Original Research Article

Antimicrobial Susceptibility Profile of Coliforms from Bovine Mastitis Cases among Pastoral Herds in Parts of Kaduna State, Nigeria: Curbing the Environmental Health Risk

ABSTRACT

Consumption of raw milk from pastoral bovines have been identified as a major source of public and Environmental Health risk in developing countries. Antimicrobial resistance is a global health challenge threatening the lives of humans and animals. The indiscriminate use and abuse of antimicrobial agents among the pastoralists on commercial animals, especially for non-therapeutic purposes has been linked by various researchers to the development of resistant strains of potentially pathogenic bacteria which are being transferred from animals to humans. In this study, the antimicrobial susceptibility profile of coliform bacteria isolated from mastitis milk of pastoral herds was investigated. Out of 147 milk samples collected and screened for subclinical mastitis, 29 (19.7%) were positive. Out of the 29 mastitis positive samples, 13 (8.8%) were positive for coliforms (6 *E. coli* and 7 *K. pneumoniae*). All the coliform isolates in this study showed 100% resistance to Penicillin and Tetracycline, and were all 100% susceptible to Imipenem. High multidrug resistance was expressed by all the isolates to Penicillin, Tetracycline and Erythromycin. All the isolates (100%) had MARI of 0.2 and above which is an indication of gross abuse of antibiotics within the studied population. However, antibiotics still effective against the coliform species tested were Imipenem (100%), Ciprofloxacin (92.3%), Gentamycin (92.3%), Chloramphenicol (84.6%), Amoxicillin/Clavulanic acid (84.6%) and Sulfamethoxazole/Trimethoprime (69.2%).

Keywords: Coliforms, Antimicrobial, Susceptibility, Mastitis, Bovines, Pastoral herds

1. INTRODUCTION

Mastitis is the inflammation of mammary gland and is a complex and costly disease in dairy herds [1, 2]. It is characterized by physical, chemical and bacteriological changes in the milk, and pathological changes in the glandular tissue of the udder [3, 4]. The occurrence of disease is an outcome of interplay between three factors: infectious agents, host resistance, and environmental factors [5].

There is agreement among authors that mastitis is the most widespread infectious diseases in dairy cattle, and, from an economic aspect, the most damaging [6, 7, 8, 9, 10, 11]. It is a global problem as it adversely affects animal health, quality of milk and the economics of milk production. It affects every country, including developed ones and causes huge financial losses [12].

The two major forms of the disease are the clinical and subclinical mastitis [13]. Clinical mastitis results in alteration of milk composition and appearance, decrease milk production, and the presence of the cardinal signs of inflammation (pain, swelling and redness, with or without heat in infected mammary quarters). It is readily apparent and easily detected [13]. In

the subclinical form of mastitis, detection of mammary quarters with the disease is more difficult because signs are not readily apparent [13].

Furthermore, Somatic Cell Count (SCC) has been accepted as the best index to use to predict udder infection in bovines, and has been used extensively as an indicator since the 1960s [13, 14]. Under field conditions, determination of somatic cell count in milk is usually done using the California Mastitis Test (CMT); In fact, CMT scores are directly related to average SCC [14]. CMT has the advantage of being inexpensive and is a test with real-time result for selection of the quarters for subsequent bacteriological examination [13].

Indiscriminate use and continuous abuse of antibiotics among the pastoralists for both therapeutic treatment of infections) and non-therapeutic (growth promoters) purposes on dairy animals has resulted to the increasing emergence of resistant strains of pathogenic bacteria, which is a great threat to human and animal health [11]. Hence, this study was embarked on to investigate the antimicrobial susceptibility profile of coliform isolates from mastitis milk samples of dairy cows among the pastoral herds in parts of Kaduna State, Nigeria.

2. MATERIAL AND METHODS

2.1 Study Area

The study was carried out in Giwa, Igabi, Chikun, Soba, Zaria, Sabongari and Birnin Gwari Local Government Areas (LGA) of Kaduna State, Nigeria (Fig. 1). These are seven out of the 23 LGAs in the state. The state lies between latitude 9.00^0 and 11.52^0 North and longitude 6.08^0 and 8.83^0 East and is 608m above sea level. The number of LGAs studied was limited by the serious security challenge in the Northern part of Nigeria. The study area has distinct wet and dry seasons within the Guinea Savannah and part of the Sudan Savannah in Nigeria. Agriculture is the main stay of the economy of Kaduna State with about 80% of the people actively engaged in farming. Another major occupation of the people is animal rearing and poultry farming. The animals reared include cattle, sheep, goats and pigs [15]. Pastoralism, Agro-pastoralism and intensive dairy farming are the predominant dairy production systems in Kaduna State. The pastoralists move around with their herds in search of fresh pasture land or grazing areas. Agro-pastoralism is practiced by farmers who grow food crops and keep livestock, while the intensive dairy farmers use part or all of their land to grow fodder crops for their dairy cattle [16].

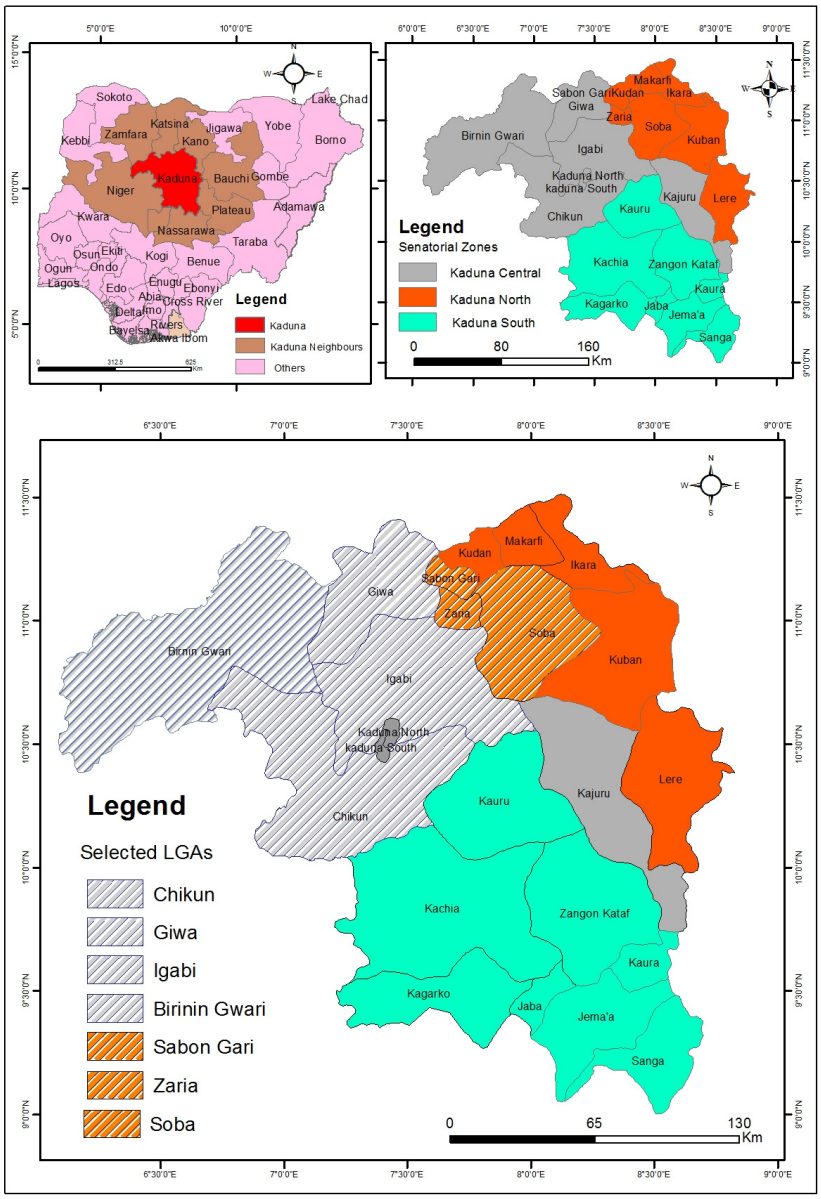

Fig. 1: The map of Nigeria and Kaduna State showing the study LGAs Adapted from the Administrative Map of Nigeria [17, 18]

2.2 Study Design

A cross-sectional study was carried out among 147 lactating bovines from 30 herds spread across seven Nomadic settlements within seven LGAs in Kaduna state between May, 2017 and July 2018 using quantitative methods of data collection.

2.3 Inclusion and Exclusion Criteria

The study population constitutes all the lactating bovines of the Nigerian indigenous breeds within the study area. All farmers/pastoralist who declined consent as well as regions within

the state that have been identified as volatile security spots were not included in this study. The animals were selected from herdsmen settlements in parts of Kaduna State, Nigeria. More so, only lactating bovines that are not currently on treatment were included in this study, while those currently undergoing any form of treatment were excluded.

2.4 Sample Size Determination and Sampling Technique

The sample size was calculated using the formula of Sarmukaddam and Gerald [19] expressed by Eq. 1. Mbuk *et al*. [11] recorded a prevalence of 10.3% for bovine coliform mastitis in Kaduna state, Nigeria which was used for sample size estimation in this study.

$$n = \frac{Z^2 p(1-p)}{L^2} \qquad (1)$$

Where:

n = is the number of samples

Z = is the standard normal distribution at 95% confidence interval = 1.96

p = is the prevalence of previous study = 10.3% = 0.103

L = is the allowable error, which is taken at 5% = 0.05

Therefore, sample size,

$$n = \frac{1.96^2 \times 0.103 \times (1-0.103)}{0.05^2} = 142$$

A sample size of 142 was estimated at 5% level of significance. A sample size of 147 was however used for ease of proportionate distribution.

A multi-stage sampling technique was used in this study. The seven LGAs were purposively selected out of 23 LGAs in Kaduna state being the LGAs with less security risks and were accessible at the time of this study. This was followed by the purposive selection of a settlement from each of the seven LGAs (total of seven settlements) based on the availability of lactating bovines that are not currently on treatment, willingness of the farmers/pastoralists to participate in the study, and accessibility of the location in order to easily transport samples collected to the laboratory for further analysis. Finally, 147 bovines were randomly but proportionately selected from all herds within the seven settlements. Bovine listing and enumeration was done to a total of 50, 30, 39, 27, 55, 40 and 68 for Settlements A, B, C, D, E, F and G, respectively out of which 24, 15, 19, 12, 26, 19 and 32 were respectively selected. A herd of bovines whose owner consented was sampled and in the event that he or she declined, the next contiguous herd of bovines was sampled. Computer generated list of random numbers from Minitab 14.2 statistical software was used to select the bovines for each of the settlements in this study.

2.5 Sample Collection and Screening for Subclinical Mastitis

Strict aseptic procedures was followed to prevent contamination with microorganisms present on the skin of udder and teats, hands of samplers and barn environment according to the methods of National Mastitis Council Guidelines described by Middleton *et al*. [20].

Prior to milk sample collection, udders and teats were cleaned using a disposable paper towel immersed in 70% ethyl alcohol and dried to avoid presence of feacal debris in the milk as it could interfere with the interpretation of CMT result. Foremilk (first jets) was discharged to reduce the contamination of teat canal. Sterile universal bottles with tight fitting cups were used. The bottles were labelled appropriately with permanent marker before sampling. To reduce contamination of teat ends during sample collection, the near teats were sampled first and then followed by the far ones. About 8mls of raw milk was aseptically collected from each bovine (2mls from each quarter). The California Mastitis Test (CMT) Reagent was used according to the manufacturer's instructions on the field to identify samples with subclinical mastitis. 2mls of milk samples was collected directly from each quarter of the udder and mixed together. 2mls of the composite milk sample was then added to 2mls of CMT reagent on the test paddle and mixed gently to observe reaction. The result was graded as described by various authors [21, 22]. All samples that tested positive for subclinical Mastitis were properly labelled and immediately transported to the Bacteriological Analysis Laboratory of the Department of Microbiology, Faculty of Life Science, Ahmadu Bello University, Zaria in an ice box for processing.

2.6 Bacteriological Analysis of CMT Positive Milk Samples

2.1.1 Inoculation of Raw Milk Samples

The CMT positive milk samples were inoculated on MacConkey agar (Oxoid, England) by streak method as described by Mekonnin *et al*. [23]. A loop full of milk sample was streaked on the agar plates aseptically using quadrant method for each sample. The plates were incubated at 37^0C and examined after 24 hours for growth.

2.1.2 Primary Isolation of Coliform Bacteria

Bacteriological analysis was focused only on the identification and isolation of Coliform bacteria. Hence, pink to red distinct colonies resulting from the utilization of lactose on MacConkey agar were presumptively considered as Coliform bacteria. The suspected isolates were sub-cultured to get pure isolates. The pure isolates were cultured on Eosin Methylene Blue Agar (EMB) which is selective and differential for Coliform bacteria. Isolates that showed metallic green sheen on EMB were presumptively considered as *E. coli*, while those with coloured appearance were considered to be other Coliform bacteria. The suspected Coliform isolates were stored in Nutrient Agar slant for further characterization and identification using the conventional biochemical tests and Microgen A+B ID Kits (UK).

2.1.3 Biochemical Characterization

All suspected coliform bacterial isolates that stained red with Gram reaction were subjected to Conventional biochemical tests. The tests conducted were: Indole, Methyl Red, Voges-Proskauer and Citrate Utilization (IMViC). The suspected coliform bacterial isolates from the tests were identified up to species level using Microgen A+B Kit (UK) in accordance with the manufacturer's instructions.

2.1.4 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was conducted for all the isolated Coliform species using disk diffusion method according to the criteria of the Clinical and Laboratory Standard Institute

[24]. Direct colony suspension of the isolates was adjusted to a turbidity equivalent to a 0.5 McFarland standard and was aseptically inoculated on Mueller-Hinton agar (Oxoid, UK) using spread plate technique. The antibiotic impregnated disks (Oxoid, UK) were aseptically fixed on the solid agar surface 15mm apart using a dispenser (Oxoid). The plates were incubated at 37^{o}C for 24 hours.

Commercially available antibiotics (Oxoid, UK) recommended as drugs of choice against *enterobacteriaceae* and those frequently used in the treatment of human and animal infections within the study area were selected. Thus, a total of ten antibiotics were used in this study. The antibiotic disks used with their various concentrations were: Amoxicillin-Clavulanic acid (30µg), Imipenem (10µg), Ciprofloxacin (5µg), Gentamycin (30µg), Chloramphenicol (30µg), Trimethoprime/Sulphamethoxazole (25µg), Erythromycin (15µg), Penicillin (10µg), Streptomycin (30µg) and Tetracycline (30 µg). Chloramphenicol was still included in this analysis because it is still being used for animal clinical in Nigeria notwithstanding the global recommendation for its removal from animal clinical.

Furthermore, the diameters of the zones of inhibition around the disks were measured to the nearest millimeter using caliper. The isolates were classified as susceptible, intermediate and resistant according to the interpretive standards of Clinical and Laboratory Standard Institute [24]. Moreover, isolates that showed resistance to two or more classes of antibiotics were considered as multidrug resistant [25, 26, 27, 28].

3. RESULTS AND DISCUSSION

3.1 Prevalence of Subclinical Mastitis and Coliforms in the Study Area

In this study, out of 147 milk samples from pastoral herds, 29 (19.73%) were positive for subclinical Mastitis out of which only 13 (8.84%) species of coliforms were isolated (six *E. coli* and seven *K. pneumoniae*). This implies that the prevalence of coliform mastitis in the study area (Parts of Kaduna state) is 8.84 %. Samples from Birnin-gwari LGA haboured the highest number of coliforms 4 (2.7%) while no coliform bacteria were isolated from samples collected from Soba Local Government Area (Table 1). *K. pneumoniae* and *E. coli* were the species associated with mastitis milk (Table 2 and Table 3).

Table 1: Prevalence of subclinical mastitis and associated coliform bacteria among the bovines studied

S/N	Local Government Area/ Settlements/Herds	No. of Lactating Bovines	No. of Bovines Examined	No.(%) of samples positive for Subclinical Mastitis	No.(%) of samples positive for coliform bacteria
1.	Giwa	50	24	6(4.1)	3(2.0)
2.	Igabi	30	15	5(3.4)	2(1.4)
3.	Chikun	39	19	3(2.0)	1(0.7)
4.	Soba	27	12	1(0.6)	0(0.0)
5.	Zaria	55	26	4(2.7)	1(0.7)
6.	Sabongari	40	19	3(2.0)	2(1.4)
7.	Birnin Gwari	68	32	7(4.8)	4(2.7)
	Total	**309**	**147**	**29(19.73%)**	**13(8.84%)**

Table 2: Biochemical Characterization (IMVIC) Of Isolates

Suspected Coliform Isolates	Indole Test	Methyl Red Test	Vogues Proskauer Test	Citrate Utilization Test	Probable Organism
C1	+	+	-	-	*Escherichia* sp
C2	+	+	-	-	*Escherichia* sp
C3	+	+	-	-	*Escherichia* sp
C4	+	+	-	-	*Escherichia* sp
C5	+	+	-	-	*Escherichia* sp
C6	+	+	-	-	*Escherichia* sp
C7	-	-	+	+	*Klebsiella* sp
C8	-	-	+	+	*Klebsiella* sp
C9	-	-	+	+	*Klebsiella* sp
C10	-	-	+	+	*Klebsiella* sp
C11	-	-	+	+	*Klebsiella* sp
C12	-	-	+	+	*Klebsiella* sp
C13	-	-	+	+	*Klebsiella* sp

Key: C1-C5 = Probable *Escherichia* species, C6-C13 = Probable *Klebsiella* species

Table 3: Microgen Tests for the Identification of the Isolates up to Species level

Presumptive Isolates	Octal number	Final Identification	Percentage Probability
EC1	04600570	*Escherichia coli* inactive	96.58%
EC2	05604520	*Escherichia coli* inactive	90.24%
EC3	04604420	*Escherichia coli* inactive	86.46%
EC4	04405421	*Escherichia coli* inactive	88.26%
EC5	07600570	*Escherichia coli*	49.76%
EC6	07601370	*Escherichia coli*	92.61%
KP1	47523766	*Klebsiella pneumonia*	99.71%
KP2	47523666	*Klebsiella pneumonia*	95.07%
KP3	47523777	*Klebsiella pneumonia*	95.2%
KP4	47523757	*Klebsiella pneumonia*	99.3%
KP5	47555777	*Klebsiella pneumonia*	87.34%
KP6	47544776	*Klebsiella pneumonia*	65.13%
KP7	47544777	*Klebsiella pneumonia*	57.67%

Key: EC1-EC6 = *Escherichia coli* (6)**;** KP1-KP7 = *Klebsiella pneumoniae* (7)

3.2 Antimicrobial Susceptibility

The coliform isolates in this study showed 100% resistance to Penicillin and Tetracycline, and were all 100% susceptible to Imipenem. High multidrug resistance was expressed by all the isolates to Penicillin, Tetracycline and Erythromycin. All the isolates (100%) had MARI of 0.2 and above which is an indication of gross abuse of antibiotics within the studied population which was further buttressed by the 100% resistance displayed against penicillin and tetracycline. However, antibiotics still effective against the coliform species tested were Imipenem (100%), Ciprofloxacin (92.3%), Gentamycin (92.3%), Chloramphenicol (84.6%), Amoxicillin/Clavulanic acid (84.6%) and Sulfamethoxazole/Trimethoprime (69.2%) (Table 4).

Table 4: Antimicrobial Susceptibility Profile of Coliform Bacterial Isolates obtained from Mastitis Milk Samples (n=13)

S/N	Antibiotic Generic Name	Dics Concentration (µg /ml)	No.(%) of Resistance	No.(%) of Intermediate Resistance	No. (%) of Susceptibility	Total (%)
01.	Erythromycin (E)	15.0	12(7.7)	0(0)	1(7.7)	13(100)
02.	Ciprofloxacin (CIP)	5.0	1(7.7)	0(0)	12(92.3)	13(100)
03.	Penicillin (PEN)	10.0*(I.U)	13(100)	0(0)	0(0.0)	13(100)
04.	Imipenem (IPM)	10.0	0(0.0)	0(0)	13(100)	13(100)
05.	Tetracycline (TET)	30.0	13(100)	0(0)	0(0.0)	13(100)
06.	Sulfamethoxazole/Trimethoprime (SXT)	25.0	4(30.8)	0(0)	9(69.2)	13(100)
07.	Chloramphenicol (C)	30.0	1(7.7)	1(7.7)	11(84.6)	13(100)
08.	Streptomycin (S)	10.0	3(23.0)	4(30.8)	6(46.2)	13(100)
09.	Amoxicillin/Clavulanic acid (AMC)	30.0	1(7.7)	1(7.7)	11(84.6)	13(100)
10.	Gentamycin(GN)	30.0	1(7.7)	0(0)	12(92.3)	13(100)

*Penicillin is given in International Units (IU).

More so, all the isolates tested exhibited six resistance patterns (A-F) according to their resistance to different antimicrobial groups (Table 5). All the isolates tested were considered multiple drug resistant (MDR) as they showed resistance to more than two classes of antibiotics tested. However, *Escherichia coli* isolate coded EC4 had the highest MAR Index of 0.9 (resistant to nine out of ten antibiotics tested), followed by EC5 that had 0.7 MAR Index (resistant to seven out of 10 antibiotics tested). *Escherichia coli* isolates coded EC1 EC2 and EC6 and *Klebsiella pneumonia* isolates coded KP4 and KP6 had MAR Index of 0.4 (resistant to four out of ten antibiotics tested). *Klebsiella pneumonia* isolates coded KP1, KP2, KP3, KP5 and KP7 had MAR Index of 0.3 each (resistant to three out of ten antibiotics tested, while *Escherichia coli* isolate with code EC2 had the least MAR Index of 0.2 (resistant to two out of ten antibiotics tested) (Fig.2).

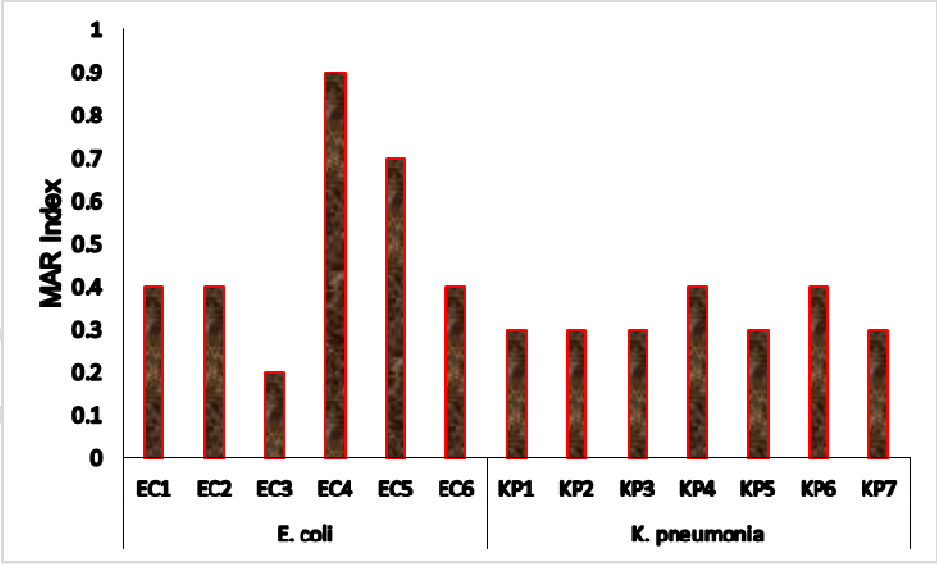

Fig. 2: Multiple Antibiotic Resistance (MAR) Index of coliform species investigated

The species of coliforms isolated in this study were *Klebsiella pneumoniae* and *Escherichia coli. Klebsiella pneumonia* was the dominant species associated with bovine mastitis in this study. This is in agreement with the work of Mbuk *et al*. [11] who isolated similar species of

these organisms in Kaduna State where *Klebsiella pneumonia* was the highest, but *Escherichia coli* was not isolated in their study. These findings also agree with Hogan and Smith [29] who reported that *Klebsiella pneumoniae* and *Escherichia coli* are the species of coliforms most frequently isolated from cases of bovine mastitis. The dominance of *Klebsiella pneumoniae* in this study agreed with the report of Podder *et al*. [30] who reported that *Klebsiella pneumoniae* is well adapted to survive in the udder and usually establishes subclinical mastitis infection of long duration which can be shed in milk, facilitating transmission to healthy animals mainly during milking process. Generally, the presence of these Coliform bacterial species in the milk is an indication of feacal and environmental contamination resulting from poor hygienic practices of rearing and milking the animals as there are no established mastitis control practices employed among the herdsmen.

The results of antimicrobial susceptibility test in this study showed that all the species of coliforms tested were sensitive to Imipenem followed by decreasing sensitivity to Ciprofloxacin, Amoxicillin/Clavulanic acid, Streptomycin, Chloramphenicol, Gentamycin and Sulfamethoxazole/Trimethoprime. This agrees with the report of Mbuk *et al*. [11] and Lira *et al*. [31] that showed similar susceptibility pattern. Susceptibility of Imipenem to all coliform species tested has proven that Carbapenems still retain considerable potency against Enterobacteriaceae. This agrees with the recommendation of CLSI [24], where this class of antibiotics was among the recommended antibiotics for treatment of infections caused by Enterobacteriaceae. High level of susceptibility to Imipenem in this study might be due to its rare use and abuse in cattle. However, it is worthy of note here that the Coliform species tested showed 1.4% intermediate resistance and 5.1% resistance to some CLSI [24] recommended antibiotics (Ciprofloxacin, Amoxicillin/Clavulanic acid, Streptomycin, Chloramphenicol, Gentamycin and Sulfamethoxazole/Trimethoprime). Therefore, irrational prescriptions and indiscriminate use of these drugs may lead to complete resistance in future [32].

However, all the species of Coliform bacteria tested were completely resistant to Penicillin and Tetracycline. This is similar to the reports of previous studies of high resistance to these same antibiotics [11, 31, 33]. The high degree of resistance observed in this study might be due to prolonged and indiscriminate usage of those antibiotics which could lead to possible resistance development in humans and animals [34, 35].

Moreover, all the *Klebsiella pneumonia* and *Escherichia coli* species in this study exhibited multidrug resistance, as they were consistently resistant to two or more classes of antibiotics among others used especially Erythromycin, Penicillin and Tetracycline. This finding agreed with the previous reports where Coliform species tested displayed multidrug resistance to Erythromycin, Penicillin and Tetracycline [11, 31]. These findings however, contradict the report of Memom *et al*. [33] where coliform species were completely resistant to Ciprofloxacin, Gentamycin, Amoxicillin and Sulfamethoxazole/Trimethoprime. However, based on this study, antibiotics still effective against the coliform species tested were Imipenem (100%), Ciprofloxacin (92.3%), Gentamycin (92.3%), Chloramphenicol (84.6%), Amoxicillin/Clavulanic acid (84.6%) and Sulfamethoxazole/Trimethoprime (69.2%). Although Chloramphenicol is prohibitive for use in animal clinicals, it is still being applied in Nigeria since there no legal framework for its prohibition yet. Since, this study has recorded high sensitivity of coliforms to Imipenem, Ciprofloxacin, Amoxicillin/Clavulanic acid, Streptomycin better than Chloramphenicol, this study joins voice to global practice to discourage the use of Chloramphenicol in animal clinical in Nigeria.

Furthermore, the result of susceptibility pattern of Coliform bacterial species obtained in this study affirms that some of CLSI [24] recommended antibiotics of choice against the treatment of infections caused by Enterobacteriaceae are increasingly becoming ineffective within the studied population. Therefore, it is very important to always conduct antimicrobial

sensitivity tests before empirical therapy is initiated to avoid resistance development to other sensitive antibiotics in future. However, based on the degree of susceptibility pattern obtained in this study, Imipenem is recommended as first line drug of choice where infection by *K. pneumonia* and *E. coli* respectively is suspected within the studied area.

4. CONCLUSION

This study concludes that the prevalence of subclinical mastitis in Kaduna State is 19.73 % while the prevalence of Coliform Mastitis is 8.84%. A low prevalence of Coliform mastitis was observed in this region, but the presence of *Klebsiella pneumonia* and *Escherichia coli* in raw milk samples of the studied bovine constitute serious environmental health risk to the consumers as the milk obtained from these herds are widely circulated and consumed without any form of treatment. They are also among the list of organisms classified as dangerous biological agents that have the potential to pose a severe threat to public health and safety by United States Public Health Services. The species of coliforms isolated in this study showed decreased sensitivity to the majority of recommended antibiotics of choice by Clinical and Laboratory Standard Institute (CLSI). This phenomenon could result to complete resistance development in future if not properly handled. The high level of resistance to some of the commonly used antibiotics by the herdsmen imply that the selection pressure imposed by the use of these antibiotics whether therapeutically in veterinary medicine or as prophylaxis in the animal production is a key driving force in the selection of antimicrobial resistance.

COMPETING INTERESTS

None

CONSENT

Ethical consent was obtained from the Postgraduate Board of the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria to undertake the study and to publish this report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee

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