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"Bacteriological analysis of street vended freshly prepared fruit juices available in Surat region."

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Abstract: *Fruit juices are natural liquids that are derived from the extraction or pressing of the fruits such as grapes, lemons, oranges, pineapple, mosambi, apple, mango etc. It is extracted mechanically or by macerating. Their demand increased because they served quickly, tasty and available at reasonable rates. Pathogenic microbes may enter in fruits during their growth and harvesting through damaged surfaces and punctures and also contaminated with microbes from raw material, juice machine, handler and unhygienic conditions. Thus the study was aimed to isolate the various etiological agentsand to analyze their antimicrobial activity, ESBL production, Biofilm production capabilities.105 various street vended fruit juice samples were collected, among which 71.42% showed positivity.Gram negative isolates predominated. Significant percentage of microbial resistance, MDR, ESBLs and biofilm producers indicated the necessity of the study.The use of good quality fruits and surface disinfection besides cleaning with pure water will certainly improve the microbiological quality of these juices.*

Key Words: *Fruit juices, antimicrobial, ESBL, Biofilm.*

1. INTRODUCTION:

Fruit juices are natural liquids, actually the extraction of the fruits. Juice is extracted mechanically or by macerating and can be extracted from various fruits such as grapes, lemons, oranges, pineapple, mosambi, mango etc. Fresh juices are of high value that enrich in natural vitamins, sugars and fibers which are necessary elements of human health. The demand of fresh juices has increased day by day and preferred throughout the world and the fresh juices sold by vendors are easily available (Ashgar et al., 2018).In developing countries, fruit juices sold street vendors are widely consumed by millions of people. All age group peoples are consumed fruit juices because of the health and nutritional benefits. The street foods are being served quickly, also tasty and it is available at reasonable rates. So it attracts all the age groups, especially the younger generations. (Suneetha et al., 2011).

Fruits have been greatly re recommended in foods due to their health –promotingg functions. They are very essential in dietary guidance because they contain high levels of minerals particularly electrolytes, vitamins such as vitamin C, and phytochemicals which act as antioxidants and fibers. Some of which include the ability to develop a blood lipid profile, detoxify the human body, and prevent vitamin deficiencies. Also, the consumption of sufficient amount of fruits lowers blood pressure, reduces the risk of some heart diseases, and prevents some kinds of cancer. (Lgiehon et al., 2020). Due to lack of good hygienic practices, the high concentration of nutritive of freshly squeezed fruit juices unfortunately provide suitable medium for growth and survival of various groups of microbes. (Uddin et al., 2017). Even improper washing of food adds numerous organisms to extracts that lead to contamination. In addition, unhygienic water that uses for dilution, dressing with ice, prolonged preservation without refrigeration are the influencing factors. Moreover, unhygienic surroundings often with swarming houseflies, fruits flies and airborne dust can also act as sources of contamination. Pathogenic microbes may enter in fruits during their growth and harvesting through damaged surfaces and punctures. Juices may be contaminated with microbes from raw material, juice machine, handler and unhygienic conditions. (Iqbal et al., 2015).The use of good quality fruits and surface disinfection besides cleaning with pure water will certainly improve the microbiological quality of these juices. For longer shelf-life and safety of the juices against fungi and molds, the use of an approved food additives could be another best option.

2. MATERIALS AND METHODS:

We have studied 105 various street vended fruit juice samples to draw the attention towards public health hazards. Samples were collected during December 2019 to March 2020.Samples of fresh fruit juices were picked up from at least 3 street vendors in each zone particularly from who's had sailing of at least 50-100 glasses/day. Various fruit juices like orange, pineapple, grapes, apple, mosambi, sugarcane, watermelon etc. were included in this study. Sample collection was done in sterile screw cap bottle and transported to the microbiology laboratory of Ramkrishan Institute. All samples were diluted 10 folds (1:10) with presterilized buffered peptone water and were filtered through

presterilized whatman No.1 filter paper to remove the solid particles. A loopful of sample was streaked aseptically on various generalized and selective media Nutrient agar, MacConkey agar, Mannitol salt agar (for bacterial agents) and Czapek-Dox agar (for fungal agents) for selective isolation and differentiation. Inoculated media were incubated at 37˚C for 24/48 hour for bacteria and 4-5 days/30˚C for fungi. Samples were observed and interpreted as positive in case of growth and negative in case of growth not observed. All isolates were identified and confirmed from their morphological, colonical, Growth and biochemical characterization using standard references (John G. Holt, Bergey`s manual of Determinative bacteriology, 11th edition, and Jean F. Macfaddin , Biochemical tests for identification of organisms, 3rd edition). Further, Antibiotic susceptibility was done for all isolates using Kirby Bauer Disc Diffusion method. Commonly used antimicrobials that were used for the study are listed in Tables.

Table 1 : Antimicrobial agents used for Bacterial isolates.

Agent	Potency (µg)	Symbol
AMPICILLIN/SULBACTAM	20	AS
CO-TRIMOXAZOLE	25	BA
PIPERACILLIN	100	PC
CEFOTAXIME	30	CF
CHLORAMPHENICOL	30	CH
CIPROFLOXACIN	5	RC
CEFTIZOXIME	30	CI
TETRACYCLINE	30	TE
OFLOXACIN	5	ZN
GENTAMICIN	10	GM
AMIKACIN	30	AK
GATIFLOXACIN	10	GK
CEPHALEXIN	30	PR
LEVOFLOXACIN	5	QB
LINEZOLID	30	LZ
CLOXACILLIN	1	CX
ROXYTHROMYCIN	15	AT
LINCOMYCIN	2	LM

Table 2: Antimicrobial agent used for Fungal Isolates.

Agent	Potency (mcg)	Symbol
FLUCONAZOLE	25	FLC
CLOTRIMAZOLE	10	CC
AMPHOTERICINB	100 unit	AP
NYSTATIN	100 unit	NS
KETCONAZOLE	10	KT
ITRACONAZOLE	10	IT

2.1. Detection of Extended Spectrum β-lactamases (ESBLs) producing microorganisms:

A well isolated colony of the test organisms was inoculated in nutrient broth and incubated at 37˚C for 18 hours for inoculum preparation. 0.1ml of culture was inoculated in previously prepared sterile M.H agar plate and spreaded properly. Then single antibiotic disc (Cefoperazone Sulbactum, Cefpodoxime) containing a known concentration of antibiotic were placed a part from each other and kept at room temperature for 20 minutes to permit the diffusion of antibiotics. After that incubation can be done at 37˚C aerobically for overnight then zone of inhibition were measured. The zone of inhibition around the Cefoperazone disc combine with Sulbactum was compared with the zone around the disc with Cefpodoxime alone. The test is positive if the inhibition zone diameter is ≤ 15 mm larger with sulbactum than without (CLSI 2012).

2.2. Detection of inducible clindamycin resistance (D-test):

Inoculum of well isolated colony of *staphylococcus aureus* from the plate incubated previously was prepared (0.5 MacFarland turbidity). 0.1ml of inoculum was spreaded over Muller Hinton Agar plates. After pre diffusion time of 15 minutes (at room temperature), the Clindamycin (CD) disc (2 mcg.) and Erythromycin (ER) disc (15 mcg.) were placed 15mm apart edge to edge manually with sterile forcep. Plates were incubated at 37˚C for overnight and the

plates were observed for the flattening of zone (D-shaped) around Clindamycin in the area between the two discs that indicated inducible clindamycin resistance (CLSI 2012).

2.3. Detection Of Biofilm Formation By Tube Method (TM):

10 ml Trypticase soya broth with 1% glucose was inoculated with a loopful of 24 hours old microbial culture and then incubated for 24 hours at 37˚C.After incubation all tubes were decant and washed with phosphate buffer saline (PBS) (pH 7.3) and drain properly and dried. Dried tubes were stained with 0.1 % safranin solution. Excess stain was removed and washed with distilled water. Tubes were than dried in inverted position and observed for biofilm formation. Results considered positive when a visible thick film lined the wall and bottom of the tube. If thin film were lined then it is considered as a weakly positive and no film line formation considered as negative result. (Christensen et al., 1982; Esteban et al., 2010).

2.4. Partial Molecular identification by 16S rRNA sequencing

Experimental Method:

DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-moleculer weight DNA has been observed. Fragment of gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose Gel. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 27F primer using BDT v3.1 cycle sequencing kit on ABI 3730x1 genetic analyzer. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs (saffron lifescience).

Phylogenetic analysis

The evolutionary history was carried out by the Neighbor-Joining method (Saitou et al., 1987). The optimal tree with the sum of branch length = 0.61301500 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches).The evolutionary distances were computed using the Maximum composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were total of 733 positions in the final dataset (Tamura et al., 2013).

3. RESULT AND DISCUSSION:

3.1. Positivity of samples:

In present study 105 fresh fruit juices samples were collected. Among all collected samples, 71.42% showed positivity with the presence of different etiological agents. Many of the samples were identified as polymicrobial. In accordance with our study, Sharma , 2013 and Uddin et al., 2017, reported, higher positivity.

3.2. Distribution of samples as per area of sample collection and type of sample:

From 105collected fruit juices samples, distributed as per typeof samples is represented below.

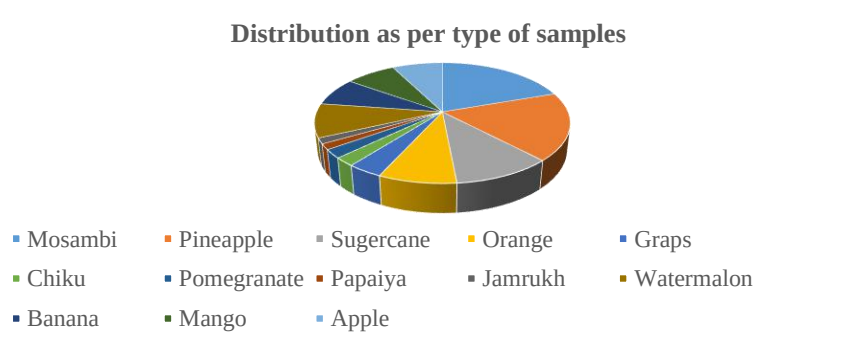


Figure1: Distribution as per the types of fruit juices samples.

3.3. Isolation and Identification of isolate:

Samples were collected, transferred to the microbiology laboratory of Shree RamKrishna Institute of Computer Education and Applied Sciences. Isolated and identification was done as per the standard microbiological procedurs. Sample were streaked on generalized media (Nutrient agar medium), differential and selective media such as (MacConkey's agar medium , Mannitol salt agar medium, Czapek Dox agarmedium). Then all isolate were

identified again from their morphological, colonical and biochemical characteristics using standard references (John G. Holt, Bergey's manual of Determinative bacteriology, 9th edition, and Jean F. Macfaddin, Biochemical tests for identification of medical bacteria, 3rd edition).

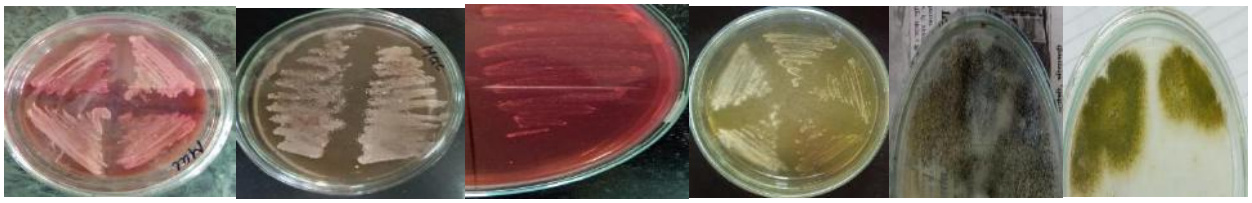


Figure 2 : Isolation of various etiological agents

From the results of various colonial and growth characterization (figure 2), total 120 isolate were observed with different colonical characterization. After their growth observed on selective/differential, generalized medium, isolate were studied for their morphological characterization and motility pattern by gram reaction and picric acid mounting and motility test by hanging drop method.

3.4. Distribution of Isolates as per Gram Reactions:

We reported the presence of 35 gram positive isolates and 75 as gram negative and 10 case of fungi. Further, among gram negative rods 51 observed as motile while 24 isolates as non –motile short rods. Our results are in accordance with Sharma, 2013.

4. DISTRIBUTION OF ISOLATES AS PER ETIOLOGICAL AGENTS:

From the result of morphological, colonical and growth characterization, isolates were successfully identified. The most predominant gram positive etiological agent we found in bacterial cases was *Staphylococcus aureus* followed by gram negative *Enterobacter spp.* and in fungal cases, *Aspergillus niger*. In contrast to our study, Reddi et al., (2015) reported *Staphylococcus aureus* as the most predominant gram positive etiological agent

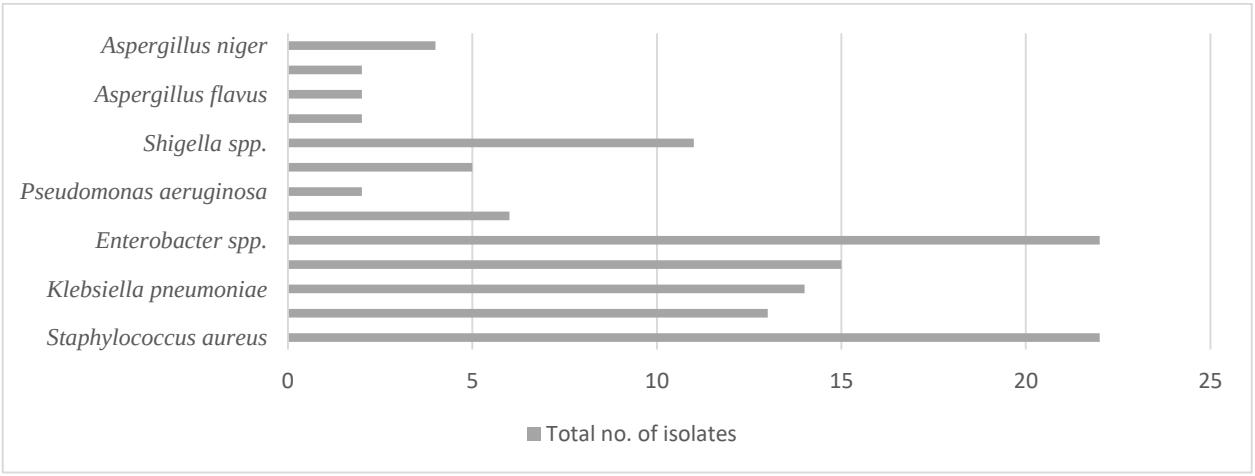


Figure 3: Distribution of different etiological agent as per frequencies.

5. ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF ISOLATES:

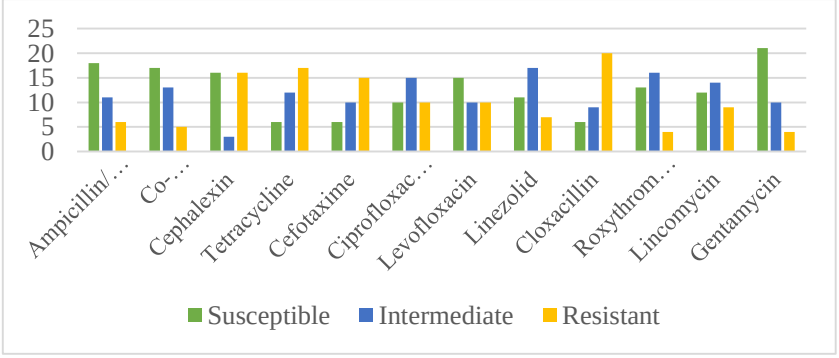


Figure 4: Resistogram of Gram positive isolates

Antibiotic susceptibility test were performed by Kirby-Bauer disc diffusion method. The results were interpreted as per guidelines provided along with the susceptibility disc. For gram positive isolates, Uddin et al., 2017 reported highest resistance against NETILMICIN (90%) followed by AMPICILLIN (84%) while we observed against the CLOXACILLIN and TETRACYCLIN.

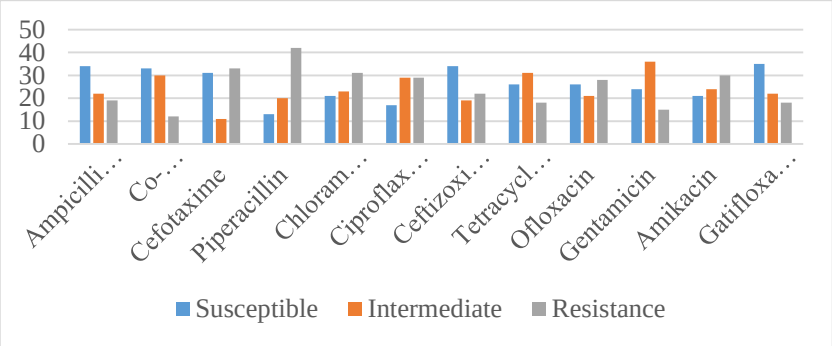


Figure 5: Resistogram of gram negative isolates.

In the present study, among the Gram negative isolates, higher resistance was observed against PIPERACILLIN and CEFOTAXIME. In contrast, Uddin et al., 2017 reported highest resistance against NALISIXIC ACID and GENTAMICIN (98% and 72 % respectively).In cases of fungal isolates, we observed higher resistances against CLOTRIMAZOLE

6. DETECTION OF MULTIDRUG RESISTANCE (MDR):

Multidrug resistant is antimicrobial resistant shown by a species of microorganism to multiple antimicrobial drugs. Such microorganism mostly result into therapeutic failure an even spread the resistant among other species of bacteria by horizontal gene transfer. In our study highest multidrug resistances found (73%) in gram negative isolates while in gram positive (23%) and in fungi (4%).

DETERMINATION OF ESBL (EXTENDED-SPECTRUM BETA-LACTAMASES) PRODUCES

ESBL is enzyme that confer resistances to most beta-lactamase antibiotics. ESBL producers are most commonly associated with therapeutic failure and therefore poor out come of contamination. All gram negative isolates were tested for ESBL production. As represented, higher percentage of isolates showed the presence of ESBLs.

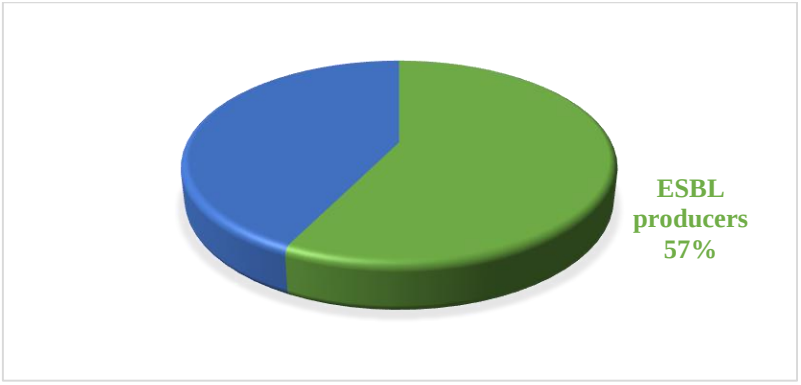


Figure 6 : Determination of ESBL (Extended-spectrum Beta-lactamases) producers

DETECTION OF INDUCIBLE CLINDAMYCIN RESISTANCE (D-TEST)

We performed D-test for gram positive isolates to analyze their sensitivity against clindamycin and to check if there is a macrolid-resistant subpopulation of bacteria present or not.

Table 3 :Susceptibility of Erythromycin & Clindamycinamong all *S. aureus* isolates

Susceptibility pattern (phenotype)	No. of isolates
ERY-S, CL-S	13

ERY-R, CL-R (constitutive, MLSB)	8
ERY-S, CL-R (D-test positive, iMLSB)	10
ERY-R, CL-S (D-test negative, MS)	4

(ERY= Erythromycin, CL= Clindamycin, R= Resistant, S= Sensitive, Constitutive MLSB= Constitutive MLSB phenotype, iMLSB= Inducible MLSB phenotype, MS= MS phenotype).

DETECTION OF BIOFILM FORMING CAPABILITIES OF ISOLATES

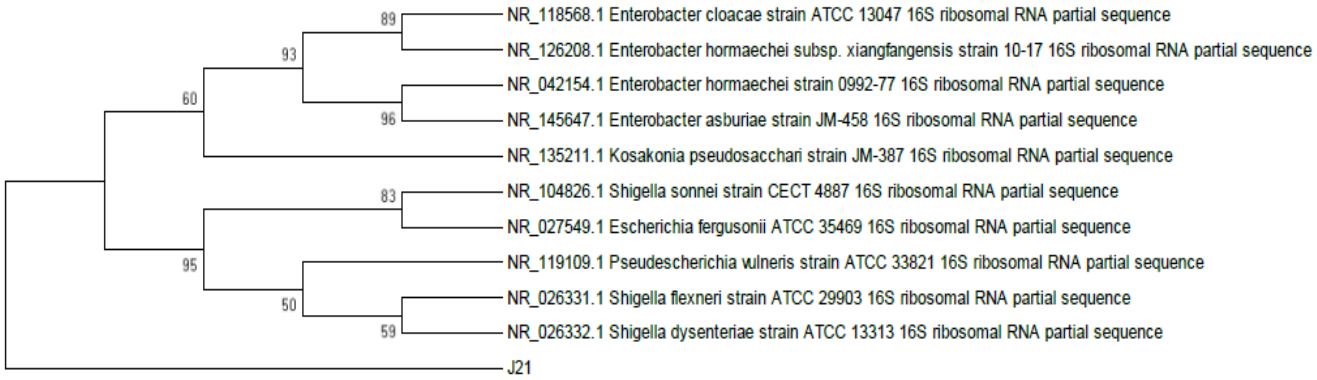
A biofilm, EPS, which is also referred to as slime may produced by many microorganisms and is one of the important virulence factor. All isolates were tested for their biofilm production capabilities by tube method. As represented, majority of the isolates were identified as higher producers

Figure 7. Biofilm producers by Tube method

7. MOLECULAR IDENTIFICATION BY 16S rRNA

In Partial Molecular Identification of the the isolate J21 was compared for homology sequence contained within large database using BLAST tool of NCBI. Partial sequence of the isolate showed identity with 16S rRNA Partial sequence of *Enterobacter cloacae* strain and identified as *Enterobacter cloacae.* From the antibiotic susceptibility patterns and biofilm production capabilities Isolate No. J21 screened for partial sequencing (16S rRNA Sequence) and phylogenetic analysis. It was carried out at Saffron Lifescience, Surat.

Phylogenetic Analysis



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