Microbiological analysis for drug resistant pathogenic microorganisms with determination of the antibacterial properties found in *Fragaria* x *ananassa* (strawberry) samples

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Strawberry is a popular fruit item consumed by people all over the world for its exceptional flavor and aroma. This fruit is being used to prepare a wide range of food items. Strawberries when consumed by people without undergoing any processing, the condition may not be good enough. People may get sick after consuming raw strawberries. Our current study identified several pathogenic bacteria which are very known in causing health related problems. *E. coli* was found in one sample (1.0×10^2 cfu/g), *Staphylococcus aureus* was commonly present in all of the five samples (up to 1.2×10^6 cfu/g), *Klebsiella* spp. was found to be present in two samples (within 1.0×10^3 cfu/g) and *Pseudomonas* spp. was present in three samples (8.2×10^4 cfu/g), respectively. All isolated bacteria showed some degree of drug resistant traits against some commonly used antibiotics. The most important and promising part of the study was to successfully find the antimicrobial activity of strawberries against seven types of pathogenic bacteria.

Fruits belong to one of the five major dietary classes of foods for getting proper and healthy nutrition. Fruits not only supply vitamins but also minerals which are essential dietary need for human. Some can cause food borne disease outbreaks if contaminated with pathogenic microbiota (1-4). Many fruits play some other roles in addition to giving us beneficial supplements. They can sometimes antagonize some pathogenic bacteria and fungi by several chemical agents like phenolic compounds, terpenoids, alkaloids etc. produced within them. Strawberry (*Fragaria* x *ananassa*) is a fruit popular for its distinct taste, aroma, color and flavor. Strawberries contain various minerals (iron, calcium, magnesium, phosphorus) and vitamins (K, E, C, A) (5-11). Beyond the beneficial activities, strawberries can also involve in causing disease if harbors adequate levels of pathogenic microorganisms. Microorganisms come in contact with strawberries in the farms through handling, soil quality, fertilizer quality, water quality, equipments used during harvesting, contact with wild life, during transportation and storage procedures (12-14). They also contain phenolic compounds among which allergic acid is produced approximately half of the total phenolics

(15, 16). It has been proved that the phenolic compounds produced by strawberries can inhibit *Escherichia coli, Salomonella* spp., *Campylobacter* spp., *Candida* spp. etc. (17-22). This is very important to get new therapeutics against these pathogens. Present study has been conducted on five strawberry samples for microbiological investigation as well as detecting antibacterial activity against some common pathogenic bacteria.

MATERIALS AND METHODS

Study area and sample processing. Five strawberry samples were collected from different super shop markets available in Dhaka city during the time span of January 2016 to February 2016. After aseptical collection of samples, they were transferred immediately to the laboratory for microbial analysis by following the procedures proposed by American Public Health Association. For both microbiological analysis and antimicrobial activity, strawberry samples were processed separately as skin and core. The skin and core separately were homogenized in normal saline followed by serial dilution up to 10^{-6} (23).

Microbiological analysis. 0.1 ml suspension of strawberry skin suspension and core suspension was introduced onto nutrient agar (NA) and Sabouraud dextrose agar (SDA) media for enumerating total viable bacteria (TVB) and Total Fungal Count (TFC), respectively (24). For identification and enumeration of specific pathogenic bacteria both skin and core suspension was added and spread onto MacConkey agar, mannitol salt agar (MSA) and cetrimide agar media which can detect *Escherichia coli*, *Staphylococcus* spp. and *Pseudomonas* spp., respectively. SDA plates were incubated at 25 °C for 48 to 72 hours. The other plates were incubated at 37 °C for 24 hours. *Eschericha coli* or *Klebsiella* spp. can be identified by distinguishable pink colony on MacConkey agar, *Staphylococcus* spp. by their yellow colony on MSA and *Pseudomonas* spp. by their greenish colored colony on cetrimide agar.

Analysis for the presence of VBNC bacteria. For detection of *Salmonella* spp. 01 ml homogenized sample of strawberry skin and strawberry core was inoculated into 9 ml selenite cysteine broth (SCB) for enrichment at 37°C for 6 hours. For detection of *Vibrio* spp., both samples from skin and core were inoculated into 9

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ml alkaline peptone water (APW) by following the same procedure as for *Salmonella* spp. After enrichment the SCB and APW media containing strawberry samples were serially diluted up to 10^{-6}. After that samples were inoculated onto *Salmonella-Shigella* (SS) agar and Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar and incubated at 37 ^{0}C for 24 hours. Black centered colonies on the SS agar is indicative for the presence of *Salmonella* spp. and the large, yellow colonies on the TCBS agar is indicative for the presence of *Vibrio* spp. Biochemical tests were performed to ensure the identity of the isolates (25).

Study of antibiotic resistance of the isolates. To determine the antibiotic susceptibility of the detected isolates from the strawberry samples, normal saline was subjected to add fresh isolates to get bacterial suspensions. After appropriate turbidity was attained, these suspensions were inoculated and spread over the Muller- Hinton agar plates using cotton swabs. Then some selected antibiotic discs were placed and incubated at 37 ^{o}C for 24 hours to get the zone of inhibition. Antibiotics used in this study of antibiotic resistance traits, are, Ampicillin (10µg), Amoxicillin (10µg), Nalidixic acid (30µg), Erythromycin (15µg), Chloramphenicol (10µg), Gentamicin (10µg), Kanamycin (30µg), Streptomycin (10 µg), Vancomycin (30µg), Ciprofloxacin (5µg), Ceftriaxone (30µg).

Determination of antibacterial activity of strawberry. To determine the antibacterial activity of the strawberry samples, agar well diffusion method was used. At first some selected bacterial isolate (*Pseudomonas* spp, *Bacillus* spp, *Salmonella* spp, *E. coli*, *Staphylococcus* spp., *Listeria* spp., *Klebsiella* spp. and *Vibrio* spp.) were taken to make their suspensions in normal saline. Then the bacterial suspensions were introduced evenly onto Mueller-Hinton agar (MHA) media using cotton swab. Then holes were made using a cork borer on the MHA to introduce strawberry samples in it with a positive (antibiotic disc-Gentamicin -GEN, 10µg) and negative control (normal saline). After incubation at 37 ^{o}C for 24 hours, plates were observed for the presence of clear zone indicating the antibacterial activity (26, 27).

RESULTS AND DISCUSSIONS

Fruits harbor many different kinds of normal microbiota as well as some pathogenic microbes under several circumstances. While eating raw, we often consume fruits along with such pathogenic and drug resistant microorganisms if not washed properly (28, 29). Different types of infectious diseases can arise from consuming the contaminated fruits. Sometimes they can provide some degree of protection against few pathogenic bacteria (30-32).

During our current study we identified a number of pathogenic bacteria in the strawberry samples (Table 1). Total viable bacterial count was determined between a range of 4.8×10^5 to 2.9×10^9 cfu per gram of sample and total fungal count was found between the range of

1.3×10^3 to 8.8×10^5 cfu/g. The indicator bacteria *E. coli* (1.0×10^2 cfu/g) was found to be present only in one strawberry sample indicating a chance of fecal contamination. The most prominent bacteria in all samples were *Staphylococcus aureus* ranging from 1.1×10^3 cfu/g to 1.2×10^6 cfu/g. *Klebsiella* spp. and *Pseudomonas* spp. were found to be present in two and three samples respectively out of five strawberry samples. The VBNC bacteria *Salmonella* spp. and *Vibrio* spp. was totally absent in all samples (Table 1).

After such result we can state that all the strawberry samples harbored *Staphylococcus aureus* which results from improper and too much handling of them. The bacteria can cause different types of food borne disease both by causing infection and intoxication (29). The environmental condition during transport and storage also encouraged the bacteria to multiply rapidly. *E. coli* in one sample indicates fecal contamination which can be due to unhygienic handling, using raw and non-treated water during any of the processes from farm to fork (30-32). The total absence of the VBNC bacteria is a good side that health problems associated with VBNC bacteria would not arise in the consumers. So it is really very important to clean fruits with fresh and pure water properly before consumption.

In recent years drug resistance problem has become a burning issue in treating diseases (33). In our study we found the bacterial isolates are resistant to several popular antibiotics (Table 2). *Klebsiella* spp. is completely resistant against one antibiotic (Imipenem), *Pseudomonas* spp. against three antibiotics (Ampicillin, Ciprofloxacin and Vancomycin), *Staphylococcus* spp. against Ampicillin, Kanamycin, Vancomycin. *E. coli* was still somewhat susceptible to the antibiotics used in this study. Most effective antibiotics for *E. coli* were Streptomycin and Vancomycin (100%), for *Staphylococcus* spp. Streptomycin and Gentamycin, for *Klebsiella* spp. Gentamycin, Imipenem and Ampicillin. *Pseudomonas* spp. was susceptible (100%) towards most of the used

TABLE 1. Microbiological analysis of strawberry samples

	Sample	TVBC (cfu/g)	Fungi (cfu/g)	*E. coli* (cfu/g)	*Klebsiella* spp. (cfu/g)	*Pseudomonas* spp. (cfu/g)	*Staphylococcus* spp. (cfu/g)
S1	Surface	1.2×10^8	3.4×10^5	0	1.0×10^2	0	1.8×10^4
	Core	2.8×10^6	8.8×10^5	0	1.0×10^3	0	1.1×10^3
S2	Surface	6.0×10^8	7.0×10^3	1.0×10^2	2.5×10^2	8.2×10^4	1.1×10^4
	Core	4.8×10^5	1.9×10^3	0	0	0	0
S3	Surface	2.5×10^8	1.6×10^5	0	0	7.9×10^3	1.2×10^5
	Core	2.2×10^8	2.2×10^3	0	0	0	1.7×10^4
S4	Surface	2.9×10^9	1.7×10^5	0	0	3.1×10^4	3.3×10^5
	Core	1.2×10^8	9.6×10^4	0	0	0	0
S5	Surface	3.5×10^8	1.4×10^5	0	0	0	1.2×10^6
	Core	4.1×10^8	1.3×10^3	0	0	0	0

TVBC= Total viable bacterial count
Vibrio spp., *Salmonella* spp. and *Shigella* spp were absent in all samples

TABLE 2. Antibiotic susceptibility of the isolates

Isolates / Antibiotics	*Klebsiella* spp N = 6		*Pesudomonas* spp. N = 5		*Staphylococcus* spp. N = 8		*E. coli* N = 3	
	R	S	R	S	R	S	R	S
AMP (10µg)	0%	100%	100%	0%	100%	0%	25%	75%
CIP (5µg)	25%	75%	100%	0%	25%	75%	70%	30%
KAN (30µg)	25%	75%	0%	100%	100%	0%	25%	75%
CEF (30µg)	50%	50%	25%	75%	75%	25%	ND	ND
AMO (10µg)	50%	50%	75%	25%	75%	25%	50%	50%
IPM (30µg)	0%	100%	50%	50%	25%	75%	25%	75%
STR (10µg)	25%	75%	0%	100%	0%	100%	0%	100%
VAN (30µg)	100%	0%	100%	0%	100%	0%	0%	100%
GEN (10µg)	0%	100%	0%	100%	0%	100%	25%	75%
NALI (30µg)	50%	50%	0%	100%	75%	25%	75%	25%
ERY (15 µg)	50%	50%	0%	100%	75%	25%	50%	50%

AMP= Ampicillin, AMO= Amoxicillin, KAN= Kanamycin, CIP= Ciprofloxacin, CEF= Ceftriaxone, NALI= Nalidixic acid, IPM= Imipenem, ERY= Erythromycin, VAN= Vancomycin, GEN= Gentamicin, STR= Streptomycin
Sensitive= S; Registrant= R

TABLE 3. Antibacterial activity of strawberry samples

Sample	Zone of inhibition (mm) against test bacteria						
	E. coli	*Klebsiella* spp.	*Salmonella* spp.	*Vibrio* spp.	*Pseudomonas* spp.	*Staphylococcus* spp.	*Listeria* spp.
Sample 1	10	22	23	22	25	19	19
Sample 2	12	20	22	19	17	17	18
Sample 3	12	15	14	15	13	13	14
Sample 4	13	14	14	17	15	15	15
Sample 5	15	15	14	16	14	20	15

antibiotics (Kanamycin, Streptomycin, Gentamycin, Nalidixic acid, Erythromycin). The resistance patters have similarity with some previous studies (28).

Resistance of pathogenic bacteria is very critical phenomena in treating diseases caused by them (29, 34-36). This resistance can rapidly transfer to susceptible bacteria making the situation more worse. So it is badly needed to follow the rules and instructions during medical treatment procedures advised or prescribed by physicians.

Many fruits exhibit antimicrobial properties towards different pathogenic bacteria. Many researches have been conducted in this aspect (28, 30-32, 34-37). Our study continued such work with strawberries to determine antimicrobial activities. After experimentation we have got a good positive result (Table 3). The strawberry samples showed a clear zone of inhibition against seven different kinds of pathogenic bacterial isolates (*E. coli, Klebsiella* spp., *Salmonella* spp., *Vibrio* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Listeria* spp.). Minimum zone of inhibition was

10 mm against *E. Coli* and the maximum zone of inhibition was 25 mm against *Pseudomonas* spp. (Table 3). The strawberry suspension contains some chemical agents naturally which can inhibit the growth of these bacteria. So strawberries can be used to prepare natural medicinal agents to treat infections caused by these bacteria.

CONCLUSION

Strawberry as a raw fruit item can cause public health hazards if good agricultural practice, proper handling, appropriate storage conditions are not provided. Because such conditions favor the contamination and rapid multiplication of pathogenic bacteria which after consumption can cause serious health related problems. Nowadays the resistance properties of such bacteria have made the treatment procedures very difficult because they are not killed any more by some antibiotics which were able to inhibit them previously. But our study determined the antibacterial properties from the strawberries which

can be used as medicinal products to inhibit several bacterial pathogens.

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