Isolation, Identification and Antibiogram of Bacteria isolatedfrom Raw Cow Milk in Dutsin-Ma, Katsina State

Abstract

The study was conducted to isolate, identify the bacteria, to know the sources of contamination of milk and antibiotic sensitivity of bacteria obtained from Dutsin-Ma, Katsina State. A total of 45 samples were collected from different locations in Dutsin-Ma such as Wednesday market, opposite FUDMA takeoff site and Hospital road. All these samples were analyzed by culturing in different media such as *Salmonella-Shigella* agar, Eosin Methylene Blue agar, Mannitol Salt Agar, Nutrient agar, Cetrimide agar, MacConkey agar. Biochemical tests were performed to identify the organism. Among 45 samples, 20 (37.7%) were *Staphylococcus spp*. Similarly, 11 (20.8%), 6 (11.3%), 4(7.5%) and 12(22.6%)were found positive for *Escherichia coli, Pseudomonas aeruginosa, Klebsiella* and *Salmonella spp.* and. respectively. Results of antibiotic sensitivity test represent that, out of ten antibiotics *Staphylococcus sp*. were very sensitive against Gentamicin (95%), Ciprofloxacin (90%), Streptomycin (70%), and highly resistant against Zinnacef (60%), Ampiclox (70%), Amoxicillin (50%). *Salmonella* sp. were highly sensitive to Pefloxacin (83.3%), Sparfloxacin (83.3%), Ciprofloxacin (95%), but resistant against Augmentin (83.3%), Streptomycin (75%), Sulfamethoxazole (66.6%). *Klebseilla spp*. were highly sensitive Pefloxacin (50%), Sparfloxacin, Chloramphenicol (75%), Ciprofloxacin (75%), but resistant toGentamicin (100%), Streptomycin (100%), Sulfamethoxazole (75%), Augmentin (75%). *Escherichia coli* were highly sensitive to Gentamicin (72.7%), Ciprofloxacin (100%), Ofloxacin (90.9%), Sparfloxacin (72.7%), but highly resistant to Sulphamethoxazole (72.2%), Whereas, *Pseudomonas aeruginosa*were highly sensitive Ciprofloxacin (100%), Chloramphenicol (66.6%), Ofloxacin (66.6%), but highly resistant against Streptomycin (100%), Augmentin 83.35%), Perfloxacin (83.3%). Data of this study suggested that raw milk contaminated with drug resistant bacteria may cause public health hazard.

Key words: Raw, Milk, Antibiotics, Resistant.

Introduction

Raw milk is obtained from cows at homes in the Fulani hamlets and villages where shelf-life and safety of the products are not considered. Milk is a major component in the human diet of vegetarian class, but it also serves as very good medium for the growth of many Microorganisms

including pathogenic bacteria [Ruegg, 2003]. Milk is a highly valuable Food, but raw milk contains and favors growth of many microorganisms. Foodborne illnesses are an important challenge to public health and cause significant economic problem in many countries [WHO. 2015]. The crucial goal of all food safety programs is to prevent food products contaminated by potential pathogens from reaching the consumer. Milk is an excellent medium for bacterial growth, which not only spoils the milk and associated products but also can cause infections in consumers. Because of the specific production, it is not possible to fully avoid contamination of milk with microorganisms; therefore, the microbial contamination of milk is an important tool in determining its quality [Srinu *et al.,* 2012]**.** During the normal milking operation however, milk is subjected to contamination from many different sources including: (1) the udder and body of cows, (2) dust from the air, (3) litter and floor (4) flies, insects and rodents (5) water supply (6) hands and clothes of the milkier (7) utensils, bottles (8) atmosphere, etc. [Ensminger *et al.,* 1994]**.** Thus, milk and the dairy products prepared from milk could be an important source of food borne pathogens (Oliver *et al.,* 2019)**.** Milk can be contaminated by various types of microorganisms such as *Streptococcus, Staphylococcus, Micrococcus, Escherichia, Bacillus, Salmonella* and *Pseudomonas sp* (Mubarack *et al.,* 2010)**.** Huge numbers of microbes can get access to milk and various milk products including *Escherichia coli,* which is an indicator of milk contamination, constituting a public health hazard [Virpari *et al.,* 2013]**.** The diseases transmissible to humans through the consumption of spoiled milk like brucellosis, tuberculosis, salmonellosis, listeriosis, *Escherichia coli* infections and many others were described extensively in 1962 by [Kapla *et al.,* 1962]**.** Antimicrobial development and eventual clinical adoption is one of the most significant issues in medical history, with engineered medicines having saved millions of lives against diseases that would have been lethal [Gajdács, *et al.,* 2012].Nonetheless,

due to the development of multidrug resistance (MDR) in these pathogens, treating infectious diseases is becoming increasingly difficult. The present study was undertaken aiming to isolate and identify the bacteria, and determine antibiotics resistant of bacteria from raw cow in Dustin-Ma, Katsina State, Nigeria.

Bacterial contamination in raw milk has been a great threat to the economic and human health which cause mild disease to life threatening illness. Raw milk can carry dangerous bacteria such as *Salmonella*, *E. coli*, *Listeria*, *Campylobacter*, and others that cause foodborne illness, often called "food poisoning." These bacteria can seriously injure the health of anyone who drinks raw milk or eats products made from raw milk. However, the bacteria in raw milk can be especially dangerous to people with weakened immune systems (such as transplant patients and individuals with HIV/AIDS, cancer, and diabetes), children, older adults, and pregnant women. In fact, Center for Disease Controlfinds that foodborne illness from raw milk especially affects children and teenagers. Hence, the need for this study.

Bacterial contamination of raw milk is of grave public health concern, especially when they are Antibiotics Resistant bacteria (ARB). This is because in addition to being a human pathogen, ARB are of health concern that can be disseminated from contaminated milk via mobile genetic elements like plasmids and transposons. Therefore, resulting to complicated, untreatable, and prolonged infections in humans, leading to higher healthcare costs and sometimes death [Ezekiel *et al*., 2019].The aim of this research is to isolate, identify and determine antibiotics resistant of bacteria from raw cow in Dustin-Ma, Katsina State, Nigeria.

Material and Methods

Sample Collection

A total of 45cow milk samples were collected randomly (using random sample collection) from 3 different locations in Dutsin-ma local government area of Katsina state, Nigeria. Nine samples were taken per week from each location for five (5) weeks. The samples were collected in sterile universal bottle and transported to the Microbiology Laboratory of the Department of Microbiology, Federal University, Dutsin-Ma for microbiological analysis.

Isolation of Bacteria

Serial dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared. Aliquots of 1ml from each dilution was inoculated into sterile nutrient agar and MacConkey agar plates using pour plate method and incubated at 37^{0}C for 24 hours. Plate counts were recorded in cfu/ml. Colonial appearance such as size, shape, consistency, color, elevation and differential characteristics such as pigmentation and the isolate was further sub-cultured in eosin methylene blue, *SalmonellaShigella*and mannitol salt agar and Gram Staining were done to further identify the isolates [Cheesbrough,2003].

Gram Staining Technique

A smear of the suspected colony from overnight culture plates were made on clean, grease – free slide. The smear was heat-fixed on slide by passing the slide briefly over Bunsen burner flame. Smear was then covered with crystal violet stain for 1 minute. Stain was removed, rinsed with tap water. Afterwards, Lugol's iodine was added for 1 minute while decolonization was carried out by addition of acetone for few seconds. Slide was quickly washed with distilled water and counter stained with Safranin for 1 minute. It was then finally flooded with water and blot dried and examined under the microscope using the oil-immersion objective lens. Suspected

Staphylococcus aureus isolates were Gram-positive cocci (appearing purples) and arranged in clusters, *Klebsiella*spp., *Escherichiacoli*, *Pseudomonas aerogenosa* and *Salmonella*spp. isolate were Gram-negative rod (appearing pink) (Cheesbrough,2003)

Biochemical Characterization and Identification

Catalase Test

A drop of 3% hydrogen peroxide solution was placed on a clean, grease-free glass slide. A loopful of overnight colony of the test organism was thereafter emulsified on the hydrogen peroxide. Observed bubble formation was regarded positive and no bubble formation was regarded negative (Cheesbrough, 2003).

Coagulase Test

One loopful of the colony was emulsified on a clean grease free glass slide. Ten microliter of citrated human plasma was added and observed for the presence of agglutination which indicates a positive reaction (Cheesbrough, 2003).

Urease Test

The media was prepared based on the manufacturer's instruction then 40% of urea solution was added and mixed well then poured in a tube and slant then the isolate was inoculated and incubated for 18-24hrs. Positive test is demonstrated by an intense magenta to bright pink color in 15-24hrs, negative test show no color (Cheesbrough, 2003).

Triple Sugar Iron Test

The media was prepared based on the manufacturers instruction poured in a test tube sterilized at 115^0C for 30minutes then it was allowed to set in the sloped form with a butt inoculation was made by stabbing trough the center of the medium to the bottom of the tube with a straight inoculation needle then the surface of the agar slant was streaked with the isolated colony.

Positive result was indicated by color change, gas production and production of H_2S (Cheesbrough, 2003).

Indole Test

The media was prepared based on the manufacturers instruction and the isolated colony was inoculated for 37^{o}C for 24-28 hours then Kovac's reagent was added to the broth culture formation of pink to red color ("cherry-red ring) in the reagent layer on top of the medium within second of adding the reagent indicate positive and no color change indicated negative (Cheesbrough, 2003)

Methyl Red Test

The broth (MRVP) was prepared according to manufacturer's instruction and the organism was inoculated then incubated for 18-24hrs then add methyl red reagent color change to red indicate positive and no color change indicate negative (Cheesbrough, 2003).

Voge's-Proskauer Test

The broth (MRVP) was prepared according to manufacturer's instruction and the organism was inoculated then incubated for 18-24hrs then add VPI and VPII reagent color change and ring formation indicate positive lack of color change indicate negative (Cheesbrough, 2003).

Citrate UtilizationTest

The media Simmons agar was prepared based on the manufactures instruction poured in tube slanted then the organism was inoculated and incubate for 18-24hrs at 37^{o}C positive result is indicated by color change green to blue (Cheesbrough, 2003).

Antimicrobial Susceptibility Testing

Isolates were screened for phenotypic resistance and susceptibility to gram positive and gram negative disc. The procedure includes inoculation of stock cultures stored at 4^{o}C on nutrient agar

slants into 10ml of nutrient broth which was then incubated overnight at 37^{o}C. Thereafter, serial dilution of 10^{1} into sterile distilled water was carried out. Afterwards, 1 ml of the culture solution was transferred into sterile petri dishes. Thereafter, sterile Mueller Hinton agar that has been cooled to 55^{o}C in water bath was poured into each and allowed to solidify. Antibiotic sensitivity disc was later placed on one of the solidified plates sterilely and both plates were incubated at 37^{o}C in an incubator overnight. Zone of inhibition seen round the antibiotic disc the following day were measured while length was categorized as resistant, intermediate and sensitive after comparing with Clinical Laboratory Standard Institute standard for each bacteria isolate (CLSI, 2021). Multidrug resistant isolates were selected based on their resistant to ≥ 3 classes of antibiotics.

Statistical Analysis

Microsoft Office Excel 2016 was used for the data analysis. The fungi isolated were recorded as frequency and prevalence. Two-Factor Without Replication Analysis of Variance (ANOVA) was used to compute and arrived at statistical decision and $p < 0.05$.

Results

Table 1: Demonstrated the mean bacterial counts of raw cow milks collected from the sample locations, in which Wednesday market of Dutsin-Ma is the highest with (8.5×106 cfu/ml), while the least mean count was shown by Hospital Road with (1.04×106 cfu/ml).

Table 2: shows the morphological characteristics of all the bacterial isolates of row cow milk using both the all-purpose and selective media used for the study.

Table 3: Demonstrated the microscopic and biochemical characteristics of five bacterial species associated with the row cow milk.

Table 4: Presents the distributions of all the bacterial species isolated from the study sites, which include Staphylococcus aureus, Salmonella species, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella species.

Table 5: shows the percentage of occurrence of bacterial species isolated from the three sample locations. Staphylococcus aureus showed the highest percentage of occurrence (37.2%) while Klebsiella spp. showed the lowest prevalence (7.5%).

Table 6: shows the antibiotic susceptibility profiles of gram-positive bacteria on different antibiotics.

Table 7: shows the antibiotic susceptibility profiles of gram-negative bacteria isolated from row cow milk on different antibiotics

Figure 1: Distribution of Isolates of Raw Cow Milk from Different Locations.

Figure 2: Percentage of Occurrence of Bacteria Species.

Table 1: Mean Value of Bacteria Load

S/No	Locations	Mean bacterial count	Percentage %
1.	Wednesday market	8.5×10^6	48.5
2.	Front of school	7.99×10^6	45.6
3.	Hospital road	1.04×10^6	5.9
	Total	**1.753×10^7**	**100**

Table 2:Morphological Characteristics of Bacteria Isolated fromCow Milk in Dutsin-Ma

Media	Morphology	Bacteria
SSA	Pink with black dot	*Salmonella*
EMB	Green with metallic sheen	*Escherichia coli*
	Pink no sheen mucoid	*Klebseilla spp*
MSA	Round transparent	*Staphylococcus aureus*
Cetrimide	Greenish-blue	*Pseudomonas aeruginosa*

Key; SSA – *Salmonellashigella* Agar, EMB – Eosin methylene blue, MSA – Mannitol salt agar

Table 3: Gram Staining and Biochemical Test Result

Bacteria	Gram Stain	Catalase	Oxidase	Urease	Citrate	Tsi	Indole	Mr	Vp	Coagulase
Escherichia Coli	-				-	+	+	+	-	
S. Aureus	+	+		+			-	+	+	+
Salmonella Spp.	-			-	-	+	-	+	-	
Klebsiella Spp.	-	+			+		-	-	+	+
P. aeruginosa	-	+	+		+		-	-	-	

- = negative + =positive, VP = Voge's Proskauer, MR = Methyl Red, TSI = Triple sugar ion test

Table 4. Distribution of Isolates of Raw Cow Milk from Different Locations

S/N	Isolate	Locations			
		Wednesday Market	**Front Of School**	**Hospital Road**	**Total**
1.	*Staphylococcus aureus*	7	8	5	**20**
2.	*Escherichia coli*	4	3	4	**11**
3.	*Pseudomonas aeruginosa*	2	1	3	**6**
4.	*Klebsiella Spp.*	2	-	2	**4**
5.	*Salmonella Spp.*	5	4	3	**12**
	Total	**20**	**16**	**17**	**53**

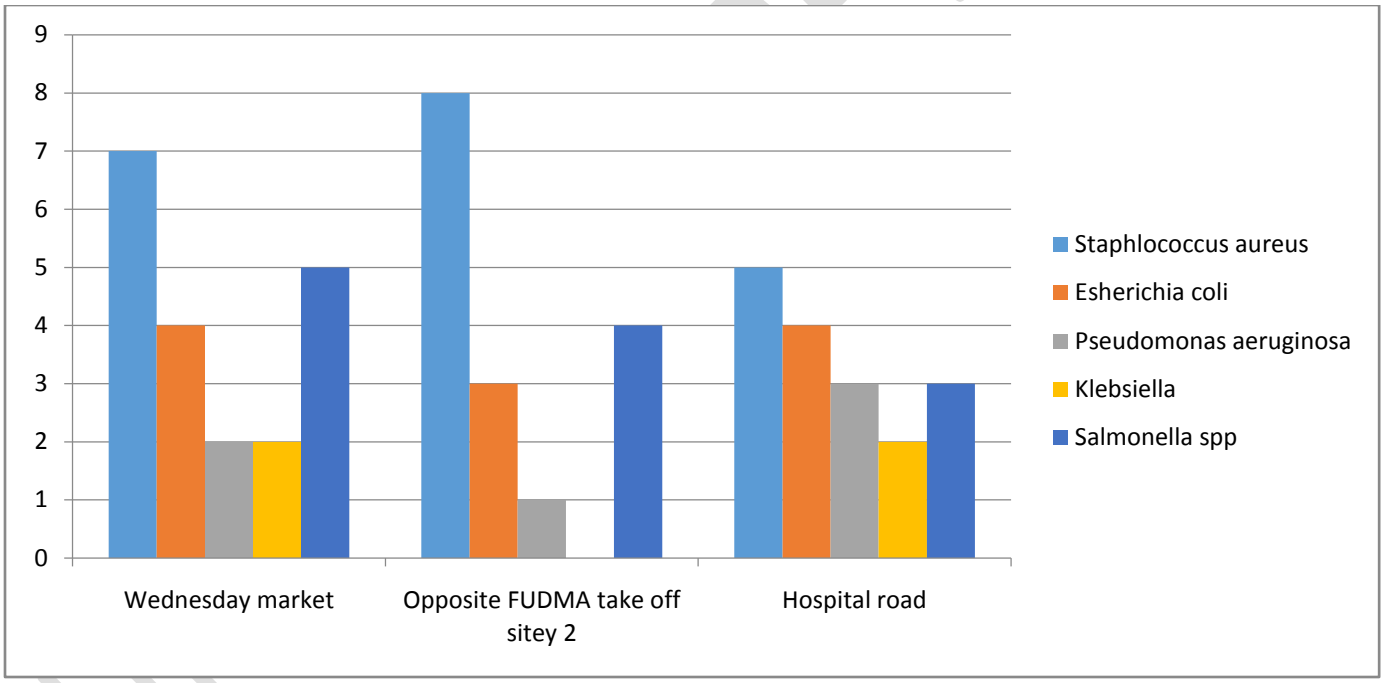

Figure 1: Distribution of Isolates of Raw Cow Milk from Different Locations

Table 5: Percentage of occurrence of Bacteria Species

S/N	Isolate	Wednesday Market	Hospital Road	Front of Fudma Take off	Percentage (%)
1.	*Staphylococcus Aureus*	7	5	8	**37.7**
2.	*Escherichia Coli*	4	4	3	**20.8**
3.	*Pseudomonas*	2	3	1	**11.3**
4.	*Klebsiella Spp.*	2	2	-	**7.5**
5.	*Salmonella Spp.*	5	3	4	**22.6**
	Total	**20**	**17**	**16**	**100**

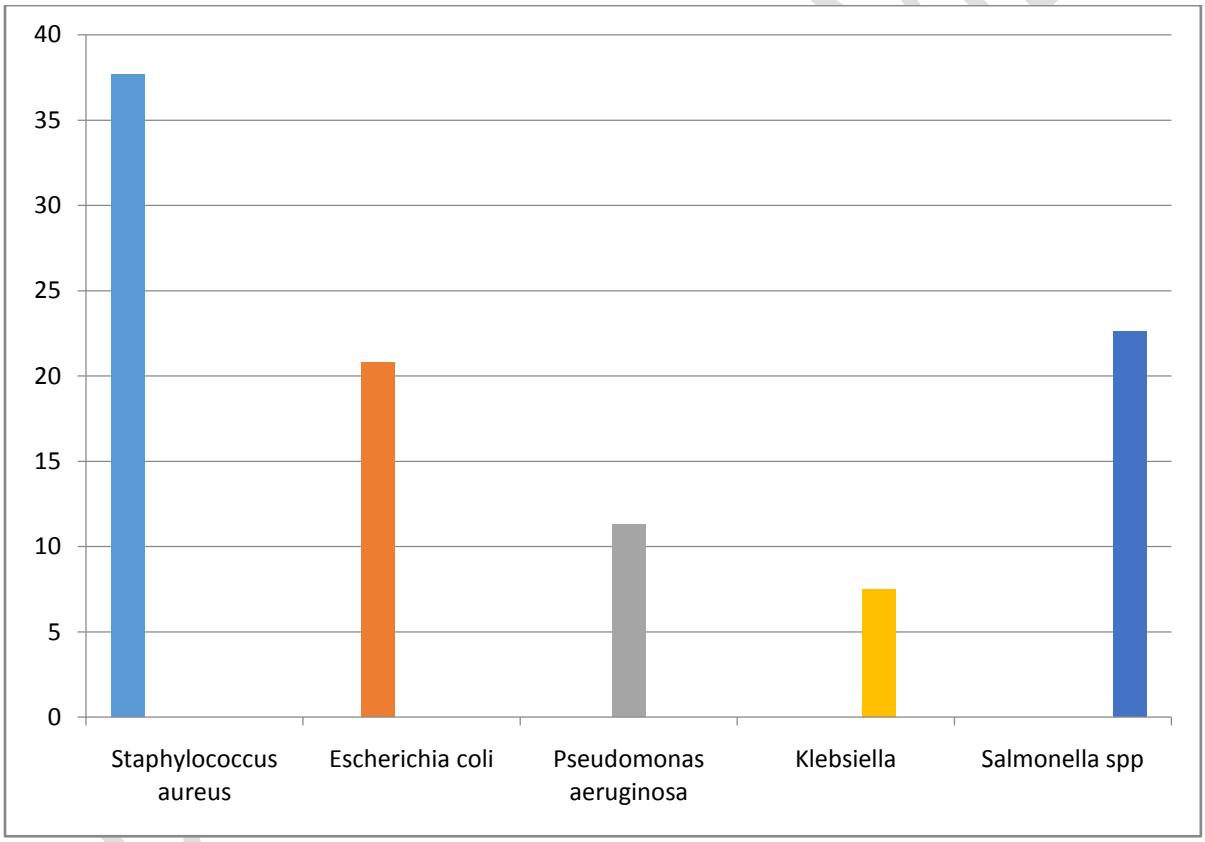

Figure 2: Percentage of Occurrence of Bacteria Species

Table 6:Antibiotics Susceptibility Pattern of Gram Positive Bacteria

Antibiotics profile (%)	Isolates		
	Staphylococcus aureus (n=20)		
	Susceptible	Intermediate	Resistance
Zinnacef	4(20)	4(20)	12(60)
Rocephin	10(20)	4(20)	5(25)
Streptomycin	14(70)	0(00)	4630)
Erythromycin	12(60)	0(00)	8(40)
Gentamicin	19(95)	0(00)	1(5)
Ampiclox	4(20)	2(10)	14(70)
Sulfamethoxazole	12(60)	0(00)	8(40)
Amoxicillin	8(40)	2(10)	10(50)
Pefloxacin	10(50)	6(30)	4(20)
Ciprofloxacin	18(90)	2(10)	0(00)

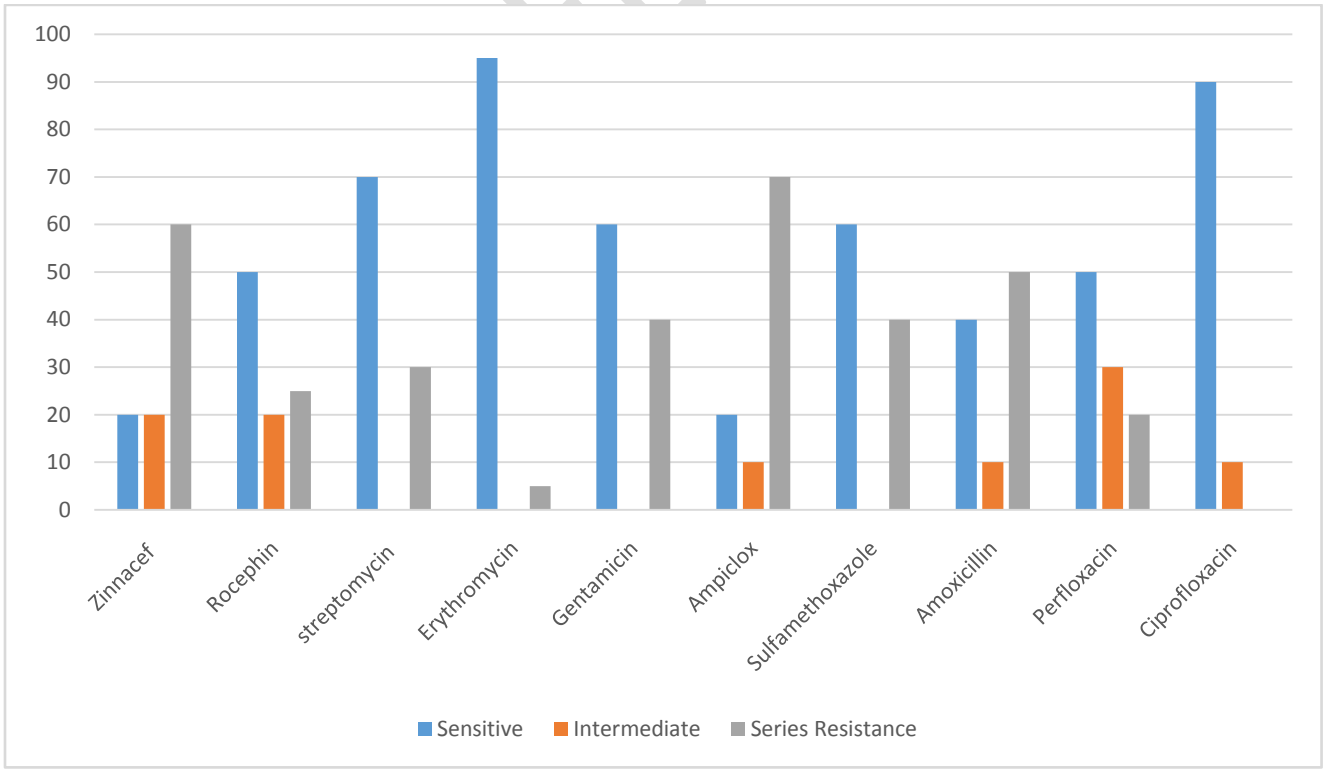

Figure 3: Antibiotics resistance profile of *Staphylococcus aureus*

Table 7: Antibiotics Susceptibility Pattern of Gram-Negative Bacteria from Raw Cow Milk

Antibiotics profile (%)	Isolates											
	***Salmonella* (n=12)**			***Klebsiella* (n=4)**			***Escherichia coli* (n=11)**			***Pseudomonas aeruginosa* (n=6)**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
AM	4(33.3)	3(25)	5(41.6)	1(25)	1(25)	2(50)	4(36.3)	2(18.2)	5(45.5)	2(33.3)	0	4(66.6)
PEF	10(83.3)	0	2(16.6)	2(50)	1(25)	1(25)	4(36.3)	5(45.5)	3(27.3)	1(16.6)	0	5(83.3)
SP	10(83.3)	1(8.3)	1(8.3)	1(25)	3(75)	0	8(72.7)	2(18.2)	1(9.09)	2(33.3)	3(50)	1(16.6)
CH	7(58.3)	3(25)	2(16.6)	3(75)	1(25)	0	6(54.5)	0	5(45.5)	4(66.6)	0	3(33.3)
GEN	6(50)	2(16.6)	4(33.3)	0	0	4(100)	8(72.7)	1(9.09)	3(27.3)	2(33.3)	1(16.6)	3(33.3)
AU	2(16.6)	0	10(83.3)	1(25)	0	3(75)	4(36.3)	2(18.2)	5(45.5)	1(16.6)	0	5(83.3)
OFX	7(58.3)	2(16.6)	3(25)	1(25)	3(75)	0	10(90.9)	1(9.09)	1(9.09)	4(66.6)	2(33.3)	0
S	2(16.6)	1(8.3)	9(75)	0	0	4(100)	2(18.2)	4(36.3)	5(45.5)	0	0	6(100)
SXT	1(8.3)	3(25)	8(66.6)	0	1(25)	3(75)	2(18.2)	1(9.09)	8(72.7)	1(16.6)	2(33.3)	3(33.3)
CPX	11(95)	0	0	3(75)	1(25)	0	11(100)	0	0	6(100)	0	0

KEY S=Sensitive, I= Intermediate, R=Resistant, AM=Amoxicillin, PEF=Pefloxacin, SP=Sparfloxacin, CH=Chloramphenicol, CN=Gentamicin, AU=Augmentin, OFX=Ofloxacin, S=Streptomycin, SXT= Sulfamethoxazole, CPX=Ciprofloxacin

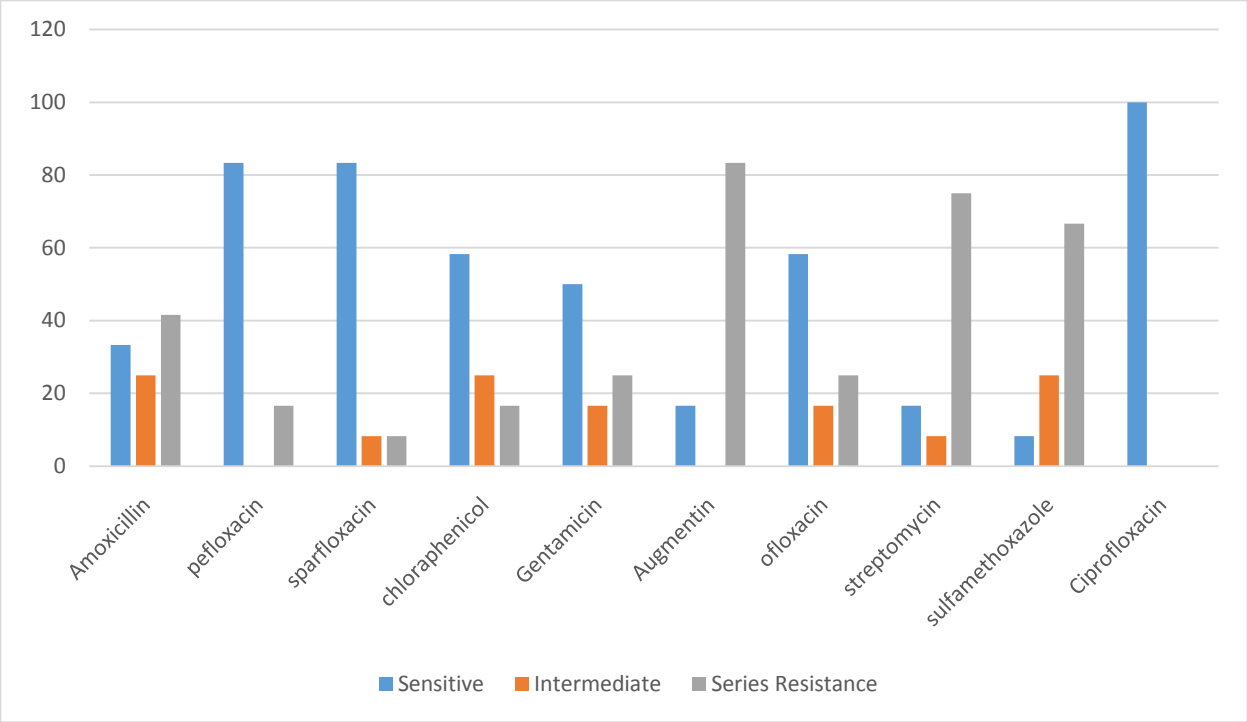

Figure 4: Antibiotics resistance profile of *Salmonella*

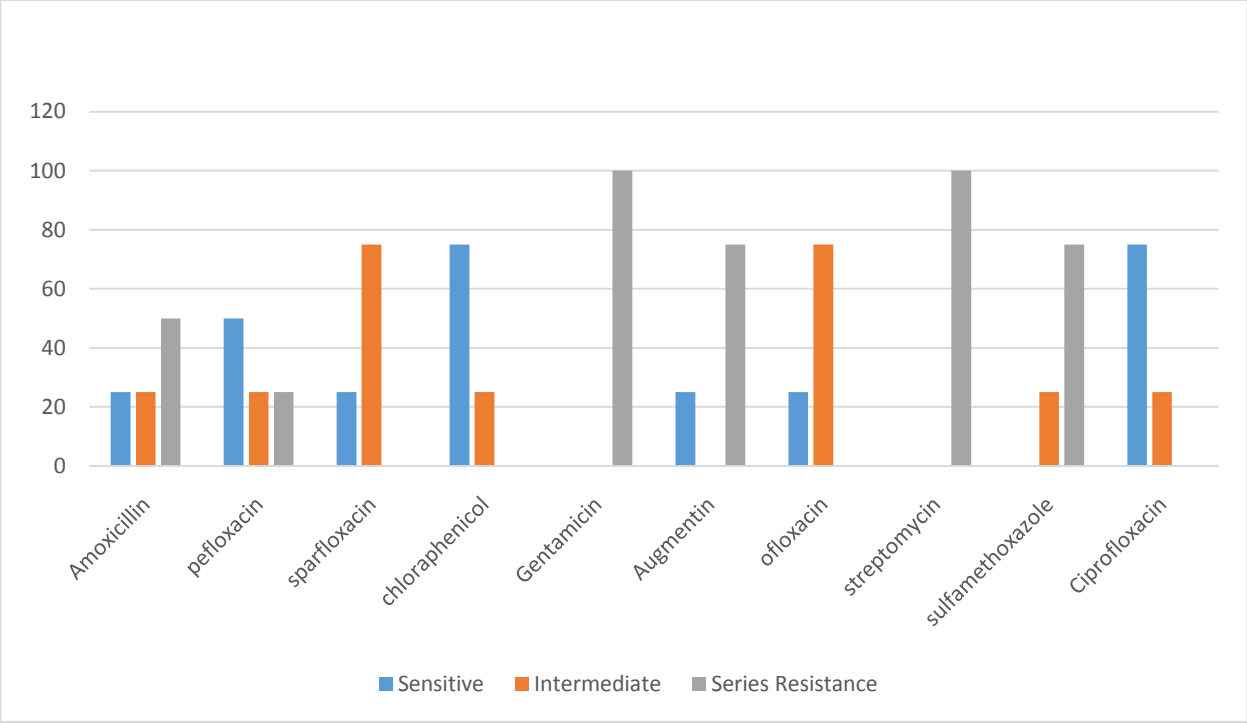

Figure 5: Antibiotics Resistance profile of *Klebsiella spp.*

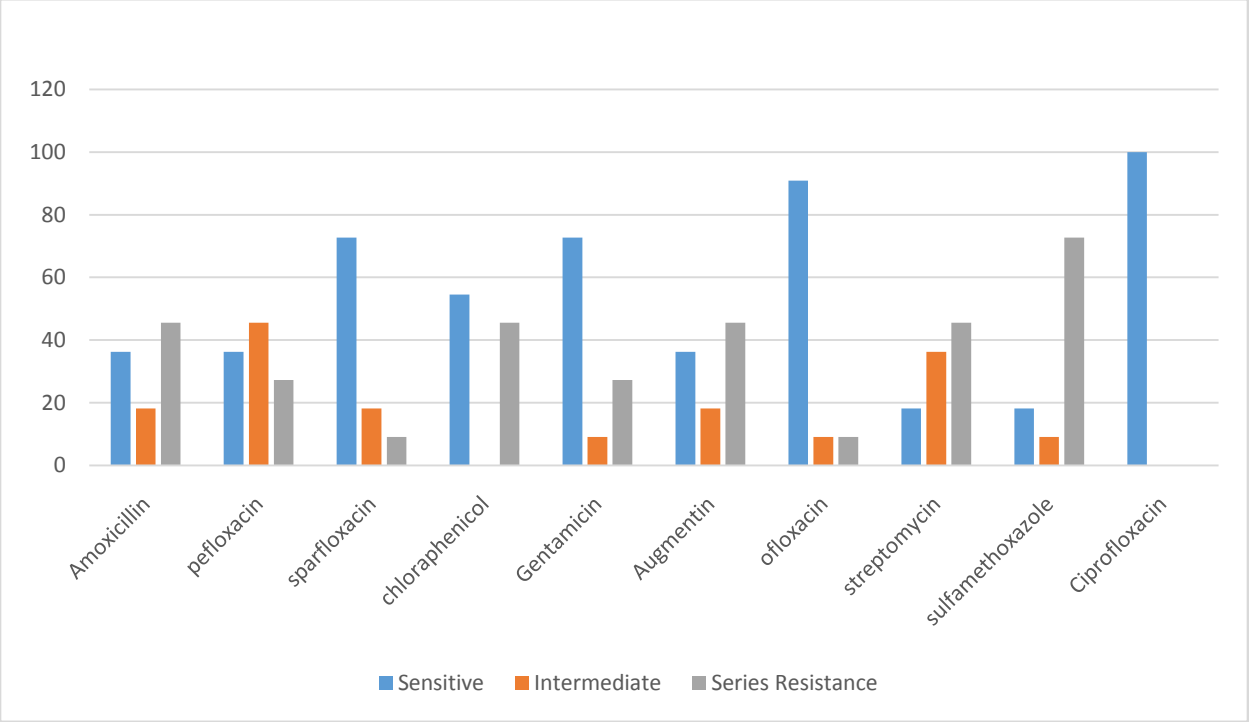

Figure 6: Antibiotics resistance profile of *Escherichia coli*

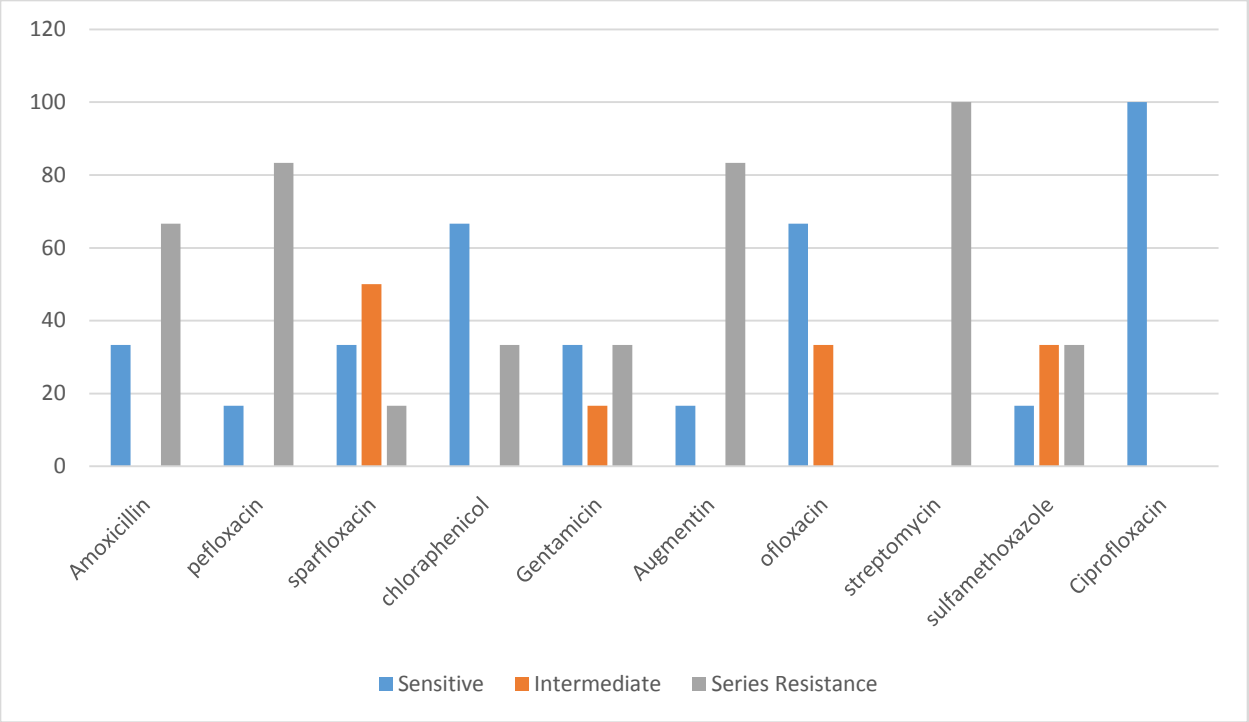

Figure 7: Antibiotics resistance profile of *Pseudomonas aeruginosa*

Discussion

The highest mean value of bacteria load of raw cow milk sample from Wednesday market as8.5x10^{6}CFU/ml (48.5%), where the moderate mean values of bacteria load from FUDMA take off campus as 7.99x10^{6}CFU/ml (45.6%) While the lowest mean value was obtained from Wednesday Market with 1.04x10^{6}CFU/ml (5.9%).

Morphological characteristics of the bacteria on Eosin Methylene Blue, Salmonella *Shigella*, Cetrimide and mannitol salt agar. On Eosin Methylene Blue agar *Escherichiacoli* and *Klebsiella* were identified,*Escherichiacoli* appeared green with metallic sheen, *Klebsiella* pink no sheen mucoid.On Cetrimide *Pseudomonas aerugenosa*appeared green. On *Salmonella-Shigella* agar

Salmonella was identified which appear colorless with black dot, and on Mannitol salt agar *Staphylococcusaureus* was identified as round and transparent.

The distribution of the bacteria in different location, Wednesday market 20 were isolated *Staphylococcusaureus*(7), *Salmonella* spp. (5), *Klebsiella* spp. (2), *Pseudomonasaeruginosa* (2) and *Escherichiacoli* (4), in hospital road 17 were isolated with staphylococcus aureus (5), *Salmonella*spp. (3), *Klebsiella*spp. (2), *pseudomonasaeruginosa* (3) and *Escherichiacoli* (4), in front of FUDMA take off campus 16 were isolated with *Staphylococcusaureus*(8), *Salmonella* (4), *Klebsiella* (0), *Pseudomonas aeruginosa* (1) and *Escherichia coli* (3). *Klebsiella* spp. werenot present in front of FUDMA take off campus.

The percentage of bacteria isolate. This study revealed that staphylococcus aureus has the highest occurrence with 37.8% followed by *Salmonella*spp.with (22.6%), *Pseudomonas aerogenosa*(11.3%),*Escherichiacoli* (20.8%) and *Klebsiella*spp.(7.5%). This finding does not agree with the findings of (Nwosu *et al*., 2017) who reported that *E. coli (*86.7%) and *Salmonella* spp.(86.7%), *Staphylococcus aureus* (80.0%), *Klebsiella* spp. (73.3%) and*Pseudomonasaeruginosa*(66.7%). Reta *et al.,* (2016) reported a prevalence rate of (24.2%) for *Staphylococcus aureus* in cow milk consumed at Jigjiga city, Ethiopia and (20.8%) prevalence rate for *Escherichia coli* reported by Makat *et al.,* (2014) isolated from locally processed Cow milk products in Nassarawa state.

The antibiotics resistantto gram positive *staphylococcus aureus*, Gentamicin, and Ciprofloxacin were sensitive with 95% and 90% respectively this does not correlate with the study of (Rokeya *et al*., 2019) which show sensitivity to ciprofloxacin 64% and gentamicin 93%. The organism was also found to be resistant to Ampiclox and Zinnacef with 70% and 60% respectively.

The antibiogram of gram negative bacteria all the isolates were highly sensitive to ciprofloxacin *salmonella* was found to be also sensitive to Perfloxacin and Sparfloxacin with 83.3% respectively, also resistance to Augmentin (83%) and streptomycin (83.3%). In a study by Makat *et al.*, (2014), *Salmonella* spp. was also sensitive to both Perfloxacin (43%) and Sparfloxacin (85.7%) and also resistant to Augmentin (14.4%) and Streptomycin (14.2%) (Makat *et al.*, 2014). *Klebsiella* was sensitive to chloramphenicol (75%) and resistance to gentamicin and streptomycin with 100%. *Escherichia coli* was sensitive to Ofloxacin (90.9%) (but resistance to Sulfamethoxazole (72.7%). *Pseudomonas aeruginosa* was resistance to streptomycin (100%) and Augmentin (83.3%). This study does not correlate with the study of (Nwosu *et al*., 2017)

Conclusion

The highest mean value of bacteria load of raw cow milk sample from Wednesday market as$8.5x10^6$CFU/ml (48.5%), where the moderate mean values of bacteria load from FUDMA take off campus as $7.99x10^6$CFU/ml (45.6%) While the lowest mean value was obtained from Wednesday Market with $1.04x10^6$CFU/ml (5.9%).

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The results obtained shows that there is presence of pathogenic microorganism that may be potential source of food borne infection that may result to food borne diseases in the consumers of these sampled products. The total viable bacteria count in all samples were above the standard, according to Nigerian Agency for Food, Drugs Administration and Control NAFDAC (2009), the microbial load limited for total liable colony count is $1.0x10^2$cfu/ml.

This study also found high levels of resistance to commonly prescribed antibiotics augmentin, streptomycin, gentamicin and sulfamethoxazole in the bacterial isolates. 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.

Recommendation

It is recommended that good sanitary measures should be taken by the people handling the cows, these measures should include proper handling of the cow, personal hygiene, treatment of udder

infection of the cow, use of hygienic milking and processing equipment, improve milk handling environment. It must be ensured that the cows are always in good health condition.

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