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ORIGINAL RESEARCH

An Arsenal of Multiple Antimicrobial Resistance, Toxins, and Virulence Factors in Gram-Negative Bacterial Isolates from Food – A Formidable Combination!

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Background: Infectious diseases caused by pathogenic members of the family *Enterobacteriaceae* cause mortality and morbidity in humans. These are mediated mainly via toxins or virulence factors in combination with multiple antimicrobial resistance (MAR) against antimicrobials intended to treat infections. Resistance can be transferred to other bacteria, possibly also in association with other resistance determinants and/or virulence properties. Food-borne bacterial infections are one of the major causes of infections in humans. The level of scientific information about foodborne bacterial infections in Ethiopia is very limited at best.

Methods: Bacteria were isolated from commercial dairy foods. These were cultured in appropriate media for identification at the family level (*Enterobacteriaceae*) based on Gram-negative, catalase-positive, oxidase-negative, and urease-negative phenotypes, followed by testing for the presence of virulence factors and resistance determinants to various antimicrobial classes using phenotypic and molecular tests.

Results: Twenty Gram-negative bacteria isolated from the foods were found to be resistant to almost all antimicrobials belonging to the phenicol, aminoglycoside, fluoroquinolone, monobactam, and β-lactam classes. All of them were multiple-drug-resistant. The resistance to the β-lactams was due to the production of β-lactamases and were also mostly resistant to some of the β-lactam/β-lactamase inhibitor combinations. Some isolates also contained toxins.

Conclusion: This small-scale study demonstrated the presence, in the isolates, of high levels of virulence factors and resistance to major antimicrobials that are in clinical use. Most treatment being empirical, there can be not only a high degree of treatment failure but also the likelihood for further development and dissemination of antimicrobial resistance. Since dairy foods are animal products, there is an urgent need to control animal-food-human transmission mechanisms, restrict antimicrobial use in animal agriculture, and improve clinical treatment from the usual empirical treatment to more targeted and effective treatment.

Keywords: dairy isolates, gram-negative bacteria, multiple antimicrobial resistance, virulence, β-lactamase inhibitor

Introduction

The hope of defeating infectious diseases following the earliest introduction of antimicrobials is being challenged because of the near simultaneous development and fast spread of multiple antimicrobial resistance (MAR). This is further compounded by antibiotic discovery not keeping pace with that of resistance development.[1,2] Many pathogenic bacteria produce β-lactamases capable of degrading β-lactams. To counter this bacterial challenge, β-lactam/β-lactamase inhibitor combinations were introduced, but pathogens respond with β-lactamase inhibitor-resistant versions of these enzymes. This combined with other β-lactam resistance mechanisms continues to diminish the treatment options for Gram-negative bacteria possessing such resistance mechanisms.[3] Major pathogenic Gram-negative bacteria belonging to *Enterobacteriaceae* also possess mechanisms to resist other classes of antimicrobials. Routes of transmission of ESBL-containing pathogens include contaminated food, person-to-person contact (eg, being a family member of a person with

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ESBL-producing *Enterobacteriaceae*), animals-to-humans, foreign travel, and antibiotic administration during hospitalization.[4–10] *Escherichia coli* are some of the most notorious enteropathogens transmitted in these ways. Several types of *E. coli* produce and secrete different virulence factors (VFs). Among these major VFs are toxins, adhesins, and hemolysins. Stx1 and Stx2, encoded by *stx1* and *stx2,* respectively, are Shiga toxins produced by certain pathogenic strains of *E. coli*. These toxins have the capacity to induce effacement of the intestinal epithelial wall and lesions, eventually causing excess fluid secretion. Other VFs include *eaeA*, which encodes for an adhesin that is required for adherence, and *hlyA*, which encodes for a hemolysin.[11]

Carbapenems represent some of the last resort drugs of choice for treating infections caused by multidrug-resistant *Enterobacteriaceae*. Mechanisms of resistance to carbapenems include production of carbapenemases. Carbapenemases include the Zn(II)-dependent enzymes – the metallo-β-lactamases (MBLs) – among which is the New Delhi metallo-β-lactamase (NDM). The MBLs are capable of hydrolyzing other β-lactams as well, including penicillins and cephalosporins, in addition to carbapenems.[12] MBLs represent one of the largest groups of carbapenemases.

Several features of NDM1 make it especially notorious: its anchorage, unlike other MBLs, to the bacterial outer membrane (which enhances its fitness and also prevents its degradability), its location on mobile genetic elements, and its association with resistance to other classes of antibiotics including the fluoroquinolones, aminoglycosides, and TMP/SMX (which suggest that these resistances are co-transferable).[13,14] The CDC lists carbapenem-resistant *A. baumannii* and ESBL-producing *Enterobacteriaceae* as urgent and serious threats, respectively.[15] The incidence of Gram-negative bacteria that are resistant to third-generation cephalosporins and carbapenems is increasing in almost all countries globally and are emerging as major causes of resistant, health-care-associated infections.[16,17] Other resistance properties possessed by some strains of *Enterobacteriaceae* include genes for macrolide resistance such as *ereA* and *ereB* coding for enzymes that hydrolyze the lactone ring of antibiotic; *mphA* for phosphorylation; and *mefA* coding for macrolide efflux.

Mortality due to multi-resistant ESBL-producing or inappropriately treated systemic infections can be high. Accordingly, the odds of mortality in patients in a tertiary care hospital in Ethiopia due to blood stream infections caused by *Enterobacteriaceae* co-resistant to multiple classes of antibiotics (third-generation cephalosporins, gentamicin, chloramphenicol, and co-trimoxazole) was reported to be extremely high (OR of 23).[18] Several other studies from Ethiopia that have reported the finding of ESBL-producing *Enterobacteriaceae* obtained from clinical specimen (blood, urine, gastrointestinal, pus, and CSF).[19–22] Most reported high-level resistance of bacterial isolates to β-lactams and other classes of antimicrobials. However, none of these studies identified ESBL types. There are other studies that reported-specific ESBLs. Accordingly, Pritsch et al[23] described three clinical isolates of *Acinetobacter baumannii* that carried the bla_{NDM1} gene from Southwest Ethiopia. These authors also described that 50% of Gram-negative clinical isolates tested positive for ESBL of CTX-M ($bla_{\text{CTX-M}}$).[24] Another group[25] reported the finding of more than three-quarters of *Klebsiella pneumonia* isolates being ESBL producers in the same study area with $bla_{\text{CTX-M}}$ and bla_{TEM} genes being the most prevalent. Resistance to other classes of antibiotics (aminoglycosides, ciprofloxacin, and sulfamethoxazole) was also found to be high (ranging from 63% to 95%).

Almost all of the studies concerning bacterial β-lactamases in Ethiopia are on clinical isolates. A search for reports on detection of β-lactamases in bacterial isolates from foods in Ethiopia did not turn out results. This study was therefore conducted to investigate and obtain a first glimpse of antimicrobial resistance and virulence in bacterial isolates in foods of dairy origin.

Materials and Methods

Isolation and Growth of Bacteria from Dairy Foods

Dairy products (milk, yogurt, and cheese) were purchased from supermarket stores. These were diluted 1:10 in sterile phosphate buffered saline (PBS), pH 7.4. Serial dilutions (10–100 µL) were spread-plated on MacConkey agar and incubated at 37°C for 24 hr. Separate colonies were randomly picked and inoculated into Luria Bertani (LB) broth for 24-hr incubation. Then aliquots were plated on agar media and incubated. Single colonies were picked and streaked on agar media followed by incubation. Single colonies were then inoculated into broth, grown for 24 hr, the broth cultures centrifuged, and the pellets resuspended in LB broth containing 15% sterile glycerol for frozen storage. Isolates were further checked for purity (absence

of Gram-positives) by Gram staining. These stocks were used for all subsequent tests, including phenotypic (biochemical, antimicrobial susceptibility [ASTs], and molecular tests). For further experimental work, frozen aliquots were grown on agar or in broth. Media used for cultivation were obtained from OXOID or Becton Dickinson.

Identification of Bacterial Isolates

Tests conducted for reasonable identification of the isolates included Gram staining, catalase and oxidase tests, motility, triple sugar iron (TSI) agar, urease production, and citrate utilization.[26] Thus, Gram-negative, catalase-positive, oxidase-negative and motile bacteria were sought for during isolation. On TSI agar, isolates that exhibited alkaline/acid or acid/acid reactions were selected. Finally, isolates were selected for further tests based on the combination of all of the following test results: Gram-negative, catalase-positive, oxidase-negative, urease-negative, motility-positive, and with or without H_2S production.

Phenotypic Antimicrobial Susceptibility Testing (AST)

AST was performed on the bacterial isolates as follows: Isolates were activated by growth on agar for 18 hr at 37°C. Bacterial suspensions were prepared in 0.85% sterile saline to match McFarland 0.5 turbidity standard and used to inoculate Mueller–Hinton agar plates (90 mm) using sterile cotton swab (Becton Dickinson) until/to make a thick/dense paste formed on the agar surface. Inoculated plates were allowed to absorb any moisture on the agar (~15 min after inoculation). Then, antimicrobial-impregnated disks (OXOID) were dispensed at a distance of 25 mm from each other. The following disks were used: amikacin (10 µg), ampicillin (10 µg), amoxicillin (20 µg), ampicillin/clavulanic acid (20/10 µg), ampicillin/sulbactam (10/10 µg), azithromycin (15 µg), aztreonam (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefoxitin (30), ceftriaxone (30 µg), cefuroxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (30 µg), gentamycin (10 µg), imipenem (10 µg), and trimethoprim/sulfamethoxazole (1.25/23.75). The plates were then inverted and placed in 37°C incubator for 18 hr, after which zones of inhibition were measured and recorded. Interpretations of the measurements were made according to Clinical Laboratory Standards Institute recommendations 2018.[27]

Molecular Antimicrobial Susceptibility Testing

Tests using PCR and targeting for the presence of some genes encoding for β-lactamases were performed. These were bla_{TEM},bla_{SHV},$bla_{CTX\text{-}M}$,bla_{KPC}, and bla_{NDM}. The primer sequences, targets, and band sizes are listed in Supplementary Table 1A. Resistance to carbapenems was tested using duplex PCR for KPC and NDM.[28,29] Tests were also conducted for molecular detection of the macrolide resistance determinants (*ereA, ereB*, *mphA*, and *mef*).[30] Isolates were classified as multidrug-resistant (MDR) as appropriate.[17]

Molecular Testing for Toxin and Virulence Determinants

The isolates were tested by PCR amplification for presence of Shiga toxins (*stx1, stx2, eaeA*, and hlyA).[31] The primers are listed in Supplementary Table 1B.

Quality Control

The following type strains were used as positive or negative controls, respectively: catalase (*Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC19615); oxidase (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922); motility (*Proteus mirabilis* ATCC 29906, *Klebsiella pneumonia* ATCC700603); urease production (*P. mirabilis* ATCC 29906, *E. coli* ATCC 25922); TSI slants (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853); Shiga toxins (*E. coli* O157, NCTC 13125, *E. coli* ATCC 25922).

Ethics

No animals or humans, or samples derived from them, were used in this study. Therefore, this study did not require ethical approval.

Results

The isolates studied in this work were selected so that they were all Gram-negative, catalase-positive, oxidase-negative and motile. On TSI agar, the isolates displayed acid/acid phenotype, but did not produce hydrogen sulfide.

There is a high level of MAR in isolates of this study (Table 1). Almost all isolates harbored two of the β-lactamases (TEM and SHV) (Figure 1). Isolates contained both of these β-lactamases, or lacked both of them. In only four of the isolates (isolates 3, 9, 11, and 18), no β-lactamases were detected. Some isolates (7, 8, 13, 14, 15, and 17) contained *stx2*, in addition to TEM and SHV. Other isolates (isolate 13 and another isolate from the same source as isolate 13) and isolate 14 contained both *hlyA* and *eaeA*, in addition to both TEM and SHV (Table 2). CTX-M was not found in any of the isolates. However, isolate 6 (a milk isolate) contained the carbapenemase KPC (Figure 1A, lane 11). All of the tested isolates, without exception, were resistant to both GEN and AZI. Similarly, isolates 10, 11, 12, 13, and 19 were found to be resistant to all tested antimicrobials except intermediate resistance to some in few cases. Even imipenem (a carbapenem) was not found to be effective, since most of the isolates showed resistance to this agent, which is considered to be one of the last resort drugs. The same pattern is seen towards ATM, to which most isolates exhibited resistance.

Direct correlations between phenotypic and genotypic tests of resistance were made difficult because resistance to β-lactams could be conferred by mechanisms other than the presence of resistance genes.

Tests conducted for detection of different macrolide resistance determinants (*ereA, ereB*, *mphA*, and *mef*) gave negative results. Still, since we have not tested these isolates for phenotypic resistance to macrolides and there are other macrolide resistance determinants, we would not be able to confirm susceptibility of the isolates to macrolides because of the absence of *ereA, ereB, mphA* and *mefA*.

W1 and W2 are tap water samples obtained from two restaurants that are several kilometers apart. These are two few samples for this type of water source but were included in these tests as lead-up for more sampling and considering these

Table 1 Antimicrobial Disk Diffusion Test Results for Isolates of This Study

Isolate	Antimicrobial														
	CHL	GEN	T/S	AK	CIP	ATM	FOX	CTX	CRO	CAZ	AML	AMP	AMC	SAM	IPM
Sau		R	I	R	R	R	S	R	I	I	S	S		S	S
Eco	I	R	I	R	R	R	R	R	R	R	I	I	I	S	R
3	S	R	S	S	S	R	R	R	S	R	S	S	R	S	R
4	R	R	I	R	R		R	R	R	R	R	R	R	R	R
5	I	R	I	S	S	S	S	S	S	S	I	I	S	S	R
6	R	R	R	R	I	R	I	R	R	R	R	R	R	S	R
7			S	**R**	S	**R**	**R**	**R**	**R**	R	S	S	S	S	S
8	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
9	S		S	R	S	R	R	R	R	R	S	S	R	S	S
10	R	R	**R**	I	R	R	R	R	R	R	R	R	R	R	R
11	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
12	R	R	R	I	R	R	R	R	R	R	R	R	R	R	R
13	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
14	S		S	R	S	R	R	R	R	S	S	S	R	S	R
15	I	R	I	R	I	R	R	R	I	I	R	R	R	I	R
16	S	R	R	S	S	S	S	R	S	S	R	R	R	S	R
17	S	R	R	S	S	S	S	S	S	S	R	R	R	S	R
18	S	S	S	S	S	**R**	S	S	S	**R**	S	S	S	S	S
19	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
20	S		S	S	S	R	R	R	I	R	S	S	R	S	

Note: The bold R indicate zero zone of inhibition. These could be considered high-level-resistant (eg, isolate 10 to T/S and isolate 18 to both ATM and CAZ).
Abbreviations: CHL, chloramphenicol; GEN, Gentamycin; T/S, trimethoprim/sulfamethoxazole; AK, Amikacin; CIP, Ciprofloxacin; ATM, aztreonam; FOX, cefoxitin; CTX, cefotaxime; CRO, cefuroxime; CAZ, ceftazidime; AML, amoxicillin; AMP, ampicillin; AMC, ampicillin/clavulanic; SAM, ampicillin/sulbactam; IPM, imipenem; S, Susceptible; I, Intermediate; R, Resistant.

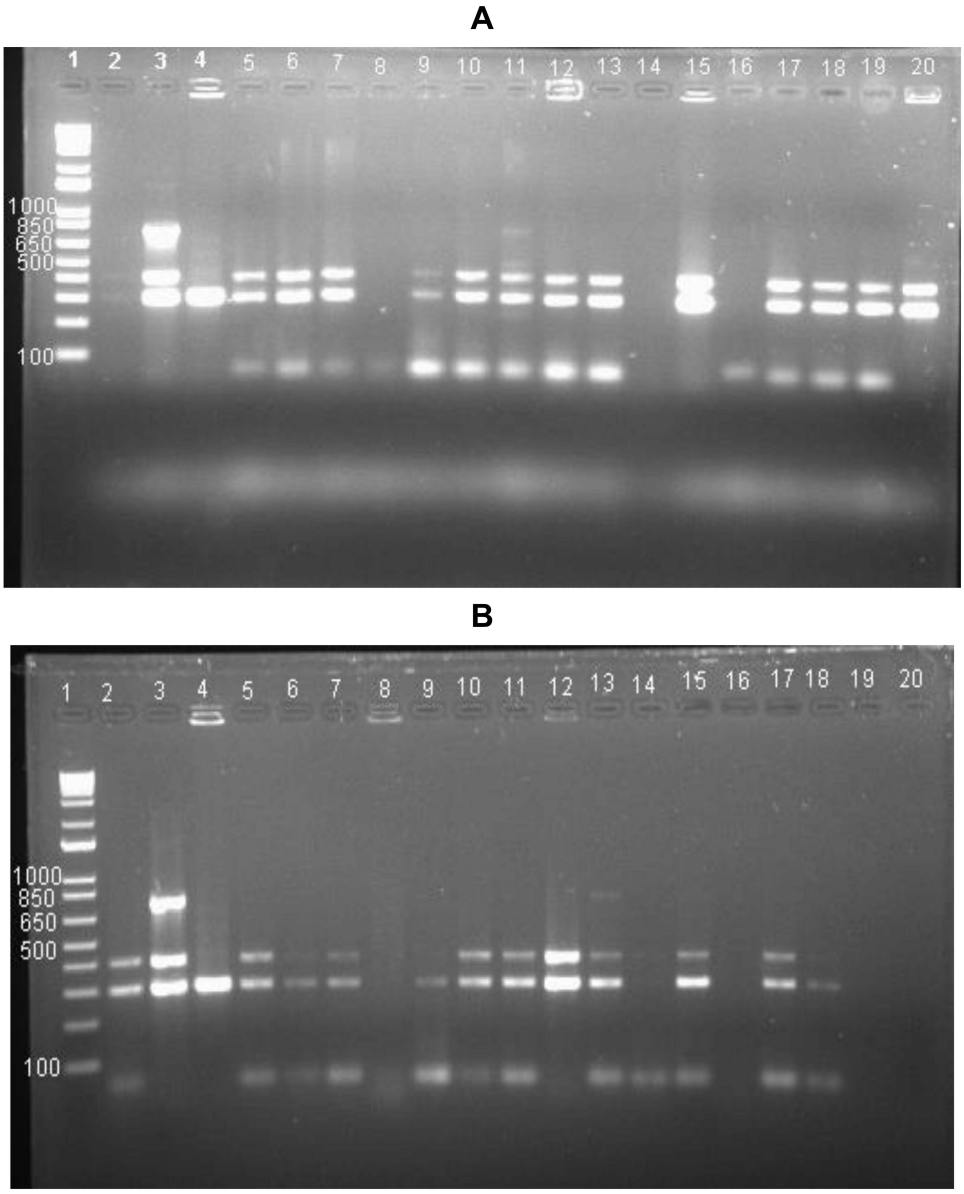

Figure 1 PCR amplification results for detection of β–lactamases. (**A**) Lane 1: MWM (1 Kb Plus Ladder), 2: positive control for SHV, TEM and KPC, 3: *K. pneumonia* ATCC700603, 4: *P. mirabilis* ATCC 29906, 5: *P. aeruginosa* ATCC 27853, 6: isolate 1, 7: isolate 2, 8: isolate 3, 9: isolate 4, 10: isolate 5, 11: isolate 6, 12: isolate 7, 13: isolate 8, 14: isolate 9, 15: isolate 10, 16: isolate 11, 17: isolate 12, 18: isolate 13, 19: isolate 14, 20: isolate 15. (**B**) Lanes 1–5: Same as A, 6: isolate 16, 7: isolate 17, 8: isolate 18, 9: isolate 19, 10: isolate 20, 11: HAb2 (cheese isolate), 12: Yogurt isolate*, 13: W1 (water isolate obtained from a restaurant), 14: bottled water isolate, 15: W2 (water isolate obtained from another restaurant), 16: *E. coli* ATCC 25922 (negative control), 17: *S. pneumonia* ATCC 49619, 18: duplicate 17: 19: H_2O (negative control). *Obtained from the same yogurt as isolate 10.

too could enter the food chain. The two samples have the same β-lactamases as the food isolates. A water isolate also contained the carbapenemase KPC (Figure 1B, lane 13).

Discussion

In this study, we tested the susceptibility of bacterial isolates from food to six different classes of antimicrobials and another nine antimicrobials consisting of β–lactams or β-lactam/β-lactamase inhibitor combinations. This study is also unique in that it described β-lactamases in conjunction with virulence factors in Ethiopia. The finding of these levels of β-lactam resistance is worrisome. Furthermore, there was only little or no difference between the susceptibilities of most isolates to β-lactams with or without β-lactamase inhibitors.

Table 2 β-Lactamases, Toxins, and Virulence Factors Detected in Bacterial Isolates

Isolate (Source)	β-Lactamase					Toxins/Virulence Factors			
	bla$_{KPC}$	*bla*$_{NDM}$	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{CTX-M}$	*aer*	*hlyA*	*stx2*	*eaeA*
1 (cheese)	–	–	+	+	–				
2 (milk)	–	–	+	+	–				
3 (milk)	–	–	–	–	–			+	
4 (milk)	–	–	+	+	–				
5 (milk)	–	–	+	+	–				
6 (milk)	–	–	+	+	–				
7[@] (milk)	–	–	+	+	–			+	
8[@] (milk)	–	–	+	+	–			+	
9 (milk)	–	–	–	–	–				
10* (yogurt)	–	–	+	+	–				
11 (milk)	–	–	–	–	–				
12 (cheese)									
13[^] (cheese)			+	+			+		+
14 (yogurt)			+	+			+		+
15 (milk)			+	+				+	
16[#] (yogurt)	–	–	+	+	–				
17[#] (yogurt)	–	–	+	+	–			+	
18 (milk)	–	–	+	+	–				
19 (cheese)	–	–	+	+	–				
20 (milk)	–	–	+	+	–				
21* (yogurt)			+	+					
HAb2 (cheese)	–	–	+	+	–				
E5[^] (cheese)			+	+			+	+	
W1			+	+					
W2			+	+					
E. coli O157**							+		+

Notes: [@]Isolates with this superscript symbol are independent isolates from the same source. *Isolates with this superscript symbol are independent isolates from the same source. [^]Isolates with this superscript symbol are independent isolates from the same source. [#]Isolates with this superscript symbol are independent isolates from the same source. **This is type strain *E. coli* O157, NCTC 13125.

Foods of animal origin, including meat, milk, and other dairy products, as well as food handlers, can harbor *Enterobacteriaceae* that can be reservoirs of ESBL and virulence genes that can have clinical consequences following consumption of such foods.[32,33] Consumption of raw milk is a known risk factor. Almost all of the products analyzed here are pasteurized products (according to the product labels). Gram negatives should not be found in such products. Moreover, these bacteria are loaded with resistance-conferring traits that spell risk to human health.

By definition, ESBLs should be inhibited by β–lactamase inhibitors such as clavulanic acid, but the vast majority of the isolates are also resistant to the β–lactamase inhibitors. This indicates that empirical treatment is likely to be not effective. Furthermore, it could fuel further development and transmission of resistance in vivo (and in other environments thereafter).

Horizontal transfer of AMR determinants has been indicated to occur in hospital and community settings. Bacteria can also acquire resistance in vivo after antimicrobial treatment.[34–36] The ubiquity of traits conferring MAR extends to other environments outside living hosts. For example, foods of animal origin may be contaminated with resistant bacteria, and these can find their way into humans.[33] On top of that, pathogenic bacteria can also carry virulence-related genes.

We have sampled only a small number of bacterial isolates. But importantly, the finding of genes encoding for β–lactamases in almost all of them tells something: not only Gram-negatives but also β–lactamases are widespread in such foods – the selection of the isolates was without bias (random). Their distribution may also extend to other

foods and habitats, such as water, to which animals have access. In this connection, and not surprisingly, ESBL-producing *Enterobacteriaceae* have been reported in drinking and wastewater samples.[37,38] This is not surprising, but the important point is resistance is brewing and it is necessary to control their further spread, since these will find their way into the food chain and eventually into humans. Here, tap water samples obtained from two restaurants several kilometers apart were tested for food isolates, and the two water samples harbored β–lactamases as well. Few tests with high positivity results are indicative of the widespread nature of the resistance problem.

There is no reliable and adequate information on use of antimicrobials in humans or animals in Ethiopia. A recent estimate of global antibiotic usage during period between 2000 and 2018 indicated that sub-Saharan Africa (Ethiopia included) has one of the lowest levels of antibiotic consumption.[39] However, these estimates are only relative and cannot be reason for deterrence from tackling antimicrobial resistance. Moreover, antimicrobial consumption in these countries is likely to increase, especially in parallel with population growth and GDP.[40]

Limitations

This study examined only a handful/few isolates per food sample (even though the finding of β-lactamases in almost all of them is indicative that more could be found if larger samples were analyzed). We were limited to conduct molecular resistance tests for all antimicrobials used in the disk diffusion tests. Moreover, we did not define the TEM or CTX-M phylogenetic groups or plasmid carriage.

Conclusions

Gram-negative bacteria with typical characteristics of Enterobacteriaceae were isolated from dairy foods. The results showed high-level phenotypic and genotypic resistance to several antimicrobial classes and are all multidrug-resistant. Moreover, the finding of other virulence factors further complicates management of food-borne infections. In conclusion, foods of animal origin must be regarded as a reservoir of ESBL-producing bacteria of clinical relevance, which might spread through the food chain. Thus, it is imperative to institute policies aimed at avoiding unnecessary or excessive use of antibiotics in both animals and humans, improving hygiene standards and ensuring drug prescriptions is efficacious as well as reducing broad-spectrum antibiotics when possible.

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Disclosure

The authors declare no conflicts of interest in this work.

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