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PATHOGENIC AND DRUG RESISTANT BACTERIA IN RAW MILK OF JESSORE CITY: A POTENTIAL FOOD SAFETY THREAT

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ABSTRACT

Milk is such a food which can meet almost all nutritional needs of human lives. Raw or unprocessed milk supports the growth of wide variety of microorganisms. The major interests of this study were examining the microbial quality of raw milk collected from different locations of Jessore city in Bangladesh and determining antibiotic susceptibility pattern of some isolated bacteria. To do so, 12 raw milk samples were collected from different areas of Jessore city. Microbial analysis comprised of enumeration of TVC (total viable count), TCC (total coliform count) and TSC (total staphylococcal count). The highest TVC, TCC and TSC were $1.95x10^9$ CFU/ml, $2.5x10^7$ CFU/ml and $1.02x10^7$ CFU/ml respectively. Prevalent bacterial populations were *Klebsiella* spp., *Enterobacter* spp., *Shigella* spp. *Staphylococcus* spp., *Escherichia coli* and *Citrobacter* spp. In order to observe the antibiotic susceptibility pattern, the antibiotic sensitivity test was performed for some randomly selected isolates of *E. coli* and *Klebsiella* spp. More than 90% isolates of *Klebsiella* spp. were found to be resistant against Erythromycin whereas more than 90% isolates were sensitive against Imipenem. On the other hand, 100% *E. coli* isolates were observed as resistant against Erythromycin and in case of Trimethopreme 100% isolates were sensitive. Multidrug resistance pattern was also found. These results suggest the necessity of hygienic practices during handling, processing and post-processing of raw milk to improve the microbiological quality and safety of raw milk.

Key words: Antibiotic resistance, Bacterial load, Milk quality.

INTRODUCTION

Milk has ~~a~~ major contribution in human diet~~.~~ It also plays as excellent culture medium for many kinds of microorganisms. Because of nutritional value, milk is called 'Ideal Food', which is synthesized in specialized cells of the mammary gland and virtually sterile when secreted into the alveoli of the udder (Tolle, 1980). However, it contains relatively few bacteria when it leaves the udder of a healthy animal and generally these bacteria do not grow in milk under the usual conditions of handling (Frazier *et al*., 2003). Several authors claimed some possibilities for contamination of raw milk. Like, during the normal milking operation, contamination occurs from the udder itself, especially the exterior of the udder and adjacent areas of udder play critical role for contamination (Bramley and McKinnon, 1990). In addition, the number and types of microorganisms in milk immediately after milking are affected by biotic factors like animal health and its cleanliness and abiotic factors such as air, soil, grass, feces, season and milking equipment (Uddin *et al.*, 2011). It is also hypothesized that differences in feeding and housing strategies of animals may influence the microbial quality of milk (Coorevits, 2008). Besides, rinsing water for milking machine and milking equipment washing may also be responsible for the presence of high number of microorganisms including pathogens in raw milk (Bramley and McKinnon, 1990). Public health problems associated with the consumption of unpasteurized raw milk and its products have been well documented (De Valk *et al*., 2000; De Buyser *et al*., 2001 and Harrington *et al*., 2002). So, examination for the presence and number of specific microorganisms is therefore an integral part of any quality control or quality assurance plan.

The detection of coliform bacteria or pathogens in milk can be used as an indicator for udder infection (mastitis), contamination in milking utensils or water supply (Bonfoh *et al.*, 2003). These infections need a wide variety of antibiotics to be treated. It is hypothesized that the indiscriminate use of antibiotics may lead to the development of multiple antibiotics resistance thereby rendering the antibiotic treatment ineffective (Johnston *et al*., 1983). According to the Infectious Diseases Report released by the World Health Organization (WHO) in 2000, drug resistant organisms are prevalent worldwide.

Over many years considerable attention has been paid to improve the quality of milk particularly the hygienic quality throughout the world. In Bangladesh, milk is produced in urban and rural areas mostly in non-organized

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way and usually supplied to the consumers in raw form. For many reasons, contamination in raw milk has never been possible to avoid yet (Uddin *et al.*, 2011 and Khaton *et al*., 2014). Due to the serious implications from the raw milk consumption and increasing concern of resistance of pathogenic bacteria to antibiotics; this current study therefore aimed to find out the prevalence and antibiotic susceptibility pattern of pathogens in raw milk supplied in the city of Jessore, Bangladesh.

MATERIALS AND METHODS

Sample collection

A total of 12 raw milk samples were collected for microbial analysis from Jessore city and its surrounding areas during the period of January to June, 2014. About 100 ml of fresh raw milk were collected in a sterile sample container using a sample collector ice box at 4°C and were transported to the laboratory without delay.

Isolation and enumeration of bacteria

The bacterial count was performed by standard plate count method (ICMSF, 1986). The microbiological conditions of safety and hygiene were then assessed using the methods recommended by International Commission on Microbiological Specifications for Foods (ICMSF, 1986). Serial dilutions of samples were made up to 10^{-7} in sterile normal water. Bacterial count was carried out by the spread plate technique. The sample (0.1ml) of each dilution was taken onto each sterile petridish and evenly spread on different culture medium and incubated at 37 °C for 24 hours. Total viable count (TVC), total coliform count (TCC) and total staphylococcal count (TSC) were done for enumeration using Nutrient Agar, MacConkey Agar and Mannitol Salt Agar respectively. Bacterial isolates were then identified according to the Bergey's manual of determinative bacteriology (Buchanan and Gibbon, 1984).

Antibiogram

Kirby-Bauer method (Bauer *et al*., 1966) was used in this study to examine bacterial susceptibility to antimicrobial agents on Mueller-Hinton agar. Tested antibiotics were Amoxycillin (AML, 10µg), Ceftriaxone (CRO, 30µg), Imipenem (IME, 10µg), Ciprofloxacin (CIP, 5µg), Streptomycin (S, 10µg), Tetracycline (TET, 30µg), Gentamicin (CN, 10µg), Nalidixic acid (NA, 30 µg), Chloramphenicol (C, 30µg), Trimethoprim-Suphomethoxasole (SXT, 25µg), Norfloxacin (NOR, 10µg) and Erythromycin (E, 15µg). The zone diameter for individual antimicrobial agent was then translated into sensitive, intermediate and resistant categories according to the interpretation table of CLSI documents on performance standards for antimicrobial susceptibility testing (CLSI, 2007).

RESULTS AND DISCUSSION

The present study was designed with a view to find out potential food safety threat associated with raw cow milk collected from Jessore city.

Large microbial load was found in almost every sample of raw milk. The standard limit for microbial counts in raw milk has been set by European Commity (EC) at $1x10^5$ CFU/ml to $5x10^5$ CFU/ml. Comparing with this, the bacteriological quality for most raw milk samples in this study was poor (Table 1). The highest total viable count (TVC) was $1.95x10^9$ CFU/ml. It may results from poor sanitary conditions of dairy yard, increasing contamination of body surface by feces and soil, lacking of washing of animal and using of unsanitary milking utensils. Even the lowest TVC count was $2.1x10^7$ CFU/ml which exceeds the limit suggested by EC. This condition indicates the poor local milking system by untrained personnel and defective supply chain. Total viable count found in this study has a strong connection with that of Uddin *et al.* (2011) who found TVC ranging 2.0 $x10^8$-2.36 $x10^9$ CFU/ml. The TVC result is also closely related to the finding of Hossain *et al.* (2010) who stated TVBC ranging $1.75x10^6$-$1.22x10^8$ CFU/ml. Finding of this research also supports the finding of Muhammad *et al.* (2009) and Yuen *et al*. (2012) who found TVC of several samples exceeding 10^6 CFU/ml and 10^7 CFU/ml respectively. However, the present study strongly differs from the findings of Tokar *et al.* (2008) who found standard plate count of raw milk 4.5 log10 which is much lower than the finding of this study. It may results from the relatively better hygienic status due to organized dairy farm production system, better quality of water and short period of transportation and preparation of sample.

The coliform count was also high in all the samples (Table 1).

Table 1. Bacterial enumeration of tested samples

No. of sample	TVC (cfu/ml)	TCC (cfu/ml)	TSC (cfu/ml)
1	9.05×10^8	1.08×10^6	8.48×10^6
2	2×10^9	5.16×10^6	1.56×10^5
3	1.95×10^9	1.5×10^7	8×10^6
4	1.03×10^9	1.5×10^7	5×10^6
5	1.14×10^9	1.7×10^6	1.2×10^6
6	2.5×10^8	1.5×10^6	6×10^5
7	6×10^9	2.5×10^7	3×10^7
8	2.7×10^7	2×10^5	1.2×10^6
9	8×10^8	1×10^6	1.2×10^5
10	1.67×10^8	5.2×10^6	1.02×10^7
11	2.1×10^7	5×10^6	9.5×10^5
12	1.5×10^8	2×10^6	5.2×10^5

Table 2. Biochemical tests for bacterial isolates

Isolates no	TSI			MIU							Probability
	Butt	Slant	H_2S	Gas	Citrate	Motility	Indole	Urease	MR	VP	
1,6,15,16,18,19,21,24,26,30,32,34, 39,42,43,44,45,48,51,53,54,59	A	A	-	+	+	-	-	+	+	-	*Klebsiella* spp.
2,8,9,11,12,13,14,20,33,36,40,46,47,50,58 ,60	A	A	-	+	+	+	-	-	-	+	*Enterobacter* spp.
3,4,5,7,10,17,22,25,27,37,41,49,55,56	A	K	-	-	-	-	-	-	+	-	*Shigella* spp.
23	A	K	-	+	+	+	-	+	+	-	*Citrobacter* spp.
28,29,31,35,38,52,57	A	K	-	+	-	+	+	-	+	-	*E. coli*

Legend: A=Acidic, K=Alkaline, MR=Methyle red, VP=Voges-Proskaur +=Positive, -=Negative

The highest coliform bacterial count was 2.5×10^7 CFU/ml. The isolation of coliforms from raw milk sample might be related to the closeness of udder to the anus of the animal since they are normal flora of the intestine and there is tendency of udder and the teat to be contaminated by the animal feces when the animal lie down on it (Edward *et al*., 2013). The lowest coliform count was 2×10^5 CFU/ml might be due to relatively better hygienic condition but still was not satisfactory. The identification of coliform bacteria, such as *E. coli*, in raw milk is a common indicator of fecal contamination. Their presence in raw milk normally associated with fecal contamination of water sources or poor hygiene practices during milking process. Irregular bathing of animal, feeding of animal in low land, muddy cow yard, unsanitary milking utensil and contamination of body surface by feces could also act as critical factors. Higher prevalence of *E. coli* was reported by many authors. In Malaysia Yuen *et al.* (2012) found the presence of *E. coli* in 47% of raw milk samples. In India Pant *et al*. (2013) found *E. coli* in 100% raw milk samples. Lower coliform count than this study was found in Muhammad *et al.* (2009) and in Uddin *et al.* (2011). *E. coli* normal flora is supposed to be harmless. But some pathogenic strains of *E. coli* can cause gastroenteritis, urinary tract infection as well as diarrhoea in infants. Although this study found

Coagulase-positive *Staphylococcus* spp. may cause human disease by the production of toxins. The formation of effective level of toxin needs a high number of microorganisms (approximately 10^5-10^6 microbes per ml of food) (IDF, 1994). In this experiment, *Staphylococcus* spp. were found in all raw milk samples The highest staphylococcal count was $3x10^7$ CFU/ml which could result from poor hygienic condition of farms of study areas, presence of subclinical mastitis or contamination from respiratory system and body surface. The lowest staphylococcal count was $1.2x10^5$ CFU/ml in this study. This finding has a close proximity with the finding of Uddin *et al*. (2011). On the other hand, Yuen *et al*. (2012) found lower count than this study in a survey hold at Malaysia. These phenomenon might due to improper milking practices among the dairy farmers. So, the samples in this research were not quite acceptable and safe to the consumers since its lowest count exceeds the standard limit. It also indicates that these samples were not prepared under good sanitation practices and were not stored in appropriate storage conditions.

Microbial characterization of randomly selected 60 isolates were performed by biochemical tests and the test results were revealed as *Klebsiella* spp., *Enterobacter* spp., *Shigella* spp., *Staphylococcus* spp., *Escherichia coli* and *Citrobacter* spp. (Table 2).

To find out antibiotic susceptibility, total 12 antibiotics were tested against 22 isolates of *Klebsiella* spp. and 7 isolates of *Escherichia coli*. The tested antibiotics were divided into mainly three groups (Table 3 and Table 4).

Table 3. Antibiotic resistance overview of *Escherichia coli* isolates

Isolates No.	Zone of inhibition (diameter in mm)											
	G-I (Cell wall synthesis inhibitor)			G-II (Protein synthesis inhibitor)					G-III (Nucleic acid synthesis inhibitor)			
	AML	CRO	IPM	CIP	S	TE	CN	E	NA	C	SXT	NOR
28	0	24	22	16	14	20	15	0	14	21	28	18
29	13	23	16	20	0	20	12	0	21	20	25	20
31	0	27	20	20	12	18	15	0	23	21	20	25
35	0	24	21	3	13	15	21	0	22	16	19	28
38	0	20	20	24	13	17	13	0	18	20	20	25
52	0	20	23	20	13	20	23	0	10	19	18	15
57	0	22	25	21	17	15	18	0	23	18	22	24

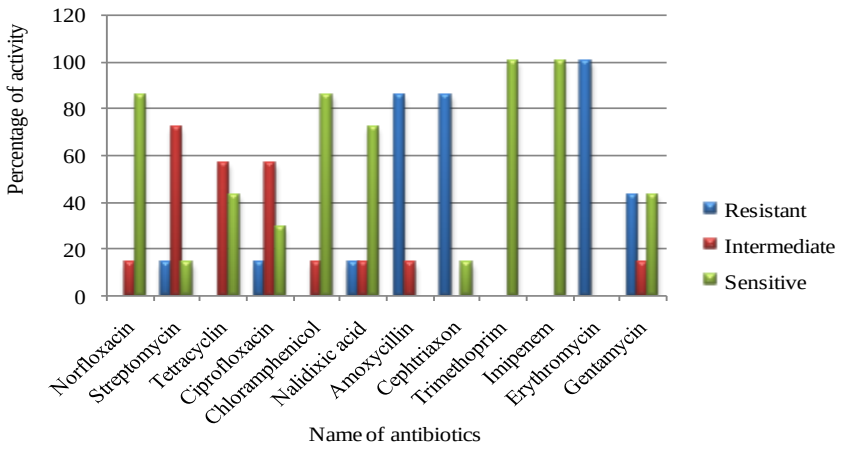

Fig. 1. Antibiotic resistance pattern of *Escherichia coli* against 12 commonly used antibiotics

In case of *Escherichia coli*, 100% isolates showed sensitivity against Trimethoprim-Sulphomethoxasole and Imipenem. It was followed by Norfloxacin and Chloramphenicol; more than 80% isolates showed sensitivity against these two antibiotics. 100% *E. coli* isolates were resistant against Erythromycin whereas 86% isolates showed resistance against Amoxycillin and Ceftriaxon. A previous study by Afroz *et al*. (2013) found *E. coli* isolates showing 88% resistance against Erythromycin, that is close to this present study. However, 60% isolates were found as intermediate against Streptomycin (Fig. 1).

Like *E. coli* isolates, *Klebsiella* spp. also showed sensitivity to a number of antibiotics (Ciprofloxacin, Tetracyclin, Norfloxacin, Imipenem, Nalidixic acid) (Fig. 2). Resistance also occured against other antibiotics like Erythromycin, Streptomycin, Chloramphenicol, Trimethoprim, Gentamycin. In a study Nipa *et al*. (2011) reported that *Klebsiella* spp. isolates showed resistance against Erythromycin, Gentamycin, Ampicillin, Chloramphenicol and Streptomycin.

Table 4. Antibiotic resistance overview of *Klebsiella* spp.

Isolates No.	Zone of inhibition (diameter in mm)											
	G-I (Cell wall synthesis inhibitor)			G-II (Protein synthesis inhibitor)					G-III (Nucleic acid synthesis inhibitor)			
	AML	CRO	IPM	CIP	S	TE	CN	E	A	C	SXT	NOR
1	0	18	25	20	15	15	17	0	19	21	18	22
6	0	0	35	30	11	0	20	0	7	10	0	28
15	0	19	24	25	15	16	18	0	19	23	19	25
16	0	21	21	23	14	15	21	0	22	20	21	25
18	13	0	21	26	20	15	23	7	15	24	0	24
19	15	25	36	25	21	14	15	0	20	21	21	15
21	0	25	23	15	12	17	14	0	15	24	25	16
24	0	28	20	18	16	19	17	0	16	22	28	17
26	0	0	0	25	0	21	0	0	22	20	25	13
30	0	26	23	28	12	18	15	0	20	22	23	26
32	0	22	24	30	12	15	14	0	19	24	0	24
34	20	17	34	26	22	17	26	23	11	24	22	26
42	0	25	35	23	13	13	24	0	15	12	23	27
43	15	23	25	29	16	15	20	0	25	25	21	25
44	0	20	30	20	14	15	15	0	21	22	20	24
45	30	23	40	27	22	25	28	15	15	20	0	27
39	0	25	24	30	19	18	20	0	21	26	25	29
51	15	18	35	25	20	20	26	26	14	25	22	29
54	0	26	25	21	19	18	26	0	21	22	23	22
53	0	18	30	30	17	19	20	0	20	28	13	23
48	12	24	21	23	22	16	20	0	25	22	22	30
59	20	23	25	26	16	17	20	10	20	27	21	30

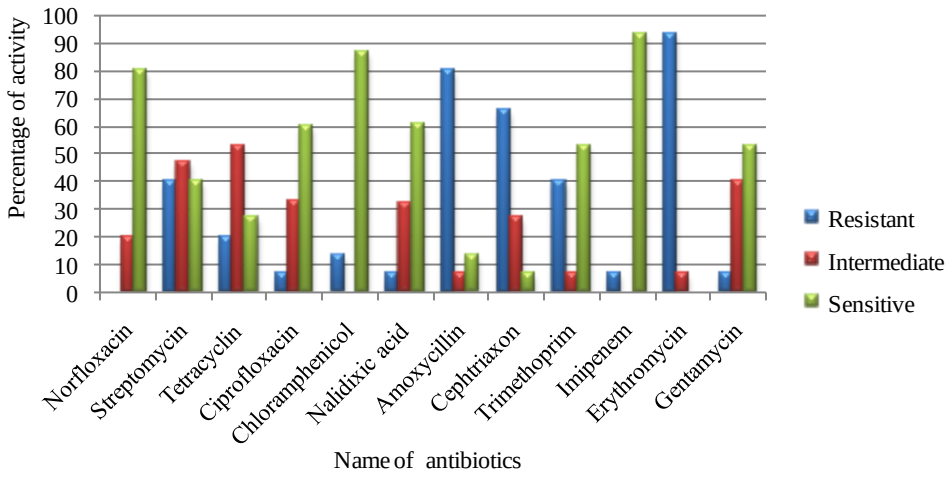

Fig. 2. Antibiotic resistance pattern of *Klebsiella* spp. against 12 commonly used antibiotics

Among 22 *Klebsiella* spp. isolates, 96% exhibited resistance against Erythromycin. However, same percentage of isolates showed sensitivity against Imipenem. Percentage of intermediate isolates remained within 55% for almost all the antibiotics tested. 80% *Klebsiella* spp. isolates were found to be resistant against Amoxycillin. This result finds similarity with Ahmed *et al.* (2014), who isolated *Klebsiella* spp., showing high resistence against Erythromycin (76.23%) and Amoxycillin (57.14%) in antibiotic susceptibility test.

Table 5 shows the antibiotic resistance pattern and multidrug resistance results in the tested isolates of *E. coli* and *Klebsiella* spp. It shows that all the isolates of *E. coli* and *Klebsiella* spp. were resistant to two or more of twelve antibiotics used here. Among 7 isolates of *E. coli*, all of them were multidrug resistant but showing their resistance patterns differently for different antibiotics (Table 5). In case of *Klebsiella* spp. all of the isolates were also found as MDR. We identified one isolate which was resistant for eight antibiotics (S-TET-E-C-NA-SXT-CRO-AML). However, results of this study differ from a previous study of Islam *et al*. (2010) who found only 3.12% isolates to be MDR but the findings of Nipa *et al.* (2011) who showed 98.06% isolates to be MDR, has a close proximity with present study. Prevalence of such a large amount of MDR bacteria in food samples is a hurdle to develop a healthy and safe living environment for human.

Table 5. Antibiotic resistance profile for *E. coli* and *Klebsiella* spp.

No. of resistant antibiotics	%		Combination of resistant antibiotics	%		Resistance classification
	E. coli	*Klebsiella* spp.		*E. coli*	*Klebsiella* spp.	
8	-	1	S-TET-E-C-NA-SXT-CRO-AML	-	4.55	MDR
6	-	2	S-IME-CN-CRO-E-AML	-	9.09	MDR
5	-	1	CIP-TET-C-E-AML	-	4.55	MDR
4	3	3	TET-CRO-C-AML	42.86	13.64	MDR
3	3	8	CRO-C-AML	42.86	36.36	MDR
2	1	7	E-AML	14.28	31.81	MDR
Total	7	22		100	100	

*MDR=Multi drug resistant

On the basis of data obtained from this study, a conclusion can be drawn that microbiological quality of most of the raw milk samples collected from different areas of Jessore city were not satisfactory as some pathogenic bacteria such as coliforms (*Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Shigella* spp., *Citrobacter* spp.) and *Staphylococcus* spp. were detected from the samples. The presence of several pathogenic bacteria along with high bacterial loads in some samples not only deteriorate the quality of raw milk but also pose safety issue to consumer. The presence of *Klebsiella* spp. in raw milk can lead to a wide range of disease states, notably pneumonia, urinary tract infections, septicemia and soft tissue infections. Pathogenic *E. coli* are occasionally responsible for product recalls due to contamination.

The major causes of microbial contamination of milk are due to milking from infected udders of the cows, unhygienic mechanical milking practices, unclean equipment or poor washing practices and improper storage conditions. Raw milk should properly be pasteurized, so that milk remains free from pathogenic microbes. Proper refrigeration temperature should also be maintained to avoid unwanted contamination.

Moreover, some of the isolated *E. coli* and *Klebsiella* spp. isolates were multidrug resistant. Their multidrug resistance pattern is a matter of great concern because these bacteria may no longer be treated with conventional therapeutic drugs and they are also capable of spreading their resistance gene to other bacterial genera. According to some recent research, the emergence of drug resistant microorganisms is one of the most serious health problems in modern society, particularly in Bangladesh (Ahmed *et al*., 2014 and Tabashsum *et al*., 2013). Frequent use of antibiotics as medicine and in food of animal has resulted an increase in prevalence of bacterial strains resistant to these antimicrobial agents (Hillier *et al*., 2002; Ahmed *et al*., 2014 and Tabashsum *et al*., 2013). Besides, post-milking contaminations with resistant bacteria from environment and food handlers also might play significant role. So, frequent use of antibiotics should be prohibited. In addition, proper training and hygiene practices during milking including post-milking process should be introduced to the dairy farmers; which could be effective to abolish the bacterial load or contamination of the raw milk.

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