, and blaCTX-M-15 are significantly associated as reported by other studies [20,21,34], suggesting that the coexistence of quinolone resistance and ESBL genes has started to become epidemic in bovine mastitis in Tunisia. The detection of such genes was very interesting due to the spread of these resistance determinants between bacteria via plasmid mobility. Nevertheless, the quinolone resistance rate (10%) revealed in our CTXβ E. coli isolates was low and our results showed much lower rates than data reported from China and India [34,38] and even lower than those from developed countries such Finland and Canada where the use of quinolones in livestock has been limited [39].

It is important to highlight the presence of class1 integrons in multidrug resistant Enterobacteriaceae with (dfrA1, dfrA17, and dfrA12) genes, conferring resistance to trimethoprim and (aadA1, aadA2, aadA5) encoding streptomycin resistance. To the best of our knowledge, the integrons were described in bovine mastitis for the first time in Tunisia. Antimicrobial resistance gene cassettes including integrons were detected in diarrheic chickens and poultry in Tunisia and Africa [11,19]. In fact, some studies were interested in integrons in Enterobacteriaceae from healthy bovine and with mastitis and only few recent reports have described integrons with different combinations of gene cassettes in Enterococcus and S. aureus in China [40,41]. These gene cassettes included in integrons remained largely unexplored in dairy cattle, suggesting the need for more studies on integrons and their circulation in livestock.

Phylogenetic typing analyses, carried out on ESBL/p(AmpC) E. coli and CTXβ E. coli isolates, revealed that most of them belong to commensal phylogroup A, followed by B1; 13 of them (11 CTXβ E. coli and 2 ESBL E. coli strains) being affiliated to pathogenic phylogroup B2. The dominance of phylogroup A in cows suffering from mastitis in Tunisia was reported by Saidani, et al. [20], and these findings agreed with previous studies that predominantly classified E. coli strains as commensal or opportunistic pathogens.
both in intramammary infections in dairy cattle or in food-producing animals [10,33,42]. Phylogenetic group analyses are an important approach to know the pathogenicity and the evolutionary relationships between different strains [43]. Indeed, identification of virulence factors revealed that strains defined as extra-intestinal pathogenic E. coli (ExPEC) and affiliated to pathogenic phylogroup B2 harbored two or three virulence genes (stx2, cnf1, fimA, and aer). The presence of cytotoxic necrotizing factor 1 (CNF1) and fimbrial adhesines type1 (fimA) led to classify E. coli isolates as ExPEC [10,44]. The absence or the low distribution of CNF genes (cnf1/cnf2) and fimA has been reported in bovine mastitis in dairy farms in Iran and Bangladesh [45,46]. In addition, the aer gene that encodes aerobactin receptor related to iron uptake was detected in our collection. Some studies reported a high prevalence of such gene in mastitis caused by E. coli in cattle [10,45].

It is important to highlight the presence of shigatoxin encoding gene (stx2) in our isolated strains. Several studies have reported shigatoxin genes (stx1/stx2), and shigatoxigenic E. coli (STEC) are considered the most pathogenic variants in bovine mastitis [8,9], although recent studies in Bangladesh and China have reported the absence of stx genes [46,47].

To our knowledge, this is the first description of STEC in clinical mastitis in dairy cattle in Tunisia. The effect of the virulence factors (VFs) of E. coli on the severity of dairy cattle suffering mastitis remains unclear. Thus, more research on different VFs of E. coli is necessary to better know the pathogenic relationship between bovine mammary infections and the severity of the clinical cases in Tunisia.

In the present study, MLST analysis was performed for ESBL/p(AmpC) E. coli and K. pneumoniae strains and revealed an extended diversity, with different STs being detected, especially in E. coli isolates (ST405, ST58, ST155, ST10, ST617, and ST399). Of note, none of our ESBL CTX-M-15 E. coli isolates belonged to ST131. Nevertheless, ST405 and ST155 were the most STs found in CTX-M-15 E. coli producers; the ST405 was considered as a vehicle driving CTX-M-15 worldwide and frequently associated with clinical human settings [32,48]. However, ST155 and ST405 were identified in healthy and sick chickens and cattle in Tunisia and Nigeria [11,19]. The ST58 and ST10 have been reported in E. coli isolates carrying CTX-M and IncFII plasmids, recovered from bovine mastitis, in France and Germany [27,28]; they were also highly distributed in livestock species in many African countries [11]. Furthermore, ST38 and ST10 have recently been related to mcr-1 positive E. coli isolates from bovine mastitis in China [14]. Recently, ST58, ST155, and ST10 were identified in ESBL E. coli isolates from diarrheic poultry in Tunisia [19]. The Detection of ST617 CMY-2 E. coli isolates in our study was supported by its presence in CTX-M E. coli producer isolates from bovine mastitis in Tunisia [20] and from companion animals and domestic farms in Tanzania [11]. The four CTXM-15 K. pneumoniae strains isolated from different farms belonged to the ST471 and ST101; the ST471 not being a frequent clone detected in bovine mastitis in farms on the coast of Tunisia [19]. This could suggest that ST471 is particularly well adapted to a bovine host. Although ST101 has been reported in K. pneumoniae isolated from milk of cows suffering from mastitis [49], there are few reports describing the genetic characteristics of K. pneumoniae strains from healthy or mastitis diseased bovine.

4. Conclusions

It is important to highlight that bovine mastitis is caused by environmental pathogens such as Enterobacteriaceae strains harboring different antibiotic resistance genes. The emergence of CTXM-15 and CMY-2 plasmids associated to PMQR and integrons in cattle in Tunisia could complicate the treatment of bovine mastitis and present zoonotic potential risk of transmission to humans. The presence of multidrug-resistant bacteria, related to virulence factors, especially shigatoxin genes belonging to different sequence types, is alarming and indicates a potential risk of the circulation of virulent and resistant clones to humans, animals, and the environment through contaminated milk or milk products. The application of good hygiene practices throughout the dairy industry and the prudent
use of antimicrobial agents against diseases affecting dairy cows are important issues that should be addressed at the global level.

5. Methods

5.1. Strain Collection

The present study was carried out on a collection of Enterobacteriaceae isolates that recovered from 300 dairy cattle with clinical mastitis in Tunisia and identified in our previously published paper [23]. This collection was composed of 14 ESBL Enterobacteriaceae isolates (10 E. coli and 4 K. pneumoniae), 3 cefotaxime resistant E. coli isolates, and 50 cefotaxime susceptible E. coli isolates. All collected isolates were conserved in skim milk at −80 °C in our strain library in the laboratory and included in this study for further characterization.

5.2. Antimicrobial Susceptibility Testing and AmpC Confirmation

Antimicrobial susceptibility testing was conducted on Mueller-Hinton agar (Biolife, Milano, Italy) plates using the agar disk diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) criteria [50]. The tested antibiotics were (µg/disc): ampicillin (10), ticarcillin (75), amoxicillin/clavulanic acid (20 + 10), cefoxitin (30), Ceftazidime (30), cefotaxime (30), Imipenem (10), ertapenem (10), gentamicin (10), tobramycin (10), streptomycin (10), nalidixic acid (30), ciprofloxacin (5), sulphonamides (200), trimethoprim/sulfamethoxazole (1.25 + 23.75), tetracycline (30), and chloramphenicol (30). E. coli strain ATCC 25922 was used as a control strain. A screening test for the detection of (AmpC) was carried out by the (DDST), according to the CLSI criteria [50].

5.3. Genomic Extraction, Polymerase Chain Reaction and Sequencing

The genomic DNA of all Enterobacteriaceae isolates was obtained using the boiling extraction method. The concentration and the purity of the extracted DNA were measured by a Nanodrop Spectrophotometer (Thermo scientific, NANODROP ONE, Waltham, Massachusetts, Etats-Unis). The detection of the different specific genes among Enterobacteriaceae isolates was performed by conventional PCR, in thermal cycler BIO-RAD T100. The reaction occurred in a 25 µL reaction mix consisting of 5 µL of 10 × Dream DNA polymerase buffer, 0.05 mM dNTPs (Thermo Scientific, France), 1U of Dream Taq DNA polymerase (Thermo Scientific, HTDS, Rue du Saule Trapu, 91300 Massy, France), 0.5 µM of each primer (Carthagenomics Advanced Technologies, Rue des Métiers, Tunis, Tunisia), and 5 µL of template DNA. The amplification program differs according to the genes sought and are presented in the relative references of each group of genes in the following paragraphs. For the visualization of PCR products, 5 µL were subjected to 1 or 2% agarose gel electrophoresis containing ethidium bromide (0.5 mg/mL). Lengths of the amplicons were determined in comparison with a 100 bp or 1 kb ladder.

The sequencing of PCR products was performed by Sanger Sequencing.

5.4. Characterization of β-Lactamase Genes and Their Genetic Environment

The genes encoding TEM, SHV, OXA-1, CTX-M, and CMY type beta-lactamases were analyzed by PCR, followed by sequencing all ESBL-p(AmpC) positive isolates [19]. Nucleotides and their deduced amino acid sequences were compared with those stored in the GenBank database to confirm the specific type of beta-lactamase genes [51]. The ISEcp1, IS26, and orf477 sequences, surrounding blaCTX-M and blaCMY genes, were analyzed by PCR, using previously described primers and conditions [31]. In addition, the genes of TEM, SHV, and OXA1 beta-lactamases were performed for all CTXS E. coli.

5.5. Characterization of Integrons and Resistance Mechanisms to Non-β-Lactam Antibiotic

The presence of the intI1 and intI2 genes (encoding class 1 and class 2 integrase, respectively) and the 3′-conserved segment (3′-CS) (qacEΔ1-sul1 genes) of class 1 integrons, in the trimethoprim-sulphamethoxazole resistant isolates, were examined by PCR [39].
Variable regions of class 1 integrons were characterized by PCR and sequencing for all intI1-positive isolates [19]. The presence of genes associated with quinolone resistance (qnrA, qnrB, qepA, and aac(6′)-Ib), streptomycin (strA and strB), sulphonamides (sul1, sul2, and sul3), and tetracycline (tetA and tetB), was determined by PCR. The aac(6′)-Ib amplicons were sequenced to identify aac(6′)-Ib-cr variants [19,31].

5.6. Virulence Factors and Phylogeny Groups

Virulence factors and phylogeny groups of E. coli isolates were screened by single or multiplex PCR assays for the presence of the eight genes encoding the following virulence factors: fimA (encoding type 1 fimbriae), papG allele III (adhesin PapG class III), cnf1 (cytotoxic necrotizing factor), papC (P fimbriae), aer (aerobactin iron uptake system) [encoding virulence factors often found in ExPEC isolates], eae (Intimin), and bfp (Type IV bundle forming pili) genes [19]. All isolates were screened for the serotypes O25a and O25b [52,53], as well as the new diffuse adhesion encoding afa operon (Gen Bank accession number FM955495), specific for isolates of O25b:H4 ST131 producing CTXM-15 [53]. In addition, E. coli strains were assigned to the phylogenetic groups A, B1, B2, or D, using a PCR strategy with specific primers for chuA, yjaA, and TspE4.C2 determinants as previously described [43].

5.7. Molecular Typing of ESBL-p(AmpC) Enterobacteriaceae Isolates

The ESBL/p(AmpC) E. coli and K. pneumoniae isolates were characterized by multilocus-sequence typing (MLST), using PCR amplification of the standard seven housekeeping loci [19]. All the amplicons were sequenced and compared with the sequences deposited in MLST database (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search, 1 May 2022) and (http://bigsdb.pasteur.fr/klebsiella/primers_used.html, 15 May 2022), specific for E. coli and K. pneumoniae, respectively.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11080948/s1, Figure S1: Antimicrobial resistance rate of 50 E. coli cefotaxime susceptible analyzed in this study; Supplementary file: Fasta form of sequence of ESBL and β-lactamases genes, gene casette of integron and sequence type genes generated in this study.

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