

Antibiotic Resistance of Different Bacterial Strains Isolated from Orange Juices

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ABSTRACT

Aim: To investigate about antibiotic resistance of different Bacterial strains isolated from Orange Juices.

Study design: Experimental.

Place and Duration of Study: Amity institute of Biotechnology, Amity University, Noida, India; March 2013 to December 2013.

Methodology: The juice samples were cultured with nutrient agar and various morphological and biochemical tests were performed for characterization. All the bacterial isolates were analysed for antibiotic sensitivity, using commonly prescribed antibiotics by Indian doctors.

Results: Out of 5 bacterial isolates from orange juice samples, 4 were multi-drug resistant.

Conclusion: In the study conducted, it was observed that more than 80% of bacterial isolates were found to be multi-drug resistant. The study shows lack of hygiene in fresh and packaged juice products.

Keywords: Antibiotics, Antibiotic resistance, Antibiotic sensitivity, Multi-drug resistant.

INTRODUCTION

Orange juice is well known for its nutritive value, mineral and vitamin content. Orange juices are sold at most of the public places for their ready consumption but there is a big issue in their handling and extraction as they could often prove to be a public health threat¹. Antibiotic resistance is the ability of microbes to resist the effects of an antibiotic. Antibiotic resistance occurs when bacteria change genetically and eliminates the effect of drugs².

There are several reports of illnesses due to the food borne diseases associated

with the consumption of fruit juices³⁻⁶. Juices have been shown to carry bacterial pathogens especially *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Staphylococcus aureus*⁷⁻⁹.

Orange juices are consumed at a very large scale. It is not only produced for domestic use but is also exported to various markets abroad. Because of the perishable nature of oranges, several small-scale industries and large scale industries in India have gone in to the processing of orange juice, such that the juice would be available

in the market throughout the year (as tetra packs). This is important in view of the fact that orange is usually available in the dry season period (October/February). On the other hand, the presence of microbial contamination in these juices and their processed products make them inappropriate for human consumption and unacceptable to the quality conscious markets. In order to assess the magnitude of the problem, a study was conducted to test randomly collected samples of fresh orange juices and widely available processed pasteurized orange juices from NOIDA region through conventional culturing techniques.

The bacterial isolates were evaluated to determine their resistance to the commonly prescribed antibiotic drugs in India. As these harmful micro-organisms have been isolated from a wide range of foodstuffs that became a major public health threat¹⁰. Orange Juices have shown to be potential sources of bacterial pathogens especially *E. coli* O157:H7, species of *Salmonella*, *Shigella* and *Staphylococcus aureus*¹¹. In several studies conducted on the resistance of food-borne pathogens, main emphasis had been found on *E. coli* and *S. aureus*¹²⁻¹⁷. However, in the present study, an attempt was made to evaluate the resistance of selected strains of all the bacterial strains that were isolated from the orange fruit products. It is believed that the results of this finding will not only add to the existing world data on bacterial resistance of food origin, but will sensitize the operators in this industry, policy makers and the regulatory agencies on the need to improve the quality of these products.

METHODOLOGY

Sample collection

Fresh and packaged orange juice product were obtained from randomly chosen vendors in the sectors-126, 125 and 18 of NOIDA region (Table 1). The

packaged orange juice product had at least 3 months to the expiry date from the period of analysis. Each juice sample was transported to the laboratory and analyzed within 2 hour.

Isolation and characterization of bacteria

Samples were serial diluted till 10^{-4} and these prepared samples were then transferred to nutrient agar medium by using pure culture techniques for isolation of bacterial strains. Sub-culturing was done to obtain pure cultures. Isolated bacteria were characterized by using gram staining and different biochemical tests like Catalase test, indol test, MRVP test, simmon's citrate agar tests, carbohydrate utilization, casein hydrolysis, amylase production etc. After the characterization process differential and selective media (EMB & MacConkey agar) were used for purification of bacterial isolates¹⁸. The isolates were identified by Bergey's Manual for Determinative Bacteriology^{1,18} (Bunchanan and Gibbons, 1974).

Antibiotic susceptibility test

100µl of culture were spread onto nutrient agar plates and 10µl of the selected antibiotics by using disc diffusion¹⁹ and by agar well diffusion method^{20,21}. Antibiotics Gentamicin, Kanamycin, Tetracycline, Amoxicillin, Ofloxacin, Levofloxacin, Nitrofurantoin and Cefuroxime were used for the study. Plates were incubated at 37°C for 24 hours and checked for the zone of inhibition for antibiotic resistance.

Plasmid isolation and gel electrophoresis

1.5ml of overnight grown bacterial culture at 37°C was taken and transferred to eppendorf tube and centrifuged at 13,000rpm at room temperature for 1 min. Then the supernatant was discarded and pellet was re-suspended in 200µl of TEG (Tris EDTA Glucose solution). Eppendorf tube was vortexed for 3 min. and 400µl of

freshly prepared solution II (Lysis solution) was added to the tube and incubated at 4°C for 2 min. 300µl of ice cold solution I (Resuspension buffer) was added to it and mixed vigorously by vortexing. The tubes were again incubated at 4°C for 5 min. and then centrifuged at 13,000rpm at 4°C for 12 min. The supernatant was transferred to fresh tube and 500µl ice chilled iso-propanol was added to it. The contents in the tube were mixed properly and centrifuged at 13,000rpm for 12 min. The supernatant was discarded and pellet was washed with 300µl of 70% ethanol. Pellet was air dried at room temperature till ethanol evaporates and was dissolved in 20µl of either TE buffer. 1% agarose gel was prepared in 0.5X TAE buffer. The samples containing plasmid was loaded into the wells and were gel electrophoresed at 50-60 volts for 1hr²².

[Solution I (Resuspension buffer) - Glucose, Tris. Cl, and EDTA

Solution II (Lysis solution) - sodium hydroxide (NaOH) and Sodium dodecyl-sulfate (SDS)]

RESULTS AND DISCUSSION

The orange juice samples contained huge amounts of bacteria ($2.7 \times 10^3 - 2.5 \times 10^5$ cfu/ml). The values obtained in the study are within the range of 10^2-10^5 cfu/ml reported for microbial populations in fruit juices²³. Presence of high bacterial count showed improper hygiene and may be a result of poor quality fruits being used.

Morphological characterization of isolated bacteria

Gram staining

Out of the five bacteria isolated, three were found to be gram negative as they appeared thick rods and cocci shaped and were pink or red in colour, whereas two were Gram positive which appeared as rods and were purple or blue in colour. (Table 2)

Biochemical characterization

Characterization of bacteria by IMViC test

Indole production

All the samples of orange juice taken were found to be indole positive. (Table 3)

Methyl-Red (MR) and Voges-Proskauer (VP)

Three samples viz. JKB2, JKB3 and JKB5 were MR positive and remaining two samples viz. JKB1 and JKB4 were MR negative, where as two samples viz. JKB1 and JKB4 were VP positive and remaining three samples viz. JKB2, JKB3, and JKB5 were VP negative. (Table 3)

Citrate utilization

All the samples viz. JKB1, JKB2, JKB3, JKB4 and JKB5 showed positive test for citrate utilization. (Table 3)

Catalase test

All the bacterial isolates were catalase positive. (Table 4)

Casein hydrolysis

Sample no. JKB5 showed positive reaction for caseinase secretions while sample no. JKB1, JKB2, JKB3 and JKB4 showed negative reaction. (Table 4)

Amylase production test (starch hydrolysis)

Sample JKB1, JKB3, JKB4, and JKB5 showed positive starch hydrolysis whereas sample JKB2 showed no starch hydrolysis. (Table 4)

Carbohydrate fermentation test

The production of air bubble in the durham tubes indicates that bacterial culture is gas producing while a change in color of the media indicates that bacterial culture is acid producing. All the five samples were producing gas while all five samples were

non-acid producers for Sucrose as carbohydrate source. For lactose all five samples were acid producer where as for Glucose only sample no. JKB1 were acid producer. (Table 5)

Growth on differential media

MacConkey agar

All the five samples showed light-pink or bright pink colored colonies giving positive result. (Table 6)

Eosin methylene blue (EMB) agar:

All sample showed growth on EMB media with pink, purple and pale pink colonies. Some of the colonies were mucoid in texture and some were dry. (Table 6)

As a result of biochemical characterization, Sample JKB1 were found negative rod, Catalase test (+), Citrate utilization (+), Indole production (+), MR test (-), VP test (+), Casein hydrolysis (-), Carbohydrate fermentation [acid producer (+) & gas producer (+)]; Sample JKB2 were found negative cocci, Catalase test (+), Citrate utilization (+), Indole production (+), MR test (+), VP test (-), Casein hydrolysis (-), Carbohydrate fermentation [acid producer (+,-) & gas producer (+)]; Sample JKB3 were found negative rod, Catalase test (+), Citrate utilization (+), Indole production (+), MR test (+), VP test (-), Casein hydrolysis (-), Carbohydrate fermentation [acid producer (+,-) & gas producer (+)]; Sample JKB4 were found positive rod, Catalase test (+), Citrate utilization (+), Indole production (+), MR test (-), VP test (+), Casein hydrolysis (-), Carbohydrate fermentation [acid producer (+,-) & gas producer (+)]; Sample JKB5 were found positive rod, Catalase test (+), Citrate utilization (+), Indole production (+), MR test (+), VP test (-), Casein hydrolysis (+), Carbohydrate fermentation [acid producer (+,-) & gas producer (+)]. On the basis of Cultural and Biochemical characterization

by Bergey's Manual for Determinative Bacteriology the isolates might be *Enterobacter* spp., *Klebsiella* spp., *Bacillus* spp. and *E. coli*.

Antibiotic susceptibility test

A total of five samples were received from different local juice shop with 5 bacteria isolated. All the bacterial isolates were highly sensitive to Levofloxacin with maximum zone of inhibition 4.8cm, also all the bacterial isolates were sensitive to Gentamicin, Ofloxacin and Kanamycin with 3.7cm, 4.5cm and 2.6cm respectively. Among the bacteria isolated only one sample viz. JKB3 was sensitive to Tetracycline with 1.5cm as its zone of inhibition. Isolates were considered multi-resistant if they showed resistance to three or more tested antibiotics. The bacterial isolates were highly resistant to Cefuroxime, Nitrofurantoin and Amoxicillin. The resistance to cefuroxime, nitrofurantoin and amoxicillin may reflect the widespread use of these antibiotics. According to results shown (Table 7) samples JKB1, JKB2, JKB4 and JKB5 were resistant to more than three tested antibiotics, thus showing multidrug-resistance. (Figure: 1)

A high level of resistance was obtained among the five isolated bacterial strains. The relatively high level of resistance to antimicrobial agents is a sign of exploitation of these agents in the environment^{14,24,25}. Antibiotic prescriptions in some hospitals are given without clear evidence of infection.

Multiple drug-resistance is an extremely serious public health issue²⁶ and it has always been related with outbreak of major epidemics throughout the world.

Plasmid isolation and gel electrophoresis

According to the results obtained after gel electrophoresis of different samples, as shown in Figure: 2 sheared

bands were observed and following possibilities can be interpreted:

- 1) It could either be a megaplasmid which is of very large size and the protocol used was for the isolation of small plasmids. The isolation of megaplasmid demand gentle handling, so large plasmid gets sheared in the absence of gentle handling. Because of presence of megaplasmid there are higher chances of reminiscence of chromosomal DNA along with plasmid DNA.
- 2) Because of the presence of antibiotic resistant genes present on transposons the resistance could be transferred to chromosomal DNA and thus not plasmid borne.
- 3) The bands of plasmid isolation could possibly be masked by bands due to chromosomal DNA.

CONCLUSION

In the study conducted, it was observed that more than 80% of bacterial isolates were found to be multi-drug resistant. These isolates were tested for commonly prescribed antibiotics, viz. Gentamicin, Kanamycin, Tetracycline, Amoxicillin, Levofloxacin, Cefuroxime, Ofloxacin and Nitrofurantoin. Maximum isolates were found resistant to β -lactam antibiotic, Cefuroxime (cephalosporin), Nitrofurantoin, Amoxicillin (penicillin) and Tetracycline. The study shows that fresh and packaged juice products are not totally safe for human consumption. Possible reason for the occurrence of human pathogens in fruits is that they cycle through the environment, using fruits as an alternative host to survive and as a vehicle to recolonize animal hosts once ingested.

The presence of the multi-drug resistant microorganisms isolated from the samples, indicates that the conditions of preparation, handling, and storage are not hygienic. To decrease microbial presence in

orange juices, correct handling practices, efficient cleaning, sanitization of containers and implementation of imperative programs are needed. Also lowering in prescription of antibiotics is recommended thus avoiding spread of multi-drug resistance among microbes. Regular monitoring of the quality of fruit juices for human consumption must be introduced to avoid any future outbreaks.

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Table 1. Details of samples collection

Sample	Source	Location 1	Location 2
Fresh orange juice	Local vender (Noida)	Sector 125	Sector 126
Packaged orange juice	Departmental store	Sector 18	Sector 125

Table 2. Gram staining

Sample	Type	Morphology	Colour
JKB1	(-)	Thick Rod	Pink
JKB2	(-)	Cocci	Pink
JKB3	(-)	Thick Rod	Pink-Red
JKB4	(+)	Rod	Purple
JKB5	(+)	Rod	Purple

*JKB = Code for Orange Juice Sample

*(+)= Gram-positive Bacteria, (-) = Gram-negative Bacteria

Table 3. IMViC test

Sample	Citrate Utilization	Indol Production	MR Reaction	VP Reaction
JKB1	+	+	-	+
JKB2	+	+	+	-
JKB3	++	+	+	-
JKB4	+	+	-	+
JKB5	++	+	+	-

*JKB = Code for Orange Juice Sample

*(+)= positive reaction, (++) = strongly positive reaction, (-) = negative result

Table 4. Biochemical activities

Sample	Catalase Test	Casein Hydrolysis	Starch Hydrolysis
JKB1	+	-	+
JKB2	+	-	-
JKB3	+	-	+
JKB4	+	-	+
JKB5	+	+	+

*JKB = Code for Orange Juice Sample

*(+)= positive reaction, (-) = negative reaction

Table 5. Carbohydrate fermentation test

Sample	Glucose		Lactose		Sucrose	
	A	G	A	G	A	G
JKB1	+	+	+	+	-	+
JKB2	-	+	++	+	-	+
JKB3	-	+	+	+	-	+
JKB4	-	+	++	+	-	+
JKB5	-	+	++	+	-	+

*A= acid producer, G = gas producer, (++) = high acid /gas producer, (+) = acid/gas producer, (-) = non-acid/ non-gas producer

Table 6. Growth on differential media

Sample	MacConkey Growth	MacConkey Colour	EMB Growth	EMB Colour
JKB1	+	Light Pink	+	Light Pink
JKB2	+	Light Pink	+	Pink-Purple
JKB3	+	Light Pink	+	Light Pink
JKB4	+	Bright Pink	+	Bright Pink
JKB5	+	Light Pink	+	Pale Pink

*JKB = Code for Orange Juice Sample

*(+)= positive reaction, (-) = negative reaction

Table 7. Antibiotic susceptibility test for bacteria isolated from orange juice sample

Sample	G	K	T	A	L	C	O	Nt.
JKB1	S (2.8cm)	S (2.4cm)	R	R	S (4.7cm)	R	S (3.8)	R
JKB2	S (3.7cm)	S (1.9cm)	R	R	S (4.7cm)	R	S (3.5cm)	R
JKB3	S (3.6cm)	S (2.6cm)	S (1.5cm)	R	S (4.8cm)	R	S (4.2cm)	R
JKB4	S (4.0cm)	S (2.2cm)	R	R	S (3.5cm)	R	S (2.7cm)	R
JKB5	S (2.6cm)	S (2.4cm)	R	R	S (4.7cm)	R	S (4.5cm)	R

*S = sensitive, R = resistant, (0.00cm) = zone of inhibition

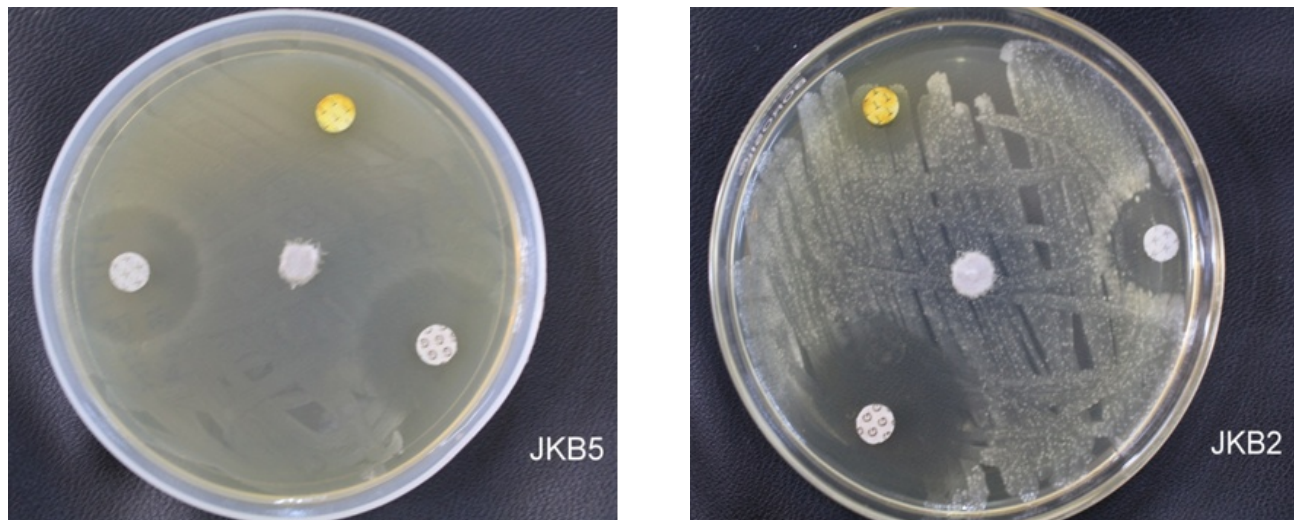


Figure 1. Zone of inhibition by antibiotics in different samples

(JKB2, JKB5 = Sample coding)

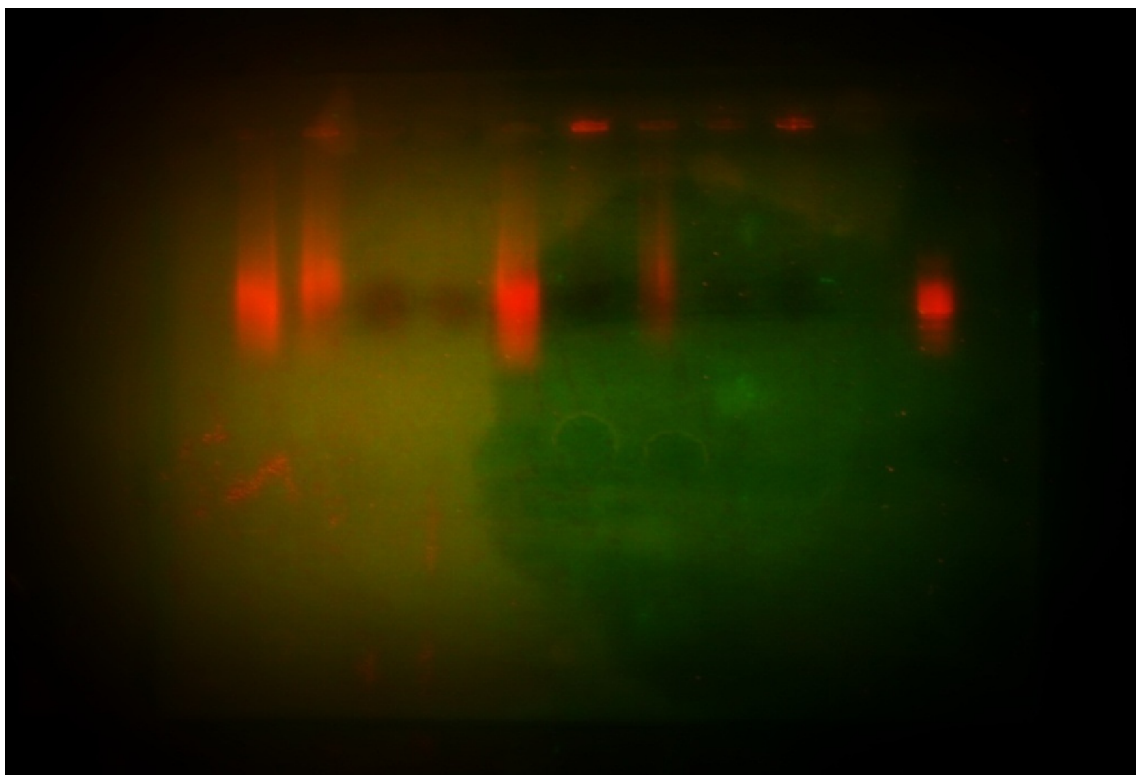


Figure 2. Gel observed under UV Transilluminator