



Antibiotic Resistance Profile of *Escherichia coli* Isolated from Bovine Subclinical Mastitis of Dairy Farms in Algeria from 2017 to 2019

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ABSTRACT

Mastitis in cows is a major problem in dairy farms leading to a decrease in the quantity and quality of milk. The aim of the present study was to examine the association between the presence of *Escherichia coli* (*E. coli*) in milk and the subclinical mastitis, and to characterize the antibiotic resistance profiles of the isolated *E. coli*. In the current study, a total of 360 cow raw milk samples from three dairy farms of the region of Algiers were analyzed. The analysis period lasted from Spring 2017 to Winter 2019. The California Mastitis Test (CMT) was applied to detect subclinical mastitis. The *E. coli* strains were isolated from milk using conventional bacteriological methods. The antibiotic resistance profile of the isolated *E. coli* strains to 12 different antibiotics was tested using the disk diffusion method. On β -lactamase-producing strains, a double diffusion test was applied to identify the Extended-spectrum β -lactamase (ESBL) phenotype. Finally, the *ctx-M* genes were amplified by PCR. Two-thirds (66.4%) of the milk samples were positive for the CMT test. A total of 97 *E. coli* strains were isolated from the milk samples, their resistance to antibiotics was tested, and 3.1% of the strains were resistant to trimethoprim-sulfamethoxazole, 6.2% to chloramphenicol, 12.3% to gentamicin, 13.4% to colistin, 23.3% to amoxicillin/clavulanate, 31.9% to kanamycin, 39.2% to enrofloxacin, 51.5% to cefotaxime, 52% to tetracycline, 57.7% to ampicillin, 74.3% to nalidixic acid, and 75.3% to amoxicillin. Furthermore, most of the *E. coli* strains (92.8%) were resistant to more than one antibiotic with a Multiple Antibiotic Resistance index ranging from 0 to 0.8. The 50 strains resistant to cefotaxime were analyzed for an ESBL phenotype. 39 of them (78%) were positive to the double-disk synergy test. Among the 39 ESBL positive strains, 27 (69.2%) were confirmed for the presence of a CTX-M gene by PCR. The present study showed that multiple drug-resistant *E. coli*, including ESBL-carriers, were frequently isolated from the milk of dairy cows in Algeria. The results underlined that the use of antibiotics on farms must be reasoned to avoid the spread of resistant strains in animals and human populations.

Keywords: Antibiotic Resistance, Cows, CTX-M gene, *Escherichia coli*, Milk, Subclinical Mastitis

INTRODUCTION

Algerian people are great milk consumers (120 l/inhabitant/year) but the local production is insufficient to fulfill this demand. The imported milk is expensive (around 1 trillion USD per year) for Algeria (MADR, 2003), therefore, Algerian authorities encourage local milk production. It is, thus, necessary to increase the local milk production yield. According to the Ministry of Agriculture and Rural Development (MADR), Algeria has a herd of 900000 dairy cows, including 23000 high potential dairy cattle (MADR, 2003). To achieve the goal of increasing the quantity and quality of milk production in Algeria, it is important to study the physiology of milk production and identify the causes that may affect it.

However, mastitis is the most expensive and the most important disease in the dairy industry. It is defined as an inflammation of one or more quarters of the mammary gland, characterized by physical, chemical, and microbiological changes in milk secretion as well as pathological changes in breast tissue (Sharif and Muhammad, 2009). The economic losses are due to the low yield of infected udders, veterinary treatments, milk seizures as well as the premature culling of cows (Peton and Le Loir, 2014).

Clinical mastitis infections are those with symptoms like udder swelling or redness that are clearly visible. On the other hand, subclinical mastitis infections do not cause any visible changes in milk or udder appearance, making it difficult to detect. Nevertheless, subclinical mastitis infections affect the dairy producer's bottom line by reducing milk production, decreasing milk quality, and suppressing reproductive performance.

Regarding the incidence of subclinical mastitis, an average annual cost of around 5000 euros has been estimated in a herd of 100 dairy cows in the Netherlands (Halasa et al., 2009). However, the presence of pathogens and/or toxins in milk as well as residues of antibiotics resulting from the treatment of mastitis can seriously compromise public health. These infections are mainly caused by coliform bacteria *Escherichia coli* (*E. coli*), staphylococci, and streptococci (Baazise, 2006). The *E. coli* is the signature of fecal contamination although *Staphylococci* and *Streptococci* are members of the skin microbiota. To fight mastitis, massive doses of antibiotics are used selecting resistant and multi-resistant bacteria in milk.

The present study was dedicated to the characterization of antibiotic-resistant *E. coli* in the milk of dairy cows in dairy farms of the Algiers region from Spring 2017 to Winter 2019.

MATERIALS AND METHODS

Ethical approval

Since the experiments were performed on naturally collected milk, no ethical question was raised by this study. The samples were taken under the supervision of a veterinarian.

Sampling

A total of 360 milk samples (50 mL) coming from 149 different cows in three different farms (Figure 1) located in the region of Algiers were analyzed. The dairy farms in Algeria were rather small (less than 100 head of cattle) and the production system is mostly semi-intensive (MADR, 2003). Except for farm 2, the cows had access to meadows. The characteristics of the sampled farms are listed in Table 1. In order to examine a possible evolution in time, certain cows were sampled several times at different time periods. Therefore, the number of milk samples was higher than the number of cows.

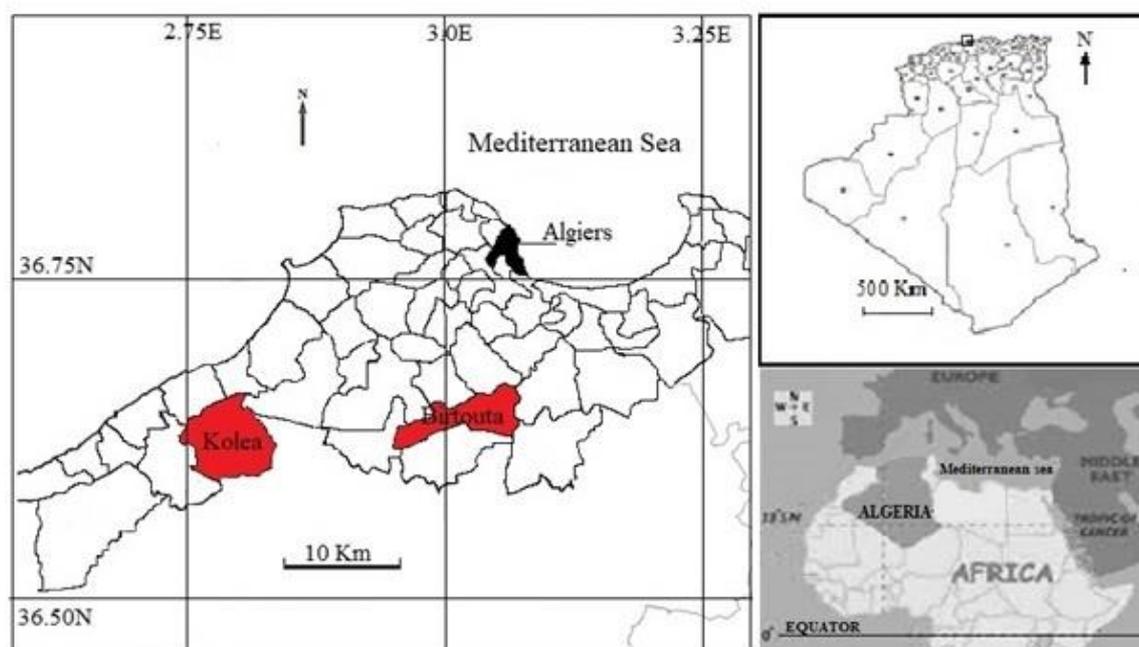


Figure 1. Localization of the sampled farms of the Algiers region

Table 1. Characteristics of the farms of the Algiers region

Farm	Name	Location (town and GPS coordinates)	Number of sampled cows	Number of milk samples	Period of sampling
Farm 1	DOUMA	Kolea, 36°36'05.3"N 2°47'00.5"E)	32	120*	March to May 2017
Farm 2	CNIAAG	Birtouta 36°39'21.8"N 3°01'27.3"E)	76	180	April to July 2019
Farm 3	ITELV	Birtouta 36°39'13.9"N 3°03'05.4"E)	41	60	April to December 2019
Total			149	360	March 2017 to December 2019

*: In this farm a sample of the bulk milk tank was also analyzed.

California mastitis test

The California Mastitis Test (CMT) was employed on farms to identify subclinical mastitis by an indirect estimation of the Somatic Cell Count (SCC) in milk (Leach et al., 2008). A four-well plastic paddle was used, one well per udder quarter of the cow to be tested. Foremilk was discarded, and then about 50-100 ml of the milk was collected into each well. An equal volume of test reagent was added (COVETO, Montaigu, France), and gently manually agitated. The reaction was scored on a scale of 0 (mixture remained unchanged) to 3 (almost-solid gel forms), with a score of 2 or 3 being considered as a positive result. A cow was considered positive if at least one quarter was positive.

Microbiological analysis

In this phase of the study, 1 ml of each milk sample was used to inoculate a sterile tube containing 9 ml of Brain Heart Infusion Broth (BHIB, Oxoid, Basingstoke, UK). The tubes were incubated for 24 hours at 37°C. For the isolation of *E. coli*, a droplet of the enrichment medium was used to inoculate a Tryptone Bile X-glucuronide (TBX) Agar (Oxoid CM0945, Basingstoke, UK) plate using a sterile loop. The plates were incubated 24 hours at 37°C. The characteristic colonies were picked and streaked on a selective medium Basing (Hektoen Agar, Oxoid PO0142, Basingstoke, UK) to obtain a pure culture. For bacterial identification, catalase, oxidase, and coagulase tests were performed (Pilet and Bourdon, 1979).

The antibiotic resistance of the isolated *E. coli* strains was analyzed using the disk diffusion method (Brown and Kothari, 1975). The diameters of inhibition were measured and compared according to the instructions by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (SFM, 2019) to determine the phenotype (sensitive, intermediate, or resistant). The used antibiotics are listed in Table 2. The antibiotics were chosen following the recommendations of the Algerian Ministry of Health (MSPRH, 2014).

Multiple Antibiotic Resistance (MAR) index was defined as dividing the number of resistant antibiotics by the number of tested antibiotics (Krumperman, 1983). For ESBL producers, the double-disk synergy test was used as previously described (Drieux et al., 2008). Commercially available disks (Oxoid, Basingstoke, UK) of Cefotaxime (CTX, 30 µg) together with 30 µg Amoxicillin-clavulanate (AMC) were placed 25 mm apart from each other on the surface of inoculated Muller-Hinton Agar (MHA) plate and incubated at 37°C for 24 hours. The test was considered positive when the decreased susceptibility to CTX and/or ceftazidime was associated with a clear augmentation of inhibition zone in front of the AMC antibiotic disk forming a characteristic “champagne cork” shape (Okoko et al., 2020). As a confirmation test, CTX (30 µg) disk and AMC (30 µg) were placed 30 mm apart on the surface of inoculated MHA, and incubated for one hour. Then, the AMC disk was removed and replaced by a CTX disk (30µg), and the plate was incubated at 37°C for 24 hours following the recommendations of the Algerian Ministry of Health (MSPRH, 2014).

Molecular detection of resistance genes

The *E. coli* strains that were positive for the double-disk synergy test (ESBL+) were analyzed for the presence of *bla*CTX-M genes using PCR as previously reported by Tabar et al. (2016). The forward and reverse primers used for PCR were *bla*-CTX-M-F 5'-ACCGCCGATAATTCGCAGAT-3' and *bla*-CTX-M-R 5'-GATATCGTTGGTGGTGCATA -3, respectively (Tabar et al., 2016). Briefly, 1 ml of an overnight *E. coli* culture in BHI (Bio-Rad, France) was used to prepare DNA by the boiling method (Djahnit et al., 2018). To amplify the CTX-M gene by PCR, 5 µl of the DNA was used. The master mix consisted of 4 µl of 5X PCR Mix (miniPCR, USA), 0.2 µl of each primer (40 µM, Eurogentec, Belgium), and 10.6 µl of DNase-RNase free sterile water (miniPCR, USA). The PCR temperature cycles were applied once (94°C for 5 minutes), followed by 40 times (94°C for 30 seconds, 58.0°C for 30 seconds, and 72°C for 30 seconds), and finally once (72°C for 5 minutes). The PCR products were analyzed by 2% gel agarose electrophoresis (Green and Sambrook, 2019). A 584 bp product was expected. Two controls were used, namely a CTX-M15+ *E. coli* strain (DSM22664, DSMZ, Germany) and a CTX-M-2+ *E. coli* strain (provided by the Belgian National reference center of antibiotic-resistant Gram-negative bacilli, Yvoir, Belgium).

Statistical analysis

The statistical analyses were performed using the following free online statistics software. MedCalc (version 20.009, Schoonjans, 2017) was used for the odds ratios. For the independence Chi-Square test, BiostatGV (version 2020) was used (Huet et al., 2021). The confidence interval for the proportions was calculated using Sample size.net (version 2021, Kohn and Senya, 2021). For the hierarchical clustering, Wessa (version 1.2.1.) was used (Wessa, 2021) with the option complete linkage. All the statistics used in this work were described in the textbook by Thrusfield and Christley (2018).

The association strength between the presence of *E. coli* in milk samples and the CMT test results was performed using the Odds Ratio (OR) calculation (Thrusfield and Christley, 2018). A case sample was defined as a sample contaminated by *E. coli* and a control sample as an *E. coli* free sample. The association among antibiotic resistance profiles was calculated using the independence Chi-square test (Thrusfield and Christley, 2018). In order to accept or

reject the null hypothesis, the following rules were adopted; p value < 0.01 was considered as a highly significant difference, p value < 0.05 was considered as a significant difference, and a p value ≥ 0.05 was considered as non-significant.

Hierarchical clustering analysis of the *E. coli* strains was made using the complete linkage method (Guess and Wilson, 2002). Briefly, a table was made with one row for each strain and one column for each antibiotic. The result of the antibiotic resistance was scored as zero for sensitive, one for resistant, and two for intermediate. The table was then used to introduce the data (as a Comma-separated Value (CSV) file) in a statistic software in R language (Wessa, 2021). The dendrogram was generated, and the length of the bars (height) indicated the distance among the strains.

Table 2. The list of used antibiotics with the disk loads and the used cut-offs

Antibiotic	Diskload (µg)	Cut-off (mm)
Ampicillin (AMP)	10	13-17
Amoxicillin (AMX)	20	13-17
Amoxicillin/Clavulanate (AMC)	30 (20-10)	13-18
Nalidixic acid (NAL)	30	13-19
Cefotaxim (CTX)	30	22-26
Gentamicin (GEN)	10	12-15
Trimethoprim- Sulfamethoxazole (SXT)	25 (1.25-23.75)	10-16
Chloramphenicol (CHL)	30	12-18
Colistin (CST)	25	10-11
Tetracycline (TET)	30	14-19
Kanamycin (KAN)	10	13-18
Enrofloxacin (ENR)	5	16-21

RESULTS

California mastitis test

In farm 1 (Douma), 120 milk sampling were collected from 32 cows. Of 120 milk samples, 78 (65%, CI 95%: 55.8-73.5) were positive to the CMT test, moreover, 31 cows (96.9%) showed at least one positive sample. In order to evaluate an evolution in time, one cow was sampled six times, 25 cows were sampled four times, two cows were samples three times, three cows were sampled two times, one cow was sampled once, and the bulk tank milk was also analyzed. Considering the obtained results, 14 out of 32 cows (43.75%, CI 95%: 26.36-62.34) presented 100% of the positive samples, 3 cows presented 75% of the positive samples, 1 cow presented 66.67% of the positive samples, 4 cows presented 50% of positive samples, 1 cow presented 33.3% of positive samples, 7 cows presented 25% of positive samples, and only 2 cows presented no positive sample. Concerning the evolution of the positivity of the samples in time, no clear pattern appeared. In farm 2 (CNIAAG), 180 milk samples coming from 78 cows were analyzed, 90 samples (50%, CI 95%: 42.5-57.5) were positive to the CMT test. Moreover, 57 out of 78 cows (73.1%, CI 95%: 61.8-82.5) showed at least one positive sample. Three cows were sampled four times, 37 cows were sampled three times, 19 cows were sampled two times and 19 cows were sampled once. Twenty-five out of 78 cows (32.1%) presented 100% of the positive samples, one (1.3%) cow presented 75% of the positive samples, one (1.3%) cow presented 66.67% of the positive samples, 7 (9%) cows presented 50% of positive samples, 17 (21.8%) presented 33.3% of positive samples, and 31 (39.7%) presented no positive samples. In farm 3 (ITELV), 60 milk sampling were performed on 40 cows; 51 samples (85%, CI 95%: 73.4-92.9) were positive. Thirty-three of the cows (82.5%, CI 95%: 67.2-92.7) showed at least one positive sample. Two cows were sampled three times, 16 cows were sampled twice, and 22 cows were sampled once. Furthermore, 31 out of 41 cows (77.5%) showed 100% of positive samples, 2 presented 50% of positive samples and 7 cows generated no positive results.

Globally, 239 out of 360 (66.39%, CI 95% 61.25-71.25) samples were positive to the CMT test, and 121 cows out of 150 (80.67%. CI 95%: 73.43-86.65) showed at least one positive sample. These results indicated a severe problem of subclinical mastitis in these farms.

Escherichia coli isolation

The results of the research on *E. coli* in the 360 milk samples (Table 3) indicated an overall prevalence of 26.9% (97/360). Therefore, the next step was to investigate if there was a correlation between CMT results and *E. coli* isolation

results (Table 4). The Odds ratio was significantly upper than one indicating an association between the presence of *E. coli* in the milk sample and the presence of a positive CMT test ($p < 0.05$).

Antibiotic sensitivity testing

The main purpose of this study was to examine the antibiotic resistance of isolated *E. coli*. The obtained results of the first serial of 12 antibiotics are summarized in Figure 2. Of the 97 tested *E. coli* strains, 1 was resistant to 10 different antibiotics, containing Ampicillin (AMP), Amoxicillin (AMX), Amoxicillin/Clavulanate (AMC), Cefotaxim (CTX), Trimethoprim-sulphamethoxazole (SXT), Chloramphenicol (CHL), Colistin sulfate (CST), Tetracycline (TET), Kanamycin (KAN), Nalidixic acid (NAL), five strains (5.2%) were resistant to eight different antibiotics (AM, AMX, AMC, CTX, CHL, K, NAL, Enrofloxacin (ENR)), nine strains (9.3%) were resistant to seven antibiotics, 22 strains (22.7%) were resistant to six antibiotics, 19 (19.6%) were resistant to five antibiotics, 11 (11.3%) were resistant to four antibiotics, eight strains (8.2%) were resistant to three antibiotics, 12 (12.4%) were resistant to two antibiotics, four (4.1%) were resistant to one antibiotic and only three strains (3.2%) were sensitive to all the tested antibiotics. Among them, two strains were isolated from two different cows of farm 2 (CNIAAG) and one strain from farm 1 (DOUMA). In other words, the MAR index was in the range of 0-0.8.

Interestingly, out of 97 strains, 85 (97.6%) different resistant types (resist types) were observed. Some clusters per farm were found as expected, but since it does not always indicate that similar resist types can be observed in different farms (Figure 3). The antibiotic resistances of the *E. coli* isolated were compared per antibiotic and among the farms in Table 5. There was a significant difference among AMP, AMC, CTX, CHL, TET, KAN, and ENR ($p < 0.01$).

The association among the resistances was also investigated (Figure 4). The results indicated that the AMP resistance profile was statistically associated with the AMC resistance, the CTX resistance, the TET resistance, the KAN resistance, and the ENR resistance ($p < 0.05$). The AMX resistance was associated with the KAN resistance, the NAL resistance, and the ENR resistance ($p < 0.01$). The AMC resistance was associated with CTX and CST resistance ($p < 0.05$). The CTX resistance was associated with the CST and the KAN resistance ($p < 0.05$). The GEN resistance was associated with the SXT resistance ($p < 0.01$). The SXT resistance was associated with the CHL ($p < 0.01$). The KAN resistance was associated with the NAL and with the ENR resistance ($p < 0.05$). The NAL resistance was associated with the ENR resistance ($p < 0.01$).

Extended-spectrum beta-lactamases

Among the *E. coli* tested samples, some seemed to potentially have an Extended Spectrum β -lactamase phenotype (ESBL). The double-disk synergy test has been performed on the 50 *E. coli* strains resistant to CTX, among which 39 strains presented a characteristic phenotype (Figure 5). The gene CTX-M encoding CTX-M β -lactamase was amplified by PCR on the 39 potentially ESBL + *E. coli*. 27 out of the 39 (69.2%) tested *E. coli* strains were CTX-M positive (Figure 6).

Table 3. *Escherichia coli* isolated strains from milk samples found in the different farms of the Algiers region

Farm*	Number of milk samples	Number of <i>E.coli</i> contaminated milk (%)
Farm 1(DOUMA)	120	45 (37.5%)
Farm 2 (CNIAAG)	180	30 (16.7%)
Farm 3 (ITELV)	60	22 (36.7%)
Total	360	97 (26.9%)

*: The farms are described in material and methods

Table 4. Association between the presence of *Escherichia coli* in milk samples and the results of California Mastitis test for cows of the studied farms of the Algiers region

	Case (<i>E. coli</i> +)	Control (<i>E. coli</i> -)
Exposed (CMT +)	74	147
Not exposed (CMT-)	23	116
Exposure rate (%)	76.3	55.9
Odds	3.2	1.3
Odds ratio (CI 95%)	2.5 (1.5-4.3)	
P-value	<0.05	

CMT: California Mastitis Test, CI: Confidence Interval, *E. Coli*: *Escherichia coli*

Table 5. Comparison of the antibiotic resistance of *Escherichia coli* per antibiotic in the different farms of the Algiers region

Antibiotic*	Farm	S	I	R	p-value (Khi2 test)
AMP	CNIAAG	3	1	27	<0.01 SS
	DOUMA	31	4	10	
	ITELV	0	1	20	
AMX	CNIAAG	4	2	25	0.1 NS
	DOUMA	12	3	30	
	ITELV	1	0	20	
AMC	CNIAAG	9	10	12	<0.01 SS
	DOUMA	32	2	11	
	ITELV	7	14	0	
CTX	CNIAAG	11	0	20	<0.01 SS
	DOUMA	24	0	21	
	ITELV	1	11	9	
GEN	CNIAAG	28	1	2	0.2 NS
	DOUMA	37	0	8	
	ITELV	19	0	2	
SXT	CNIAAG	23	8	0	0.07 NS
	DOUMA	27	15	3	
	ITELV	19	2	0	
CHL	CNIAAG	21	9	1	<0.01 SS
	DOUMA	41	0	4	
	ITELV	20	0	1	
CST	CNIAAG	23	0	8	0.04 SI
	DOUMA	38	3	4	
	ITELV	20	0	1	
TET	CNIAAG	17	3	11	<0.01 SS
	DOUMA	12	11	22	
	ITELV	2	0	19	
KAN	CNIAAG	17	1	13	<0.01 SS
	DOUMA	40	1	4	
	ITELV	4	2	15	
NAL	CNIAAG	4	2	25	0.2 NS
	DOUMA	12	3	30	
	ITELV	2	0	19	
ENR	CNIAAG	4	14	13	<0.01 SS
	DOUMA	24	9	12	
	ITELV	1	6	14	

S: sensitive; R: resistant; I: intermediate; SI: significant; SS: very significant; NS: not significant. * AMP: Ampicillin, AMX: Amoxicillin, AMC: Amoxicillin/Clavulanate, NAL: Nalidixic acid, CTX: Cefotaxim, GEN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, CHL: Chloramphenicol, CST: Colistin, TET: Tetracycline, KAN: Kanamycin, ENR: Enrofloxacin

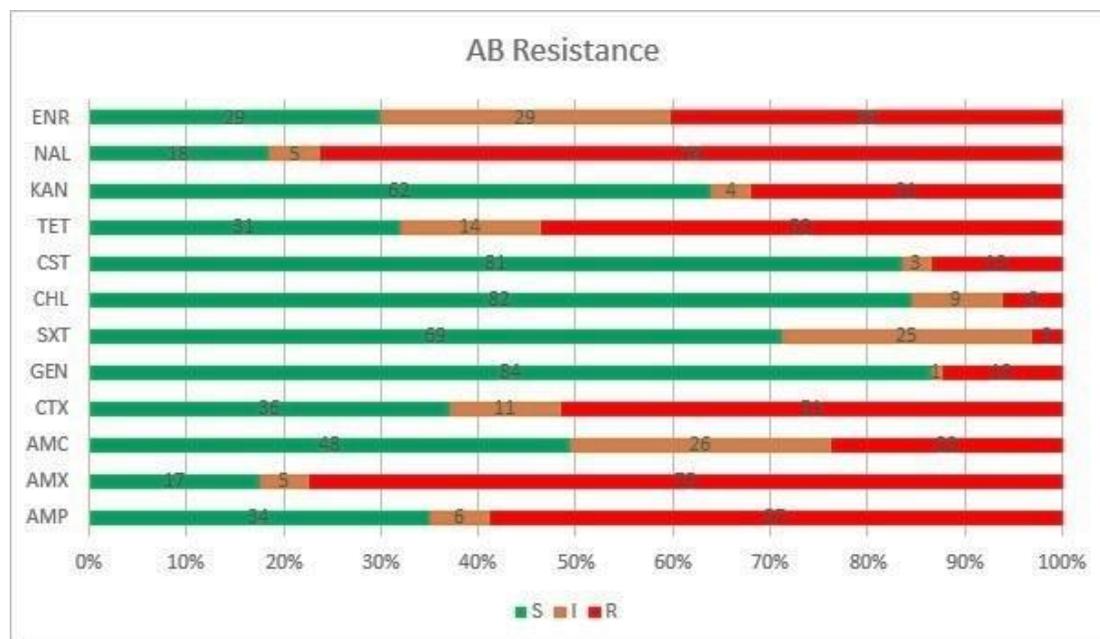


Figure 2. Resistance profile for 12 different antibiotics of the 97 *Escherichia coli* isolates from the farms of the Algiers region. AMP: Ampicillin, AMX: Amoxicillin, AMC: Amoxicillin/Clavulanate, NAL: Nalidixic acid, CTX: Cefotaxim, GEN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, CHL: Chloramphenicol, CST: Colistin, TET: Tetracycline, KAN: Kanamycin, ENR: Enrofloxacin, S: Sensitive, R: Resistant, I: Intermediate.

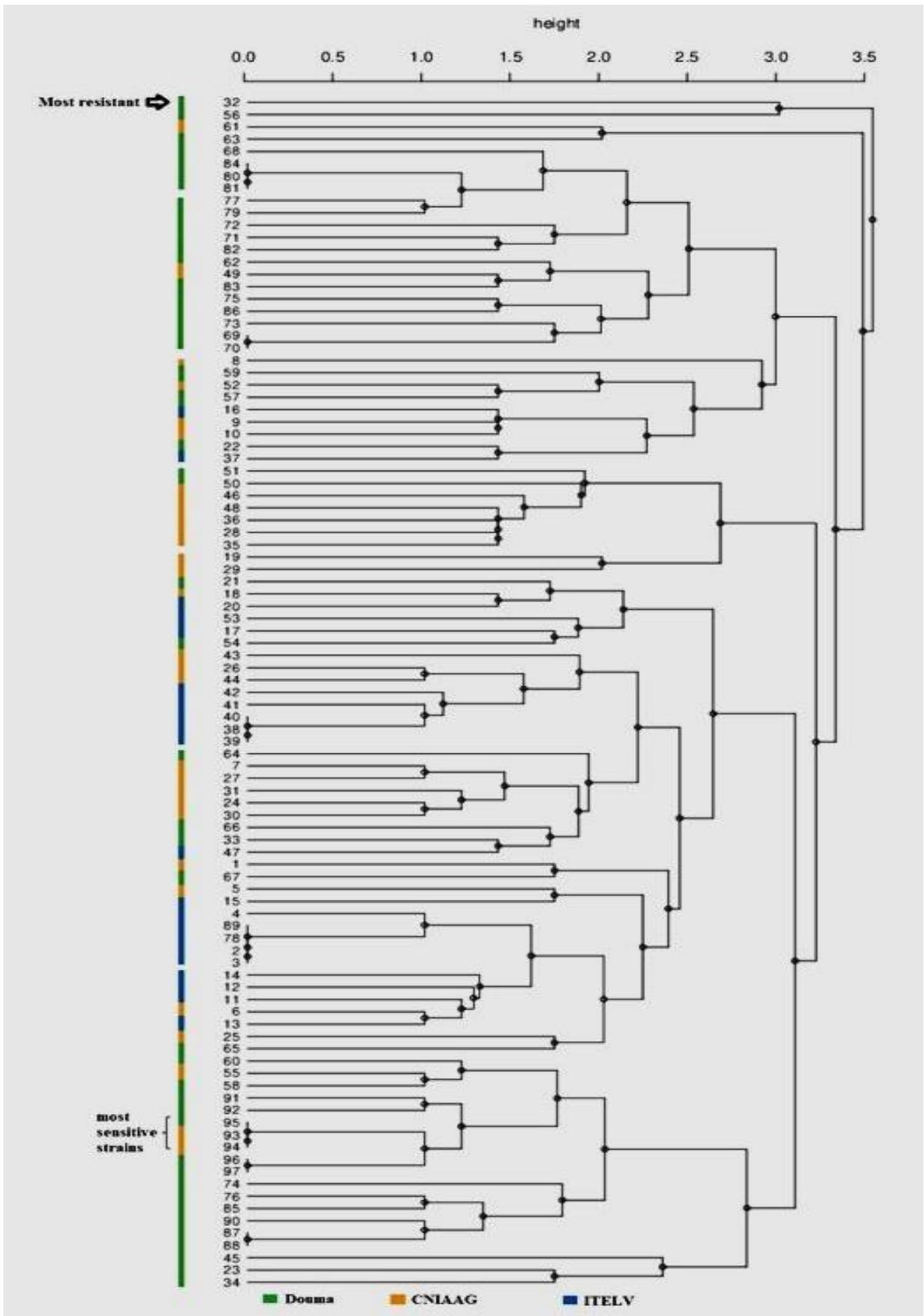


Figure 3. Dendrogram comparing the antibiotic resistance profiles of the 97 *Escherichia coli* strains. The *Escherichia coli* isolates were numbered from 1 to 97. The colors indicated the farm where the *Escherichia coli* strain was isolated. The length of the lines is proportional to the distance among the strains (Defays, 1977)

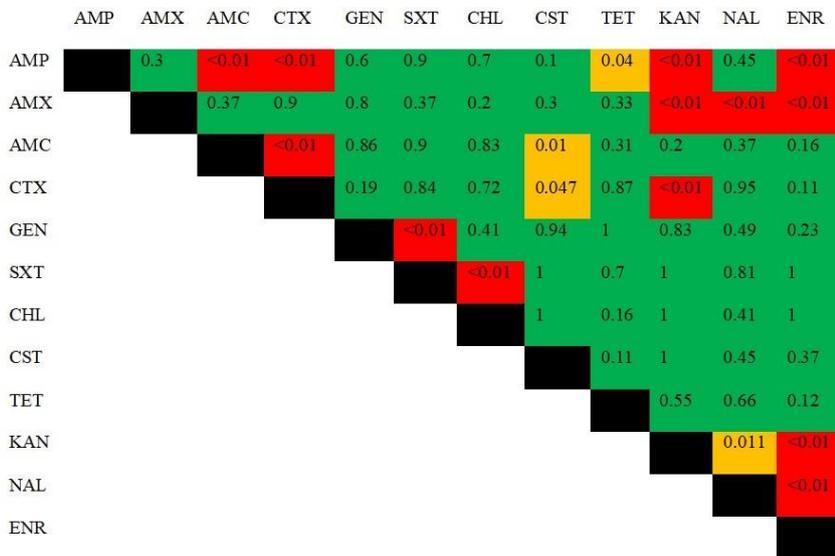


Figure 4. Associations among resistances. An independence chi 2 test has been applied. The p values of the Chi 2 test were indicated. In green ($p \geq 0.05$); there was no association, in orange ($0.05 < p < 0.01$), there was a significant association; and in red ($p < 0.01$), there was a significant association. AMP: Ampicillin, AMX: Amoxicillin, AMC: Amoxicillin/Clavulanate, NAL: Nalidixic acid, CTX: Cefotaxim, GEN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, CHL: Chloramphenicol, CST: Colistin, TET: Tetracycline, KAN: Kanamycin, ENR: Enrofloxacin.

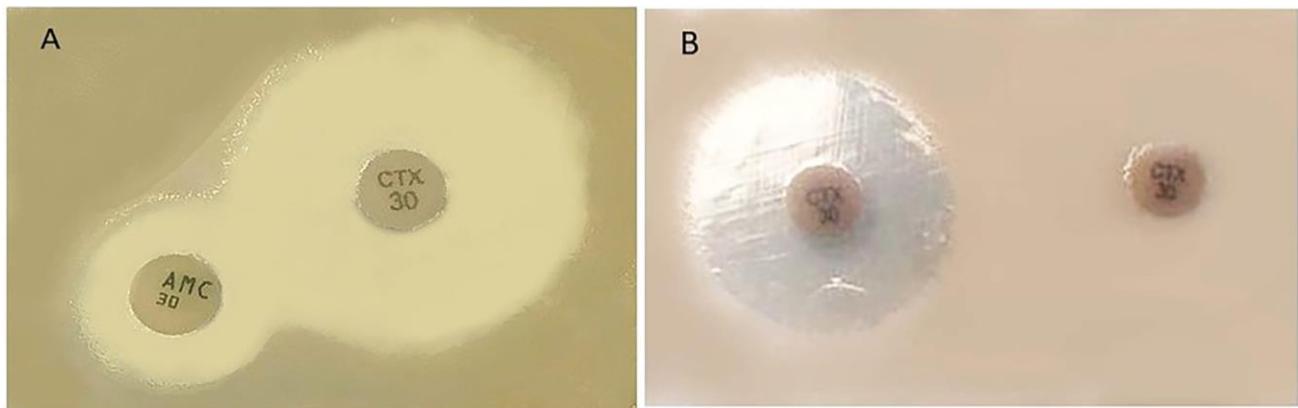


Figure 5. ESBL phenotype identification using synergy and double disk profiles. **A:** Synergy profile between AMC (30 µg) and CTX (30 µg). Due to clavulanate inhibition of ESBL; **B:** Double disk profile. The CTX (30 µg) disk on the left showed a classical inhibition phenotype. The CTX (30 µg) disk on the right showed no inhibition due to the pre-incubation for one hour with an AMC (30 µg) disk.

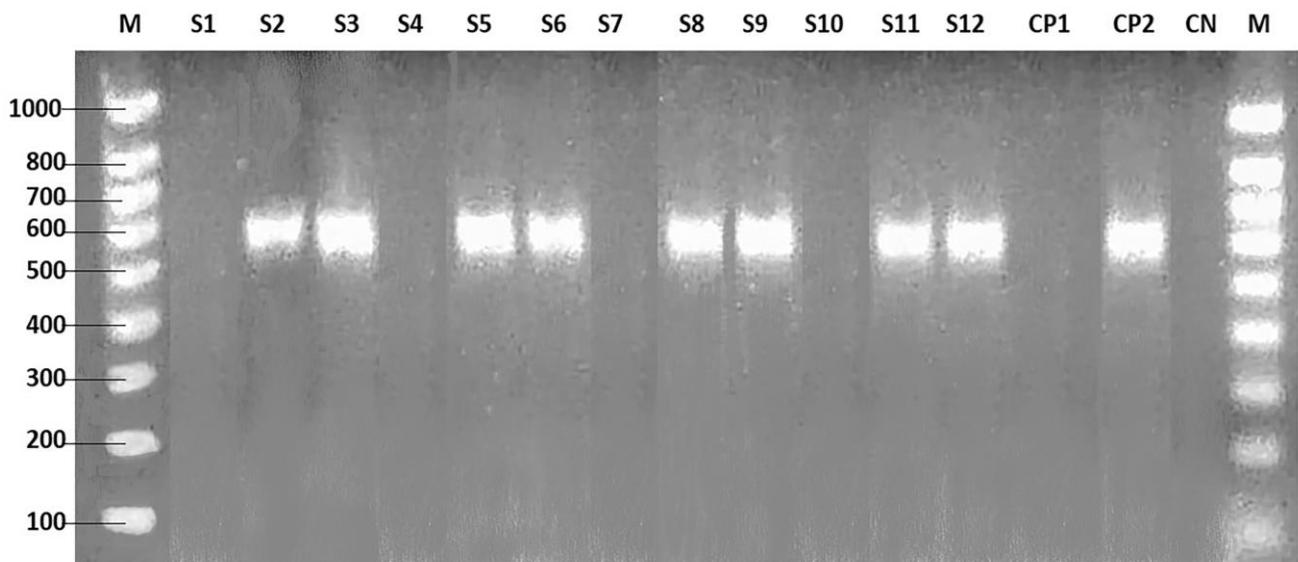


Figure 6. Agarose gel electrophoresis (2%). M: molecular weight ladder (bp). S1, S4, S7, S10: negative samples. S2, S3, S5, S6, S8, S9, S11, S12: positive samples. CP1: CTX-M-1 + strain; CP2: CTX-M-2 strain; CN: Negative PCR control.

DISCUSSION

Mastitis is a common inflammation of the cow udder leading to defect in milk production quantitatively and qualitatively leading to economic losses. In the present study, 239 (66.4%) cows presented a positive CMT test which was in accordance with the previous study in Algeria where 79% of the tested cows were CMT + (Asnour et al., 2012), and with the study on milk quality in West Algeria (Aggad et al., 2009). *Escherichia coli* can harbor and transmit resistance to antibiotics. The virulence and the antibiotic resistance of bacteria are often linked (Cepas and Soto, 2020). *Escherichia coli* is one of the bacteria causing mastitis along with Staphylococci and Streptococci (Burvenich et al., 2003). Given its origin, it is not normal to find this bacterium in milk indicating fecal contamination.

In the current study, the prevalence of *E. coli* in milk samples was 26.94%. When this prevalence was compared with the prevalence of other studies of the literature, the present prevalence is high. For instance, a prevalence of 6.5% was found in Jordan (Ismail and Abutarbush, 2020), 10% in Mexico (Olivares-Pérez et al., 2015), 11.1% in China (Yu et al., 2020), 15.5% in Belgium (Verbeke et al., 2014) and 7% in Egypt (Ameen et al., 2019). Interestingly, the present *E. coli* prevalence in the current study was approximately close to the prevalence (26%) found in another recent study in Algeria (Tahar et al., 2020) or a study in Ethiopia (27.3%) (Haftu et al., 2012), and lower than the prevalence found in Tunisia (31.7%) (Saidani et al., 2018).

A positive association (OR > 1) was observed between the presence of *E. coli* and a CMT+ test. Indeed, *E. coli* is a bacterium often found in mastitis (Burvenich et al., 2003). The hygiene of the farm is very often involved in the contamination of milk by *E. coli*. Heavily soiled litter is a major source of contamination (Magnusson et al., 2007). Decontamination of udders during milking is especially important to eliminate bacterial contaminations as much as possible. Therefore, the presence of *E. coli* in milk is an indicator of the lack of hygiene during milking (Saidani et al., 2018). The generalization of antibiotic therapy has produced spectacular results in the treatment of mastitis. But it has an unfortunate corollary, the selection of Multi-Drug Resistant (MDR) germs. In addition, previous studies have shown that the use of antibiotics for many years has acted as a selection agent of an antibiotic-resistant bacterial population on the farm prior to antibiotic use for a new clinical purpose (Avrain et al., 2003; Boonyasiri et al., 2014).

Food-producing animals harbor bacteria in their intestinal tract include potentially pathogenic bacteria for humans. These bacteria could serve as reservoirs of resistance determinants that could spread throughout the food chain, reducing the effectiveness of antimicrobials used in human and veterinary medicine (Gouvêa et al., 2015; Manyi-Loh et al., 2018).

The obtained results of the current study revealed a wide variation in resistance to the tested antibiotics. The resistance phenotype ranged from 3.1% (SXT) to 75.3% (AMX). It is worth mentioning that most of the *E. coli* strains (90/97, 92.8%) were MDR with a MAR index ranging from 0 to 0.8 with one strain resisting to 10 out of the 12 tested antibiotics. The presence of MDR strains was due to the significant pressure exerted by the extensive use of antibiotics on the farms analyzed. The appearance of MDR strains was favored by different mechanisms, including a resistance mechanism leading to the resistance of more than one antibiotic (efflux pump), the horizontal transfer of resistance by mobile genetic elements such as plasmids or transposons, and the presence of strains with a high mutation rate (Chang et al., 2015).

Recently in Algeria, multidrug-resistant *E. coli* in cows with clinical mastitis in 42 different dairy farms have been investigated (Tahar et al., 2020). The antimicrobial susceptibility testing was performed by the disk diffusion method on 52 *E. coli* strains. The observed resistances were AMX (86.5%), TET (75%), AMC (59.6%), SXT (36.5%), doxycycline (13.5%), and ciprofloxacin (13.5%). Multidrug resistance was observed in 38.4% of isolates (Tahar et al., 2020). In a similar study in Egypt (Ameen et al., 2019), the *E. coli* strains isolated from cow milk were resistant to AM (33%), SXT (23%), and TET (16%) or AMC (6.6%). In Switzerland (Nüesch-Inderbinen et al., 2019), the *E. coli* strains isolated from cow milk were resistant to AM (22%), TET (16%), SXT (9.8%), gentamicin (3.7%), or to AMC (2.4%). The observed differences in the different studies were mostly dependent on the used antibiotics in the farms. Therefore, SXT was the best choice in the present study, although AMC was the most appropriate choice in the Egyptian and Swiss studies.

The study of resistor types of the different strains underlined the existence of clusters in the same farm. Nevertheless, some similar resistance profiles were present in different farms. It is related to the fact that the same antibiotics were used in different farms (Österberg et al., 2016). When the resistance per antibiotic was compared among the farms, significant differences ($p < 0.05$) were observed for the prevalence of the resistance to several antibiotics (AMP, AMC, TET, KAN, and ENR). Therefore, the circulating strains were not the same for the different farms. It is due to the used antibiotics in the farms, and to the fact that the sampling period was not the same for all the farms. When the resistance is considered by the antibiotic family, the results were observed as follows; quinolones, NAL, and ENR (a fluoroquinolone) were tested. In fact, 74.3% and 39.2% of the *E. coli* strains were resistant to nalidixic acid and ENR, respectively. The resistances were significantly associated ($p < 0.01$), which is logical since the mechanisms of resistance were similar (mutations chromosomal genes *gyrA* and *parE*). The high levels of resistance can be related to the large use of ENR by the veterinarians in the sampled farms (personal communication).

For fluoroquinolones, a recent study in Algeria indicated 13.5% of the resistance of *E. coli* strains isolated from cow milk against ciprofloxacin (Tahar et al., 2020). In that study, the sampled region was located 200 km southeast of the current sampling region. The differences can be related to the fact that different antibiotics were used by veterinarians in this region. For example, in this study (Tabar et al., 2020), the most prevalent resistance was resistance to AMX (86.5%), TET (75%), and AMC (59.6%). In Tunisia, in a similar study, 11% of the *E. coli* isolates were resistant to ENR (Saidani et al., 2018).

For sulfonamides, the resistance against SXT in the present study was low (3%) compared to the data of the literature for comparative studies. For example, the resistance rate was 23.3% in Egypt (Ameen et al., 2019) or 17.8% in Tunisia (Burvenich et al., 2003). But the results indicated 25% of intermediate resistance which is concerning.

Nevertheless, a low rate of resistance was also found in Canada (5.8%) (Saini et al., 2012). For TET, the resistance rate was 52%. In the same country for a similar survey, the resistance rate was 75% (Tahar et al., 2020). The same high level of resistance rate was observed in Tunisia (46.6%) (Saidani et al., 2018) or Brazil (92%) (Rangel and Marin, 2009). Tetracycline represents one of the oldest molecules used, both in therapy and preventively, generating remarkably high resistance.

Nevertheless, in regions where this antibiotic was used less, lower resistance rates were recorded, for instance, the rates were reported as 2.6% in Canada (Saini et al., 2012), 12% in China (Yu et al., 2020), as well as 16.66% (Ameen et al., 2019) and 27.5% in Egypt (Ombarak et al., 2018). Tetracycline has been used inappropriately in animal feed in many countries, leading to the development of resistance. This has prompted some countries to prohibit their use in animal feed, such as in European countries (OJEU, 2003).

For aminoglycosides, KAN and gentamicin were tested. Following the classification (Saberfar et al., 2008), the results indicated that the resistance rate of the strains found in the current study for KAN goes to group II of middle resistance with a resistance level of 31.9%. The resistance to KAN was significantly different ($p < 0.01$) among the tested farms with a farm presenting 71.4% of resistant *E. coli*. Kanamycin resistance was significantly associated ($p < 0.02$) with the resistance to beta-lactam antibiotics (AMP, AMX, and CTX), and to resistance to quinolones (NAL and ENR). These associations need to be investigated because these antibiotics belong to different families and the resistance mechanisms are different. Moreover, such association was not found for gentamycin.

The high sensitivity of *E. coli* strains to gentamicin was due to the non-use of this antibiotic in veterinary treatments in Algeria, and therefore, no selection of resistant strains. The prevalence of KAN-resistant *E. coli* isolated from cow milk was within the range of 2.8% in China (Yu et al., 2020), 4.1 % in Egypt (Ombarak et al., 2018), and 6% in France (Botrel et al., 2010).

For gentamicin, a rate of 12% was recorded which was close to what has been recorded in Tunisia (19.5%, Saidani et al., 2018), in Egypt (13.3%, Ameen et al., 2019), and In China (12%, Yu et al., 2020). In addition, the present results were extremely low compared to those reported in Brazil (Rangel and Marin, 2009), Lebanon (Barbour et al., 2015), or Iran (Fazel et al., 2019), where the reported rates were 67.9%, 77%, and 49.2%, respectively. For polymixins, colistin (polymyxin E) has been tested. Colistin is a cationic polypeptide antibiotic that interacts with the outer membrane of gram-negative bacteria. Despite colistin once was avoided due to its nephro- and neurotoxicity, this drug has now become a last-resort antimicrobial agent for treating life-threatening infections caused by MDR gram-negative bacteria. However, in November 2015, the first plasmid-borne colistin resistance gene *mcr-1*, encoding a phosphoethanolamine transferase, was detected in livestock and raw meat samples as well as humans in China (Liu et al., 2020).

In the current study, 13.4% of the *E. coli* strains were resistant to colistin. Colistin resistance was associated ($p < 0.05$) with CTX resistance. Interestingly, colistin and ESBL resistance are plasmid-borne, and can be co-transferred as previously described in China (Liu et al., 2020). It will be, therefore, interesting to study the plasmid profiles of the isolated strains to identify the genetic support of the colistin resistance.

β -lactams are classical antibiotics interfering with cell wall synthesis. In the present study, several β -lactams were tested; AMP, AMX, AMC, and CTX. 75.3% of the *E. coli* strains were resistant to AMX, 57.7% to AM, 51.5% to CTX, and 23.3% to AMC. Therefore, β -lactam resistance is common in the analyzed farms. The resistance of *E. coli* strains to AMP ranged widely, 2.6% in Canada (Saini et al., 2012), 22% in Switzerland (Nüesch-Inderbinen et al., 2019), 31.7% in China (Yu et al., 2020), 33.3% in Egypt (Ameen et al., 2019), and 59% in Brazil (Rangel and Marin, 2009).

The resistance to AMX was 86.6% in a similar study in another region of Algeria (Tahar et al., 2020), confirming that the *E. coli* strains resistant to AMX are common in cow milk suffering from mastitis in Algeria. Nevertheless, the use of a combination of AMC dropped the resistance rate to 23.7% in the current study and to 59.6% in the other Algerian study (Tahar et al., 2020), which was confirmed in other studies conducted in Brazil (Rangel and Marin, 2009) and Egypt (Ameen et al., 2019) decreasing from 58% to 14% and 33.3% to 6.7%, respectively.

For CTX, the resistance rate was 4.5% in Egypt (Ombarak et al., 2018) and 18.1% in China (Yu et al., 2020). Therefore, the observed resistance rate in the current study was quite high indicating that *E. coli* strains producing β -lactamases were common in Algeria. This increase is particularly worrying because third-generation cephalosporins have been classified as critically important antibiotics (Collignon et al., 2009). Among the different mechanisms involved in β -lactams resistance, the production of a β -lactamase, an enzyme hydrolyzing the β -lactam ring of the β -lactams, is common. The emergence of ESBL and the widespread of this type of resistance is a major concern in animal and human health. Extended Spectrum β -lactamases are the enzymes having the ability to hydrolyze the β -lactam ring of broad-spectrum β -lactams such as oxyimino-cephalosporins, including cefotaxime, ceftriaxone, and ceftazidime. These antibiotics are the so-called “third-generation cephalosporins”. Because diverse types of ESBLs genes are usually plasmid-borne, and since the plasmids can be transferred among various species of Gram-negative bacteria, the genes for ESBLs can be acquired by this horizontal transfer mechanism (Kawamura et al., 2017).

In the present study, by using the double-disk synergy test, among 50 *E. coli* strains resistant to CTX, 39 (78%) presented a classical synergy pattern of ESBL strains. Widespread resistance to newer generations of cephalosporins is attributed largely to the spread of CTX-M type Extended-spectrum β -lactamases (ESBLs) in Gram-negative bacteria, especially in *Escherichia coli*. As a result, the rapid global dissemination of the ESBL-producing *E. coli* is an emerging public-health concern (Pitout and Laupland, 2008).

In the current study, by using classical PCR (Tabar et al., 2016), the CTX-M gene has been identified in 27 (69.2%) of the strains. Besides, CTX-M and ESBL can be TEM-derived or SHV-derived (Kawamura et al., 2017), so it is possible that the ESBL *E. coli* strains are of these types. Moreover, the used PCR amplified mainly the CTXM-2 family genes (Figure 7). However, CTX-M-type ESBLs have been roughly divided into four groups based on the sequence

similarity in amino acid residues; CTX-M-1 group, CTX-M-2 group, CTX-M-9 group, and CTX-M-8/CTX-M-25 group (Naas et al., 2007; Kawamura et al., 2017). Therefore, it is also possible that the mentioned ESBL strains were from another CTX-M group. For example, CTX-M-15 which is common at this moment is a part of CTX-M-1 group and was the most isolated CTX-M gene in dairy cattle (Afema et al., 2018).

CTX-M-1	TCGGGAGGAAGACTGGGTGTGGCATTGATTAACACAGCAGATAAATTCGCAAATACTTTAT	251
CTX-M-9	AGCGGAGGGCGGCTGGGCGTCGCGCTCATCGATAACCGCAGATAATACGCAGGTGCTTTAT	289
CTX-M-2	TCGGGAGGTTCGGCTTGGCGTTGGCGTGATTAACACCGCCGATAAATTCGCAGAT	194
CTX-M-25	AGCGGGGACGACTGGGTGTGGCGTTGATTAACACCGCCGATAAATTCGCAGACGCTCTAC	289
	* * * * *	
CTX-M-1	CGTGCTGATGAGCGCTTTGGCGATGTGCAGCACCAGTAAAGTGATGGCCGTGGCCGCGGTG	311
CTX-M-9	CGCGGTGATGAACGCTTTCCAAATGTGCAGTACCAGTAAAGTTATGGCGGCCGCGCGGTG	349
CTX-M-2	CGTGCCGATGAACGTTTTTGGCGATGTGCAGTACCAGTAAAGTGATGGCGGCCGCGCGGTG	254
CTX-M-25	CGCGCCGACGAGCGTTTTTGGCATGTGCAGCACCAGTAAAGTGATGGCGGTAGCGCGGTG	349
	* * * * *	
CTX-M-1	CTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCT	371
CTX-M-9	CTTAAGCAGAGTGAAACGCAAAAGCAGCTGCTTAATCAGCCTGTGAGATCAAGCCTGCC	409
CTX-M-2	CTTAAACAGAGCGAGAGCGATAAGCACCTGCTAAATCAGCGCGTTGAAATCAAGAAGAGC	314
CTX-M-25	CTTAAGCAAAGTGAAACGCAAAAGGGCTTGTGAGTCAGCGGTTGAAATTAAGCCCTCA	409
	* * * * *	
CTX-M-1	GACTTGGTTAACTATAATCCGATTGCGGAAAAGCACGTCGATGGGACGATGTCCTGCGCT	431
CTX-M-9	GATCTGGTTAACTACAATCCGATTGCCGAAAACACGTCACGGCACAATGACGCTGGCA	469
CTX-M-2	GACCTGGTTAACTACAATCCCATTGCGGAGAAAACACGTTAACGGCAGATGACGCTGGCT	374
CTX-M-25	GACTTGATTAATAACAACCCATTGCGGAAAACACGTCATGGCACGATGACATTCGGG	469
	* * * * *	
CTX-M-1	GAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGATTTCT	491
CTX-M-9	GAGCTGAGCGCGGCCGCGTTCAGTACAGCGACAATACCGCCATGAACAAATGATTGCC	529
CTX-M-2	GAGCTTAGCGCGAGCGCGCTGCAGTATAGCGACAATACTGCCATGAATAAGCTGATTGCC	434
CTX-M-25	GAGTTGAGCGCGGCCGCGCTACAGTACAGCGATAATACTGCCATGAATAAGCTGATTGCC	529
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CTX-M-1	CACGTTGGCGGCCCGGCTAGCGTACCGCGTTTCGCCGACAGCTGGGAGACGAAACGTTTC	551
CTX-M-9	CAGCTCGGTGGCCCGGGAGGCGTGACGGCTTTTGCCTCGCGGATCGGGGATGAGACGTTT	589
CTX-M-2	CATCTGGGTGGTCCCATAAAGTGACGGCGTTTGTCTCGTTCGTTGGGTGATGAGACCTTC	494
CTX-M-25	CATCTCGGGGGCCGGATAAAGTGACGGCATTTGCCCGTACGATTGGCGATGACACGTTTC	589
	* * * * *	
CTX-M-1	CGTCTCGACCGTACCGAGCCGACGTTAAACACCGCCATTCGGGGCGATCCGCGTGATACC	611
CTX-M-9	CGTCTGGATCGCACTGAACCTACGCTGAATACCGCCATTCGGGGCGACCCGAGAGACACC	649
CTX-M-2	CGTCTGGACAGAACCAGCCACGCTCAATACCGCCATTCAGGGCGACCCGCGTGATACC	554
CTX-M-25	CGGCTCGATCGTACCGAGCCGACGCTCAACACCGCGATCCCGGGCGACCCGCGGATACC	649
	* * * * *	
CTX-M-1	ACTTCACCTCGGGCAATGGCGCAAACCTCTGCGTAATCTGACGCTGGGTAAAGCATGGGT	671
CTX-M-9	ACCACGCCGCGGGCGGATGGCACAGACGTTGCGTCAGCTTACGCTGGGTGATGCGCTGGGC	709
CTX-M-2	ACCACGCCGCTCGCGATGGCGCAGACCCCTGAAAATCTGACGCTGGGTAAAGCGCTGGCG	614
CTX-M-25	ACCACGCCGTTAGCGATGGCGCAGGCTCTGCGCAATCTGACGTTGGGCAATGCCCTGGGT	709
	* * * * *	
CTX-M-1	GACAGCCAACGGGCGCAGCTGGTGACATGGATGAAAGGCAATACCACCGGTGCAGCGAGC	731
CTX-M-9	GAAACCCAGCGGGCGCAGTTGGTGACGTGGCTCAAAGGCAATACGACCGGCGCAGCCAGC	769
CTX-M-2	GAAACTCAGCGGGCACAGTTGGTGACGTGGCTTAAAGGCAATACTACCGGTAGCGCGAGC	674
CTX-M-25	GACACTCAGCGTGCAGCTGGTGATGTGGCTGAAAGGCAACACCACCGGCGCTGCCAGC	769
	* * * * *	
CTX-M-1	ATTCAGGCTGGACTGCCTGCTTCTGGGTGTGGGGGATAAAAACCGGCAGCGGTGACTAT	791
CTX-M-9	ATTCGGGCCGGCTTACCGACGTCGTGGACTGCAGGTGATAAGACCGGCAGCGCGACTAC	829
CTX-M-2	ATTCGGGCCGGTCTGCCGAAATCATGGGTAGTGGGCGATAAAAACCGGCAGCGGAGAT	734
CTX-M-25	ATTCAGGCAGGGCTACCCACATCGTGGGTGTGCGGGGATAAAAACCGGCAGCGCGGTTAT	829
	* * * * *	
CTX-M-1	GGCACCACCAACGATATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCTGGTC	851
CTX-M-9	GGCACCACCAATGATATTGGCGGTGATCTGGCCGAGGGTCGTGCGCCGCTGGTTCTGGTG	889
CTX-M-2	GGCACCACCAACGATATCGCGGTGATCTGGCCGAAAAGATCGTGCGCCGCTGATTCTGGTG	794
CTX-M-25	GGTACGACGAATGATATCGCGGTATTGGCCGAAAAGGTCGCGCGCCGCTCGTTCTGGTG	889
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Figure 7. Alignment of the main CT-X-M sequences using ClustalOmega software. CT-X-M-1 (genbank NG_048897.1), CTX-M-2 (genbank NG_048968.1), CTX-M-9 (genbank NG_049043.1), and CTX-M-25 (genbank NG_048974.1). The sequences of the used primers were highlighted. The identities were indicated by a star. The numbers corresponded to the number of the genbank sequences.

CONCLUSION

In conclusion, the findings of the current study have indicated that cows producing milk are commonly positive to CMT test, and subclinical mastitis is an underestimated problem in Algeria. *Escherichia coli* is frequently isolated in cow milk indicating an improper hygiene level in the farms and an inappropriate disinfection procedure at the milking.

Antibiotic resistance is a major concern in animal and human medicine. The increase of MDR strains in animals and humans leads more and more to therapeutic dead ends. Most of the *E. coli* isolates were found as MDR, and a particular focus was put on ESBL. Of the studied *E. coli*, 40% were ESBL-carriers. Although animals should be cared for, the sparing use of antibiotics is recommended. In any case, the use of antibiotics as a growth promoter should be prohibited.

DECLARATIONS

Author's contributions

Ghallache Loubna and Mohamed-Cherif Abdellah conceived the study design, carried out laboratory work, participated in data analysis and interpretation, drafted the manuscript. Ghallache Loubna and Mohamed-Cherif Abdellah contributed equally to this work. China Bernard conceived the study design, participated in data analysis and interpretation, revised the manuscript. Benfodil Karima conceived the study design, drafted and revised the manuscript. Miroud Kamel revised the manuscript. Ait-Oudhia Khatima conceived the study design, took part in the coordination and management as well as field studies, participated in data analysis and interpretation, and revised the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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