Microbiological analysis and determination of antibacterial activity of apple samples collected from local markets in Dhaka city, Bangladesh

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Present study attempted to detect the prevalence of contaminating microorganisms in 10 categories of local market apple samples commonly consumed in Dhaka city, Bangladesh. A significant number of total viable bacteria were quantified up to 10^6 cfu/g in these samples. *Staphylococcus* spp. was the predominant isolate within a range of 1.0×10^4– 3.2×10^6cfu/g whereas *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Shigella* spp., *Pseudomonas* spp. and *Vibrio* spp. were also quantified. Fungal contamination (within the range of 10^5 cfu/g – 10^7cfu/g) was also observed. Alarmingly most of the bacterial isolates present in the samples were proved to be multi-drug resistant. Antibacterial activity of the samples was tested against some common bacterial isolates but was found to be ineffective.

Apples are a well-off source of different sorts of phytochemicals including flavonoids (e.g., catechins, flavanols etc) with other phenolic compounds like epicatechin- procyanidins available in the skin, core and pulp of different types of apple. Current research is trying to investigate whether nutrients and/or phytochemicals in apples may impinge on the risk of some types of cancers. Apples become readily contaminated at different stages during its production such as growing, harvesting, processing, storing, shipping or preparing (1-3). Presence of bacterial isolates possess a potential threat of food borne illness to consumers. Pathogens such as *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Aeromonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Vibrio* spp. and *Pseudomonas* are responsible for various disease, morbidity and mortality. *Salmonella* spp. alone is responsible for 1.3 billion cases of salmonellosis and 3 million deaths (4, 5).

One of the most important health related concern on fruit consumption lies on the occurrence of drug resistant microorganisms which is known to broadly hamper the infectious disease medication that is mainly based on the use of antibiotics (6-11). In recent years, many antibiotics have lost their efficiency due to development of resistant strains (12) and through the resistance genes expression and also due to natural mutations within the microbial populations (13).

This situation has lead to the development of many adverse effects of antibiotics including hypersensitivity, immune-suppression and allergic reactions (14) and increasing interest in mounting alternative antimicrobial drugs which are highly effective and non toxic, derived entirely from natural sources (15). Ultimately, scientists are gaining interest towards natural products that have been the scientific basis of remedial effects (16).

According to the World Health Organization, about 80% of the world's population still depends entirely on plants for their medication. In both developing and developed nations medicinal plants are the main source of drugs. Beside plants, fruits also have been studied by the researchers for the detection of many active phytochemicals such as polyhenols, anthocyanins, flavonoids, terpenoids, carotenoids, cumarins, saponin and vitamins that are richly present in fruits (17).

Based on these facts and considerations, present study aimed to determine and enumerate microorganisms causing the spoilage of apples with their drug-resistance traits and to assess the anti-bacterial activity of the samples studied.

MATERIALS AND METHODS

Selection of study area & collection of fruit samples for investigation. Samples were collected from some popular super shops from five areas of Dhaka city such as Rampura, Khilgao, Shantinagar, Moghbazar and Mailbagh. Samples were collected in different time intervals and transported to the laboratory as soon as possible according to the method suggested by American Public Health Association (18). For the identification and enumeration of pathogenic bacteria and fungi, at first 10 g of each sample was taken, then blended with 90 ml normal saline (pH 7.8) and diluted up to 10^{-4} and then dilutions were used for plating purposes according to the standard guideline (19).

Isolation and enumeration of spoiling microorganisms. The primary inoculation was performed by the conventional culture technique with the addition

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of 0.1 ml of each sample onto nutrient agar (NA) and Sabouraud's Dextrose Agar (SDA) for the determination total viable bacterial count (TVBC) and total fungal load respectively, following the spread plate technique (19). The plates were incubated at 37 $^\circ$C for 24 hours and at 25 $^\circ$C for 48 hours for TVBC and fungal load respectively. From the dilutions 10^{-3} and 10^{-5}, the sample (0.1 ml) was spread onto MacConkey agar for the enumeration of coliforms (especially, *Escherichia coli* and *Klebsiella* spp.) and fecal coliform, respectively. Afterward, the MacConkey agar plates were incubated at 37 $^\circ$C for 24 hours. An aliquot of 0.1 ml of diluted sample was spread onto Mannitol Salt Agar (MSA) and *Pseudomonas* agar for the isolation of *Staphylococcus* spp. And *Pseudomonas* spp. respectively and incubated at 37 $^\circ$C for 24 hours. For the enumeration of *Listeria* spp., 0.1 ml of suspension was spread onto the *Listeria* identification media and plates were incubated at 37 $^\circ$C for 24 hours. *Listeria* spp. was identified as blue-green colonies on *Listeria* identification agar media with a further confirmation by biochemical tests (20).

Enrichment for enumeration of *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. Prior to quantify the relatively stressed cells or the viable but non-culturable (VBNC) microbial cells, 1 ml of sample was transferred into 9 ml of selenite cysteine broth (SCB) and alkaline peptone water (APW) for the enrichment of *Salmonella, Shigella*, and *Vibrio* spp. respectively and incubated at 37 $^\circ$C for 6 hours (8, 21, 22). After incubation, the samples were diluted up to 10^{-5} and then 0.1 ml of samples from 10^{-3} and 10^{-5} dilutions were spread onto *Salmonella-Shigella* (SS) agar and Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar for the isolation of *Salmonella and Shigella* spp, *and Vibrio* spp. respectively. Plates were incubated at 37 $^\circ$C for 48 hours for the detection of typical colonies. Finally, all the isolates were biochemically examined following standard procedures (8, 19, 23)

Antibiotic susceptibility test. All the bacterial isolates were examined for antibiotic susceptibility traits (either drug resistant or sensitive) against 16 antibacterial drugs (including first, second and third generation drugs) by disc diffusion assay on Mueller-Hinton agar (Difco, Detroit, MI) against commonly used antibiotics following the standard protocol (24, 25). Antibiotic discs included trimethoprime/sulfamethoxazole (25 µg), erythromycin (15 µg), amoxicillin (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), streptomycin (10 µg), ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), cefixime (5 µg), polymixin b (300 units), kanamycine (30 µg), vancomycine (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), azythromycine (15 µg) and penicillin G (10 µg).

Determination of antibacterial activity of apple samples. Antibacterial activity was determined by using the agar well diffusion methods. (26, 27). Normal saline suspensions of test organisms (*Pseudomonas* spp., *Listeria* spp., *Aeromonas* spp., *Vibrio* spp., *Salmonella* spp., *Klebsiella* spp., *Staphylococcus aureus*, *E. coli*) consisting of 10^6 cells/mL (compared with McFarland standard) were introduced on separate Muller- Hinton agar (MHA) and lawns were prepared. After drying, sterile cork borers were used to create 8 mm^3 wells. A volume of 100 µL of each sample (with a concentration of 11µg/µL) was poured into separate wells, dried and then incubated at 37 $^\circ$C for 12-18 hours. Normal saline and Chloramphenicol (10 µg) were used as positive and negative control, respectively. Presence of clear zone around the sample solution (if any) indicated the presence of antibacterial activity.

RESULTS AND DISCUSSIONS

Fruits are mostly eaten raw. So they may lead to the outbreaks of human diseases causing the overall public health at a serious threat. Many health-related problems are associated with the propagation of etiological agents as well as their drug-resistance abilities. Based on these facts, the current study aims to find out the total viable bacterial count and pathogenic load of various types of apples consumed in Bangladesh, drug resistance properties of the suspected pathogens, and finally to determine the antibacterial activities of these apple samples.

Prevalence of pathogenic microorganisms. Hazard Analysis and Critical Control Point-Total Quality Management (HACCP-TQM) technical guidelines rates microbial quality for raw foods containing aerobic plate count of $<10^4$ cfu/g as "Good", 10^4-5×10^6 cfu/g as "Average", 5×10^6-5×10^7 cfu/g as "Poor" and $>5\times10^7$cfu/g as "Spoilt". Several previous studies showed that *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio* spp. and fungi were frequent in a variety of fruits and vegetables samples (14, 22). However, in this study, after enrichment *Vibrio* spp. was estimated within a range of $3.5x10^3$-$3.2x10^6$ cfu/g while *Salmonella* and *Shigella* spp. were found in between $2x10^4$-$5.6x10^6$ cfu/g and $1x10^4$-$2.8x10^6$ cfu/g, respectively (Table 1). Presence *E. coli*, *Salmonella* spp. and *Shigella* spp. which are often in connection with poor sanitary practices and they put a pointer to a potential risk of food borne illness to consumers (5). Surprisingly total viable bacterial load (TVBC) in all the samples observed in this study was almost same, near about 10^6 cfu/g whereas coliform, *Pseudomonas* and fungal count, all were within the range of 10^6 cfu/g. Coliforms are indicator organisms and counts of 10^4-10^6 cfu/g reported in this work are a cause for concern, since the fruits are usually consumed without further processing. *Staphylococcus* spp. was present within the range of 1×10^4-3.2×10^6 cfu/g. *Staphylococcus aureus* is common food contaminants from man and the environment, their presence in food however, need to be controlled because they have been reported as cause of major food borne illnesses (4). The presence of these pathogenic organisms revealed the possibility of spreading enteric diseases to the consumers. Fungus presence indicates the presence of various mycotoxins in the fruit samples that may pose severe threat to the human health. A study performed by Maria et al., (28) showed variable fungal growth in between 3.8×10^4-5.9×10^4 cfu/g. Overall, fungal growth of the apple samples in this study was 10^6 cfu/g in average (Table 1). The fungal isolates of apple fruits in another study *Aspergillus* spp; *Penicillium* spp, *Rhizopus*, *Mucor* spp are common environmental contaminants.

So, this study demonstrated the presence of different types of microorganisms with a high load in the apple samples. There are so many possibilities by which pathogens may come in contact with the fruits such as the crop land, organic fertilizers, irrigating water, packaging materials, transport vehicles etc. Besides, unhygienic personnel practicing and processing of the fruits and their storage in such a condition which favors microbial growth might also be responsible for such spoilage of fruits. The contaminating pathogens are the reason for various types of enteric diseases as well as serious intoxications in human health.

Antibiotic susceptibility patterns of pathogens found in apple samples. Drug resistance is a serious problem in these days that is becoming more and more threat for the global public health. The antibiogram study showed, although some of the isolates were susceptible towards some antibiotics, several other antibiotics were proved ineffective, indicating the risk of the emerging resistant isolates causing health hazards. Most of the isolates were

TABLE 1. Isolation and enumeration of microorganisms in the local market apple samples

Apple sample	Total viable bacteria (cfu/g)	Fungi (cfu/g)	*Escherichia. coli* (cfu/g)	*Klebsiella* spp. (cfu/g)	*Salmonella* spp. (cfu/g)	*Shigella* spp. (cfu/g)	*Staphylococcus spp* (cfu/g)	*Pseudomonas spp* (cfu/g)	*Vibrio spp* (cfu/g)
Golden delicious 1	3.8×10^5	2.2×10^5	6.0×10^5	0	2.8×10^6	1.6×10^4	1.08×10^6	1×10^5	9.2×10^5
Golden delicious 2	5.2×10^6	9×10^5	1.5×10^5	1.0×10^5	2.0×10^4	2.0×10^5	2.8×10^5	5×10^5	1×10^4
Red Delicious 1	4.4×10^5	3.6×10^6	4.2×10^5	0	3.8×10^5	2.8×10^6	5.4×10^5	6.0×10^6	5.2×10^5
Red Delicious 2	3.5×10^5	4.5×10^5	2×10^5	0	0	1×10^4	2.3×10^5	8×10^5	3.2×10^6
Honey Crisp 1	1.6×10^6	8.4×10^5	4.5×10^5	2.2×10^5	0	3×10^4	1.3×10^6	1.2×10^6	1.4×10^5
Honey Crisp 2	2.2×10^6	3.12×10^6	7.2×10^5	0	6.2×10^5	2.8×10^6	1×10^4	3.2×10^6	2.8×10^6
Fuji	4.7×10^6	4.4×10^6	1.6×10^6	0	4.9×10^6	0	3.2×10^6	6×10^4	4.2×10^6
Gala	3.8×10^5	5.4×10^7	1.8×10^5	0	3.7×10^5	0	4.1×10^4	4.2×10^5	3.5×10^3
Braeburn	3.1×10^6	3.1×10^6	8.2×10^5	0	5.6×10^6	0	2×10^4	4.2×10^6	8.6×10^5
Paula Red	3.5×10^5	9.8×10^5	2.2×10^5	3.5×10^5	0	1.2×10^5	6×10^4	9×10^5	1×10^4

TABLE 2. Antibiogram of the different bacterial isolates collected from apple samples.

Antibiotic	*E. coli*	*Klebsiella* spp.	*Pseudomonas* spp.	*Salmonella* spp.	*Staphylococcus* spp.	*Listeria* spp.	*Vibrio* spp.	*Bacillus* spp.
Gentamicin (CN) 10 μg	20mm (S)	17mm (S)	20mm (S)	19mm (S)	14mm (I)	17mm (S)	23mm (S)	17mm (S)
Ampicillin(AMP) 10 μg	13mm (R)	12mm (R)	11mm (R)	9mm (R)	13mm (R)	12mm (R)	34mm (S)	8mm (R)
Amoxicillin (AML) 10 μg	12mm (R)	26mm (S)	13mm (R)	15mm (I)	12mm (R)	11mm (R)	16mm (I)	9mm (R)
Chloramphenicol (C) 30 μg	26mm (S)	27mm (S)	30mm (S)	25mm (S)	19mm (S)	20mm (S)	20mm (S)	23mm (S)
Cefixime (CFM) 5 μg	12mm (R)	16mm (I)	13mm (R)	16mm (I)	14mm (R)	15mm (R)	17mm (I)	12mm (R)
Ciprofloxacin (CIP) 5 μg	21mm (S)	21mm (S)	28mm (S)	30mm (S)	27mm (S)	17mm (I)	27mm (S)	20mm (I)
Erythromycin (E) 15 μg	26mm (S)	11mm (R)	20mm (S)	20mm (S)	13mm (I)	18mm (S)	14mm (I)	25mm (S)
Imipenem (IPM)10 μg	31mm (S)	21mm (I)	24mm (S)	17mm (R)	40mm (S)	15mm (R)	17mm (R)	28mm (S)
Nalidixic acid (NA) 30 μg	11mm (R)	15mm (I)	25mm (S)	27mm (S)	14mm (I)	12mm (R)	13mm (R)	9mm (R)
Ceftazidime (CAZ) 30 μg	18mm (I)	17mm (R)	15mm (R)	17mm (I)	12mm (R)	14mm (R)	19mm (I)	18mm (I)
Cefuroxime (CXM) 30 μg	15mm (I)	16mm (I)	13mm (R)	14mm (R)	13mm (R)	12mm (R)	17mm (I)	9mm (R)
Vancomycin (VA) 30 μg	11mm (R)	9mm (R)	11mm (R)	14mm (I)	8mm (R)	12mm (I)	9mm (R)	13mm (I)

TABLE 2. Antibiogram of the different bacterial isolates collected from apple samples (continued)

Antibiotic	*E. coli*	*Klebsiella* spp.	*Pseudomonas* spp.	*Salmonella* spp.	*Staphylococcus* spp.	*Listeria* spp.	*Vibrio* spp.	*Bacillus* spp.
Penicillin (P) 10 µg	13mm (R)	12mm (R)	11mm (R)	9mm (R)	8mm (R)	12mm (R)	11mm (R)	8mm (R)
Oxacillin (OX) 1 µg	14mm (R)	12mm (R)	11mm (R)	9mm (R)	7mm (R)	12mm (R)	13mm (R)	7mm (R)
Tetracycline (TE) 30 µg	15mm (S)	15mm (S)	22mm (S)	25mm (S)	13mm (I)	9mm (R)	14mm (I)	12mm (I)
Azithromycin (AZM) 15 µg	17mm (S)	17mm (S)	11mm (R)	15mm (S)	18mm (S)	9mm (R)	16mm (S)	28mm (S)
Penicillin G (P G) 10 µg	15mm (R)	14mm (R)	13mm (R)	14mm (R)	13mm (R)	12mm (R)	11mm (R)	9mm (R)
Streptomycin (S) 10 µg	9mm (R)	8mm (R)	7mm (R)	8mm (R)	9mm (R)	15mm (S)	9mm (R)	14mm (S)
Ceftriaxone (CRO) 30 µg	19mm (R)	18mm (R)	17mm (R)	16mm (R)	15mm (R)	14mm (R)	20mm (I)	18mm (R)

R= Resistant; S= Sensitive; I= Intermediate

showed that *Staphylococcus* spp. were highly resistant against ampicillin, amoxicillin, penicillin. vancomycin, enoxairtfce, cefixime, oxacillin, ceftazidime, oximecefur and streptomycine. This result is quite similar with this study (Table 2). High resistance of *Vibrio* spp. against ampicillin, chloramphenicol, nalidixic aciderythromycin, ciprofloxacin, penicillin, streptomycin, vancomycin, cefixime and cefuroxime has been indicated by Acharjee et al. (8), whereas in this study variable susceptibility pattern were obtained against the different antibiotics for Salmonella and *Vibrio* isolates (Table 2). *Pseudomonas* spp and *Klebsiella* spp. were also found to be resistant against most of the antibiotics (Table 2).

Antibacterial activity of apple samples. Antimicrobial activity was determined by using the agar well diffusion methods as previously demonstrated (26) against eight common bacterial laboratory isolates (*Bacillus* spp., *Pseudomonas* spp., *Vibrio* spp., *Escherichia* coli, *Klebsiella* spp., *Staphylococcus* spp., *Listeria* spp. and *Salmonella* spp.). Previous study showed that, the apple skin portions were found to exhibit moderate anti-bacterial activity against *Pseudomonas* spp. and *Listeria* spp. with a relatively lower activity against *Klebsiella* spp.; while the flesh portions showed to moderately active against *Pseudomonas* spp. and *Staphylococcus* spp. and arelatively lower activity against *Listeria* spp. and *Aeromonas* spp. (29). In the current study no antibacterial activity was observed to be present in the experimented samples.

CONCLUSION

Overall, the findings of this study clearly indicate a complete bacteriological profile of local market apples, which is of public health significance. Further studies with some advanced molecular settings for the better detection of the pathogens as well as some good solvent extraction methods need to be established to use apple as a potential therapeutic agent which may reduce the use of conventional drugs for the remedy of various diseases.

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