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A MICROBIOLOGICAL INVESTIGATION ON MILK BASED SWEETS WITH SPECIAL REFERENCE TO *Escherichia coli*

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ABSTRACT

The present study was undertaken to assess the microbial load of milk made sweets sold by twelve sweet shops of Chandigarh. Some commonly consumed sweets (Plain Burfi, Milk Cake, Kala Kand, Gulab Jamun, Chaina Murgi, Gajar Burfi, Khoya Burfi) were tested and E.coli were isolated from them. The total bacterial count and gram negative count ranged between 10.1x104-6.7x105 and 4.4x103 – 3.7x105 for Plain Burfi, 5.8x103-3.0x105 and 3.9x102-6.8x104 for Milk Cake, 4.5x104-8.9x106 and 4.1x103-9.1x105 for Kala Kand, 3.4x104-9.2x105 and 1.7x103-2.9x105 for Gulab Jamun, 3.0x103-2.9x105 and 1.7x102-8.9x104 for Chaina Murgi, 1.5x104-3.3x106 and 5.1x103-9.4x105 for Gajar Burfi and 1.5x103-5.1x104 and 2.1x102-17.7x103 for Khoya Burfi respectively. Khoya Burfi showed the lowest counts while Gajar Burfi samples exhibited the highest counts. E.coli was isolated in 89.3% of all the samples screened. The highest percent isolation of E.coli from sweet samples was in Plain Burfi (100%), Kala Kand (100%) Gulab Jamun (100%), Gajar Burfi (100%) and least in Khoya Burfi (66.7%). Salt aggregation test (SAT) for hydrophobicity revealed that 97.33% strains were hydrophobic, indicating a high level of hydrophobicity. Maximum antibiotic sensitivity was noticed towards Chloramphenicol (82.67%) and minimum towds Ampicillin (25.33%). Multidrug resistance was shown by 35 (46.67%) strains. This organism is significant from public health point of view as it has been associated with the onset of food poisoning in human beings. However a large volume of these products are produced in unorganized sector, unbranded, with little precautions of food safety and quality. This investigation is a factual documentation of such a finding and suggestions as to the methods needed to improve the safety and quality.

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KEY WORDS:
***E.coli*, Total Bacterial Count, Gram Negative Count, Milk Made Sweets, Public Health**

INTRODUCTION

Food is a major source of hazards to human health and food-borne disease is globally the single most common illness. Deeply concerned by this, the Fifty-third World Health Assembly adopted a resolution calling upon the World Health Organization (WHO) and its Member States to recognize food safety as an essential public health function (WHO 2002). In India, it is estimated that 20% of deaths among children under five are caused by diarrheal disease (WHO 2006), 70% of these being associated with unsafe food or water (Unnevehr and Hirschhorn 2000). So, to combat these food borne disease, ensuring that food is microbiologically safe, is an essential element of public health.

Due to complex biochemical composition and high water activity milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms. Therefore in the processing of milk, some of them may produce undesirable effects and some micro-organisms produce food infections which can either carry the pathogens that will increase the likelihood of infection of the consumer`s food (Soomro et al. 2002). Milk is supposed to constitute a complex ecosystem for various microorganisms including bacteria. Milk products like cheese and curd are widely consumed and market for them has existed in many parts of the world for many generations. There is an increase demand by the consumer for high quality natural food, free from artificial preservatives, and contaminating microorganisms. Contamination of milk and milk products, with pathogenic bacteria is largely due to processing, handling, and unhygienic conditions (Singh and Prakash 2008).

The contamination of milk and milk products is largely due to human factor and unhygienic conditions. Usually milk is contaminated with different kinds of microorganisms at milk collecting places. Milk is a major part of human food and plays a prominent role in the Punjabi diet. Approximately 50 percent of the milk produced, is consumed as fresh or boiled, one sixth as yoghurt or curd and remaining is utilized for manufacturing of indigenous varieties of milk products and milk made sweets (Randhawa and Chahal 2008).

The manufacture of these products is based on traditional method without any regard to the quality of raw material used and/ or the hygienic quality of the products. Under such conditions many microorganisms can find access to the milk products. Among all micro-organisms Escherichia coli is frequently contaminating organism, and is reliable indicator of fecal pollution generally in insanitary conditions of water, food, milk and other dairy products (Soomro et al. 2002; Benkerroum et al. 2004). Presence of E. coli in milk products indicates the presence of enteropathogenic

microorganisms, which constitute a public health hazard. Enteropathogenic E. coli can cause severe diarrhoea and vomiting in infants, and young children (Singh and Prakash 2008). In 2011, Europe faced outbreak of food poisoning in which 18 people were killed and more than 2000 were infected with Enteropathogenic E.coli (Ropeik 2011). E.coli was isolated from milk products like Mawa/Khoa, Dahi, cheese and Gulabjaman (Soomro et al. 2002; Singh and Prakash 2008). So, keeping in mind the above facts the present study was designed to determine total bacteria count, gram negative bacteria count and to isolate the E. coli from milk made sweets sold under market conditions at Chandigarh.

MATERIAL AND METHODS

SELECTION OF SAMPLES

Twelve sweets shops were surveyed to determine the hygiene standards maintained in their shops. Samples of commonly consumed milk made sweets collected from sweet shops of Chandigarh, selected by random sampling method. All the samples were immediately brought to the laboratory in sterile tubes for further processing and analysis.Various milk made sweets sampled for analysis for were: Plain Burfi, Milk Cake, Kala Kand, Gulab Jamun, Chaina Murgi, Gajar Burfi, Khoya Burfi.

CHEMICALS AND MEDIA

Media used for cultivating microorganism and biochemical tests were bought from Hi-media laboratory in the dehydrated form which were reconstituted as per given instructions.

MICROBIOLOGICAL ANALYSIS

One gram of the sample was weighed under aseptic conditions near the flame, homogenized in a sterile pestle and mortar and added to 9 ml of the sterile normal saline blank. Various dilutions of these were used for determination of viable count by standard plate count method using nutrient agar for determination of total bacterial count and Mac Conkey`s agar for the gram –negative count. The desired dilutions from samples in saline were poured in petri dishes and swirled with melted media. After solidification, the plates were incubated in an inverted position at 37°C for 36-48 hours. The numbers of colonies were counted & the final count or colony forming unit/ml (CFU/ml) was determined by multiplying the number of colonies in the plate by dilution factors.

ISOLATION OF E.COLI

The swabs, food samples and water samples were streaked on Mac Conkey`s agar and the non mucoid lactose fermenting colonies were picked up and culture purified by the plate streak method on EMB agar.

IDENTIFICATION OF ISOLATE

The purified cultures were identified on the basis of colony morphology, gram staining and specific bio chemicals tests. Classification of bacteria was done based on Bergey's Manual of Systematic Bacteriology 8th edition (Orskov 1984).

BIOCHEMICAL TESTS FOR IDENTIFICATION OF E.COLI

INDOLE PRODUCTION

This test demonstrates the ability of bacteria to decompose the amino acid tryptophan to indole. The organisms were first grown in peptone acid water medium taken in small tubes, which were incubated at 37oC over night. To these tubes 0.5 ml of kovac's reagent was added and the tubes shaken gently. A positive reaction was indicated by development of red after a minute (Dash et al. 2012).

METHYL RED TEST

The isolates were grown in tubes containing glucose phosphate peptone water. Few drops of methyl red indicator were added to each tube and the result read immediately after mixing. A positive while a yellow colour indicated a negative reaction (Dash et al. 2012).

VOGES-PROSKAUR TEST

The ability of organisms to produce acetyle methyl carbinol or its reduced product 2, 3 butylene glycol was tested by growing the organisms in glucose phosphate peptone water at 37oC for 24 hours. Then, 0.6 ml alpha napthol solution was added to culture tubes followed by 0.2 ml of 40% aqueous KOH. The tubes were shaken and allowed to stand for a few minutes. A positive reaction was indicated by the development of a pink colour within 5 minutes, which later changed to crimson red after a lapse of 20 minutes (Dash et al. 2012).

CITRATE UTILIZATION TEST

The ability of organisms to utilize citrate as the sole carbon and energy source for growth was tested by using Kosser's liquid citrate medium. The medium prepared as slants in small tube was stabbed with the organism and incubated at 37oC for 24 hours. A positive reaction was represented by a change in the colour of the medium from green to blue (Dash et al. 2012).

Surface hydrophobicity
Salt aggregation test (SAT) was used to assess the relative surface hydrophobicity of E.coli isolates. Bacterial cell suspensions were mixed with equal volumes of ammonium sulfate of various molarities (0.02–3.2 mol/l). The lowest concentration of ammonium sulfate that produced visible aggregation was scored as the SAT hydrophobicity value (Ljungh and Wadstrom 1982). The aggregation in saline solution used as a control was regarded as autoaggregation.

Antibiotic sensitivity profile of E.coli isolates
E. coli was assessed for their sensitivity against different antibiotics viz., Ampicillin (35 mcg), Gentamicine (30 mcg), Erythromycin (15 mcg), Chloramphenicol (30 mcg) and Nalidixic acid (30 mcg) as reported previously (Bauer et al. 1966).

A loopful of the test organism was transferred to 3 ml sterile peptone water tubes, which were incubated for 4 hours at 37oC. 0.1 ml of growth was spread on a nutrient agar plate. The antibiotic discs were incubated at 37oC for 24 hours. Distinct zones of inhibition of growth were observed around respective discs indicating the susceptibility of the organism to the same (Bauer et al. 1966).

RESULTS AND DISCUSSION

The present research findings pertain to the determination of total bacteria count, gram negative bacteria count and isolation of E. coli in commonly consumed seven milk made sweets sold under market conditions. A total of 84 samples, twelve samples of each sweet from different shops were collected randomly and analyzed microbiologically for bacterial count and isolation of E. coli.

TOTAL BACTERIAL AND GRAM NEGATIVE BACTERIAL LOAD

As shown in Table 1, the total bacterial counts of Plain Burfi samples ranged between 10.1x104-6.7x105 CFU/gm and the gram negative counts ranged between 4.4x103-3.7x105CFU/gm. The total bacterial counts of Milk Cake ranged between 5.8x103-3.0x105 CFU/gm and the gram negative counts ranged between 3.9x102-6.8x104 CFU/gm. The values for total counts in Kala kand were between 4.5 x 104-8.9x106 CFU/gm where as the gram negative counts were between 4.1x103-89.1x105 CFU/gm. The total counts observed in Gulab Jamun ranged between 3.4x104-9.2x105 CFU/gm and the gram negative counts ranged between 1.70x103-2.9x105 CFU/gm. Total bacterial counts in Chaina Murgi ranged between 3.0x103-2.9x105 CFU/gm where as the gram negative counts ranged between 1.7x102-8.9x104 CFU/gm. In Gajar Burfi, the total bacterial counts ranged between 1.5x104-3.3x106 CFU/gm and the gram negative counts ranged between 5.1x103-9.4x105 CFU/gm. The total bacterial counts in Khoya Burfi ranged between 1.5x103-5.1x104 CFU/gm where as the gram negative counts ranged between 2.1x102-17.7x103 CFU/gm. Similarly, Umoh et al. (1990) revealed that the mean Staphylococcus count for the fermented milk products collected ranged between 4.5 X 103 - 4.3 X 104 CFU/ml and the mean aerobic mesophillic plate count ranged between 5.6 X 105 to 2.7 X 106 CFU/ml. Standard plate count (SPC) for Kala Kand and Pedha samples were 450 X 106/ml and 5.1 X 106 CFU/ml respectively (Bhatt 2001). Improper handling of milk by personnel in sweet shops and water used in these shops could be other contributing factors to the high bacterial load of milk sweets. High bacteria counts result due to the contaminating micro-organisms in fresh produce. With the start of human handling, further contamination begins and it continues till the product is being handed over to the consumer (Frazier and Westhoff 1988).

The comparison of the total and gram negative counts from various samples of sweets collected from different sweet shops is illustrated in Table 2. It was observed that range of total bacterial count and gram negative count was highest for Gajar Burfi i.e. 1.5x104-3.3x106 CFU/gm and 5.1x103-9.4x105 CFU/gm respectively whereas Khoya Burfi had lowest total and gram negative bacterial count i.e. 1.5x103-5.1x104 CFU/gm and 2.1x102 -17.7x103 CFU/gm respectively. Coliform indicated the lowest count (7.5x103 CFU/g) and the highest (5.3x106 CFU/g) in burfi whereas 6.5x103 and 5.2x106 CFU/g in khoya for selected sample by Farzana et al. (2009). Similarly, Varga (2007) also found that approximately 14% of total collected dairy samples were failed to meet the legal requirements in terms of overall hygienic quality.

ISOLATION OF E. COLI

The percentage isolation of E. coli from various sweets is shown in Table 3. E.coli was isolated in 89.3% of all the samples screened. It was observed that the highest percent isolation of E. coli was shown by samples of Plain Burfi, Kala Kand, Gulab Jamun and Gajar Burfi (100%) where as the lowest was shown by samples of Khoya Burfi (66.7%). Similarly, Maity et al. (2010) isolated E.coli in 27.91% samples of Chhana based milk products. Recent studies in other countries indicate similar results in other dairy products. In Brazil high levels of faecal contamination (95.5%) was detected in cheese samples, and Enteropathogenic E. coli (EPEC) was isolated from 21.1% of the samples (Araujo et al. 2002). Similar reports were found in Iraq where 40.5% of cheese samples were contaminated with EPEC strains (Abbar and Kaddar 1991). In 2011, total 142 cases of food poisoning have been reported in which 1 death case was recorded in India (Anonymous 2011). According to Microbial Food Safety Regulation of India, E. coli must be absent in 1g sample of Khoya/ Chhana/ Paneer as per the Prevention of food adulteration rules, 1956. . Poor personal hygiene, use of unhygienic water and unhygienic surroundings act as source of contamination (Chukuezi 2010; Mensah et al. 2002). So, presence of the E. coli may cause food poisoning and pose a threat to public health.

SURFACE HYDROPHOBICITY

Hydrophobicity is the chief compound to determine organism's pathogenicity. Cell-cell adhesion is of fundamental importance in a biological system. In our study, 97.33% of E.coli showed positive hydrophobicity. A large number of E.coli isolates (53.33%) from sweets showed aggregation even at the minimum concentratin (i.e. 0.02M) of ammonium sulphate, indicating high degree of pathogenicity (Table 4). There are various mechanisms by which cells can bind to each other. Marshal et al. (1994) and Ludwica et al. (1984) reported cell surface hydrophobicity to be a major event in the attachment of bacteria to host cells which explains the degree of pathogenicity of the strains isolated. Hydrophobicity allows the organism to approach the negatively charged surface of animal cells there by contributing to an infection (Diniz et al. 2000).

With the increase in the concentration of ammonium sulphate, the rates of hydrophobicity also increased from 53.33% at 0.02 M to 97.33% at 3.2 M concentration. At 3.2 M concentration, maximum aggregation (100%) was observed in isolates of Plain Burfi, Milk Cake, Kala Kand, Gulab Jamun and Chaina Murgi, where as minimum (87.5%) was seen in case of Khoya Burfi (Table 4). In the present study, high hyprophobicity was seen in 53.33% isolates. Thus, such high degree of hydrophobicity, as reported in this study are indicative of possible infections outbreaks due to high level of pathogenic strains. A study conducted by Bansal and Kaul (2004) also revealed similar results where 86% E.coli were found to give positive SAT test.

ANTIBIOTIC SENSITIVITY PROFILE OF E.COLI ISOLATES

Antibiotics are the chemical agents which interfere with the growth and activity of micro organisms. They are used to prevent

the transmission of disease and infection. In response to the wide spread use of antibiotics, pathogens have developed a high level of resistance to antibiotics. The extensive use of antibiotics has enabled the disease causing bacteria to develop several highly effective ways of resisting these drugs. (Ogbodo et al. 2011). The present study has investigated the sensitivity of E.coli strains towards five antibiotics. Without adequate knowledge of the sensitivity pattern of gram negative bacteria which are the most common infectious agents, any infective treatment would be impossible (Sharma 1998).

It was observed that antibiotic sensitivity of E.coli isolates was maximum for Chloramphenicol (82.67%) followed by Nalidixic acid (81.33%), Gentamycin (77.3%), Erythromycin (46.67%) and Ampicillin (25.33%) (Table 5). Resistance observed in large number of isolates may be attributed either to an increase in pool of transmissible genes (R-Plasmids) in the community (Johnston et al. 2005) or to widespread use of antibiotics which may provide selection pressure in favour of the resistant strains (Maillard 2005).

In this study, multi drug resistance was observed in 53.8% of isolates obtained from sweets (Table 6). Multiple drug resistance in pathogenic E.coli was also observed by Bass et al (2001). Possession of R-plasmid as a wide spread phenomenon in most of the Enterobacteriaceae is reported to be the major cause of multiple drug resistance (Wain and Kidgell 2004). Results showed a significant ($p \leq 0.05$) correlation between multi drug resistance and hydrophobic organisms (Table 7). This shows that those two characters may be located on the same plasmid as all these properties are plasmid mediated.

CONCLUSION

The findings of the present study revealed that E. coli is frequently occurring organism in indigenous milk made sweets such as Plain Burfi, Kala Kand, Gulab Jamun and Gajar Burfi. The method of their manufacturing, handling, sale and transportation of these products are entirely based on the tradition. As observed during sample collection, the handling of sweets with bare hands, non-usage of aprons, and absence of hair covering and handling of money during serving might also contribute to poor hygienic conditions. Such system could pose favourable environment for bacterial contamination. Thus, more hygienic preventive measures are required to reduce the bacterial contamination, so as to increase the wholesomeness of these products.

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Table 1. Total bacterial and gram negative bacterial load found in milk made sweets collected from various sweets shops

Sweet Shops	Total bacterial count (CFU/gm) Gram negative bacterial count (CFU/gm)							Gram Negative Bacterial Count (CFU/gm)						
	Plain Burfi	Milk Cake	Kala Kand	Gulab Jamun	Chaina Murgi	Gajar Burfi	Khoya Burfi	Plain Burfi	Milk Cake	Kala Kand	Gulab Jamun	Chaina Murgi	Gajar Burfi	Khoya Burfi
1	11.6 x104	5.7x 104	4.8x 105	1.70 x105	3.0 x 104	1.20 x106	14.0 x103	7.4x 103	4.4x 103	25.8 x103	12.8x 104	6.7 x102	9.4 x105	8.1 x102
2	7.1 x105	5.9x104	8.5 x105	4.8 x105	2.9 x105	3.0 x105	5.1 x104	3.7x105	4.1x103	7.8x104	3.4 x104	1.1 x104	6.2 x104	3.1 x103
3	4.2 x105	6.5x104	1.66 x106	1.35 x105	1.1 x105	11.5 x104	1.41 x104	23.5 x104	5.8x103	11 x104	5.8 x104	2.8 x103	11.3 x104	3.4 x103
4	3.7 x105	3.0x105	8.9 x105	3.1 x105	3.0 x103	5.3 x105	2.85 x104	5.5x104	6.8x104	9.1 x104	2.91 x105	1.7 x102	5.6 x104	3.6 x103
5	5.5 x105	6.4x104	1.1 x106	18.8 x104	12.1 x104	8.8 x105	3.2 x104	29x104	4.2x103	9.0 x105	1.70 x103	2.3 x103	7.2 x104	2.9 x103
6	3.1 x105	5.8x103	4.2 x105	1.76 x105	4.2 x104	1.21 x106	3.3 x104	7.4x103	4.2x102	22.7 x104	12.1 x104	7.2 x103	9.6 x105	17.7 x103
7	6.7 x105	6.2x104	9.9 x105	3.1 x105	2.76 x105	3.3 x105	1.52 x104	29.1x 104	3.3x103	8.2 x104	13.3 x104	1.1 x104	6.6 x104	3.2 x103
8	3.2 x105	14.0x 104	8.9 x105	9.2 x105	1.46 x105	1.4 x106	4.2 x104	5.5x104	7.2x103	7.5 x105	3.4 x104	7.8 x104	2.95 x105	14.2 x103
9	3.3 x105	1.49x 105	1.10 x106	1.53 x105	1.63 x105	1.13 x106	3.2 x104	4.2x104	9.9x103	4.9 x105	7.7 x104	8.9 x104	2.21 x105	2.1 x103
10	6.3 x105	1.12x 105	4.5 x104	4.5 x105	1.2 x104	3.3 x106	1.5 x103	5.1x104	4.2x103	4.1 x103	5.1 x104	2.1 x103	2.1 x105	2.1 x102
11	10.1 x104	5.8x103	4.2 x105	3.4 x104	3.1 x104	5.3 x105	3.3 x104	4.4x103	3.9x102	22 x104	3.1 x103	1.3 x103	5.1 x103	3.1 x103
12	5.7 x105	6.3x104	1.12 x106	6.6 x105	2.1 x105	2.1 x105	2.8 x104	7.7x103	3.3x103	9.0 x105	4.6 x104	3.4 x104	5.6 x104	2.9 x103

Table 2. Comparison of the total bacterial and gram negative bacterial counts of various sweets

Sweets	Range of Total Bacterial Counts	Range of Gram Negative Counts
Plain Burfi	$10.1x10^4$-$6.7x10^5$	$4.4x10^3$-$3.7x10^5$
Milk Cake	$5.8x10^3$-$3.0x10^5$	$3.9x10^2$-$6.8x10^4$
Kala Kand	$4.5 x10^4$-$8.9x10^6$	$4.1x10^3$-$9.1x10^5$
Gulab Jamun	$3.4 x10^4$-$9.2x10^5$	$1.7x10^3$-$2.91x10^5$
Chaina Murgi	$3.0 x10^3$-$2.9x10^5$	$1.7x10^2$-$8.9x10^4$
Gajar Burfi	$11.5 x10^4$-$3.3x10^6$	$5.1x10^3$-$3.3x10^5$
Khoya Burfi	$1.5 x10^3$-$5.17x10^4$	$2.1x10^2$-$17.7x10^3$

Table 3. Percent isolation of E. coli from various sweets collected from sweet shops

Sweets	Number of Samples	No. of E. coli Isolated	Per-centage
Plain Burfi	12	12	100
Milk Cake	12	10	83.3
Kala Kand	12	12	100
Gulab Jamun	12	12	100
Chaina Murgi	12	09	75
Gajar Burfi	12	12	100
Khoya Burfi	12	08	66.7
Total	**84**	**75**	**89.3**

Table 4. Surface hydrophobicity of E. coli isolates at varying concentrations of (NH4)2SO4

Sample	No. of E. coli isolates	No. of E. coli isolates showing aggregation of different con-centration of (NH4)2SO4			
		0.02M	0.2M	1.8M	3.2M
	tested upon				
Plain Burfi	12	5	8	10	12
Milk Cake	10	4	8	9	10
Kala Kand	12	5	8	10	12
Gulab Jamun	12	5	6	10	12
Chaina Murgi	9	6	7	8	9
Gajar Burfi	12	8	10	10	11
Khoya Burif	8	7	8	8	7
Total	**75**	**40**	**55**	**65**	**73**
Percentage		**53.33%**	**73.3%**	**86.67%**	**97.33%**

Table 5. Sensitivity of E. coli isolated from sweet samples towards different antibiotics.

Sample	No. of E. coli isolates tested upon	Sensitivity towards				
		Erythro-mycin (30 mcg)	Ampicillin (35 mcg)	Chloram-phenicol (30 mcg)	Gentamy-cin (30 mcg)	Naidixic acid (30 mcg)
Plain Burfi	12	5	3	10	9	**10**
Milk Cake	10	5	2	9	7	**9**
Kala Kand	12	6	4	10	8	**11**
Gulab Jamun	12	6	3	9	10	**8**
Chaina Murgi	8	4	3	7	6	**7**
Gajar Burfi	12	5	2	11	11	**10**
Khoya Burif	8	4	2	6	7	**6**
Total	75	35	19	62	58	**61**
Percentage		46.67%	25.33%	82.67%	77.33%	**81.33%**

Table 6. Distribution of E. coli isolates according to their multi - drug resistance to antibiotics

Sample	No. of E. coli isolates tested upon	No. of antibiotics to which isolates were resistant					
		None	One	Two	Three	Four	Five
Plain Burfi	12	2	--	4	--	--	--
Milk Cake	10	--	6	--	1	--	--
Kala Kand	12	--	6	6	2	--	--
Gulab Jamun	12	--	8	5	1	--	--
Chaina Murgi	9	2	5	6	--	--	--
Gajar Burfi	12	4	7	3	1	--	--
Khoya Burif	8	--	--	4	2	--	--
Total	**75**	**8**	**32**	**28**	**7**	--	--

Table 7. Correlation of multi-drug resistance with cell surface hydrophobicity.

Total no. of stains	No. of multi drug resistant strains	Cell surface Hyrophobicity		Chi-square value	Evaluation
		Positive	Negative		
75	35	73	2	9.67	Significant*