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Isolation and Identification of Pathogenic *Escherichia coli*, *Klebsiella* spp. and *Staphylococcus* spp. in Raw Milk Samples Collected from Different Areas of Dhaka City, Bangladesh

Md. Aftab Uddin,[1] Hasan Md. Motazzim-ul-Haque,[1] Rashed Noor[1‡]

[1]*Department of Microbiology, Stamford University Bangladesh*

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The present study was undertaken with the aim of investigating the bacteriological quality of locally available raw milk. A total number of 22 raw milk samples were collected from Dhaka city and its surrounding areas during the period from October 2009 to November 2009. The analysis comprised enumeration of total viable bacterial count (TVBC), total coliform count (TCC) and total staphylococcal count (TSC) for the determination of sanitary quality. The highest TVBC, TCC and TSC were 2.36 x10^9 cfu/ml, 2.0 x 10^8 cfu/ml & 4.7 x10^7 cfu/ml, respectively. In order to observe the antibiotic-resistance pattern, the antibiogram assay was carried out. All *Escherichia coli* isolated from raw milk exhibited 100% resistance against Rifampin (R) and Tetracycline (TE) and 50% resistance against Nalidixic Acid (NA) but were 100% sensitive against Imipenem (IPM). *Klebsiella* spp. exhibited 100% sensitivity against Imipenem (IPM). *Staphylococcus* spp. isolated from raw milk exhibited 100% resistance against Rifampin (R) and Nalidixic Acid (NA), but were 100% sensitive to Gentamicin (CN) and Imipenem (IPM). This survey indicates that most of the raw milk samples were not satisfactory in course of public health standard as some pathogenic bacteria were detected from these samples. Frequent use of antibiotics should be prohibited since antibiotic resistant strains are continuously increasing.

Milk and milk products are ideal foods for all age groups in both rural and urban people all around the world. Milk is defined to be the lacteal secretion, practically free from colostrums, obtained by the complete milking of one or more healthy cows, 5 days after and 15 days before parturition, which contains not less than 8.5 percent milk solids and not less than 3.5 percent milk fat (1). Milk constituents include water (87.20%), protein (3.50%), fat (3.70%), milk sugar or lactose (4.90%), ash (0.70%) and dry matter (12.80%) (2).

Milk and milk products consist of high moisture, are nearly neutral in pH and are rich in vitamins. Hence, milk easily favors the growth and multiplication of many bacteria, even pasteurized or refrigerated. These bacteria may significantly influence the quality of the milk and milk products. Milk contains relatively few bacteria when it is secreted from the udder of a healthy animal. However, during milking operations, it gets contaminated from the exterior of the udder and the adjacent areas, dairy utensils, milking machines, the hands of the milking man, from the soil and dust. In these way bacteria, yeasts and molds get entry into the milk and thus constitute the normal flora of milk.

Milk might also be contaminated with pathogenic bacteria or bacterial toxins which may serve as vehicle for the transmission of diseases to humans such as salmonellosis, diarrhea, food poisoning, tuberculosis etc. Still other microorganisms are used in the preparation and preservation of milk products, such as yogurt, cheese etc (3).

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder (4). Beyond this stage of milk production, microbial contamination can generally occur from three main sources (5); from the udder, from the exterior of the udder and from the surface of milk handling and storage equipment. Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, feces and grass (6). The number and types of micro-organisms in milk immediately after milking are affected by factors such as animal and equipment cleanliness, season, feed and animal health (7). It is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk (6). Rinsing water for milking machine and milking equipment washing may also be responsible for the presence of high number of micro-organisms including pathogens in raw milk (5). Gram-negative bacteria usually account for more than 90% of the microbial population in cold raw milk that has been stored (8-11).

‡Corresponding Author. Mailing address: Dr. Rashed Noor, Department of Microbiology, Stamford University Bangladesh, 51, Siddeswari Road, Dhaka, Bangladesh. Phone: +88-02-8354577 (Ext-472), Fax: +88-02-9143531, Email: noor.rashed@yahoo.com .

The Gram-negative flora is composed mainly of psychrotrophic species of *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium*, *Flavobacterium* and *Enterobacter* (8 - 11). Presence of enterotoxigenic and antimicrobial resistant strains of *S. aureus* has become remarkably widespread in foods. This requires a better control of food contamination sources and distribution of antimicrobial resistant organisms (12). The quality and safety of milk and milk products are determined by the presence of indicator bacteria and other bacteria in lesser number. According to European Union Standards for Raw Milk Production, the bacterial count should be less than 10^5cfu/ml. So microbiological assessments have an important role to play in the dairy industry to protect the public health and can reduce economic losses by the early detection of inadequate processing, packaging or refrigeration. This study investigates the microbiological quality and safety of locally produced raw milk.

MATERIALS AND METHODS

Sample Collection. Twenty two raw milk samples were collected for microbial analysis from Dhaka city and its surrounding areas. The sampling period was from October 2009 to November 2009. Milk samples were collected in sterile container (bottles). About 100 ml of fresh raw milk were collected in a sample container using a sample collector ice box at 4 °C and were transported to the laboratory without delay.

Isolation of microorganisms from raw milk samples. Serial dilutions of samples were made up to 10^{-7} in sterile normal saline.

Enumeration of Microorganism. The bacterial count was performed by standard method (13). The microbiological condition of safety and hygiene were then assayed using the methods recommended by International Commission on Microbiological Specifications for Foods (ICMSF).

Total Viable Bacterial Count. The total viable 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 the solid nutrient medium and incubated at 37 °C for 24 hours. The plates were screened for the presence of discrete colonies after incubation period and the actual numbers of bacteria were estimated as colony forming unit in per ml (cfu/ml). Then the results per dilution were recorded. Quantitative analysis for the presence or absence of specific microorganisms was done by plating on selective media. Total coliform count (TCC) and total staphylococcal count (TSC) were done in the same way using MacConkey agar medium and Mannitol Salt Agar medium, respectively. All the viable counts were the average of at least three independent experiments. Bacterial isolates were then identified according to the Bergey's manual of determinative bacteriology (14), and manual for the identification of medical bacteria (15).

Antibiogram. Kirby-Bauer method (16) was used to examine bacterial susceptibility to antimicrobial agents. Shortly, after 16-18 h of incubation muller hinton agar plates (containing pure culture and antibiotic discs) were examined and the diameters of the zones of complete inhibition were measured in millimeter scale. The zone diameter for individual antimicrobial agents was then translated into susceptible, intermediate and resistant categories according to the interpretation table of the Becton Dickinson Microbiology Company, USA.

RESULTS AND DISCUSSION

Total Viable Bacterial Count (TVBC). The total viable bacterial count is the number of bacteria in a sample that can grow and form countable colonies on Nutrient agar after being held at 37 °C for 24 hours. The highest total viable bacterial count (2.36×10^9 cfu/ml) was found in sample no-10, collected from Uttara and lowest total bacterial count was 2.0×10^8 cfu/ml, which had been collected from Mohammadpur (Table 1).

The variation in TVBC of the milk may be due to the hygienic maintenance during milking. Aaku et al. (2004) and Arenas et al. (2004) reported on 5.5×10^6 cfu/ml and 10^6 to 10^7 cfu/ml of the total number of micro-organisms in pooled raw milk (17,18), respectively, which is lower than this experiment. Lee et al. (1996) conducted an experiment in Seoul of Korea and found that the bacterial count in raw milk ranged from 4×10^6 to 2.7×10^7 cfu/ml (19).

Total Coliform Count (TCC). The presence of coliform bacteria, such as *E. coli*, in milk is a common indicator of fecal contamination. *E. coli* was isolated from 14 milk samples out of 22 raw milk samples. The highest coliform bacterial count was found in sample no. 11, collected from Tongi (8×10^6 cfu/ml) and lowest total coliform count was 1.0×10^4 cfu/ml which was collected from Asulia (Table 1). Higher prevalence of *E. coli* was reported by many authors. In Egypt, Aly and Galal, (2002) showed the presence of *E. coli* in raw milk and the number reduced in the heat treated one (20). In India, the raw milk and products were heavily contaminated by *E. coli* (21).

Total Staphylococcal Count (TSC). Coagulase-positive staphylococci may cause human disease through the production of toxins. The formation of effective levels of toxin requires a high number of microorganisms (approximately 10^5–10^6 micro-organisms per ml of food) (22). In this experiment, staphylococci were found in 11 raw milk samples out of 22 tested samples. The highest staphylococcal count was found in sample no. 10, collected from Uttara (4.7×10^7 cfu/ml). On other hand no coliform and pathogenic staphylococci were found in five milk samples (samples no- 5, 14, 16, 18 and 22) which indicated good microbiological quality (Table 1). So the samples were acceptable and were safe to the consumers and these samples were prepared under good sanitation practices and stored in appropriate storage conditions.

Antibiogram of the Isolates. Antibiotic resistance has increased worldwide that leading to failures in treatment of human infectious diseases. Resistance against antibiotics by pathogenic bacteria is a major concern in the anti-infective therapy of both humans and animals. The Kirby-Bauer disk diffusion test was used in this experiment to determine whether the isolated organisms were susceptible or resistant to a selection of antimicrobial agents. Antibiotic resistance pattern of *E. coli*, has been shown in Fig 1. *E. coli* isolated from raw milk exhibited 100% resistance against Rifampin (R) and Tetracycline (TE), also 50% resistant to Nalidixic Acid (NA) but 100% sensitive against Imipenem (IPM).

TABLE 1. Total viable bacterial count (TVBC), total coliform count (TCC) and total staphylococcal (TSC) count in raw milk samples.

Number of samples	Areas of sample collection	Total viable bacterial count (TVBC) cfu/ml	Total coliform count (TCC) cfu/ml	Total staphylococcal count (TSC) cfu/ml
S-1	Gabtoli	5.2×10^8	2.0×10^6	2.0×10^6
S-2	Nikunja (1)	8.2×10^8	4.0×10^6	6.0×10^6
S-3	Farmgate	2.2×10^9	Nil	6.0×10^6
S-4	Mohammadpur	2.0×10^8	3.0×10^6	4.0×10^6
S-5	Banasree	9.6×10^8	Nil	Nil
S-6	Rampura	1.45×10^9	7.0×10^6	1.3×10^7
S-7	Mirpur	1.55×10^9	2.0×10^6	Nil
S-8	Nikunja (2)	1.6×10^9	4.0×10^6	Nil
S-9	Kuril	2.24×10^8	1.3×10^5	1.40×10^6
S-10	Uttara	2.36×10^9	2.0×10^5	4.70×10^7
S-11	Tongi	1.55×10^9	8.0×10^6	3.6×10^5
S-12	Mohakhali	7.3×10^8	Nil	4.0×10^6
S-13	Newmarket	9.4×10^8	1.0×10^5	Nil
S-14	Khilkhet	1.35×10^9	Nil	Nil
S-15	Asulia	1.27×10^9	1.0×10^4	Nil
S-16	Basabo	1.55×10^9	Nil	Nil
S-17	Khilgaon	7.6×10^8	2.0×10^5	Nil
S-18	Tejgaon	1.43×10^9	Nil	Nil
S-19	Dhanmondi	1.53×10^9	5.0×10^6	1.2×10^7
S-20	Badda	2.24×10^9	Nil	5.3×10^6
S-21	Santinagar	1.36×10^9	5.0×10^5	Nil
S-22	Gazipur	1.58×10^9	Nil	Nil

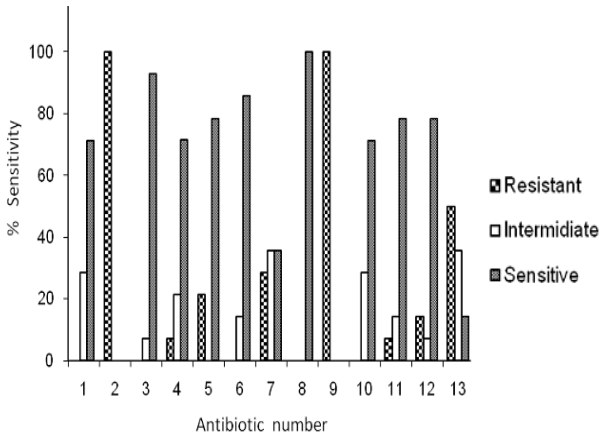

FIG. 1. Antibiotic sensitivity pattern of *E. coli*

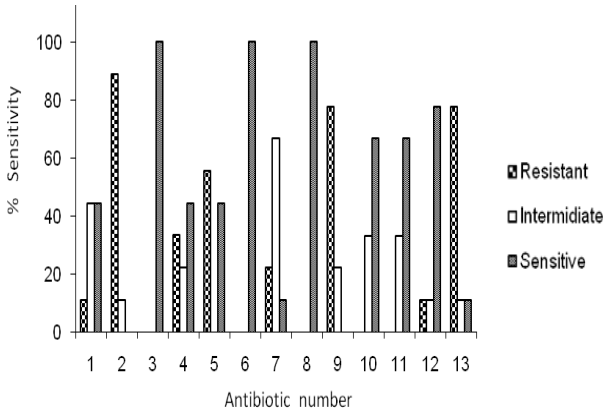

FIG. 2. Antibiotic sensitivity pattern of *Klebsiella* spp.

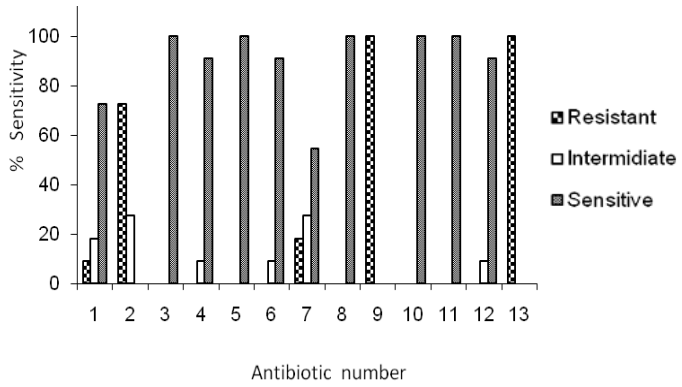

FIG. 3. Antibiotic sensitivity pattern of *Staphylococcus* spp.

Note: 1. Streptomycin (S), 2. Tetracycline (TE), 3. Gentamicin (CN), 4. Nitrofurantoin (F), 5. Chloramphenicol (C), 6. Amikacin (AK), 7. Niomycin (N), 8. Imipenem (IPM), 9. Rifampin (R), 10. Kanamycin (K), 11. Ciprofloxacin (CIP), 12.Trimethoprim-Sulphamethoxazole (SXT), 13. Nalidixic Acid (NA).

Susceptibility of isolated *E. coli*, *Klebsiella* and *Staphylococcus* spp. to different antimicrobial agents were measured *in vitro* by the Kirby-Bauer method. It allowed rapid determination of the efficacy of drug by measuring the zone of inhibition that result from diffusion of the antimicrobial agent into the medium surrounding the disc. Commercially available antimicrobial discs were used for the test.

Fig. 2 shows antibiogram profile of *Klebsiella* spp. These isolates exhibited 100% sensitivity against IPM and high resistance against TE, NA and R. *Staphylococcus* spp. isolated from raw milk exhibited 100% resistance against R and NA, but 100% sensitive to CN, C, IPM, K and CIP (Fig. 3).

CONCLUDING REMARKS

From the present study, it can be concluded that the microbiological quality of most of the raw milk samples collected from different areas of Dhaka city were not satisfactory as some pathogenic bacteria such as *E. coli*, *Klebsiella* spp. and *Staphylococcus* spp were detected from the samples. The presence of *S. aureus* and *Klebsiella* spp. will render milk unfit for human consumption, since sufficient number of these organisms will cause infection and intoxication. Multiplication and production of *S. aureus* would however, depend upon environmental factor like time, temperature, relative humidity and duration of storage and food factors, potential water activity (a_w), moisture contents, nutrients present, additives used and associated microflora like *S. aureus* and coliforms and fecal coliforms (23). Raw milk should be pasteurized, so that milk remains free from pathogenic microbes. Proper refrigeration temperature should be maintained. Frequent use of antibiotics should be stopped as antibiotic resistant strains are continuously increasing.

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