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Juice Sample from Street Side Shop

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Abstract: ***Five crowded juice centres which are present in Peenya (Bangalore) was selected and freshly prepared orange juice sample was collected from each of these places respectively and examined microbiologically. Macroscopic and microscopic features were determined. To identify the morphological features, gram staining was carried out to differentiate between gram positive and negative bacteria. Five biochemical tests were performed i.e. catalase, oxidase, starch hydrolysis, MR and VP. These tests helped in the identification of bacteria. Molecular identification like DNA isolation, polymerase chain reaction, gel elution, Sanger PCR, bioinformatics analysis was done for bacteria characterization. The organisms encountered include Escherichia coli, Vibrio cholerae, Klebsiella pneumoniae, Shigella, Citrobacter. Antibiotic susceptibility test was done using 10 different commonly used antibiotics, Amoxicillin, Azithromycin, Doxycycline, Norfloxacin, Cephalexin, Ciprofloxacin, Cefpodoxime, Cefixime, Amoxicillin + Clavulanate, Metronidazole. The size of zone of inhibition (diameter of areas without bacterial growth) was estimated in mm. It was evident, that the most susceptible antibiotic is Cephalexin with a zone of inhibition of 50mm. The least resistant is Cefpodoxime with 13mm zone of inhibition.***

Keywords: Street side shop, Peenya, resistance, susceptibility, antibiotics

1. Introduction

Fruit drinks are popular among people of all ages all year, especially during the hot seasons. They are made by pressing the fruit pulp and combining it with a substantial amount of water, ice, syrup, and other components that vary according to the recipe (7). They are high in vitamins, minerals (calcium, phosphorous, sodium), and bioactive compounds (flavanone glycosides, hydroxycinnamic acid, antioxidants) that help to improve human health by maintaining blood lipid profiles in hypercholesterolemic patients, inhibiting breast cancer, urinary tract infections, and congestive heart failure (13). These juices are typically sold in congested places such as retail malls, schools, universities, hospitals, and offices (4).

The link between food intake and human diseases was recognized very early, with Hippocrates (460 B.C.) reporting that there is a substantial link between food consumption and human sickness (8). There have been over 200 distinct food-borne illnesses documented (10). Globally, contamination of fruit juices with pathogenic microorganisms has been linked to epidemics of infectious illnesses with significant morbidity and mortality (1, 3, 12, 9). If the fruits used to make juice have damaged skin surfaces, they may get contaminated with ambient microflora, including pathogenic microorganisms. Furthermore, ingredients used in juice preparation, such as ice and water, equipment used during preparation, the hygienic condition of the juice maker, the surface where the fruits are processed, and so on, can affect juice quality and, if not properly maintained, can transfer pathogenic microorganisms to the final juice. If this occurs, customers will suffer from food-borne gastrointestinal illnesses and, in some cases, death (2). Escherichia coli, Vibrio cholerae, Klebsiella pneumoniae, Shigella, and Citrobacter are among the harmful bacteria discovered in fruit juices.

The current study was designed to determine the presence of pathogenic bacteria found in fresh vended orange juice and its drug resistance traits sold in different areas of Peenya in Bangalore city to identify the risk of public health.

2. Materials and Methods

4 samples of freshly prepared orange juice were collected from different vendors around Peenya, Bangalore. The samples were collected in sterile tubes and processed in the laboratory within an hour. They were labelled as 1A, 1B, 1C 1D, 1E, 2A, 2B, 2C, 3A, 3B, 3C 4A, 4B and 4C.The orange juice samples were serially diluted by taking 1ml of orange juice tube 1 and 900 microliter of water in tube 2,3,4,5 respectively. From tube 1, 100 microliter of orange juice was added to tube 2 and again 100 microliters of tube 2 was added to tube 3 and so on. This was repeated for all the samples. 4g of LB media was made up to 100 ml by adding water and was autoclaved, which was later poured on to clean petri plates and was allowed to solidify. The tubes 2 (10^{-2}) and 4 (10^{-4}) were streaked on these petri plates. This was kept is an incubator at 37°C for 24 hours for the colonies to be formed.

Pure colonies

The agar solution was poured into petri plates after marking the colonies and was allowed to solidify. 1ml of nutrient broth was added to Eppendorf tubes labelled as 'SJS a' to 'SJS e'. The colonies were again placed on the agar plate and the nutrient broth was added. This agar plate was incubated at 37°C for 24 hours.The microscopic analysis was done after gram staining to determine if the bacteria was gram positive or gram negative. The macroscopic observation of the colonies was done wherein, the shape, size, color, texture and opacity were noted down. If the size of colony was greater than 1mm it was classified as large, less than 1mm as small. The shape of colony observed were round, rhizoidal, irregular, irregular donut.

Biochemical Tests

MRVP broth into poured into five sterile test tubes. The test organisms are into the respective test tubes. All the test tubes are incubated at a temperature of 35 degrees Celsius for 48 hours.For the Methyl Red test, 3-5 drops of methyl red are added to each test tube containing the test organisms and further observed for colour change in the medium.

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For the Voges-Proskauer (VP) test, 12 drops of V-P solution I (naphthol solution) and 2-3 drops of V-P solution II (40% potassium hydroxide) is added to each test tube containing the test organism. Each test tube is secured with a cotton plug and mixed for 30 seconds. The test tubes are kept undisturbed for 10-15 minutes and observed for colour change.

Catalase test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. A loop or sterile wooden stick is used to transfer a small amount of colony growth in the surface of a clean, dry glass slide. A drop of 3% H2O2 is placed on the glass slide and observed for the evolution of oxygen bubbles.The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result.

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase. Flood the growth with oxidase reagent and observe for the development of a deep indigo blue in and around the colonies. The clear oxidase reagent turns blue when cytochrome oxidase adds electrons to the chemical instead of to oxygen, as it normally does. Development of the blue colour is positive for presence of the enzyme cytochrome oxidase. The colour change does not occur in a negative test.

The fifth test was starch hydrolysis test. Using a sterile technique, make a single streak inoculation of organism to be tested into the centre of labelled plate. Incubate the bacterial inoculated plates for 48 hours at 37°C.Following incubation, flood the surface of the plates with iodine solution with a dropper for 30 seconds. Pour off the excess iodine. Examine for the clear zone around the line of bacterial growth.

Molecular Identification

DNA Isolation: The sample obtained is centrifuged at 10,000 rpm for one minute.1ml of CTAB buffer is added to the pellet and kept for lysis for 30 minutes at 60°C.Centrifugation is carried out for 10 minutes at 10,000 rpm after adding Chloroform and isopropanol in the ratio 24:1.Centrifuge again for 5mins at 10,000 rpm.Add 1ml of isopropanol and precipitated DNA is obtained after keeping it overnight at 20°C.

DNA Columns: 750µl of the precipitated DNA is taken and centrifuged at 10,000rpm for 1 minute.The flow is discarded and the whole step is repeated again. This is now washed with 700µl of wash buffer at 10,000 rpm for 1 minute. The flow is discarded. The same step is repeated with 600µl wash buffer.Dry spin is carried out at 10,000 rpm for 2 minutes.20µl elution buffer is added to a fresh tube to the center of the column and kept for 3minutes, after which it is centrifuged at 10,000 rpm for a minute.1µl of RNAse is added and kept for 30 minutes at 37°C.Agarose gel electrophoresis is carried out at 95V and 250mA by adding 1% agarose.

Polymerase Chain Reaction: Denaturation is carried out i.e., initial and final at 95°C and for 5 minutes and 30 minutes respectively. Annealing is carried out at 58°C and 72°C for 1 minute each. Extension is carried out at 72°C for 1 and 5 minutes.16s rRNA gene was amplified through a total of 35 cycles and gel electrophoresis was performed to cut the band and gel elution was carried out. The band size was 1200 bp.

Gel Elution: 450 µl of gel elution buffer is added and kept for 20 minutes at 55°C.After reaching room temperature, 200µl of isopropanol is added after which it is passed through column and spun at 10,000 rpm for 1 minute.600µl of wash buffer is added and spun at 10,000 rpm for 1 minute. The flow is discarded. Dry spin is carried out at 10,000 rpm for 2 minutes. The flow is discarded. 20 µl of elution buffer is added and spun at 10,000 rpm for a minute. 20 µl of pure DNA fragment is obtained. The DNA fragment is subjected to denaturation which is carried out i.e., initial and final at 95°C and for 5 minutes and 30 minutes respectively.Annealing is carried out at 58°C for 30 minutes. Termination is carried out at 60°C for 1 minute. The product is linked in 31 30XL genetic analyzer. Electropherogram is obtained in. ab1 format which was observed in Finch TV. This is converted to FASTA and pdf files using sequence scanner. BLAST results are obtained using FASTA sequence and phylogenetic analysis is determined.

The organisms selected were *Salmonella* (1A), *Shigella*(1B), *Klebsiella* (2A), *Vibrio spp* (3A) *and Enterobacteria* (4C).Amongst these, Klebsiella was selected owing to be the deadliest and causing pneumonia, bloodstream infections, meningitis, and urinary tract infections.

Amongst these, we selected Klebsiella as it was the deadliest pathogen causing diseases likesepticemia, liver abscesses, and diarrhea in humans, pneumonia in humans.

Antibiotic Susceptibility test

4 g of LB media and 0.5 g of agar in 100ml of water was used to prepare agar plates. After it solidified, the agar plate surface, was inoculated by spreading a volume of microbial inoculum over entire Agar surface. Then a hole with diameter 6-8 mm was punched aseptically. 100 micro liter of antibiotic was introduced in these wells. These plates were thenincubated at 37°C for 24 hours. The size of zone of inhibition (diameter of areas without bacterial growth) was estimated in mm.

3. Results

Macroscopic analysis was determined for the shape, size, color, texture and opacity. The shape of colony observed were round, rhizoidal, irregular, irregular donut. The color observed was mostly greenish sheen, and a very few being colorless. Texture was mucoid / butyrous. The opacity of colonies was either opaque or translucent.

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Table 1: Macroscopic Analysis of samples

SAMPLES	MACROSCOPIC FEATURES					MICROSCOPIC FEATURES
	SIZE	SHAPE	TEXTURE	COLOUR	OPACITY	
1A	Small	Round	Mucoid Butyrous	Greenish Sheen	Opaque	Cocci
1B	Large	Irregular	Mucoid Butyrous	Greenish Sheen	Opaque	Rod shaped
1C	Large	Rhizoidal	Mucoid Butyrous	Greenish Sheen	Opaque	Rod shaped
1D	Large	Irregular	Mucoid Butyrous	Greenish Sheen	Translucent	Rod shaped
1E	Large	Irregular Donut	Mucoid Butyrous	Greenish Sheen	Opaque	Rod shaped
2A	Small	Round	Mucoidal	Greenish Sheen	Opaque	Rod shaped
2B	Large	Irregular	Mucoidal	Greenish Sheen	Opaque	Rod shaped
2C	Small	Rhizoidal	Mucoidal	Greenish Sheen	Opaque	Rod shaped
3A	Large	Round	Mucoidal	Greenish Sheen	Opaque	Rod shaped
3B	Irregular	Large	Mucoidal	Greenish Sheen	Opaque	Rod shaped
3C	Large	Irregular	Mucoidal	Greenish Sheen	Opaque	Rod shaped
4A	Small	Round	Mucoidal	Greenish Sheen	Opaque	Rod shaped
4B	Large	Round	Mucoidal	Greenish Sheen	Translucent	Rod shaped
4C	Irregular	Large	Mucoidal	Greenish Sheen	Opaque	Rod shaped

Microscopic analysis was carried out for all the samples after gram staining. All organisms were found to be gram negative indicating a pink or a purple color.

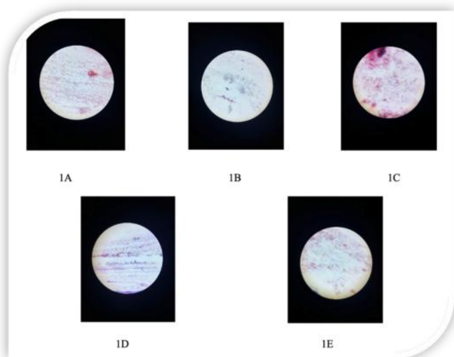

Figure 1: Microscopic observation of samples 1A-1E

Biochemical Tests

All samples were positive for catalase and VP test. Oxidase and MR test were negative.Samples were positive for starch hydrolysis except 2A.

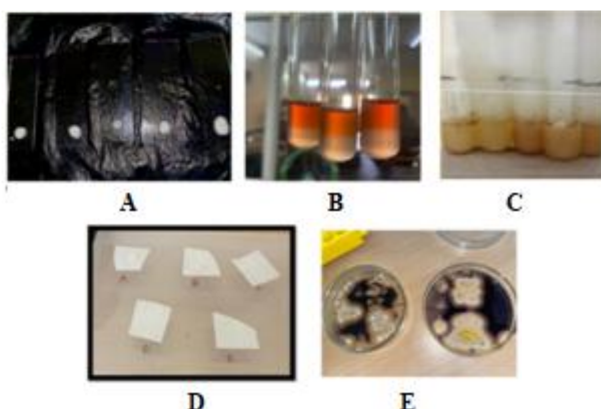

Figure 2: A) Catalase test B) VP test C) Oxidase test D) MR test E) Starch hydrolysis test

Gel Electrophoresis

Bands obtained from gel electrophoresis were of 0.5 bp.

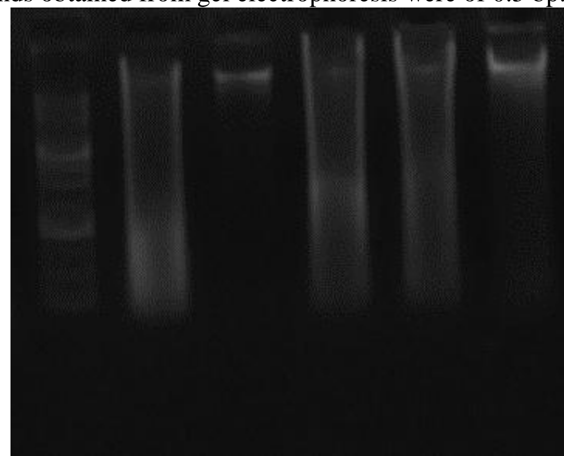

Figure 3: Bands obtained from gel electrophoresis

Bioinformatic Analysis

Electropherogram was obtained from Finch Tv. The peaks represent the amount of A, G, T and C nucleotides present. The X axis gives the DNA fragment size in base pairs and the Y axis highlights the florescence units.

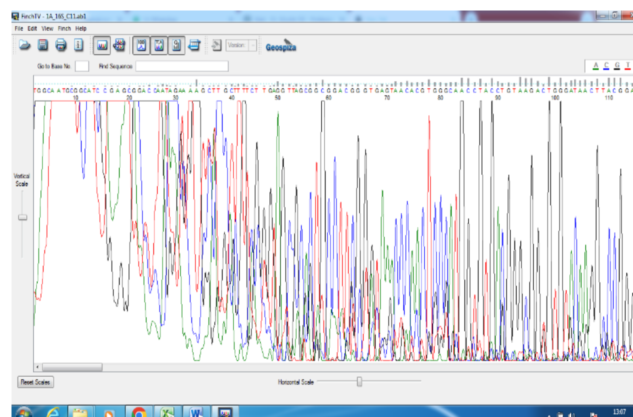

Figure 4: Electropherogram for Klebsiella

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The FASTA sequences for *Klebsiella* was as follows
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGT
CGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGAC
GAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTG
CCTGATGGAGGGGGATAACTACTGGAAACGGTAGC
ACTGCAGTGGGGTAACGGCTCACCTAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGG
GTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTG
AGGTTAATAACCTCATCGATTGACGTTACCCGCAGA
AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTG
GGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGG
ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATT
CGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGT
AGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGGAGGAATACCGGTGGCGAATGCACGTTCGATTTG
GAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTA
ACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAAC
GCGAAGAACCTTACCTGGTCTTGACATCCACAGAAC
TTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGA
GACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGT
GAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTC
AAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTG
GGGATGACGTCAAGTCATCATGGCCCTTACGACCAG
GGCTACACACGTGCTACAATGGCATATACAAAGAGA
AGCGACCTCGCGAGAGCAAGCGGACCTCATAAAAC
TGCAGTCCGCCCGTCACACCATGGGAGTGGGTTGC
AAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGC
TTACCACTTTGTGATTCATGACTGGGGTG

Hit obtained from BLAST of the obtained sequence was *Klebsiella sp.* E2A2 16S ribosomal RNA gene, partial sequence; had a percent identity of 97.47% with E value 0.0 and query coverage of 88%. From the phylogenetic tree analysis, it was found that the test sample was confirmed to be *Klebsiella sp.*

Figure 5: Phylogenetic tree construction

Antibiotic Susceptibility Test:
Bacteria was found to be the least resistant to Cefpodoxime and most susceptible Cephalexin.

Figure 6: Results for Antibiotic Susceptibility test

The zone of inhibition was determined for a total of 10 antibiotics.

Table 2: Results of zone of inhibition formed

S. No.	Antibiotics	Zone of Inhibition (in mm)
1	Amoxicillin	16
2	Azithromycin	32
3	Doxycycline	28
4	Norfloxacin	36
5	Cephalexin	50
6	Ciprofloxacin	40
7	Cefpodoxime	13
8	Cefixime	18
9	Amoxicillin+ Clavulante	18
10	Metronidazole	19

4. Discussion

Street food vendors sell products to millions of LMIC consumers at a reasonable cost, thus contributing to the economic growth in these countries. However, such healthy input to the economy can be jeopardized by the unsafe hygiene practices of the vendors, as they create opportunities for infectious diseases. In India, the Food Safety Standard Authority of India (FSSAI) in collaboration with the Ministry of Skill Development and Entrepreneurship, launched a "clean street food" education program to train 20,000 roadside vendors about safe food preparation (6). According to WHO, unsafe practices such as chewing or smoking tobacco do not only affect the health of the vendors, but also put consumers at risk of exposure to toxic chemicals (e.g., second hand tobacco smoke exposure containing polycyclic aromatic hydrocarbons) (14). The results of the current study are in agreement with the findings of the previous studies, and therefore, justify why it is so important to implement educational programs for street food vendors across India and in other developing countries. In our study, it is encouraging that more than 60% of vendors from each market were found to wash hands with soap and water. However, several other unsafe behaviours, including frequent itching of the body, wiping of hands on their dirty clothes, and scalp scratching during the preparation of juice samples were also observed, which could potentially induce heavy microbial contamination in the juice products. The importance of source water and waste disposal are crucial in offering safe juice products to consumers (5). Several studies in India have reported the significant role of water quality, as water is used for diluting the raw juices (11). Our study observed that almost 100% of

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vendors used water from municipal sources, and bought ice from local vendors. Consequently, high frequencies of *E. coli, Salmonella, Klebsiella, Shigella, Citrobacter, Vibrio cholerae* were detected in juice samples in these two market areas.

There are several potential limitations in our study. This was an exploratory study, and therefore no absolute conclusive statement can be drawn from the results. The major goal of the study was to generate pilot data regarding the antibiotic resistant bacteria present in fruit juice products prepared and sold by the street food vendors in a crowded city in India (i.e., Bengaluru). Our sample size was also restricted, since we lacked adequate funding. In future, we plan to expand the study with a larger sample size, including a comparison group, with a collection of samples over different seasons of the year. We plan to use high throughput analytic methods to elucidate the flux of antibiotic resistant bacteria in future large-scale studies.

5. Conclusion

Fruit juices are very popular drink with adequate number of vitamins and minerals which is very important for our health. But this nutritious substance can also be harmful for the health if it harbours adequate number of pathogenic bacteria. The microbiological quality of fruit juices served by street vendors in Peenya was not within the acceptable limits, as reflected in our study. Furthermore, water quality used for the preparation of juice products was compromised. Thirdly, the fruits, environmental factors, mixer used played a role in the contamination. Bacterial strains isolated from juice samples were resistant to some common and essential antibiotics, indicating a pressing public health concern. Based on our findings, future studies should focus on developing awareness programs to educate and improve hygiene and food handling behaviours practiced by fruit juice vendors.

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