ORIGINAL RESEARCH PAPER
Volume-6 | Issue-11 | November-2017 | ISSN No 2277 - 8179 | IF : 4.176 | IC Value : 78.46
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

PREVALENCE OF MULTI-DRUG RESISTANT BACTERIA IN BETEL (PIPER BETEL L) LEAF-WASHED-WATER OF THE ROAD SIDE 'PAAN' STALL IN NORTHERN WEST BENGAL

Biological Science

Swapan Kumar Chowdhury Department of Botany, Sreegopal Banerjee College, Bagati, Mogra, Hooghly, Pincode-712148, West Bengal, India.

Dilip Hore Dalanath High School, Jalpaiguri, West Bengal, India.

Sujit Kumar Mahanta Indranarayanpur High School, Gangarampur, West Bengal, India.

Brinda Adhikari Department of Biological Sciences, University of Manitoba, Winnipeg, MBR3T2 N2,Canada.

Mahua Mukherjee RT Girls High School, Suri, West Bengal, India.

Shriparna Mukherjee Department of Botany, P.D. Womens' College, Jalpaiguri, West Bengal, India.

Ranadhir Chakraborty* Department of Biotechnology, University of North Bengal, Raja Rammohanpur, P.O.-NBU, Dist- DarjeelingW.B., India. *Corresponding Author

ABSTRACT

Bacterial pathogens causing acute diarrheal diseases and enteric fever (typhoid) are the most prevalent microbial contaminants of water and water drenched fresh produce in India. Fresh betel leaf is chewed and consumed as raw by a large proportion of Indian population. People buy betel leaf as 'paan' from non-hygienic road-side stalls for direct consumption. Betel leaves are always drenched with contaminated water, as an outcome, can be a widespread medium of transmission of enteropathogenic bacteria. The present study was aimed to determine level of enteric bacterial contamination in betel leaf-washed-water, and assess the antibiotic resistance profiles (ARPs) of the isolates. The principal objective was to reveal the presence of antibiotic-resistant enteric bacteria and document their resistance pattern in order to create awareness among the authorities of the regulatory bodies concerned with public health and hygiene and 'paan' consumers in general. Mean Probable Number (MPN) tests confirmed the presence of total coliform (TC), fecal coliform (FC) in each of the 'paan'-washed-water samples tested. Type of enteric bacteria and their abundances were determined by dilution plating on different selective plates. Heterotrophic bacterial count in the water samples ranged from 5.85 x 10^5 to 2.78 x 10^6
c.f.u. ml^{-1}. Bacteria resistant to all the five antibiotics tested were found in three of five samples. Presence of significantly small proportion of antibiotic-sensitive bacteria in the water samples (one of them contained no sensitive bacteria) is considered to be very alarming.

KEYWORDS

Betel leaf wash water, Road side Pan Stall, Pathogenic Bacteria, Multiple-antibiotic- resistant bacteria, coliform bacteria.

1. Introduction

The *Piper betel* L. (Betel leaf) is the leaf of a vine belonging to the Piperaceae or the Black pepper family (Gunther, 1952), which includes pepper and Kava. An excellent mouth freshener, the deep greenyoung heart shaped leaves of Betel (*Piper betel* L.) vines are popularly known as 'Paan' in India. It is related with every human life. This edible leaf is traditionally used for chewing as mouth-freshner in their natural raw condition. The active constitution of betel oil belongs to the primarily class of benzene compounds. Though particular emphasis has been placed on chavibetol (betel-phenol; 3-hydroxy-4-methoxyallylbenzene), it also contains chavicol (p-allyl-phenol; 4-allyl-phenol), estragole (p-allyl-anisole; 4-methoxy-allylbenzene), eugenol (allylguaiacol; 4-hydroxy-3-methoxy-allylbenzene; 2-methoxy-4-allyl-phenol), methyl eugenol (eugenol methyl ether; 3,4-dimethoxy-allylbenzene), and hydroxycatechol (2,4-dihydroxy-allylbenzene) (CSIR,1984). Despite these, there are several terpenes and terpenoids found in the betel oil. There are two mono-terpenes, p-cymene and terpinene, and two mono-terpenoids, eucalyptol and carvacrol, and two sesquiterpenes, cadinene and caryophyllene. Fathilah et al. (2000) have reported that the crude aqueous leaf extract of Piper betle L. exhibits antibacterial activity towards *Streptococcus mitis, Streptococcus sanguis* and *Actinomyces viscosus*, some of the early colonizers of dental plaque.

'Paan'chewing is much popular among the village people than the urbanites. The most probable place of origin of betel vine is Malaysia (Chattopadhyay and Maity, 1967) and also grows in South and South –East Asia (India, Sri Lanka and Bangladesh). There are about 100 varieties of betel vine available throughout the world, of which about 40 varieties are found in Indian sub-continent and about 30 varieties are reported from West Bengal (Guha 1997, 2006; Maity 1989; Samanta 1994).. It grows best under the shaded, tropical forest ecological conditions with humidity and temperature ranging from 40-80 % and 15-40 °C, respectively. A well-drained fertile sandy or sandy loam or sandy clay soil with pH range of 5.6 –8.2 is considered suitable for its cultivation (CSIR, 1969; Guha and Jain, 1997). However, in the areas with lower rainfall (1500 – 1700 mm) the crop is cultivated with small and frequent irrigations, i.e. every day in summer and every 3-4 days in winter, whereas adequate drainage is required during the rainy season (Jana, 1995; Mishra et al., 1997). In different corner of the country it is also known as Nagaballi, Nagurvel, Saptaseera, Sompatra, Tamalapaku, Tambul, Tambuli, Vaksha Patra, Vettilai etc (CSIR, 1969; Guha, 1997). The best betel leaf is the "Magadhi" variety (from the Magadha region) grown near Patna in Bihar, India. In Kerala, the famous variety of betel leaf is from Venmony near Chengannurand it is called "Venmony Vettila". Betel leaf cultivated in Tirur,in Kerala, and Hinjilicut in Odisha are of fine quality. Betel leaves exported from Tirur are famous in Pakistan as "Tirur 'paan'". The vast economic potentiality of the crop can be adequately established by the fact that about 15-20 million people consume betel leaves in India on a regular basis (Jana, 1996) besides those in other countries of the world which may include over 2 billion consumers. Further, as far as the national employment generation is concerned, it is estimated that about 20 million people derive their livelihood directly or indirectly, partly or fully from production, processing, handling, transportation and marketing of betel leaves in India, which includes about 5 million workers from West Bengal (Jana, 1995; Jana, 1996).

The two-key current health problem facing the under-development countries are poor nutrition with in adequate water supply and sanitation. It is this problem that underlay the poor condition of the host

and hence high level of infectious diseases. It has been estimated that as much as 80 % of diseases in under developed countries are associated with water. Few people have access to an adequate quantity or quality of water supply or to an effective sewage disposal system. These applies to both crowded urban and the rural areas and the result is the high level of fecal matter contamination and related diseases such as hookworm, cholera and chronic dysentery. The unhygienic condition under which people live produces adverse effect on the health of the individuals. Generally speaking, the clerical staff, office bearers, porters, cart pullers and others are more liable to enteric diseases as they often take food and drinks outside as well as accustomed to chewing 'paan' from road side 'paan' stalls and there by become more prone to infection. Very often 'paan' sellers do not follow the general guidelines of personal cleanliness, thus creating conditions favorable for the transmission and proliferation of water borne diseases. The negligence to follow the general rule of personal hygiene is not entirely due to ignorance, but also due to the traditional habits and customs of the people. Due to scarcity of filtered water in summer, people have to use unfiltered water for cleaning purposes. The common practice of washing 'paan' leaves with the contaminated water may also help in the spread of enteric diseases among the consumers.

Due to the serious implications of consuming contaminated 'paan', the present work was aimed to conduct a pilot survey on 'paan'-washed-water samples from five locations in North Bengal.

MATERIALS AND METHODS

Sample collection:

The water samples were collected from road side 'paan' stalls of five different localities, namely, Islampur, Bagdogra, Shivmandir, Jalpaiguri and Coochbehar of Northern part of West Bengal. For collection of water used for soaking betel leaves, sterilized bottles were used. During water collection from the tumbler, the bottles were opened under water (at a depth of 15 – 30 c.m.), rinsed thoroughly with the sample water and were half filled by opening and closing the bottles underneath. The collected samples were transported to the laboratory in ice box. Analyses were performed within 1 to 8 hours after collecting the samples.

Determination of most probable number (MPN Test):

Most Probable Number (MPN) is a method used to estimate the concentration of viable microorganisms in a sample by means of replicate liquid broth growth in ten-fold dilutions. LB medium was prepared in single (1X) and double (2X) strength concentrations. 2X LB medium was dispensed in five tubes (10ml in each tube) and 1X medium was dispensed in ten tubes (10 ml in each tube) and a Durham tube was inserted in each tube in inverted position. Five 2X tubes and ten 1X tubes were taken for each water sample to be tested.

Using a sterile pipette, 10 ml of water was added to each of the five tubes containing 2X LB medium. Similarly 10 ml of water was added to each of the five tubes containing 1X LB medium and 0.1ml of water to remaining five tubes containing 1X LB medium. All the tubes were incubated at 37°C for up to 48 hrs. The numbers of tubes giving positive results were compared with a standard chart and the result was scored.

Preparation of antibiotic stock solutions and antibiotic plates:

Antibiotic stock solutions were prepared by dissolving measured amounts of respective antibiotic to its suitable diluents. Antibiotic powders were weighed to 0.1mg accuracy; liquids were quantified by micropipette. The antibiotics used were ampicillin (100µg ml^{-1}), chloramphenicol (100µg ml^{-1}), kanamycin (50µg ml^{-1}), streptomycin (100µg ml^{-1}) and tetracycline (20µg ml^{-1}). The desired concentrations of the antibiotics (diluted from the stock) were stirred into the melted agar at approximately 45°C and immediately poured into Petri-dishes. These LB agar plates containing standard concentration of a respective antibiotic were stored at 4°C and were used within seven days of preparation.

Enumeration of total heterotrophic load, and determination of abundances of enterobacteria in selective plates:

Total heterotrophic load of bacteria in Betel leaf-washed water samples was enumerated by standard plate count (SPC) method. The standard plate count values provide density of aerobic and facultative bacteria in water sample which can grow at 37 0C. Serial dilutions of water samples were made in sterile 0.5% NaCl solution, which served as diluents of a known volume. Once diluted, 0.1 ml of the suspension was spread uniformly on Luria Bertani (LB) agar plates with a glass spreader. The plates were incubated at 37°C overnight. The standard bacterial count of the suspension was obtained by multiplying the number of colonies per plate by reciprocal of the dilution.

Selective microbiological media, obtained from HiMedia India, were used for the isolation of different enterobacterial isolates like *Escherichia coli, Salmonella sp., Shigella sp., Enterococcus* sp. (especially representatives of the genera involved in causing diarrhea, dysentery and typhoid fever). The media used were ENDO-agar, EMB-agar, McConkey agar, Xylose Lysine Deoxycholate agar (XLD), Leifsons Deoxycholate agar (LDA), Pfizer Selective Enterococcus agar (PSE) and Bismuth Sulphate Agar (BSA). ENDO – Agar is recommended for the confirmation of the presumptive- test for members of the coliform group. Tubes of liquid media showing positive ('+'ve) presumptive reaction for the presence of coliform bacteria were sub-cultured on two ENDO –Agar to observe lactose fermenting coliform red colonies with metallic sheen; and colourless colonies for the lactose non – fermenters. EMB-Agar was used for the isolation and differentiation of Gram negative enteric bacilli. The MAC- Conkey medium was used for coliform counts. XLD agar was used for selective isolation and enumeration of *Salmonella typhi* and other *Salmonella* sp. LDA medium was used for selective isolation and differentiation of *Salmonella* species and *Shigella* species. PSE agar was used for selective isolation and cultivation of *Enterococci*. BSA was used for isolation and preliminary identification of *Salmonella* typhi and others Salmonella sp.

0.1 ml aliquots from serially diluted tubes of the water samples (from the same dilution series of the samples described above) were spread onto these selective agar plates and incubated overnight at 37°C for getting the total count of specific bacterial isolates.

.

Determination of multiple antibiotic resistance(s) (MAR) profile of the isolates appeared on the selective plates:

The bacterial colonies that appeared on seven different selective agar plates (ENDO-agar, EMB-agar, McConkey agar, Xylose Lysine Deoxycholate agar, Leifsons Deoxycholate agar, Pfizer Selective Enterococcus agar and Bismuth Sulphate Agar) were picked up randomly with sterile tooth picks and transferred to gridded LB agar plates containing no antibiotic. These plates were considered as master plates and were incubated for 24 hours at 37°C. These master plates were then replicated onto LB agar plates, each containing a single antibiotic of desired concentrations. The replicated plates were incubated at 37°C for approximately 24 hours and drug resistance was determined. The isolates were considered resistant to multiple antibiotics, only if their growth on the presence of antibiotic were as well developed as their growth on the control plates. Any sign of inhibition or sensitivity was considered to be indicative of nonresistance

3. Result & Discussion:

During this study, 'paan'-washed water samples (from the tumbler where 'paan' is being washed before it is prepared and given to the customers) were collected. The collected samples were analyzed to evaluate the incidence of fecal coliform and fecal Enterococci. Besides calculating the MPN value and total heterotrophic bacterial load, the presence of specific pathogenic organism in different selective plates were determined. The present study has also undergone experiments to determine the antibiotic resistance profile(s) of the isolates.

3.1. MPN Test for determining Total Coliform (TC), Fecal Coliform (FC), and Fecal Streptococci (FS) count of the 'paan'-washed water sample:

In this study, a total of five samples have been collected from Shibmandir (sample-1), Bagdogra (sample-2), Jalpaiguri (sample-3), Islampur (sample-4) and Siliguri (sample-5). The collected samples were analyzed in the laboratory for MPN test. Statistical tables (MPN tables) are utilized to determine the number of bacteria present and the range in the 95% confidence interval based on the number of positive culture tubes. The results obtained were as follows:

Sample- 1 and 5: Results have shown positive (combination: 5-5-5)

for TC and FC but in case of FS the positive combination was 5-4-2. The MPN index for TC and FC against 5-5-5 combination is ^{3}1600 and 95% confidence limit in lower and upper shows nil. But in case of FS, MPN index is 220 and 95% confidence limit in lower and upper shows 100, 580 respectively.

Sample-2 and 3: Results have show positive (combination: 5-5-5) for TC, FC, and FS. The MPN index against 5-5-5 combination is ^{3}1600 and 95% confidence limit in lower and upper shows nil.

Sample- 4: Result has shown positive (combination: 5-5-5) for TC and FC but in case of FS the positive combination was 4-4-0. The MPN index for TC and FC against 5-5-5 combination is ^{3}1600 and 95% confidence limit in lower and upper shows nil. But in case of FS, MPN index was 34 and 95% confidence limit in lower and upper shows 16, 80 respectively.

The name "coliform" is given to a whole group of bacteria which can occur in water and indicate potential health problems. Total coliform (TC) constitutes all of the coliform bacteria while a fraction of it is Fecal coliforms (FC). Both TC and FC are the representatives of a large family of bacteria known as the enterics. However, most of the enterics do belong to the TC group, but very few belong to the FC group, which is considered much graver from the hygienic point of view. Fecal coliforms are more or less specialized types of bacteria and are dominated by *Escherichia* coli which colonizes healthy human intestine and passes out in good numbers in the fecal material. These can be counted in water by using the fecal coliform tests and the counts are usually given as FC cells in 100 ml of water. Human feces tend to have much more FC than Fecal Streptococci (FS). The FS tend to be more common in animal feces and so comparing the numbers of FC to FS (FC: FS ratio) is reflective of the source of contamination whether the water has been polluted with human fecal wastes (>2:1 ratio) or animal wastes (<1:1). The total coliform (TC) group contains a wider variety of bacteria including Escherichia coli and a broad spectrum of the enteric bacteria. These enteric bacteria are able to grow frequently in the intestine, but can also grow to a variable extent in the environment. In consequence, the TC count does not necessarily relate specifically to fecal pollution, but to the bacterial loading of enterics within the water source. Since the count is dominated by the bacteria which can occur in the intestine, it is used as a broader spectrum test for fecal pollution in a water system. From the five water samples tested, it can be inferred that all of them contained fecal bacteria.

3.2 Quantification of total heterotrophic load of collected 'paan'-washed water sample(s) and load of enterobacterial isolates that appeared on different selective plates:

The maximum and minimum counts of heterotrophic bacteria and cell densities obtained on PSE and MAC plates were obtained from sample 3 and 1 respectively (Table 1). Highest and the least density of bacteria on XLD, BSA, and LDA plates was observed in the sample 3 and 4 respectively (Table 1). Sample no. 1 and 5 have shown maximum and minimum counts respectively on EMB plates (Table 1). The manifestation of different types of enteric bacteria in the selective plates used in this study has presented in the Table. 2.

Table1: Total heterotrophic load of five different water samples and total count of enteric bacterial isolates appeared on different selective plates

Sample no.	Total Heterotrophic Load (c.f.u/ml)	XLD (c.f.u/ml)	BSA (c.f.u/ml)	LDA (c.f.u/ml)	PSE (c.f.u/ml)	ENDO (c.f.u/ml)	MAC (c.f.u/ml)	EMB (c.f.u/ml)
1	5.85×10^{5}	1.05×10^{4}	1.12×10^{4}	7.0×10^{3}	4.3×10^{2}	3.8×10^{3}	5.16×10^{4}	9.68×10^{4}
2	6.64×10^{5}	3.14×10^{4}	1.02×10^{5}	2.7×10^{4}	2.2×10^{3}	4.05×10^{5}	1.34×10^{5}	9.64×10^{4}
3	2.78×10^{6}	4.12×10^{4}	1.12×10^{5}	3.62×10^{4}	4.3×10^{3}	3.5×10^{3}	5.69×10^{5}	7.2×10^{4}
4	1.21×10^{6}	3.3×10^{3}	2.18×10^{2}	2.02×10^{3}	7.3×10^{2}	3.05×10^{4}	1.31×10^{5}	8.5×10^{4}
5	2.32×10^{6}	1.84×10^{4}	4.30×10^{3}	2.69×10^{4}	3.19×10^{3}	3.75×10^{4}	1.05×10^{5}	7.1×10^{4}

Table 2: Growth of specific bacterial isolates on selective media

Media	*E. coli*	*E. aerogens*	*K. pneumoniae*	*S. faecalis*	*S. aureus*	*S. typhi*	*Shigella sp.*
ENDO-Agar	+	+	-	-	-	-	-
EMB- Agar	+	+	+	-	-	-	-
Mc-Conkey Agar	+	+	+	+	+	-	-
XLD Agar	-	-	-	-	-	+	-
LDA Agar	-	-	-	-	-	+	+
PSE- Agar	-	+	-	-	-	-	-
BSA	-	-	-	-	-	+	-

3.3 Multiple antibiotic resistance profile (MRP) of the bacterial isolates appeared on the selective media

Analysis of Sample -1

BSA – Agar:

Altogether 75 bacterial colonies were picked up from the BSA plate and their resistance profile was determined following replica plating method. 4 isolates (5.33%) were resistant against all the antibiotics used in this study (ACKST), 5 isolates (6.66%) exhibited quadruple resistance (ACST and ACKT), 26 isolates (34.66%) were resistant to three different antibiotics (ACT and AST), 18 (24.0%) exhibited double resistance (AT, AC. AS and CT), 19 isolates (25.33%) were found to resist only one antibiotic (A and S) and 3 isolates (4.0%) were sensitive to all antibiotics used in this study (Fig. 1).

XLD – Agar:

A total of 73 isolates were picked up from XLD agar plate. 6 isolates (8.21%) exhibited quintuple resistance (ACKST), 38 isolates (52.05%) exhibited resistance to 4 different antibiotics (ACST and ACKT), 24 isolates (32.87%) were resistant to three different antibiotics (ACT and AST) Triple, 4 isolates (5.47%) exhibited resistance to two different antibiotics (AT, AC. AS and CT). Only a single sensitive isolate was obtained (Fig. 1).

LDA – Agar:

A total of 83 isolates were screened for multiple antibiotic resistance profile. Only one isolate was quintuply resistant (ACKST), 11 isolates (13.25%) were quadruply resistant (ACST and ACKT), 68 isolates (81.92%) were triply resistant (ACT and AST), 2 (2.40%) were double resistant (AT, AC. AS and CT) and single isolate (1.20%) responded to single antibiotic (Fig. 1).

MAC – Agar:

From McConkey agar plate 77 isolates were screened for multiple antibiotic resistance profile determination. Single isolate was quintuple (ACKST) resistant, 23 isolates (29.87%) exhibited quadruple (ACST, ACKT) resistance, 40 isolates (51.94%) exhibited resistance triply (ACT, AST), 5 (6.49%) were doubly resistant (AT, AC, AS and CT), 2 (2.59%) were single resistant (A and S) and 6 (7.79%) isolates were found sensitive (Fig. 1).

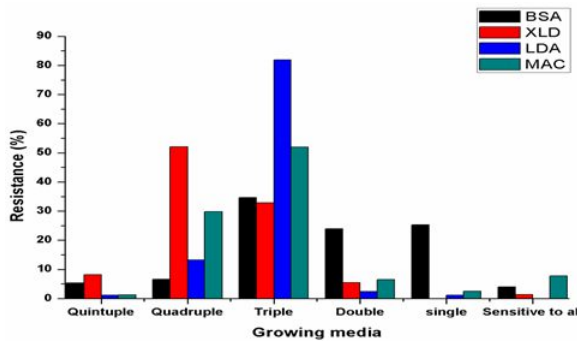

Fig. 1: Graphical representation of resistance with antibiotic combinations at different growth media with isolates derived from sample1

Analysis of Sample -2

MAC – Agar:

From McConkey agar plate 71 isolates were screened for multiple

antibiotic resistance profile determination. 51 isolates (71.83 %) exhibited resistance triply (ACT, AST), 8 (11.26 %) were doubly resistant (AT, AC, AS and CT), 2 (2.81%) were single resistant (A and S) and 10 (14.08 %) isolates were found sensitive. Here quintuply and quadruply resistant isolates were not found (Fig. 2).

BSA – Agar :
Altogether 74 bacterial colonies were picked up from the BSA plate and their resistance profile was determined following replica plating method. 48 isolates (64.8 %) exhibited quadruple resistance (ACST and ACKT), 15 isolates (20.27 %) were resistant to three different antibiotics (ACT and AST), 2 (2.70 %) exhibited double resistance (AT, AC. AS and CT), 6 isolates (8.10 %) were found to resist only one antibiotic (A and S) and 3 isolates (4.05 %) were sensitive to all antibiotics used in this study (Fig. 2).

XLD – Agar:
A total of 74 isolates were screened for multiple antibiotic resistance profile. 22 isolates (%) were triply resistant (ACT and AST), 44 (59.45%) were double resistant (AT, AC. AS and CT) and 3 isolates were sensitive against all five antibiotics used in this study. None of the isolates were quintuply or quadruply resistant (Fig. 2).

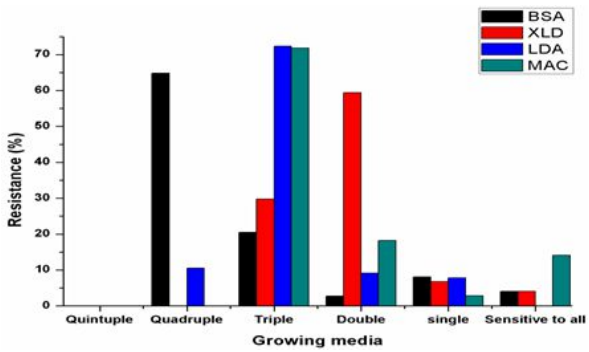

Fig. 2: Graphical representation of resistance with antibiotic combinations at different growth media with isolates derived from sample 2.

Analysis of Sample 3
MAC – Agar:
From McConkey agar plate 77 isolates were screened for multiple antibiotic resistance profile determination. Single isolate was quintuply (ACKST) resistant, 52 isolates (67.40%) exhibited resistance triply (ACT, AST), 13 (16.88 %) were doubly resistant (AT, AC, AS and CT), 9 (11.68 %) were single resistant (A and S) and 2 (2.59 %) isolates were found sensitive. None of the isolates were quadruply resistant (Fig. 3).

BSA – Agar:
Altogether 76 bacterial colonies were picked up from the BSA plate and their resistance profile was determined following replica plating method. 4 isolates (5.26 %) were resistant against all the antibiotics used in this study (ACKST), 3 isolates (3.94 %) exhibited quadruple resistance (ACST and ACKT), 30 isolates (39.47 %) were resistant to three different antibiotics (ACT and AST), 13 (17.10 %) exhibited double resistance (AT, AC. AS and CT), 11 isolates (14.47 %) were found to resist only one antibiotic (A and S) and 19 isolates (25 %) were sensitive to all antibiotics used in this study. None of the isolates were quintuply resistant (Fig. 3).

LDA – Agar:
A total of 75 isolates were screened for multiple antibiotic resistance profile. Two isolates (2.66 %) were quintuply resistant (ACKST), 4 isolates (5.33 %) were quadruply resistant (ACST and ACKT), 30 isolates (39.9 %) were triply resistant (ACT and AST), 16 (21.33 %) were double resistant (AT, AC. AS and CT) and 23 isolates (30.66 %) responded to single antibiotic (Fig. 3).

XLD – Agar:
A total of 73 isolates were picked up from XLD agar plate. 40 isolates (51.89 %) were resistant to three different antibiotics (ACT and AST) Triple, 24 isolates (32.87 %) exhibited resistance to two different antibiotics (AT, AC. AS and CT). 10 isolates (13.69 %) were found to resist single antibiotic. Three sensitive isolates were obtained. None of the isolates were quintuply or quadruply resistant (Fig. 3).

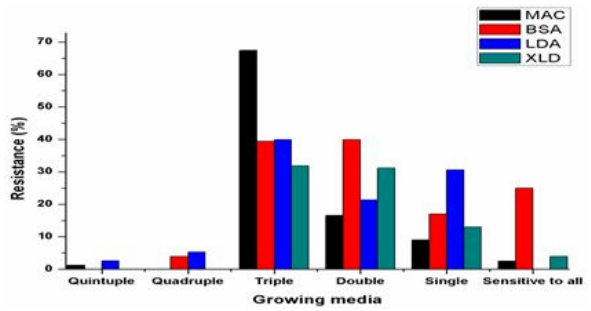

Figure-3: Graphical representation of resistance with antibiotic combinations at different growth media with isolates derived from sample 3

Analysis of Sample 4
MAC – Agar:
Total of 32 isolates were screened for the presence of multiple antibiotic resistance profile. 14 isolates (43.29%) were triply resistant. A single isolate exhibited Quintuple resistance (ACKST), 5 (15.62%) were quadruply resistant, 9 (28.12 %) doubly resistant and 3 isolates (9.37 %) exhibited single resistance.

BSA – Agar:
39 isolates were screened. 14 isolates (41.17%) were triply resistant (ACT and AST), 11 isolates () were found to resist two different antibiotics (AT, AC. AS and CT) and only 9 isolates were singly resistant (A and S). None of the isolates were quintuply or quadruply resistant. Isolates, sensitive to all tested antibiotics were also absent.

LDA – Agar:
Of the 20 isolates screened, 8 isolates (40 %) were found to resist two different antibiotics (AT, AC. AS and CT). 20 % (4 isolates) of the screened isolates were quintuply (ACKST) resistant. Two isolates were triply resistant (ACT and AST) and 6 isolates exhibited resistance to single antibiotic (A and S).

XLD – Agar:
Altogether 67 isolates were screened and multiple resistance profile was scored. 82% (55 isolates) of the isolates exhibited resistance to single antibiotic (A and S) and 17.91 % (12 isolates) were doubly resistant (AT, AC. AS and CT).

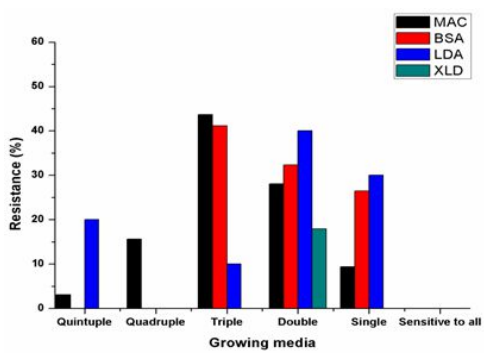

Figure-4: Graphical representation of resistance with antibiotic combinations at different growth media with isolates derived from sample 4.

Analysis of Sample-5:
MAC – Agar:
Total 30 isolates were screened. 14 isolates (46.6%) were triply resistant (ACT and AST), 12 isolates (%) were doubly resistant (AT, AC. AS and CT) and only 4 were resistant to four different antibiotics (ACST and ACKT).

BSA – Agar:
Total of 61 isolates were screened. 59 isolates (96.7%) were triply

resistant (ACT and AST). Single isolate was quadruply resistant (ACST and ACKT) and single isolate showed resistance to two different antibiotics (AT, AC. AS and CT).

LDA – Agar:

Altogether 20 isolates were screened. 8 isolates (40%) were doubly resistant (AT, AC. AS and CT), single isolate was quadruply (ACST and ACKT) resistant, 7 isolates (35 %) were triply resistant (ACT and AST) and 4 (20%) were resistant to singly antibiotic (A and S).

XLD – Agar:

Fifty isolates were screened. 17 isolates (56%) were quadruply resistant, 4 isolates were singly resistant.

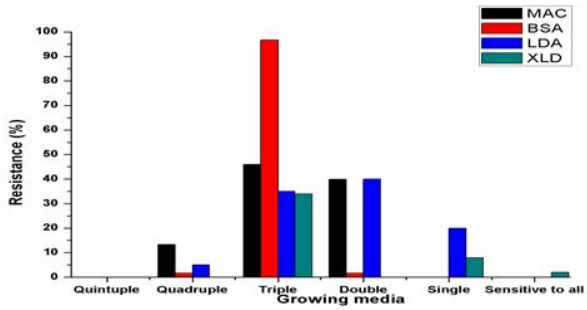

Figure-5: Graphical representation of resistance with antibiotic combinations at different growth media with isolates derived from sample 5.

In India, the average number of cases reported per year, for acute diarrheal diseases during the years 2011 -2013, was 1,08,98,435; of which 19,06,047 y^{-1} (17% of the total) was from West Bengal. The average number of deaths per year resulting from for acute diarrhoeal diseases in India and West Bengal in particular, during the same period, was 1,484 and 290 respectively. Deaths due to enteric fever (typhoid) have come down to <400 y^{-1} in India, but average number of cases reported per year, during the period - 2011-13, was 13,59,087 of which 1,26,351 cases y^{-1} are from West Bengal (pib.nic.in /newsite/printRelease.aspx?relid=106612). Bacterial infections cause the more serious cases of diarrhea. Typically, infection with bacteria occurs after eating contaminated food or drinks (food poisoning). Chewing raw 'paan' contaminated with bacteria, as demonstrated in this study, could be an important cause for bacterial diarrhea among paan chewers of West Bengal. Treatment of diarrheal patients with antibiotics would be more cumbersome if the infection is caused by the multiple-antibiotic-resistant pathogen. In this study, sample 4 (collected from Islampur) possessed the greatest risk as it was devoid of any sensitive bacteria (Fig. 4). Quintuple-antibiotic-resistant bacteria (resistant to all the five antibiotics tested) was not found in the samples 2 and 5; while the other samples were found, although in lesser quantities compared to representations of quadruple/ triple/ doubly-antibiotic resistant enteric bacteria, to bear bacteria resistant to all the antibiotics used in this study (Fig. 1 – 5). Hence, the study reveals the extent of microbial contaminations in 'paan' and emphasizes for an intervention by the public health regulatory bodies of India that looks after the food safety and adulterations.

4. Conclusion:

From this study it is concluded that the water used by 'paan' vendors of road side 'paan' stall is contaminated with many antibiotic resistant pathogenic bacteria like *Salmonella typhi, S. faecalis, S. aureus and E. aerogenes, Shigella sp., K. pneumoniae, E. coli, P. mirabilis* and Enterococci from different 'paan' wash water sample. Selective media were used to isolate and identify putative pathogenic bacteria from the 'paan'-washed-water samples. The present study also emphasizes the danger of transmission of potentially pathogenic bacteria from 'paan' drenched with contaminated water to human beings in causing various water-borne-diseases.

Acknowledgement: The authors are grateful to the University of North Bengal for providing grant for the purchase of consumables for this research.

REFERNCES

[1] Gunther E. The Essential Oil, New York; D Van Nostrand & Co. Inc., 1952; 5:160-161.
[2] Guha P. Paan Theke Kutir Silpa Sambhabana, (In Bengali). Exploring Betel Leaves for Cottage Industry, In: Krishi Khadya-O- Gramin Bikash Mela –A Booklet published by the Agricultural and Food Engineering Department, IIT, Kharagpur, India, 1997; 15-19
[3] Guha P. Betel leaf: the neglected green gold of India, Agricultural and Food Engineering Department, IIT, Kharagpur, India. J.Hum. Ecol, 2006; 19 (2): 87-93
[4] Maity S. Extension Bulletin: The Betelvine, All India Coordinated Research Project on Betelvine, Indian Institute of Horticultural Research, Hessarghatta, Bangalore, India, 1989.
[5] Samanta C, Paan chaser samasyabali-o-samadhan: Ekti samikkha (In Bengali), A Report on the Problems and Solutions of Betel Vine Cultivation. A booklet published by Mr. H. R. Adhikari, C-2/16, Karunamoyee, Salt Lake City, Kolkata-64 (WB), India, 1994.
[6] INDIA. (CSIR), The Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products - Raw Materials Vol viii: ph-re. India: Council for Scientific and Industrial Research (CSIR), 1969; 394.
[7] Guha P, Jain RK. Status Report on Production, Processing and Marketing of Betel Leaf (Piper betle L.), Agricultural and Food Engineering Department, IIT, Kharagpur, India, 1997.
[8] Jana BL. Gram Banglar Arthakari Phasal-Paan (In Bengali). Betel Leaf: A Cash Crop of Villages of Bengal. Asaboni, Flat 203, 184, B. B. Chatterji Road, Calcutta, 1995.
[9] Jana B.L. Improved technology for betel leaf cultivation. A paper presented in the "Seminar-cum-Workshop on Betel Leaf Marketing", held at State cashew nut farm, Directorate of Agricultural Marketing, Digha, Midnapur (W. B.), India, June 5-6,1996.
[10] CSIR. The wealth of India, 1984; 8-87.
[11] Mishra D, Mishra SK, Acharya A, Das JN. Annual Report of All India Coordinated Research Project on Betelvine. Department of Plant Pathology, College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar, India, 1997.
[12] Fathilah AR, Bakri MM, Rahim ZHA. Pascasidang Simposium Sains Kesihatan Kebangsaan ke-3. Fakulti Sains Kesihatan Bersekutu, UKM; 2000: 216-219.
[13] Chattopadhyay SB, Maity S. Diseases of Betelvine and Spices.ICAR, New Delhi, 1967.
[14] Tong Z, Sadowsky MJ. A selective medium for the isolation and quantification of Bradyrhizobium japonicum and Bradyrhizobium elkanii strains from soils and inoculants, Appl. Environ. Microbiol, 1994; 60-581.
[15] Endo. S, Zentralbl, Bakteriol.Abt. 1, Orig, 1904; 35-109.
[16] Levin M, Schoenlein H.W. A compilation of culture media for the cultivation of microorganisms.The Williams & Wilkins, Baltimore, Md; 1930.
[17] Clesceri L.S, Greenberg A.E, Eaton A.D. Standard methods for the examination of water and wastewater, 20th ed. Washington, D.C, American Public Health Association; 1998.
[18] Marshall R.T. Standard methods for the examination of dairy products, 16th ed. Washington, D.C, American Public Health Association; 1992.
[19] APHA, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, American Water Works Association and Water Pollution Control Federation, 20th ed., Washington; 1998.
[20] Marshall R.T. Standard methods for the examination of dairy products, 16th ed. Washington, D.C, American Public Health Association; 1993.
[21] Downes, Ito ED. Compendium of methods for the microbiological examination of foods, 4th ed. Washington, D.C, American Public Health Association; 2001.
[22] Baron, Spilman, Carey. Bacteriol. Proc. 59th Gen. Meet. Soc. Am. Bacteriologists, Abstr. G7; 1959: 29.
[23] Luis M, De LA, Maza, Pezzlo, Marie T, Janet T, Shigei, Peterson, Ellena M. Color Atlas of Medical Bacteriology, Washington, D.C, ASM Press; 2004 :103.
[24] APHA, American Public Health Association, Compendium of Methods for the microbiological Examination of Foods. - 3rd Ed; 1992.
[25] Bhat P, Raina D. Comparative evaluation of deoxycholate citrate medium and xylose lysine deoxycholate medium in the isolation of shigellae. Am. J. Clin. Pathol, 1975; 64-99.
[26] Dunn C, Martin, W. J. Comparison of media for isolation of Salmonellae and Shigellae from fecal specimens,European Pharmacopeia II, Chapter VIII, 10. Appl. Microbiol, 1971; 22-17.
[27] Rollender W, Beckford O, Belsky R D, Kostroff B. Comparison of xylose lysine deoxycholate agar and Mac-Conkey Agar for the isolation of Salmonella and Shigella from clininal specimens, Am. J. Clin. Pathol, 1969; 51-284.
[28] Kwon-Chung KJ, Bennett JE, Medical mycology, 1st edn. Philadelphia: Lea & Febiger, 1992.
[29] Taplin D, Zaias N, Rebell G, Blank H. Isolation and recognition of dermatophytes on a new medium (DTM), 1969 Feb;99(2):203-9.
[30] Larone DH, Medically important fungi. A guide to identification, 3rd ed., Washington, D.C, ASM Press, American Society for Microbiology, 1995.