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Antibiotic susceptibility of enterobacteriaceae species isolated from mastitic milk in Algeria

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ABSTRACT

Objective: To determine: species distribution of 35 Enterobacteriaceae isolates involved in bovine mastitis; and antimicrobial susceptibility pattern of all isolates from geographical areas in Algeria. **Methods:** Enterobacteriaceae species identification was performed based on conventional biochemical techniques and using the API 20 E test. The antibiotic susceptibility was determined according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS). In total, 35 Enterobacteriaceae species: *Escherichia coli* (*E. coli*) (n=5), *Klebsiella oxytoca* (*K. oxytoca*) (n=5), *Enterobacter cloacae* (*E. cloacae*) (n=4), *Klebsiella ornithinolytica* (*K. ornithinolytica*) (n=4), *Kluyvera* spp (n=4), and *Hafnia alvei* (*H. alvei*) (n=3), were the most commonly identified. Enterobacteriaceae isolates were the most resistance to Amoxicillin+Clavulanic Acid (74.28%), Ampicillin (28.57%) and Amoxicillin (28.57%) followed by Tetracycline (20%) and Céfodiaxin (14.7%). **Conclusions:** We conclude that Enterobacteriaceae species from bovine milk presented significantly distinct antimicrobial resistance profiles, evaluated by phenotypic test, which has implications for treatment and management decisions.

1. Introduction

Bovine mastitis is the most common disease in dairy cows worldwide which occurs primarily in response to intra-mammary bacterial infection[1]. It has remained as a major challenge to the worldwide industry despite the widespread implementation of mastitis control strategies. It continues to be one of the economically most important diseases of dairy cattle[2,3]. Approximately 60 percent of clinical mastitis cases are caused by environmental organisms, especially *Escherichia coli* (*E. coli*). Unfortunately, the increase in dairy size, the type of confined housing, increased milk production, increasing dry matter intake and manure production, genetic pressure towards production and lowered disease resistance are all modern dairy management realities that work against the control of coliform mastitis.

Coliform mastitis is the most common form of clinical mastitis, the incidence of which has been found to increase in many herds and countries during recent decades[4]. *E. coli* are the most commonly implicated coliform species in mastitis for which the primary source is the bovine feces, cow's environment and infect the udder via the teat canal [4,5].

These bacteria originate from immediate environment of the cow since practically all the area in and around the cow are contaminated by manure. The factors are further complicated in urban situations of dairy farming where over crowding, lack of hygiene, poor ventilation, improper

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flooring etc, are some of the predominating factors. Coliforms often produce an acute form of mastitis[6].

Antimicrobial therapy is the primary tool for the treatment of mastitis. It has been used extensively over the past 45 years in control of bovine mastitis and is the most common method employed at present to treat mastitis in Algeria. The problems associated with antibiotic therapy are its failure due to multiple etiology, drug resistance and non-co-operation of farmers due to expensive treatment.

In other hand, it has been established that mammary infections, including *E. coli* are also associated with biofilm formation[7]. Hence, the organisms within biofilms are resistant towards the host immune response as well as antibacterial agents.

Therefore, it is necessary to select suitable antibiotics, preferably after antibiotic susceptibility testing and using such antibiotics at an adequate dose for sufficient duration to ensure effective treatment and control of coliform mastitis but, little work has been carried out in our region, regarding the isolation and antibiogram of these bacteria causing mastitis.

Keeping this information in background, the present study was designed to study the Enterobacteriaceae species distribution and to identify and determine the antibiotic susceptibility of different Enterobacteriaceae species isolated from bovine mastitis in Algeria.

2. Material and methods

2.1. Sources of milk samples

Milk samples from clinical and subclinical bovine mastitis were collected from different dairy farms located in and around center region of Algeria. Milk samples were aseptically collected from the affected quarters of the cows before any antimicrobial treatment and cultured using standard methods.

2.2. Cultural examination and identification of isolates

California Mastitis Test (CMT) positive milk samples were collected from lactating cows on private farms. Individual mammary quarter milk samples were aseptically collected into sterile vials just before milking. The samples were transported in a cool box at 4 ℃ to the laboratory. The CMT and bacteriological analyses were initiated within 24 h after sampling. For the isolation and identification of Enterobacteriaceae, ten micro liters of each milk was streaked onto blood agar plates supplemented with 5% sheep blood nutrient agar and MacConkey's agar plates and all plates were incubated at 37 ℃ for 24 to 48 h.

Grown colonies were analyzed based on colony morphology, gram-staining and Enterobacteriaceae species further identification was performed based on conventional biochemical techniques as described by Cruickshank *et al*[8] and using the API 20 E test kit (BioMerieux, France) as described by the manufacturer.

2.3. Antibiotic susceptibility test

Using the disc diffusion method on Mueller Hinton Agar (Oxoid) the antibiotic susceptibility test was carried out and interpreted by the consideration of the National Committee for Clinical Laboratory Standards guidelines-NCCLS[9]. The procedure followed is briefly described here. Enterobacteriaceae isolates were grown overnight on blood agar and colony suspension was prepared using the sterile saline water equivalent to a 0.5-McFarland standard.

Suspension (100 μL) was spread over the media plate and antibiotic disc was transferred aseptically on the surface of inoculated media plate. These plates were incubated afterward at 35 ℃ for 24 h.

Commercially available antimicrobial sensitivity discs (Oxoid) were used: Chloramphenicol (C)(30 μg), Colistin (CS)(50 μg), Amoxicillin + Clavulanic Acid (AMC)(10 μg), Ampicillin (AMP)(10 μg), Cefodiazin (CZ)(30 μg), Amoxicillin (AM)(30 μg), Ceftiofur (XNL) (30 μg), Cefoxitin (FOX) (30 μg), Cefotaxim (CTX) (30 μg), Gentamicin (G)(10 μg), Neomycin (N) (30 μg), Sulfamethoxazole-trimethoprim (SXT) (25 μg), Tetracycline (TE)(30 μg), Enrofloxacin (ENR)(5 μg), Flumequin (FT) (30 μg) and Nalidixic acid (NA) (30 μg).

Quality controls were run using *E. coli* ATCC 25922. Cultures were classified as sensitive, intermediate and resistant on the basis of the diameter of the zone of inhibition[10]. Isolates resistant to three or more antimicrobials were considered multi-resistant[11].

2.4. Statistical analysis

The scored antibiogram data were analyzed with a computer package STATISTICA. Data on antimicrobial susceptibility was compared by *Chi*-square test for independence.

3. Results

The results of identified Enterobacteriaceae species are presented in Table 1. On the basis of biochemical characteristics, 16 different kinds of Enterobacteriaceae isolates were identified and among these *E. coli* were the most isolated.

Table 1
Species of Enterobacteriaceae isolated from bovine mastitis milk samples.

Bacteria species	Bacteria isolated	
	Number	%
E. coli	5	14.3
K. oxytoca	5	14.3
E. cloacae	4	11.4
K. ornithinolytica	4	11.4
K. spp	4	11.4
H. alvei	3	8.5
S. arizonae	1	2.8
P. aeroginosa	1	2.8
Y. pestis	1	2.8
A. hydrophila	1	2.8
F. horyzihabitans	1	2.8
E. sakazaki	1	2.8
E. asburiae	1	2.8
B. agrestis	1	2.8
E. americana	1	2.8
P. spp	1	2.8
Total	35	100

In total, 35 Enterobacteriaceae species recovered were *E. coli* (14.3%) and *Klebsiella oxytoca* (*K. oxytoca*) (14.3%), the most frequently identified, followed by *Enterobacter cloacae* (*E. cloacae*) (11.4%), *Klebsiella ornithinolytica* (*K. ornithinolytica*) (11.4%), and *Klebsiella ornithinolytica* (*K. ornithinolytica*) (11.4%). A small percentage of other Enterobacteriaceae species were also isolated.

The result of antibiotic susceptibility tests of 35 Enterobacteriaceae isolates against to 16 antibiotics are shown in Table 2.

In general, the antibiotic susceptibility test of the Enterobacteriaceae species showed the highest rates of susceptibility against Neomycin (100%), Gentamycin (100%), and Trimethoprim+Sulphamethoxazole (97.1%), followed by Chloramphenicol (94.3%), Colistin (94.3%), and Ceftiofur (91.4%) and whereas, these isolates were the most resistance to Clavulanic Acid+ Amoxicillin (74.3%).

Considering individual enterobacteria species, antibiotic susceptibility between individual species showed some difference. For example, *E. coli* was 100% sensitive to the majority of tested antibiotics (FOX, N, SXT, ...). Other strains were also sensitive to various antibiotics (Table 3 and 4) except for AMC.

Among the 35 isolates, only one was susceptible to all the antibiotics used in this study and rest were observed susceptible against at least one antibiotic.

Based on the disk diffusion test, Enterobacteriaceae isolates were predominantly sensitive to lot of antibiotics selected (Table 2 and 3). However, 28.6% of the Enterobacteriaceae species were multi-resistant against three or more antimicrobials (Table 4). In that regard, multiple resistance to AMC, TE, and AMX in the disk diffusion test was confirmed.

Table 2
Antibiotic sensitivity pattern of Enterobacteriaceae isolated from bovine mastitis.

Antibiotics	Profile	Susceptible		Resistant		Intermediate	
	Break* points	Number	%	Number	%	Number	%
CTX	≤14-23≥	29	82.8	0	0	6	17.1
AMC	≤13-18≥	8	22.8	26	74.3	1	02.8
FOX	≤14-18≥	29	82.8	5	14.3	1	02.8
SXT	≤10-16≥	34	97.1	1	02.8	0	0
N	≤13-18≥	35	100	0	0	0	0
TE	≤14-19≥	28	80	7	20	0	0
XNL	≤17-21≥	32	91.4	0	0	3	08.6
AM	≤13-17≥	21	60	10	28.6	4	11.4
CZ	≤14-18≥	28	82.3	5	14.7	1	2.9
AMP	≤14-21≥	21	60	10	28.6	4	11.4
G	≤12-15≥	35	100	0	0	0	0
FT	≤21-25≥	30	85.7	3	08.6	2	05.7
ENR	≤16-21≥	32	91.4	0	0	3	08.6
NA	≤13-19≥	31	88.6	2	05.7	2	05.7
C	≤12-18≥	33	94.3	2	05.7	0	0
CS	≤10-13≥	33	94.3	2	05.7	0	0

*Zone of inhibition (mm) by disk diffusion method susceptibility interpretative guidelines based on NCCLS (2002).

CTX: Cefotaxim; AMC: Amoxicillin + Clavulanic acid; FOX: Cefoxitin; SXT: sulfamethoxazole-trimethoprim; N: Neomycin; TE: Tetracycline; XNL: Ceftiofur; AM: Amoxicillin; CZ: Cefodiazin; AMP: Ampicillin; G: Gentamicin; FT: Flumequin; ENR: Enrofloxacin; NA: Nalidixic acid; C: Chloramphenicol; CS: Colistin

Table 3

Percentage of susceptibility of 35 Enterobacteriaceae species isolates from bovine mastitis to selected antimicrobials based on diameter of inhibition zone.

Enterobacteriaceae strains	Total	Number of susceptible isolates (n, %)															
		CTX	AMC	FOX	SXT	N	TE	XNL	AM	CZ	AMX	G	FT	ENR	NA	C	CS
E. coli	5	4(80)	1(20)	5(100)	5(100)	5(100)	5(100)	4(80)	4(80)	5(100)	4(80)	5(100)	5(100)	5(100)	5(100)	5(100)	5(100)
K. oxytoca	5	4(80)	0(0)	5(100)	5(100)	5(100)	5(100)	5(100)	3(60)	5(100)	1(20)	5(100)	5(100)	4(80)	4(80)	5(100)	5(100)
E. cloacae	4	4(100)	0(0)	2(50)	4(100)	4(100)	2(50)	4(100)	4(100)	2(50)	1(25)	4(100)	2(50)	3(75)	4(100)	4(100)	4(100)
K. ornithinolytica	4	3(75)	0(0)	3(75)	4(100)	4(100)	4(100)	3(75)	3(75)	4(100)	1(25)	4(100)	4(100)	3(75)	3(75)	3(75)	3(75)
K. spp	4	3(75)	0(0)	2(50)	4(100)	4(100)	2(50)	4(100)	2(50)	2(50)	2(50)	4(100)	2(50)	4(100)	4(100)	4(100)	4(100)
H. alvei	3	3(100)	3(100)	3(100)	3(100)	3(100)	1(33)	3(100)	0(0)	2(67)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)
S. arizonae	1	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
P. aeroginosa	1	0(0)	0(0)	0(0)	0(0)	1(100)	1(100)	1(100)	0(0)	0(0)	1(100)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)
Y. pestis	1	0(0)	0(0)	1(100)	1(100)	1(100)	0(0)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	0(0)
A. hydrophila	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
F. horyzihabitans	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
E. sakazaki	1	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
E. asburiae	1	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
B. agrestis	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
E. americana	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
Pantoea spp	1	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
Total	35	29(13)	8(5.2)	29(13.8)	34(15)	35(16)	28(13.3)	32(14.6)	21(8.65)	29(13.7)	21(12)	35(16)	30(14)	32(15.3)	31(13.6)	33(15)	33(14.8)

CTX: Cefotaxim; AMC: Amoxicillin + Clavulanic acid; FOX: Cefoxitin; SXT: sulfamethoxazole–trimethoprim; N: Neomycin; TE: Tetracycline; XNL: Ceftiofur; AM: Amoxicillin; CZ: Cefodiazin; AMP: Ampicillin; G: Gentamicin; FT: Flumequin; ENR: Enrofloxacin; NA: Nalidixic acid; C: Chloramphenicol; CS: Colistin

Table 4

Resistance patterns of 35 multidrug resistant Enterobacteriaceae species isolated from bovine mastitis in Algeria.

Multidrug resistance pattern	Number of resistant isolates (n=35)	Resistance (%)
AMC	11	31.4
AM	5	14.3
AMC–AMX	5	14.3
AM–AMX	1	2.9
AM– TE	2	5.7
AMC–FOX–CZ	1	2.9
AMC–NA–C–CS	1	2.9
AMC–TE–CS	1	2.9
AMC–TE–AMX	1	2.9
AMC–TE–AMX– CZ	1	2.9
AMC–TE–FOX–AM–FT	1	2.9
AMC–TE–CZ–FT	1	2.9
AMC–FOX–AMX	1	2.9
AMC–FOX–AMX–CZ	1	2.9
AMC–FOX–SXT–AM–CZ–FT–NA–C	1	2.9
All susceptible	1	2.9

AMC: Amoxicillin + Clavulanic Acid; FOX: Cefoxitin; SXT: sulfamethoxazole–trimethoprim; TE: Tetracycline; AM: Amoxicillin; CZ: Cefodiazin; G: Gentamicin; FT: Flumequin; NA: Nalidixic acid; C: Chloramphenicol; CS: Colistin

4. Discussion

In this study, *E. coli* was observed to be the predominant between Enterobacteriaceae species. This is consistent with other studies[12]. High prevalence of *E. coli* in mastitis milk samples has also been reported by Deborah *et al.*[13] and Balakrishnan *et al*[14].

The predominance of coliform mastitis pathogens is due to the presence of environmental factors such as poor hygienic conditions, warm and humid weather, and the general lack of farm cleanliness and sanitation, which may account for the observed high prevalence of environmental pathogens[6]. On the other hand, deficient milking procedures, poor hygiene and treatment practices could contribute to a high prevalence of these contagious pathogens, as *E. coli* originates from the cow's environment and infect the udder via the teat canal[4].

That these species were implicated in bovine mastitis in the present study implicates alternate environmental sources of Enterobacteriaceae capable of inducing intra-mammary infection and mastitis.

Little published local information on antimicrobial susceptibility of pathogens responsible for bovine intra-mammary infections in Algeria is available. Thus, there is need to routinely investigate and record the epidemiology of bovine mastitis and antibiogram sensitivity of bacterial isolates in various parts of Algeria.

In general, the antibiotic susceptibility test of the Enterobacteriaceae species showed high rates of susceptibility against various antibiotics except against antibiotics from beta lactam. These antibiotics should be

used cautiously in the treatment of bovine mastitis coliform unless antimicrobial sensitivity testing is performed.

Considering the results based on the most isolated individual species in this study, FOX, SXT, N, TE, XNL, CZ, AMX, C, FT, ENR, NA, C and CS were 100% effectives on *E. coli* whereas, CTX and AMC were 80% and 20% effectives, respectively. In addition to the most resistant specie to the majority of antibiotics was *Pseudomonas aeroginosa* (*P. aeroginosa*) . Moreover, *Flavimonas horyzihabitans* (*F. horyzihabitans*) strain showed susceptibility to all antibiotics used in this study.

The differences of antibiotic susceptibility between individual species may arise from several reasons. For instance, the geographical variations in resistance profiles of Enterobacteriaceae species have a considerable impact on antimicrobial prescription.

For example, the rate of AMC resistance (74.3%) observed in this study is much higher than those reported by Malinowski *et al.* [15](16.4%). From the above antibiotic resistance pattern it can be concluded that indiscriminate and frequent use of these antibiotics in animals could be the reason for their ineffectiveness against Enterobacteriaceae species.

For the others antibiotics, the results of present study showed some similarities with previous studies[16-18].

On the other hand, some authors reported high percentage of resistance to ampicillin and tetracycline[19, 20]. These authors found that the examined strains were genotypically different, multidrug resistant, and carried multiple resistance genes.

Hence, because of the differences occur in the efficacy of antibiotics against individual bacteria species, the identification of bacterial agents has been recommended along with the antibiotic susceptibility test[21].

The most important factor in the control of coliform mastitis is the emergence of multiple drug resistant strains. Resistance to more than three antimicrobials was high among Enterobacteriaceae species, which is consistent with other studies on isolates from bovine mastitis[12]. Development of multiple drug resistance among most of these species may be related to transmission of R factor[22] which is extrachromosomal genetic determinants 'plasmids'. *E. coli* species often contain multiple plasmids that may contain any number of antibiotic resistant genes[23].

This is consistent with a high genetic diversity of *E. coli* associated with mastitis, as reported by Srinivasan *et al.*[20].

Antimicrobial resistance is influenced by the different practices of administering antimicrobial products (dosage, timing, frequency, type and frequent change of types) [24]. Our data on the susceptibility to commonly used antimicrobials support this.

For example, amoxicillin-clavulanic acid combination, ampicillin and tetracycline are heavily used for the treatment of bacterial infections across all animal species in Algeria (personal observations), while tetracycline is used in many of the intramammary formulations. This is reflected in the higher prevalence of resistance to AMC, ampicillin and tetracycline.

Likewise, gentamicin and neomycin (both as intramammary formulations), are expensive and quite new in the Algerian livestock industry and therefore less frequently used, and this difference is reflected in a lower prevalence of resistance.

The resistance observed for some antibiotics (for example AMC) may also be related to the long period of inappropriate use of antimicrobial products, under-dosing or incomplete treatment of animals, since in Algeria these are dispensed without a prescription. So, it is recommended that intensive farmer education programs are needed to improve udder health in dairy herds in Algeria.

The present study showed the antimicrobial susceptibilities of Enterobacteriaceae species isolated from bovine milk samples collected from lactating cows in the center region of Algeria. In this study, relatively high numbers of different individual Enterobacteriaceae species have been isolated and identified. These results also indicate that Enterobacteriaceae species are susceptible to various antibiotics except for AMC with high resistance rate which is intensively used in the prevention and treatment of mastitis without any antibiogram test.

It is concluded that intensive farmer education programs are necessary to improve udder health in the dairy sector in Algeria.

Hence, this study emphasizes judicious selection of antibiotics, preferably after antibiotic sensitivity testing and using such antibiotics at an adequate dose for sufficient duration to ensure effective treatment and control of coliform mastitis.

Conflict of interest statement

We declare that we have no conflict of interest.

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