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## Assessment of Bacterial Contamination and Antibiotic Susceptibility Patterns of Bacteria Isolated from Milk collected from Biharwe in Mbarara District: Cross-Sectional Study

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### ABSTRACT

**Introduction:** Bacterial contamination in milk is increasingly becoming a global health threat that is predisposing humans to various food borne pathogens. This study is carried out to isolate and identify the possible common bacteria which cause milk contamination and the risks associated with it in Biharwe.

**Methods:** A cross-sectional study was carried out in Biharwe small holder dairy farms, Kashari Sub County, Mbarara district, from March 2022 to October 2022 to assess bacterial contamination, isolate, identify, and test antibiotic susceptibility patterns of organisms. A total of 20 milk samples were randomly collected from 20 privately owned small holder dairy farms with healthy lactating animals. Isolation and identification of organisms was carried out by using biochemical tests. The bacteria so identified and their isolation rate were *E. coli* (20%), *Citrobacter freundii* (40%), *Enterobacter Cloacae* (30%), *Enterobacter agglomerans* (10%). These are indicative of significant contamination of milk and important human pathogens. The antibiotic susceptibility test was performed on Mueller-Hinton agar by the Kirby-Bauer disk diffusion method. About 100 % of *E. coli* isolates was sensitive to Tetracycline with 0% resistance, 75% of *Citrobacter freundii* was sensitive to Tetracycline with 25% resistant isolates, 100% of *Enterobacter cloacae* was sensitive to Tetracycline with 0% resistance, and 100% of *E. agglomerans* was sensitive with 0% resistance. Overall 90% of the isolates were sensitive to Tetracycline with only 10% resistance. All the bacterial isolates were 100% sensitive to Gentamicin with 0% resistant. 100% of *Citrobacter freundii*, *Enterobacter cloacae* and *Enterobacter agglomerans* isolates were resistant to Ampicillin while only 50% of *E. coli* isolates was sensitive to Ampicillin. 100% of *Enterobacter cloacae*, *E. coli* and *Enterobacter agglomerans* isolates were sensitive to Streptomycin

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while 75% of *Citrobacter freundii* was sensitive and 25% was resistant to Streptomycin. *Citrobacter freundii* was the only isolated organism resistant to more than one antibiotic (100% resistant to Ampicillin and 25% resistant to Tetracycline and Streptomycin). Therefore, the pattern of antibiotic susceptibility tests among the isolates is statistically significant in Gentamicin (P=0.00).

**Conclusion:** This study revealed that raw cow's milk in the study area could be an important source of infection with a wide range of organisms, particularly gram-negative bacteria. An important source of microbial contamination of milk is fecal pollution probably cow dung. There is need for instituting effective control measures to protect public health. This includes mandatory milk pasteurization by traders and improved hygienic handling of containers during milking, ensuring milking is not done on floors with cow dung.

**Keywords:** Pathogens; Cow's milk; *E. coli*; Tetracycline; Contamination

## INTRODUCTION

Milk and dairy products are important source of vital nutrients for human beings. The unique composition and properties make milk an excellent medium for bacterial growth and source of bacterial infection. Milk borne pathogenic bacteria pose a serious threat to human health, and constitute about 90% of all dairy related diseases. *Staphylococcus aureus*, *Salmonella* spp., and *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Campylobacter* are the main microbiological hazards associated with raw milk consumption. Microbiological status of raw milk is affected by several factors including a health status of the animal, farm management practices, environmental hygiene and poor temperature control. In some countries with low socio-economic status, income growth and urbanization has led to almost doubled consumption of milk and dairy products [1-4].

Unlike in developed countries, the dairy industry in most African countries is underdeveloped, dominated by unpasteurized milk and informal markets.

*Escherichia coli* (*E. coli*) strains are one of the most important causes of food borne diseases around the world milk and dairy products are one of the main sources of transmission of the *E. coli* strains into the human. *E. coli* is a gram-negative, non-sporulating, flagellated, rod shaped and facultative anaerobic bacterium which belongs to Enterobacteriaceae family. Enterohemorrhagic *E. coli* (EHEC) strains are a sub type of the Vero (Shiga) toxin (Vtx or Stx) producing *E. coli* (VTEC or STEC). EHEC bacteria are causative agents of severe syndromes including Hemolytic Uremic Syndrome (HUS), Thrombotic Thrombocytopenic Purpura (TTP), Hemorrhagic Colitis (HC) and bloody and non-bloody diarrhea [5-10].

## MATERIALS AND METHODS

### Study Design and Setting

A cross-sectional laboratory study design. The study was conducted in randomly selected small holder dairy farms of Biharwe, Kashari Sub County, Mbarara district. The study area was selected based on the abundance of dairy farms in the area.

### Study Population

A cross-sectional study was carried out in Biharwe small holder dairy farms, Kashari Sub County, Mbarara district, from March 2022 to October 2022 to assess bacterial contamination, isolate, identify, and test antibiotic susceptibility patterns of organisms. A total of 20 milk samples were randomly collected from 20 privately owned small holder dairy farms with healthy lactating animals. Isolation and identification of organisms was carried out by using biochemical tests.

Healthy cows of breeds, crossbreed (Friesian, Big Kyogi) and local Ankole Cattle, were included during the study period.

### Sample Size

Sample size was determined using Krejcie and Morgan table; our population size (N) of 20 randomly selected farms with healthy cows gave us the required sample size (S) as 20.

### Study Procedure

#### Bacterial detection and isolation from milk samples

**Sample preparation and incubation:** Three test tubes were dispensed with 9 ml of normal saline. Threefold serial dilutions of the sample from  $10^{-1}$  to  $10^{-3}$  were done. Then, 1 ml of the milk sample was added into the 9 ml of normal saline ( $10^{-1}$  dilution). Then, 1 ml of the resulting solution was transferred into a second tube containing 9 ml ( $10^{-2}$  dilution). The procedure was repeated for the last dilution.

After the serial dilutions, 1 ml of the diluted milk sample was added into a sterile petri dish. Then approximately 15 ml of plate count agar (46°C) was poured into inoculated petri dish. The inoculum and the medium were carefully mixed by rotational movement of the petri dishes in the opposite directions 10 times before incubation. After complete solidification, all the petri dishes were inverted and placed in the incubator at 35°C for 48 hours to allow for bacterial growth. By using a bacterial colony counter, the number of colony forming units was counted.

The number of counted bacteria was expressed in colony forming units per ml using the following formula:

Number of bacteria=Number of Colony Forming Unit (CFU)/  
Volume plated (ml) × total dilution factor

### Coliform Count

The dilutions and inoculation was done as for the total bacteria count except that here we used L-EMB agar.

### Isolation and Identification of *Escherichia coli*

The presence of *E. coli* and coliforms was detected on L-EMB agar after incubating at 35°C for 24 hours. The growth of purple colonies with dark centers and greenish metallic sheen on L-EMB medium was considered a positive reaction. The biochemical reactions; indole reaction, citrate reaction and lactose fermentation were then performed on positive cultures.

**Antibiotic sensitivity testing:** Using the Kirby-Bauer disc diffusion method, the Antimicrobial Susceptibility Test (AST) was performed on Mueller-Hinton agar by the disk diffusion method. Isolated organisms were transferred to 5 ml of 0.9% saline water. The turbidity was measured using densitometry and adjusted to 0.5 McFarland. After measuring the turbidity, a sterile cotton swab was dipped into the suspension and then Mueller-Hinton agar plate was inoculated by rotating 60°. Antimicrobial discs were applied to the media using a disc dispenser and then incubated at 37°C for 16-18 hrs. Measurement of the zones of clearance was done by using a ruler. The criteria used to select the antibiotic agents tested were based on the availability and frequency of prescription for the management of bacterial infections in animals. Four

antibiotics were selected, Ampicillin 3 units; tetracycline, 30 µg; gentamicin, 120 µg; Streptomycin 25 units (OXOID discs), were used during measuring the zone of clearance. Standard breakpoints were interpreted based on the clinical and laboratory standards institute, and *E. coli* ATCC.

### Statistical Analysis

Laboratory data was captured in Microsoft Excel office (version 2019) to eliminate errors that would have been made when using manual calculations and then transferred to SPSS computer software version 20. Accordingly, descriptive statistics such as percentages and frequency distribution was used to describe bacterial isolates and antimicrobial susceptibility which was expressed as percent of resistant, intermediate and susceptible. P-value <0.05 was taken as cut off for statistical significance [11-15].

## RESULTS

### Isolated Bacterial Species

Four bacterial targets were identified in the milk sampled in the study area. The bacteria so identified and their isolation rate were *E. coli* 2 (20%), *Citrobacter freundii* 4 (40%), *Enterobacter cloacae* 3 (30%), *Enterobacter agglomerans* 1(10%). These are indicative of significant contamination of milk and important human pathogens [Table 1](#).

**Table 1:** Isolated bacteria.

S/No.	Isolates	Frequency (n)	Percentage (%)
1	<i>Escherichia coli</i>	2	20
2	<i>Citrobacter freundii</i>	4	40
3	<i>Enterobacter cloacae</i>	3	30
4	<i>Enterobacter agglomerans</i>	1	10
	Total	10	100

### Growth of Microorganisms on Plate Count Agar and L-EMB Agar

All the samples cultured on plate count agar with no dilution (N) yielded no bacterial growth. On the first dilution ( $10^{-1}$ ), 5% bacterial growth was yielded and 95% absence of growth, second dilution ( $10^{-2}$ ) and third dilution ( $10^{-3}$ ) yielded 10% bacterial growth and 90% absence of growth.

Samples cultured on L-EMB agar with no dilution (N) yielded no bacterial growth. On the first dilution ( $10^{-1}$ ), 10% bacterial growth (5% coliforms and 5% *E. coli*) was yielded and second dilution ( $10^{-2}$ ) yielded 5% bacterial growth *i.e.* Coliforms and no *E. coli*. Third dilution ( $10^{-3}$ ) yielded 5% bacterial growth (coliforms) ([Table 2](#)).

**Table 2:** Growth of microorganisms on plate count agar and L-EMB agar plate count agar.

S/No.	Growth	Frequency	Percent	Valid percent	Cumulative percent
N	No growth	20	20	100	100

10	No growth	19	95	95	95
	<i>Escherichia coli</i>	1	5	5	100
100	No growth	18	90	90	90
	<i>Enterobactercloacae</i>	1	5	5	95
	<i>Enterobacteragglomerans</i>	1	5	5	100
1000	No growth	18	90	90	90
	<i>Citrobacterfreundii</i>	2	10	10	100
L-EMB agar (coliforms)					
N	No growth	20	100	100	100
10	No growth	19	95	95	95
	<i>Citrobacterfreundii</i>	1	5	5	100
100	No growth	19	95	95	95
	<i>Enterobactercloacae</i>	1	5	5	100
1000	No growth	20	100	100	100
L-EMB agar ( <i>E.coli</i> )					
N	No growth	20	100	100	100
10	No growth	19	95	95	95
	<i>Citrobacterfreundii</i>	1	5	5	100
100	No growth	20	100	100	100
1000	No growth	19	95	95	95
	<i>Citrobacterfreundii</i>	1	5	5	100

### Biochemical Tests

In this study, milk samples were cultured and four bacterial isolates were obtained. The isolates were differentiated on

the basis of cultural studies and primarily by biochemical characteristics such as Simon's Citrate test, motility test, hydrogen sulfide gas production, Indole, and acid production tests (Table 3).

**Table 3:** Biochemical tests.

S/No.	TSI		SIM media			SCA	Microorganism	
	Slope/Butt	Gas	H <sub>2</sub> S	Indole	Motility	H <sub>2</sub> S		
1810	A/A	+	-	+	+	-	-	<i>E.coli</i>
1100X	A/A	+	-	+	+	-	+	<i>Enterobacter Cloacae</i>
14100	A/K	+	+	+	+	+	-	<i>Citobacter freundii</i>
1810Y	A/K	+	+	+	+	+	-	<i>Citrobacter freundii</i>
1100	A/A	+	-	+	+	-	+	<i>Enterobacter Cloacae</i>
1810Z	K/A	+	+	+	+	+	-	<i>Citrobacter freundii</i>
19100	K/A	+	+	+	+	+	+	<i>Enterobacter agglomerans</i>
181000	A/A	+	-	+	+	-	+	<i>Enterobacter cloacae</i>

1810X      A/A      +      -      +      +      -      +      *Enterobacter Cloacae*

Key: A/A: Acid-Acid; A/K: Acid-Alkaline

### Antimicrobial Susceptibility of the Bacterial Isolates

The antimicrobial susceptibility tests of the bacterial isolates were variable. About 100 % of *E. coli* isolates was sensitive to *Tetracycline*, 75% of *Citrobacter freundii* was sensitive to *Tetracycline* with 25% resistant isolates, 100% of *Enterobacter cloacae* was sensitive to *Tetracycline* and 100% of *E. agglomerans* was sensitive with 0% resistance. Overall 90% of the isolates were sensitive to *Tetracycline* with only 10% resistance. All the bacterial isolates were 100% sensitive to

*Gentamicin*. 100% of *Citrobacter freundii*, *Enterobacter cloacae* and *Enterobacter agglomerans* isolates were resistant to Ampicillin while only 50% of *E. coli* isolates was sensitive to Ampicillin. 100% of *Enterobacter cloacae*, *E. coli* and *Enterobacter agglomerans* isolates were sensitive to Streptomycin while 75% of *Citrobacter freundii* was sensitive and 25% was resistant to Streptomycin. *Citrobacter freundii* was the only isolated organism resistant to more than one antibiotic (100% resistant to Ampicillin and 25% resistant to *Tetracycline* and *Streptomycin*) (Tables 4 and 5).

**Table 4:** Summarizes the pattern of Antibiotic susceptibility test among isolated organisms, S: Sensitive, R: Resistance.

	Isolates							
	<i>Escherichia coli</i>		<i>Citrobacter freundii</i>		<i>Enterobacter cloacae</i>		<i>Enterobacter agglomerans</i>	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Tetracycline	100	0	75	25	100	0	100	0
Ampicillin	50	50	0	100	0	100	0	100
Gentamicin	100	0	100	0	100	0	100	0
Streptomycin	100	0	75	25	100	0	100	0

**Table 5:** Showing summary of antibiotics activity on the isolates significant level  $P < 0.05$ , S: Sensitive, R: Resistance.

Antibiotics	Isolates		
	S (%)	R (%)	P-value
Tetracycline	90	10	0.644
Gentamicin	100	0	<0.01
Ampicillin	10	90	0.217
Streptomycin	90	10	0.644

Therefore, the pattern of antibiotic susceptibility tests among the isolates is statistically significant in Gentamicin ( $P=0.00$ ).

## DISCUSSION

The Coliform bacteria isolated from milk samples were *E. coli* (20%), *Citrobacter freundii* (40%) and *Enterobacter Cloacae* (30%). These were higher than those in a certain study conducted by Seraphine Nkie Esemu, et al. *Enterobacter cloacae* (12.6%), *Escherichia coli* (7.0%) and *Citrobacter freundii* (0.4%). The differences in the relative occurrence of

Coliform bacteria could be due to differences in bacterial load of the various coliforms in the various environmental sources. In addition, majority of Coliform isolates from a study conducted using the raw milk consumed in West Bengal, India were *E. coli*, *Enterobacter specie*, *Klebsiella specie*, *Serratia specie* and *Citrobacter*, which closely agree to the findings from our study.

The high bacterial contamination rate of milk in our study implies that milk can pose health risks to consumers. If milk is not handled properly and in hygienic condition, it will support the growth of pathogenic micro organisms leading to

transmission of zoonotic and food borne diseases that can compromise the health of the population. Therefore, to prevent contamination of milk by pathogenic micro-organism, improving animal health, environmental hygiene, dairy farming practices, milk handling, transportation and storage practices are required.

Our findings revealed that gentamicin (100%) was the only antibiotic with no resistance by *Coliforms*. Low resistance to gentamicin (10.2%) by isolated *Coliforms* was also recorded in some study conducted. Susceptibility was highly shown to Gentamicin (100%) among the identified *Enterobacteriaceae* in this study, which is similar to Gentamicin reported in West Bengal, India.

*P. agglomerans*, until recently known as *Enterobacter agglomerans* and its new nomenclature is not yet widely in use. It is an opportunistic pathogen and, when introduced into the organs of humans or other animals, may cause severe and occasionally fatal infections. The most serious infections are in individuals with underlying diseases and in the young persons. Since clinical reports involving *P. agglomerans* are typically of polymicrobial nature, confirmed virulence of *P. agglomerans* is difficult to reveal. Infections caused by this organism often involve patients that are already affected by diseases of other origin, and isolates are rarely conserved for confirmatory analysis. *P. agglomerans* is ubiquitous in nature and it has been isolated from a wide variety of ecological niches and from different kinds of specimens from humans and animals. *P. agglomerans* is mostly isolated from powdered infant formula in developed and developing countries through the world.

Generally, although some coliform bacteria exhibited low resistance to some of the antibiotics tested and could be recommended as a drug of choice for coliform associated infections, the pattern of resistance differed with specific coliform genera. According to world health organization reports, the resistance of *Escherichia coli* to Ampicillin is pervasive in a range of 0.0%–98.0% in Africa. For example, in this study, resistance against Ampicillin for *E. coli* was 50%, this may be due to prolonged and indiscriminate usage and prescription of the antibiotic which often leads to possible resistance development in animals. Resistance to Streptomycin and Tetracycline was exhibited by 25% and 25% of *Citrobacter freundii* respectively. Thus, it is important to isolate the coliform and perform an antibiotic susceptibility test, if possible, before any antibiotic therapy [16-19].

## CONCLUSION

This study revealed that raw cow's milk in the study area could be an important source of infection with a wide range of organisms, particularly gram-negative bacteria. An important source of microbial contamination of milk is fecal pollution probably cow dung. There is need for instituting effective control measures to protect public health. This includes mandatory milk pasteurization by traders and improved hygienic handling of containers during milking, ensuring milking is not done on floors with cow dung.

The occurrence of multi-drug resistant *Citrobacter freundii* should be under consideration during selection of antibiotics for treatment of encephalitis in cattle. Furthermore, dairy cows become infected with *Citrobacter freundii*, therefore diagnosis does not have implication for treatment only but also it indicates zoonotic transmission since it becomes reservoir for human infection.

## RECOMMENDATIONS

In practice, indiscriminate use of drugs should be controlled and farmers should use Gentamicin for *Enterobacteriaceae* related infections as no organism was resistant to it. Veterinarians, livestock extension workers and farmers should implement *in-vitro* susceptibility testing prior to the use of antibiotics for treatment of infections in cows. Further studies that could incorporate isolation of milk contaminating bacteria to the species level should be done to evaluate the imminent danger posed by microbes from raw milk.

## LIMITATIONS

This study didn't comprehensively examine all important bacterial contaminants of milk and factors responsible for milk contamination.

Some of the farmers could not be available at the stipulated time of milk sample collection.

Some of the consented farmers later denied us access into their farms as it rained heavily and they could not be available at the time of sample collection.

The microbiology laboratory of Mbarara university of science and technology was under renovation for some weeks and this put our laboratory work on hold.

## ETHICAL CONSIDERATIONS

We sought consent from the farmers who participated in the study.

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We thank Mbarara district agricultural department for providing a suitable environment for our research activities.

## AUTHOR CONTRIBUTIONS

All authors made significant contribution to the work reported in all areas of the study design, execution, and acquisition of data, analysis, execution, interpretation and all other areas.

(1, 2, 3, 4) Conceived and collected data, (2, 3, 4, 5, 6) edited the paper, (1) wrote the first draft of the paper, (1, 2, 3, 4, 5, 6) wrote the paper and (1, 2, 3, 4, 5, 6) agreed with conclusions of this work.

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