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Original Article

Antibiotics and metals resistant coliform contents of fruit cocktail samples collected from Lahore

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Abstract
In this study, fruit cocktail samples from Lahore, were screened for coliforms' enumeration. Thirty one coliforms strains were isolated and 90%, 83% and 80% of them appeared resistant to vancomycin, oxacillin, and amoxicillin, respectively. Whereas 65% and 17% were found resistant to nalidixic acid and rifampicin, respectively. Out of these isolates 23% expressed resistance to Cr and 20 % to Cu, whereas all were susceptible to Hg and Ag. The zones of inhibition Hg and Ag were from 24 to 42mm and 12 to 22 mm, respectively. Anthropogenic and urban origin coliforms isolates could be traced due to their resistances against certain antibiotics and heavy metals resistance. The samples might have been contaminated with enteric pathogens which too had resistance to antibiotics.
Key words: *E.coli* in cut fruits, *Klebsiella* in cut fruits, *Salmonella* in cut fruits, *Shigella* in cut fruits

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INTRODUCTION

Fresh fruits and vegetables are essential components of human diet as there is ample evidence of health and nutritional benefits associated with them. Accordingly consumption of fresh or minimally processed fruits and vegetables has undergone a sharp increase (Olivas and Barbosa-Canovas, 2005). On the other hand less processed vegetables, sprouts and fruits have been reported for out breaks of infectious diseases in developing as well as developed countries (WHO, 1998; Burnett and Beuchat, 2001). Fruit cocktail locally called as 'fruit chat' comprising salted small pieces of banana, papaya, guava and apple with lemon juice are much popular among consumers in Pakistan as well as in many parts of India. "Fruit chat" is a nutritious midday meal or is served as an accompaniment to meals. Like all other street foods during the processing as well serving "fruit chats" are exposed to abundant contaminations and are involved in causing food borne illnesses to the consumers (Kumar *et al*., 2006). It is well known that minimally processed fresh cut fruits and vegetables provide a good substrate for microbial growth (Nguyen-The and Carlin, 1994). Such substrates may allow proliferation of human pathogenic organisms like enterohemorrhagic *E. coli* and *L. monocytogenes* (Christison *et al.,* 2008).

Animal manure is a major source of macro and micro nutrients and its usage as fertilizer without any treatment is a common practice (Madison *et al*. 1995). A well known source of food borne pathogenic bacteria is bovine manure (Zschock *et al.,* 2000; Wells *et al.,* 2001) its usage without destroying pathogens obviously enhances the likelihood of vegetables' contaminations cultivated in manure fertilized soils (Ingham *et al.,* 2004). It is believed that preharvest contamination generates significant public health risks (Beuchat, 2002; Wachtel *et al.,* 2002; Stine *et al.,* 2005).

Cross contamination of bacterial and viral pathogens at domestic level and in food service establishments is thought to be a major contributing factor for sporadic and epidemic food borne illness (Bloomfield and Scott, 1997; Guzewich and Ross, 1999). Above referred studies suffice to indicate microbial contamination of vegetables and fruits due to use of manure fertilizers and contaminated water for irrigation and washing purposes. Further unhygienic processing of raw produce especially of the cut-surfaced fruits and

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vegetables, contaminates the material with microbes of anthropogenic origins. “Fruit Chat” is a street vended popular food of almost all cities of Pakistan. During its processing boiled and fresh vegetables and fresh fruits are cut into small pieces, mixed with certain dairy products and sugars and sold up to several hours after their initial processing. Moreover, majority of the shops do not refrigerate the food after its preparation and due to high ambient temperature initial bacterial contamination multiply. This study was thus undertaken to assess the faecal contamination of some certain of the food sampled collected from different areas of Lahore. The information obtained in this study clearly demonstrate that the popular street food was heavily contaminated by the coliform bacteria resistant to certain antibiotics and heavy metals as well. Transferring of enteric pathogens and spread of the allied infections amongst the consumers can easily be speculated. Prompt and strict measures to control the objectionable microbial contents of such food is strongly recommended.

MATERIALS AND METHODS

Fruit cocktail samples were collected at morning and evening hours, from different areas of Lahore viz. University of the Punjab, Quaid-e-Azam Campus Bridge, Mughalpura, Main Bazar Anarkali, Johar Town, and Sabzazar Colony in sterile glass bottles. The morning samples were soon transported to the laboratory and screened for inoculating on Eosin Methylene Blue (EMB) agar. While, the evening collections were preserved overnight in refrigerator before processing.

Three dilutions for each sample were made by mixing 0.1ml of a sample slurry in 9.9ml of sterile water. Second and third dilutions of $1:10^4$ and $1:10^6$ were then prepared by making serial dilutions, similarly. The EMB agar (Oxoid) was suspended in water according to the manufacturer’s instructions and autoclaved for 15 min. at 151b/inch^2 and allowed to cool around 50^{o}C. About 20ml of it was then poured in each sterilized Petri plate and allowed to solidify in the plates. The prepared medium was clear and had a reddish brown appearance. On solidified EMB agar plates, 0.1ml of a given dilution was inoculated uniformly employing sterile bent glass rod while working within a laminar air-flow system. The plates were then incubated at 37^{o}C up to 24 hours and then observed for colonies’ appearance. Plates expressing 30 to 300 colonies were selected for enumerating colony forming units (CFU). Colony characteristics *viz.,* elevation, margin, consistency and opacity were determined by studying well separated colonies (Pelczar *et al*. 1986). For pure culturing a portion from a representative bacterial colony of a morphologically recogrnizable category was disposed on the surface of the medium EMB agar and streaked in quadrant manner. The plates were incubated for 24 hours at 37^{o}C in inverted position.

To ensure purity, a well separated colony from the culture was picked by a sterilized transferring loop from the selective agar plate and streaked on a nutrient agar (Oxoid) plate. Growth from the nutrient agar plates was restreaked on the selective agar plates. The pure culture thus obtained assured its purity (Black, 1996).

The pure cultures were then preserved by growing them on the nutrient agar slants following by immersion in presterilized paraffin oil, poured ½ inch above the slants’ upper margin. Overnight incubated nutrient broth grown bacterial cultures were processed for Gram’s staining, motility, oxidase and catalase tests as described by Benson (Benson, 1994). While the indole, methyle red, citrate and Voges proskaure tests were performed as described by Collins and Lyne (Collins *et al.,* 1995).

For heavy metal sensitivity tests, Whatman filter paper discs of 9mm diameter were autoclaved and then loaded with 5µl of 1 % aqueous solutions of $CuSO_4$, $K_2Cr_2O_7$, $AgNO_3$ and $HgCl_2$. Then discs were placed onto the surface of inoculated nutrient agar plates. After incubation at 37^{o}C for 24 hours, the plates were noticed for zones of growth inhibition/ retardation.

For antibiotics’ susceptibility pattern determination oxacillin (OX1), chloramphenicol (C30), nalidixic acid (NA30), rifampicin (RD30), vancomycin (VA30) and amoxicillin (AML 25) sensitivity discs (Oxoids) were placed on to freshly inoculated nutrient agar plates. After incubation at 37^{o}C for 24 hours the plates were observed for zones of growth inhibition/ retardation.

RESULTS AND DISCUSSION

Screening of the fruit chat samples on EMB agar plates revealed that they were heavily contaminated with coliform bacteria (Table I).

The coliforms' contaminations of the food samples were represented by *Klebsiella*, *Salmonella* and *Enterobacter* species. Sample 1 collected at morning and evening hours showed 1395 x 10^4 and 9.25 x 10^4 C.F.U./ml, respectively (Table I).Among the morning samples the isolate of *Salmonella sp*. was found resistant to the antibiotics vancomycin, oxacillin and amoxicillin. While from the evening samples *Klebsiella* isolate showed resistance to vancomycin, oxacillin and amoxycillin. *Enterobacter* sp. was found resistant to the concentration of Cr tested (Table III). For sample 2 *Salmonella* sp. was recovered from material sampled at evening (Table I).C.F.U /ml of the sample ranged from 0.2 x 10^4 to 0.4 x 10^4. The strain 2E1 of the species *Klebsiella* sp. was found resistant to the antibiotics vancomycin, oxacillin and amoxicillin. The growth was retarded in the presence of chromium (Table III).

Table I: Colony forming units (C.F.U) of the bacteria isolated on EMB agar from the "fruit chat" samples

Sample No (site of collection)	C.F.U X10^4/ ml Morning (Evening)	Isolate	Colony features	
			Diameter (Colour)	Elevation(Shape)
1 Punjab University	1395 (9.25)	*Salmonella* sp.-1M3	0.80 (pink)	Convex (round)
		Klebsiella sp.-1E1	1.75 (pink nucleated)	Convex (L-form)
		Enterobacter sp.-1E6	0.35 (pink nucleated)	Convex (L-form)
2 (Mughalpura)	0.2 (0.4)	*Klebsiella* sp.-2M2	0.5 (pink)	Convex (round)
		Salmonella sp.-2E1	3.0 (pink nucleated)	drop like (L-form)
3 (Anarkali)	38.7 (16.3)	*E.coli*-3M1	0.8 (green sheen)	drop like (round)
4 (Johar Town)	70 (352)	*E.coli*- 4M2	1.5 (green sheen)	drop like (round)
		Shigella sp.-4E1	1.0 (colorless)	Convex (round)
		Salmonella sp.- 4E2	1.3 (colorless)	drop like (round)
5 (Sabzazar Colony)	2.10 (1.0)	*Salmonella* sp.- 5M2	1.1 (colorless)	Raised (round)
		E.coli- 5E1	1.8 (green sheen)	Raised (L-form)

Table II: Characteristics[a] of the bacteria isolated on EMB agar from the "fruit chat" samples

Isolate	Physiochemical tests					
	Indole	Methyle Red	Citrate test	Oxidase test	Catalase test	Motility
Salmonella sp.-1M3	-ve	+ve	+ve	-ve	+ve	-ve
Klebsiella sp.-1E1	+ve	-ve	+ve	-ve	+ve	-ve
Enterobacter sp.-1E6	-ve	-ve	-ve	+ve	-ve	-ve
Klebsiella sp.-2M2	-ve	-ve	-ve	+ve	+ve	+ve
Salmonella sp.-2E1	-ve	-ve	+ve	-ve	+ve	-ve
E.coli-3M1	+ve	+ve	+ve	+ve	+ve	+ve
E.coli- 4M2	+ve	-ve	+ve	-ve	+ve	+ve
Shigella sp.-4E1	-ve	+ve	-ve	-ve	-ve	-ve
Salmonella sp.- 4E2	-ve	+ve	+ve	-ve	+e	+ve
Salmonella sp.- 5M2	-ve	+ve	+ve	+ve	+ve	+ve
E.coli- 5E1	-ve	+ve	-ve	-ve	+ve	+ve

a: All the isolates described in the table were found –ve for Gram's reaction and Voges Proskauer test.

Table III: The drug and heavy metal resistance of coliforms bacteria isolated from the fruit chat samples.

Coliform isolate	Antibiotics						Heavy metal (1%)			
	OX1	C30	NA30	RD30	VA30	AML25	$HgCl_2$	$K_2Cr_2O_7$	$AgNO_3$	$CuSO_4$
Salmonella-1M3	R	28	28	13	R	R	30	33	13	15
Klebsiella-1E1	R	26	25	10	R	R	31	31	13	14
Enterobacter-1E6	8	25	18	22	21	20	32	R	13	(19)
Klebsiella-2M2	8.4	27	22	18	20	16	28	16	14	(14)
Salmonella-2E1	R	27	22	11	R	R	30	(30)	13	11
E.coli type-3M1	R	26	24.6	R	R	8.8	26	R	14	11
E.coloi type-4M2	R	32	15	13	R	R	36	(32)	20	R
Shigella-4E1	12	32.4	30	26	25	30	(35)	R	18	17
Salmonella-4E2	R	20	19.5	17.5	18	R	30	(13)	16	11
Salmonella-5M2	R	R	16	13	R	R	29	(28)	12	R
E.coli-5E1	R	25	19.3	17(RD5)	22	R	29	14	18	11

Values represent diameter (mm) of zone of growth inhibition.
R= the isolate was found resistant for the application.

Morning and evening collections of sample 3 showed 38.7 x 10^4 and 6.3 x 10^4 C.F.U./ml, respectively. Three strains of *E.coli* and one of *Klebsiella* were isolated from the morning sample. One strain each of *Salmonella* and *Enterobacter* appeared from the evening sample (Table I). The strain E.*coli* was resistant to the antibiotics vancomycin, oxacillin and rifampicin as well as to the Cr (Table III).

Morning and evening collections of sample No.4 showed 70 x 10^4 and 352 x 10^4 C.F.U./ml, respectively. Five coliform isolates represented by *E*. *coli*, *Salmonella* and *Enterobacter* sp. were recovered from the morning, while *Klebsiella*. *Shigella* and *Salmonella* sp. were isolated from the evening samples (Table I). The *E.coli* showed resistance against the antibiotics vancomycin, oxacillin and amoxicillin. The *E.coli* isolate showed resistance to Cu while *Shigella* to Cr (Table III).

Morning and evening collections of sample 5 showed 2.1 x 10^4 and 1.0 x 10^4 C.F.U./ml, respectively. *E.coli* and *Salmonella* sp. were isolated from each of the morning and the evening samples (Table I). The *Salmonella* isolate showed resistance against vancomycin, oxacillin, amoxicillin and chloramphenicol. Whereas *E.coli* isolate was resistant to oxacillin and amoxicillin. The *Salmonella* isolate showed resistance to Cu (Table III). Three of the five samples indicated several folds higher coliform contents for the morning collections than those obtained at evening. It is known that bacterial content of such foods derives from initial levels of contamination, whereas cross-contamination during processing and handling of the produce nutritional, nature of the substrate in terms of supporting growth of the microorganisms concerned, incubation period and temperature exert great influences in this regard (Beuchat and Ryu, 1997; Nwachukwu *et al.,* 2008). Different components of the food are routinely prepared several hours before serving. Therefore, it may be speculated that keeping the product at relatively higher ambient temperature; which favors the microbial growth, vigorous traffic activities creating suspended atmospheric particulates and aerosols might have caused highest coliform content of three of the morning samples. C.F.U. for the fruit chat samples ranged from 0.2 x 10^4 to 1395 x 10^4 and 0.4 x 10^4 to 352 x 10^4/ml, for the morning and evening samples, respectively. While the remaining two samples depicted coliform titres at evening hours.

Different factors might be attributed to explain the differing results. For, instance more traffic at evening hours for these two localities might be traced as one of the causes of higher bacterial load. Food-borne disease outbreaks indicate importance of screening various vegetable products, especially the ones which are vulnerable to exposure of microbes and are used in raw form or to prepare products not

involving cooking or other means of reducing bacterial load. Increasing number of food borne diseases' outbreaks has been associated with consumption of fresh products (De Rover, 1998). In this country untreated domestic sewage effluents are used to irrigate vegetables in suburban areas. Consequently, such vegetables are heavily contaminated with coliform content. Qazi *et al.* (2006) described that samples of *Brassica oleracea*, a commonly used salad vegetable, collected from different locations within the city Lahore were heavily contaminated with coliform bacterial content. Leaves of the vegetable showed upto 80.1×10^5 C.F.U./cm^2. Haemolytic bacterial isolates as well as antibiotic resistant were not uncommon.

The total sixty eight strains of coliforms were represented by *Enterococcus, Bacillus, Bordetella, Enterobacter, Clostridium* and *Acetobacter* genera. Not only the fresh samples of above referred vegetable collected from the city were found contaminated by the coliform bacteria rather water of the canal passing through the city (Qazi *et al*., 2005), another popular street food locally called as Dahi Bhallay; a fresh and boiled vegetables cocktail (Qazi and Qureshi, 2002) and even suspended particulate matter of the city have been shown to harbor objectionable levels of cultivatable coliforms (Qazi, *et al.,* 2002). For irrigation with untreated wastewater laiden with domestic sewage represents an important source of pathogenic microbes present on vegetables. For example association has been linked between levels of coliform and incidence of *Salmonella* (Goyal *et al.,* 1977).

The food samples reported in this communication indicated presence of *E.coli, Klebsiella, Shigella, salmonella* and *Enterobacter sp.* As the popular food is consumed more or less routinely thus many enteric infections can be speculated to be prevailing in the population. These coliforms also showed resistance to the antibiotics oxacillin, chloramphenicol, nalidixic acid, rifampicin, vancomycin and amoxicillin. Presence of antibiotic resistant microbes in the food samples verified not only their anthropogenic source but also threw light that they might had come from infected persons who had taken these antimicrobials indiscriminately including low dose intake for longer period of time. Importance of educating public and farmers in particular about the risk involved in the use of contaminated water and untreated manure is established (Cordelia *et al.,* 2003). Measures to minimize the risk of microbial contamination at all points from the field to the table through good agricultural practices and good manufacturing practices would be the most effective strategy to assure that fresh produce is safe for human consumption (Blumenthal *et al.,* 2000). Considering coliform content of the sample worked out in this study, the probability of enteric pathogens warrants for health risks to the consumers.

Moreover, antibiotics resistance of the coliform describes their clinical relevance and is suggestive to speculate that many of the potential pathogens, traveling along this contaminated food, may be drug(s) resistant too. Heavy metals' resistance nature of the coliform may indicate contamination of the environment to which these microorganisms could be associated. Strict measure to prepare and store the produce are recommended to be taken seriously by local public health authorities for controlling the enteric infections spread by such contaminant foods.

Conclusively the fruit cocktail locally known as fruit chat evaluated during this study indicated objectionable levels of coliform contaminations. Resistance of the bacterial isolates to Cu and Cr and some commonly used antibiotics show their longer anthropogenic and industrial association. Higher C.F.U. of coliforms in majority of the samples reflected that the processing and handling of the food is below the desired level of hygienic standards. As enteric infectious diseases including hepatitis are very common in this country, it is thus recommended that public higher authorities should take strict and prompt actions for controlling the routes of such food borne infectious diseases.

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