Original Article

Journal of Food Safety and Hygiene

journal homepage: http://jfsh.tums.ac.ir

Antibiotic resistant bacteria in raw cow milk and milk products retailed in the northern region of Ghana; a food safety challenge

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ARTICLE INFO

Article history:
Received 23 Oct 2019
Received in revised form
21 Dec 2019
Accepted 28 Dec 2019

Keywords:
Antimicrobial resistance;
Bacteria;
Raw milk;
Northern Ghana

ABSTRACT

The presence of antimicrobial resistant foodborne bacteria is a major food safety challenge for food that is consumed raw. Abuse and overuse of antibiotics in the agriculture sector has been identified as a contributory factor to the rising threat of antibiotic resistance. In many developing countries where milk is marketed and consumed raw through informal channels, the occurrence of bacterial contamination is high and poses a major public health risk. This situation is exacerbated when caused by antimicrobial resistant bacteria. Hence this study was conducted to determine the antimicrobial resistant pattern of bacteria in raw cow milk and milk products retailed in the Northern Region of Ghana. Antibiotic resistance profiles were established for 150 bacteria isolates *(Escherichia coli, E. coli* O157:H7*, Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp. *Shigella* spp. and *Proteus* spp.) obtained from the culture of raw milk (n=210) and milk products (n=60) retailed within the Northern region of Ghana. Susceptibility to nine antimicrobials commonly used in veterinary and human medical practice was performed on all the isolates using the agar disc diffusion method according to Clinical and Laboratory Standards Institute guidelines. Isolates showed highest resistance to Nalidixic acid followed by Chloramphenicol, Gentamicin, Trimethoprim-sulfamethoxazole and Ceftriaxone but were most susceptible to Ciprofloxacin and Ampicillin. About 25 – 47.6 % of *Staphylococcus aureus* showed resistance to Cefoxitin. Milk and milk products sold in the northern region of Ghana are contaminated with bacterial pathogens with high levels of antimicrobial resistance. A one health approach is required to curtail the threat of antibacterial resistant bacteria in the food chain.

Citation: Vicar KE, Feglo KP, Acquah KES, Williams W, Saba SKC, Mensah GI. **Antibiotic resistant bacteria in raw cow milk and milk products retailed in the Northern Region of Ghana; a food safety challenge.** J food safe & hyg 2019; 5(4): 206-213

1. Introduction

Dairy products are easy conduits for transmission of food borne pathogens (1-8) with milk and milk products being major sources of human infection with antimicrobial resistant pathogens. (4, 9-16).

In the dairy industry, antimicrobial agents are administered extensively to prevent, control, or treat infection in animals and to serve as feed and growth enhancers (14). Uncontrolled administration of these antimicrobials to cattle may lead to bacterial pathogens developing resistance (14). The consequence of this action may be the transfer of antimicrobial resistant genes from one bacterium to another. Subsequently resistant bacteria end up in the food chain (14,17). Resistant foodborne bacteria have become a food safety challenge that demands a one-health based solution. Many bacterial infections that were previously treatable have over the years developed resistance to several antimicrobial agents (18,19). Each day, the number of bacteria resistant to all known antibiotics

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increases, leading to the prediction that by 2050, "humanity may return to an era without antibiotics"(20). To address this issue with urgency, the growing threat of antimicrobial resistance has been recognized as a priority topic for human development by the General Assembly of the United Nations, on the same scale as global warming (21).Nowhere is this phenomenon greater than in developing countries where the antimicrobial consumption patterns in agriculture vary across regions and countries, and even antimicrobials that have been banned in other countries, including the developed countries, are still being used (22,23). Unlike in most developed countries where antibiotics can only be obtained with prescription from a clinician, in many developing countries, they are readily available over the counter, through unregulated supply chains as well as purchased without prescriptions (24). The occurrence of resistant bacteria in raw milk has become a great challenge in many developing countries especially among the immunocompromised and children living in these countries who are consumers of raw milk (13). Apart from uncontrolled food exchange that has contributed to the spread of antimicrobial resistance, the veterinary services in Ghana for several years could not prioritize antimicrobial resistance surveillance. Hence there was no acceptable national standards for antibiotic residue in veterinary and aquaculture products. Also, no testing for antimicrobial residue was done and no regulation on minimum allowable weaning period before slaughter and processing. The lack of regulation and testing can also contribute to the overall resistance of microorganism (25).
In Ghana, antimicrobial resistance has been reported in food products such as meat, fish, chicken and raw milk (5,26). In a study on prevalence of antibiotic resistant bacteria in milk sold in Accra, 26.42% of resistant isolates were found in unpasteurized milk all of which were multi-drug resistant (26). In a study on prevalence of antibiotic resistant bacteria in milk sold in Accra, 26.42% of resistant isolates were found in unpasteurized milk all of which were multi-drug resistant (27). In this same study, high Ceftriaxone resistance of 87.5% and 75% was detected in *Escherichia coli* (*E. coli)* and *Klebsiella* spp. respectively. It is also worth noting that in the same study a 100% resistance to ampicillin, tetracycline, chloramphenicol, cefotaxime and cotrimoxazole were reported among bacteria isolated (27). The Northern region of Ghana is a significant contributor to the annual milk production and also demand (5,26) yet the limited data on antimicrobial resistance in raw milk excludes the Northern region.
In our previous study to evaluate raw milk quality, Mensah *et al* 2018 (26) found the quality of raw milk sold in the northern region of Ghana compromised by several bacteria pathogens including *E. coli,* (*E. coli O157:H7, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* spp. *Shigella* spp., and *Proteus* spp and antibiotic residues at the farm level (26). The aim of this study was to determine the antibiotic resistant profile of these bacteria responsible for the contamination.

2. Materials and methods

2.1. The study Area

This study was carried out in Tolon, Central Gonja, East Gonja, Kumbungu, Sagnarigu, Savelugu and Tamale metro districts in the northern region of Ghana, selected because of their high milk production and supply chain to the local community. In consultation with district veterinarians, 30 kraals in each district were purposely selected based on the size of kraal, accessibility and proximity to market sites. The samples were collected between March and September 2017 when milk production peaks as a result of increase in feed. Milking is done in the kraals where the ground is covered with cow dung mixed with urine and flies can be seen everywhere.

2.2. Sample collection

A total of 210 kraals were visited. From each kraal, 50 ml of raw milk was aseptically withdrawn from the container containing the pooled milk into sterile screw cap falcon tubes.

A total of 60 milk products including wagashie (15), cottage cheese (15), bruchina (15) and fresh yoghurt (15) were purchased from various sale points randomly. Each sample was labelled and placed in a cool box with icepacks (to maintain temperature at 4^{o}C) and sent to the Microbiology laboratory at the University for Development Studies (UDS) for bacteriological analysis within 24 h of milking.

2.3. Culturing, Isolation and identification of Bacteria

Each sample was homogenized and 100 µl (after 1 in 10 serial dilution has been performed) as indicated by Mensah *et al* 2018 (26) was sub-cultured onto MacConkey agar, blood agar, Baird parker agar and Salmonella-Shigella (SS) agar (Oxoid® Ltd., Basingstoke, Hampshire, England) and incubated at

37°C for 18-24 h. Characterization of bacterial isolates was carried out using colonial morphology, microscopic techniques and biochemical tests including gram's reaction, coagulase test, oxidase test, Oxidation–Fermentation test, catalase test and 3 % KOH tests. *E. coli* was specifically tested for using *E. coli* Chromogenic agar (ECC chromo Selective-85927, Sigma-Aldrich, Germany). Dark blue-violet colonies were confirmed as E. coli when the colonies turned cherry red colour upon the addition of Kovac's reagent. The confirmed *E. coli* isolates were further tested for the presence of *E. coli* O157:H7 using the latex slide agglutination kit (DR0120, Oxoid). For isolation of Salmonella and Shigella, distinct pale and colourless colonies on MacConkey and on Salmonella-Shigella agar were tested biochemically on Kligler iron agar, urea agar, and Simmons citrate agar (all obtained from (Oxoid® Ltd., Basingstoke, Hampshire, England). The isolates with reaction result typical of Salmonella colonies were sub cultured on XLD media and incubated at 37°C for 24 h. Red colonies with black centers after the incubation period were identified as Salmonella spp. and speciation was done using Oxoid rapid latex agglutination test kit (Oxoid® Ltd., Basingstoke, Hampshire, England)

Staphylococcus aureus (*S. aureus*) colonies appeared as black or grey colored colonies on Baird parker agar were then picked and streaked on nutrient agar for coagulase test. Staphylase Test (Oxoid DR0595A), a rapid test kit for the detection of coagulase positive *S. aureus* was used according to manufacturer's instruction.

2.4. Antimicrobial Susceptibility Testing Antimicrobial susceptibility tests were performed on all the isolates by agar disc diffusion method according to Clinical and Laboratory Standards Institute (28) guidelines. The stored isolates (previously stored in 20% glycerol) were brought to 37°C (by incubation) to activate the microorganisms. The activated bacteria were inoculated onto Nutrient agar (NA) plates and incubated at 37°C for 24 h after which antimicrobial susceptibility tests were performed on Muller-Hinton (MH) agar. The MH agar plates which contained 4 mm depth of agar were warmed to room temperature in the incubator with the lids open for 10–15 min for excess moisture to be absorbed into the medium.
The microorganisms were tested for susceptibility to antibiotics commonly used in veterinary and human practice namely; Gentamicin (GN:10 µg), Chloramphenicol (CHL: 30 µg), Ceftriaxone (CRO: 30 µg), Tetracycline (TET: 30 µg), Ampicillin (AMP: 10 µg), Ciprofloxacin (CIP : 5 µg) and Cefoxitin (FOX: 30 µg).
Purified colonies were homogeneously suspended in screw cap tubes filled with 2 ml sterile normal buffered saline and turbidity adjusted to an equivalent of a 0.5 McFarland standard using Vitek colorimeter (Lenexa, Kansas, USA). Sterile cotton-tipped swabs were dipped into the homogenized suspensions and excess fluid removed by pressing and rotating the swab against the side of the tube above the level of the suspension. The swabs were then evenly spread on the entire surface of the MH agar to produce a confluent lawn of bacterial growth. The inoculated MH agar plates were left to dry for 5 min before placing the antibiotic discs on the surface using sterile forceps. The plates were inverted and incubated aerobically at 35°C for 16 to 24 h. The plates were examined for clear zones of inhibition around the discs after the incubation period. The Diameter of each inhibition zone was measured in millimeter (mm) using a caliper, and the results recorded. Zone sizes were classified as resistant (R), intermediate (I) and sensitive (S) according to the general guidelines prepared by CLSI (28)

2.5. Data collation and statistical analysis
All data was entered in Microsoft office excel 2016 (Microsoft corporation) and presented in summary tables and charts. Data were also presented as frequencies and percentages.

3. Results
Generally, the bacteria isolated from raw milk and milk product showed varied resistance to the antibiotics tested. Isolates showed highest resistance to Nalidixic acid (78.6 – 83.6%) followed by Chloramphenicol (60.7-83.6%), Gentamicin (57.1 - 79.5%), Trimethoprim-sulfamethoxazole (67.9 - 78.7%), Ceftriaxone (57.1 - 72.1%), Tetracycline (46.4 - 72.1%) and Ampicillin (28.6 - 66.4%) as shown in Table 1 and 2.
About 25 – 47.6 % of *S. aureus* showed resistance to Cefoxitin as *Salmonella* and *Shigella* species showed the least resistance of 0.0 % and 14.3% respectively to Trimethoprim-sulfamethoxazole.
Enteric bacteria isolated from raw milk showed high resistance to some broad-spectrum antibiotics. *E. coli*, *Salmonella* and *Shigella* species showed 100% resistance to Chloramphenicol.
Multidrug resistance of 66.4% and 53.6% were recorded among isolates detected from raw milk and milk products respectively (Table 3).

4. Discussion

Bacterial contamination of raw milk is of grave public health concern, especially when the bacteria strains are antibiotic resistant. This is because in addition to being pathogenic to humans who consume raw milk, antibiotic resistant bacteria are also widely disseminated in the environment via animal wastes. These may cause complicated, untreatable, and prolonged infections in humans, leading to higher healthcare costs and sometimes death (22, 26, 29, 30). In this study, the high resistance detected among *Escherichia coli* isolated have been reported in other studies in Africa. In Nigeria, Reuben and Owuna 2013 (31) detected 100% resistance of *E. coli* isolated from raw milk to tetracycline. Also, Bonyadian et al 2014 (10) have reported that *E. coli* isolates from raw cow milk and unpasteurized cheeses showed resistance to Ampicillin (66%), Ggentamicin (69.6%), and Ciprofloxacin (56.7%) (10). However, studies done in the northern region of Ghana by Frederick et al 2016 (32) reported low resistance of *E. coli* isolates from milk and hands of milkers in Nyankpala community to Gentamicin (25.93%), tetracycline (29.63%) and Ceftriaxone (37.04). *E. coli* isolates from raw cow milk, yoghurt and cheese were also reported to have low resistance to Gentamicin (6.81%) and Tetracycline (56.8%) (33) compared to what this study has revealed. In this study we equally detected low resistance of *E. coli* to Ciprofloxacin (26.7 – 33.3 %) but high resistance to Ampicillin (20.0 %). Another important pathogen detected is *S. aureus.* Coagulase-positive *S. aureus* is a pathogenic strain that produces enterotoxin responsible for food poisoning, gastroenteritis and toxic shock syndrome in humans throughout the world (13, 18, 34). Many studies have reported the resistance profile of *S. aureus* to Ciprofloxacin, and Gentamicin (3, 16, 35, 36). This study detected high resistance of *S. aureus* to Nalidixic acid (79.3%), Cefoxitin (75.9%), Ciprofloxacin (65.5%), Gentamycin (62.1%) and Chloramphenicol (55. 2%). These results are in contrast to that of (37) who detected a rather high sensitivity towards Ciprofloxacin, Cefoxitin, Gentamycin and Chloramphenicol. The antimicrobial susceptibility pattern in the present study differs from that of *S. aureus* detected in the study by Alamin *et al* 2013 (38) (38) which was sensitive to Ciprofloxacin (77.8%), Gentamycin (88.8%), and Tetracycline (77.8%). However, similar to this study, it was found high resistance to Ampicillin, Tetracycline, Chloramphenicol, Trimethoprim-sulfamethoxazole in *S. aureus* isolates from milk (27).

High Staphylococcal-resistance detected in this study is a cause for public health action. Methicillin resistant *S. aureus* (MRSA) strains are now reportedly being isolated in livestock and milk, posing a threat over a potential spread of MRSA to consumers through the food chain (39). MRSA bears the mecA gene which alters penicillin binding proteins (PBP) having low affinity for all beta-lactam antimicrobials (19). Hence, transmission of MRSA strains through the food chain will contribute to the growing problem of antimicrobial-resistance.

Salmonella spp. isolated from raw milk and milk product showed high resistance to Chloramphenicol (100.0%) and Ampicillin (90-100.0%) respectively but low resistance to Ciprofloxacin (0-10%) and Gentamycin (20.0%). Different resistant patterns of *Salmonella* species have been reported in various studies. Teshome et al in 2016 (37) reported that 95.0% of *Salmonella* spp. were resistant to Ciprofloxacin and 75.0% resistant to Gentamycin and Chloramphenicol. Addis et al in 2011 (1) and Tadesse and Dabassa 2012 (15) also reported that *Salmonella* spp. from raw milk and dairy products showed low resistance to Tetracycline (12.0 %) and Ampicillin (33.3%) which correlates with the findings in this study. In our study, a lower percentage of *Salmonella* spp. were resistant to Ampicillin (25.0%) and Tetracycline (16.7%). *Salmonella* associated infections that results from resistant bacteria become difficult to treat or sometimes impossible to cure so this is encouraging. The resulting rise in morbidity and subsequent mortality will necessitate development of new antibiotics that are costly. For instance, antibiotic resistance in *Salmonella* has been associated with higher frequency and duration of hospital stays, longer illness, a higher risk of invasive infection and a two-fold increase in the risk of death in the two years after infection. Compared with infections susceptible to antibiotics, infections with antibiotic-resistant S. Typhimurium are associated with an increased risk of invasive disease and death (1).

Shigella species resistant to Ceftriaxone and Chloramphenicol was 100%. However, *Shigella* species showed low resistance to Ciprofloxacin (14.3%) and Trimethoprim-sulfamethoxazole (14.3%) hence may be the drugs of choice when shigellosis is diagnosed. This pattern of resistance in milk borne isolates may be due to the export and import of many food products between Ghana and other countries. Food imports and exports pose a high risk of introducing new resistant infectious agents into regions that have not been exposed to such pathogen type. Most of the bacteria isolated were resistant to all the antibiotics tested is a

public health concern. The possible reason for the high resistance of the isolated bacteria to the antimicrobials may due to indiscriminate use of antibiotics by livestock keepers (40). This is shown clearly from the results where milk samples positive for antibiotic residues harbored more resistant pathogens compared to the samples with no detectable antimicrobial residues (Table 3). This is as a result of limited veterinary extension services, farmers' inadequate knowledge on animal health and easy access to antibiotics in livestock markets (41). Bacteria may have developed resistance through the inappropriate use of the antibiotics in cattle and the acquisition of resistance genes (14,40,42).

Table 1. Antibiotic-resistance profiles of bacteria pathogens from raw milk

Bacteria species	N	Number (%) of resistant bacteria																	
		NA		GEN		CIP		TET		CHL		AMP		COT		CTR		FOX	
		n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)		(%)	*n*	(%)	*n*	(%)	*n*	(%)
E. coli	30	30	(100.0)	30	(100.0)	8	(26.7)	28	(93.3)	30	(100.0)	19	(63.3)	30	(100.0)	28	(93.3)		NT
E. coli 0157:H7	5	5	(100.0)	5	(100.0)	2	(40.0)	2	(40.0)	5	(100.0)	1	(20.0)	5	(100.0)	4	(80.0)		NT
Salmonella sp.	10	7	(70.0)	2	(20.0)	1	(10.0)	2	(20.0)	10	(100.0)	9	(90.0	0	(0.0)	10	(100.0)		NT
Shigella sp.	7	1	(14.3)	2	(28.6)	1	(14.3)	2	(28.6)	7	(100.0)	7	(100.0)	1	(14.3)	7	(100.0)		NT
Proteus sp.	6	5	(83.3)	6	(100.0)	5	(83.3)	4	(66.7)	5	(83.3)	6	(100.0)	6	(100.0)	5	(83.3)		NT
P. aeruginosa	9	5	(55.6)	5	(55.6)	1	(11.1)	4	(44.4)	9	(100.0)	5	(55.6)	8	(88.9)	5	(55.6)		NT
K. pneumoniae	34	30	(88.2)	33	(97.1)	34	(100.0)	34	(100.0)	24	(70.6)	24	(70.6)	34	(100.0)	19	(55.9)		NT
S. aureus	21	19	(90.5)	14	(66.7)	17	(81.0)	12	(57.1)	12	(57.1)	10.0	(47.6)	12	(57.1	10	(47.6)	10	(47
Total	122	102	(83.6)	97	(79.5)	69	(56.6)	88	(72.1)	102	(83.6)	81	(66.4)	96	(78.7)	88	(72.1)	10	(8.2

AMP: Ampicillin; COT: Trimethoprim-sulfamethoxazole e; FOX: Cefoxitin; CTR: Ceftriaxone: Chloramphenicol; NA: Nalidixic acid; CIP: Ciprofloxacin: GEN: Gentamicin; TET: Tetracycline: NT: Not Tested

Table 2. Antibiotic-resistance profiles of bacteria pathogens from milk products

Bacteria species	N	Number (%) of resistant bacteria																	
		NA		GEN		CIP		TET		CHL		AMP		COT		CTR		FOX	
		n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
E. coli	12	12	(100.0)	8	(66.7)	4	(33.3)	6	(50.0)	7	(58.3)	5	(41.7)	12	(100.0	11	(91.7	NT	
Salmonella sp.	2	2	(100.0)	0	(0.0)	0	(0.0)	0	(0.0)	2	(100.0)	2	(100.0)	0	(0.0)	1	(50.0	NT	
P. aeruginosa	2	1	(50.0)	0	(0.0)	0	(0.0)	2	(100.)	2	(100.0)	0	(0.0)	2	(100.0)	0	(0.0)	NT	
K. pneumoniae	4	3	(75.0)	4	(100)	1	(25.0)	3	(75.0)	2	(50.0)	1	(25.0)	1	(25.0)	2	(50.0	NT	
S. aureus	8	4	(50.0)	4	(50.0)	3	(37.5)	2	(25.0)	4	(50.0)	0.0	(0.0)	4	(50.0)	2	(25.0)	2	(25.0)
Total	28	22	(78.6)	16	(57.1)	8	(28.6)	13	(46.4)	17	(60.7)	8	(28.6)	19	(67.9)	16	(57.1)	2	(7.1)

AMP: Ampicillin; COT: Trimethoprim-sulfamethoxazole e; FOX: Cefoxitin; CTR: Ceftriaxone: Chloramphenicol; NA: Nalidixic acid; CIP: Ciprofloxacin: GEN: Gentamicin; TET: Tetracycline: NT: Not Tested

Table 3. Multidrug resistance detected among bacteria isolated

	Raw Milk			Milk Products		
Isolates	No. of isolates	MDR	(%)	No. of isolates	MDR	(%)
E. coli	30	21	(70.0)	12	4	(33.3)
E. coli 0157:H7	5	2	(40.0)	0	0	0
Salmonella sp.	10	4	(40.0)	2	1	(50.0)
Shigella sp.	7	3	(42.9)	0	0	0
Proteus sp.	6	2	(33.3)	0	0	0
P. aeruginosa	9	7	(77.8)	2	2	(100.0)
K. pneumoniae	34	24	(70.6)	4	2	(50.0)
S. aureus	21	18	(85.7)	8	6	(75.0)
Total	122	81	(66.4)	28	15	(53.6)

5. Conclusion

The study found high levels of resistance to commonly prescribed antibiotics in the bacteria isolates from raw milk. This calls for strengthening of regulations that cover the sale, distribution, dispensing, and prescription, of veterinary antibiotics. This is because antibiotic resistant bacteria may cause complicated, untreatable, and prolonged infections in humans, leading to higher healthcare cost and sometimes death. Fortunately, Ghana, which hosted the Global AMR call to action agenda in November 2018, like many developing countries has developed an AMR action plan (launched in April 2018) that seeks to address the challenges posed by this menace through public engagement and training of farmers of antimicrobial resistance (AMR) and antimicrobial usage (AMU).

Conflict of interest

The authors declared that they have no conflict of interest.

Acknowledgements

We are grateful to the Animal Research Institute (ARI), the Veterinary Services Directorate (VSD) and the Ministry of Food and Agriculture (MOFA) of the study districts for sample collection support. The corresponding author is a postdoctoral fellow of DELTAS Africa Initiative [Afrique One—ASPIRE/DEL-15-008]. Afrique One—ASPIRE is funded by a consortium of donors including the African Academy of Sciences (AAS), Alliance for Accelerating Excellence in Science in Africa (AESA), the New Partnership for Africa's Development Planning and Coordinating (NEPAD) Agency, the Wellcome Trust [107753/A/15/Z] and the UK government. This work was carried out in collaboration with all authors. Authors GIM and EKV designed the study, wrote the protocol and prepared the first draft manuscript. Author EKV did the sample collection. Authors EKV SEKA and WW performed the bacteria culture and isolation and antimicrobial susceptibility testing. Authors EKV, GIM, PKF and CSKS performed the data analysis. Authors EKV, GIM and PKF revised the manuscript. All authors read and approved the final manuscript.

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