

Prevalence of Microbial Loads on Betel Leaf with Special emphasis on Multidrug Resistance Salmonella spp and its Public Health Implications

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Abstract— Presence of pathogen especially *Salmonella* spp in the Betel leaves suspended export of Betel leaf in Europe. Bangladesh has a subtropical monsoon so the present study was undertaken to determine Microbial loads of Betel leaf on the basis of seasonal variation (rainy and winter season). A total of 50 Betel leaf samples were collected from five sources (betel field, transport, whole seller, local shop, betel leaf washing water used in local shop). Highest TVC (total viable count) were counted from local shop sample (5.3×10^5 CFU/ml) and the lowest TVC was found from field sample (2.5×10^3 CFU/ml). This study results showed that during rainy season (July-October) TVC count was higher than winter season (November-February). From this study 10 genera of bacteria, were isolated from betel leaf such as *E.coli*, *Vibrio* spp, *Bacillus* spp, *Pseudomonas* spp, *Klebsiella* spp, *Salmonella* spp, *Shigella* spp, *Staphylococcus* spp, *Enterococcus* spp and *Proteus* spp) and 5 genera of fungus (e.g. *Aspergillus* spp, *Fusarium* spp, *Rhizopus* spp, *Zygosaccharomyces* spp and *Rhizoctonia* spp) were isolated. Out of 184 isolates we found the following percentage of isolated microorganisms: 17.9% in betel leaf field, 19.5% in Transport, 19.5% in wholesaler, 28.8% in local shop and 14.3% in betel leaf washing water from local shop. Antibiotic sensitivity test showed that all of the isolates were resistant to Bacitracin, Penicillin, Vancomycin, Erythromycin and against other 5 antibiotics (Azithromycin, Gentamycin, Cephalexin, Ciprofloxacin and Chloramphenicol) isolates showed Resistant, Moderate and Sensitive Results. Data of this study suggest that Betel leaves from different source could harbor multidrug resistant bacteria specially *Salmonella* spp which underscore the need of implementation of hygienic practices during production, harvesting,

transportation, storage, selling and preparation of Betel leaves to safeguard public health.

Keywords— Antibiotic Sensitivity, Betel leaf, Drug Resistance, Seasonal Variation, TVC.

I. INTRODUCTION

Botanical name of betel vine is *Piper betel*. In Bangladesh, it is known as 'paan'. It is available in many Asian countries including Bangladesh. The betel plant originated from the South and South East Asia. The betel leaf is cultivated in most of South and Southeast Asia. Betel leaves has good export potential and Bangladesh exports betel leaves to the countries like Pakistan, India, Indonesia, Malaysia, Burma and Thailand. The harvested leaves are consumed locally or exported to other parts of Asia, the Middle East, Europe, and the United States.

In Bangladesh, farmer prepares a garden called a barouj in which they grow betel. The barouj is fenced with bamboo sticks and coconut leaves. The soil is plowed into furrows of 10 to 15 meters length, 75 centimeters in width and 75 centimeters' depth. Oil cakes, manure, and wood ash are thoroughly incorporated with the topsoil of the furrows. The creeper cuttings are planted at the beginning of the monsoon season. The harvest lasts for 15 days to one month. Betel plays an important role in the economy of rural Bangladesh. In some regions betel leaf cultivation is the main source of income for farmers. A total of 2,825 hectares of land is dedicated to betel vine farming. The average production cost for these betel farms in Bangladesh are about Tk. 300,000 per hectare, and the farm owners can earn a profit of over Tk. 100,000 per hectare. Betel vine is an important medicinal and recreational plant in Southeast Asia.

Betel leaf (Paan) export to the European and Middle Eastern countries stood at over US \$ 31 million in 2012.

Detection of *Salmonella* bacteria in betel leaf from Bangladesh in the UK prompted the European Union to suspend imports. Expatriates from Bangladesh and India are the primary customers of betel leaf in European countries. Saudi Arabia and the USA are other big markets for betel leaf [1]. The government has taken an initiative to produce bacteria-free betel leaf in order to resume its export.

The surface of Betel leave can be contaminated with microbial pathogens by polluted air, water and soil, during pre-harvest stage. Packaging materials used for carry and storage at Betel leaf, moisture content and water used for washing of Betel leaf are important sources of contamination during post-harvest stage [2].

1.1 Rationality of the Study

According to the Food Standards Agency, UK- Since October 2011 there have been several food safety notifications concerning the presence of a range of pathogenic *Salmonella* strains found in foodstuffs containing or consisting of betel leaves originating in or consigned from Bangladesh. There is a temporary suspension of imports of betel leaves from Bangladesh until 30 June 2018 [3]. So it's high time to develop methods for controlling *Salmonella spp.* in the Betel leaf for local consumption.

Detection of *Salmonella* bacteria in betel leaf from Bangladesh in the UK prompted the European Union to suspend imports. Expatriates from Bangladesh and India are the primary customers of betel leaf in European countries. Saudi Arabia and the USA are other big markets for betel leaf [4].

The surface of Betel leaves can be contaminated with microbial pathogens by polluted air, water and soil, during pre-harvest stage. Packaging materials used to carry and storage Betel leaf, moisture content and water used for washing are important sources of contamination during post-harvest stage [5].

1.2 Prevalence of microorganism in food (leaf) products

Post- harvesting the spoilage of betel leaves accounts for the post-harvest loss in the range of 35%–75% respectively [6][7].

A comprehensive microbiological investigation of pathogen causing leaf diseases has been conducted to isolate, classify and characterize micro flora of Betel leaf. *Xanthomonas compestris* pv. *Betticola* bacteria have been identified previously from damaged Betel leaves [8].

Across Trinidad the prevalence and microbial load of *Listeria spp.*, *Escherichia coli* O157 and *Salmonella spp.* was determined in the products of supermarkets. The microbial load was assessed using the total aerobic plate count (TAPC) per g/ml of foods and prevalence of *Escherichia coli* O157 and *Salmonella spp.* were determined using conventional methods. For *Listeria*

monocytogenes, immune magnetic separation (IMS), TECRA (enzyme-linked immune sorbent assay, ELISA) and conventional methods were used. The \log_{10} mean \pm SD TAPC per g or ml was highest for vegetables (11.0 \pm 11.6), and lowest for seafood (5.2 \pm 5.7) ($p < 0.05$). The prevalence of *L. monocytogenes* was 1.7%. Sixteen (4.5%) of 153 samples yielded *E. coli* but all samples were negative for *Salmonella spp.* and *E. coli* O157 [9]

A significant bacterial count (CFU g) was detected in jhal-muri (1.66x10⁶ CFU g), betel-leaf (1.49x10⁶ CFU g), hog-plum (1.87x10⁶ CFU g), sweet (3.39x10⁶ CFU g) and bun (3.11x10⁶ CFUg). Sola (6.24x10⁶ CFU g), cup-cake (6.19x10⁶ CFU g), peaju (4.96x10⁶ CFU g), sheek-kabab (2.63x10⁶ CFU g) an vhel-puri (1.96x10⁶ CFU g) found to be contaminated with moderate bacterial count whereas, in singara (8.93x10⁶ CFU g) and somosa (4.11x10⁶ CFUg) load was found. Jhal-muri, hog-plum, betel-leaf, peaju, sheek-kabab, singara and vhel-puri were found to be 100 % contaminated with coliforms with an unacceptable range, as compared to somosa (75 %), sola (50 %) and bun (25 %). But cup-cake and sweet were free from contamination with coliforms (0 %). So among the 48 RTE food samples, 29.16 % of them did not contain coliforms. It was found that, sola (6596 CFU g), hog-plum (6197 CFU g), betel-leaf (3856 CFU g) and jhal-muri (2312 CFU g) were hazardously contaminated with fungi [10], when evaluated bacterial loads in salad vegetables using spread plate agar dilution method was done. Bacterial loads ranged from 1.6 x 10⁶ to 2.9 x 10⁸ CFU/g. *Escherichia coli*, *Klebsiella* and *Enterobacter* were amongst the Coliforms (lactose fermenters), while *Proteus*, *Pseudomonas aeruginosa*, *Salmonella* and *Shigella* were non-lactose fermenters associated with the samples.

Salmonella spp. is an important zoonotic pathogen that cause an estimated 1.4 million illness, 16000 hospitalization and between 400 to 600 deaths annually in the united states alone [11][12]. *Salmonella* can produce invasive infections that lead to sepsis and death. Young children, the elderly and those with compromised immune systems are especially susceptible to severe disease.

The prevalence of multidrug resistant among *Salmonella* strain has increased over the past two decades [13][14][15], making treatment failures more common among those with serious disease. In addition, infections with resistant strains of *Salmonella* tend to be more severe and lead to higher rates of hospitalization than those caused by susceptible strains [16][17][18][19]. And multidrug - resistant strains of zoonotic *Salmonella spp.* present on ready-to-eat Paan is a public health concern. It may be one of the factors responsible for the hyper endemic status of salmonellosis [20]. People generally acquire salmonellosis through foodborne exposure,

although direct contact with infected animals is another possible route [21][22]. The outcome of different experiments showed that the best season for longer storage of betel leaves in any of the form which may be petiolated or depetiolated is winter seasons i.e. December-January [23] .

Fungus is any member of the group of eukaryotic organisms that includes microorganisms such as yeasts and molds as well as the more familiar mushrooms. These organisms are classified as a kingdom, Fungi which are separate from the other eukaryotic life kingdoms of plants and animals. Fungi spoiling organisms are silently invading, acidifying, fermenting, discolouring, and disintegrating microbes that render corn such as maize, wheat, barley etc. Fungi spoilage is caused by two factors, (biotic) living which includes insects, birds, rodents and microorganisms and (non-biotic) non-living which includes temperature, humidity and time. The world is concerned with food safety that has enhanced interest in fungal and subsequent food spoilage. Contamination with mould causes deterioration of product which affects human and animal health. [24]. Fungal spoilage of corn reduces the nutritional value and palatability of the feed, thereby increasing its allergic potential and may result in mycotoxic contamination [25].

II. RESULTS

2.1 Collection and transportation of samples

A total of 50 betel leaf samples were collected from Different sources (betel field, transport, whole seller, local shop, betel leaf washing water used in local shop) on the basis of seasonal variation (rainy and winter season). Individual sample placed in the sterile container. The samples were transported carefully to the Bacteriology laboratory for bacteriological analysis.

2.2 Processing of betel leaf samples

The betel leaf samples in polythene-bag were washed with sterile PBS (phosphate buffered saline). One Betel leaf was washed with 20ml of sterile PBS. A 5 fold serial dilution of the washed samples was prepared in nutrient broth.

2.3 Determination of Total Viable count (TVC) of betel leaf

A total of 0.1 ml 10 fold diluted sample (10^{-1} to 10^{-6}) was transferred and spreaded onto nutrient agar (NA) and incubated at 37°C for 24-48 hours. TVC was determined by using the following formula

$$\text{CFU/ml} = \text{Number of colonies/ml} \times \text{dilution factor}$$

Table.1: Total Viable count of betel leaf

Betel leaf source	Sample	Seasonal variation		TVC, CFU/ml
		Winter	Rainy	
Route level(field)	1,6,11,16,21	1.68 X 10^4	1.96 X 10^4	1.82 X 10^4
	26,31,36,41,46			
Transport	2,7,12,17,22	1.32 X 10^5	1.55 X 10^5	1.41 X 10^6
	27,32,37,42,47			
Whole seller	3,8,13,18,23	2.7 X 10^5	2.58 X 10^5	2.64 X 10^6
	28,33,38,43,48			
Local shop	4,9,14,19,24	4.42 10^4	4.18 X 10^5	2.31 X 10^6
	29,34,39,44,49			
L.S.W.W	5,10,15,20,25	1.51x 10^5	2.46 X 10^5	1.98 X 10^6
	30,35,40,45,50			

* L.S.W.W= Local Shop Washing Water

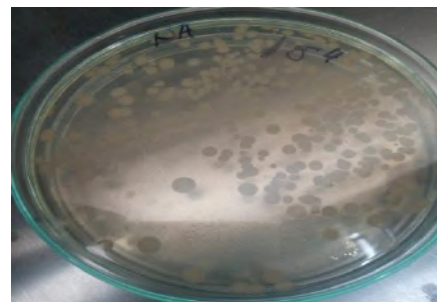


Fig1: Growth of microorganism on Nutrient Agar medium (TVC)

2.4 Statistical Data Analysis for significance level

Test for two independent Samples/Two –tailed test was performed to show statistical significance (Table-2).

A one way ANOVA (Table-3, 4) followed by Analysis of the differences between the categories Fisher (LSD) test (Table-5) were also used. We also conducted t Paired test, ANOVA test followed by Fisher (LSD) to find out whether our calculated value had any significance.

Table2: Test for two independent Samples/Two-tailed test, 95% confident interval on the difference between the means of different collection site

Variable	Rainy	Variable
Observations	25	Observations
Obs. with missing data	0	Obs. with missing data
Obs. without missing data	25	Obs. without missing data
Minimum	10000	Minimum
Maximum	630000	Maximum
Mean	219440	Mean
Std. deviation	187260.754	Std. deviation
Difference	96480	Difference
t (Observed value)	2.182	t (Observed value)
t (Critical value)	2.011	t (Critical value)
DF	48	DF
p-value (Two-tailed)	0.034	p-value (Two-tailed)
Alpha	0.05	Alpha

t-Paired test interpretation

H0: The difference between the means is equal to 0.

Ha: The difference between the means is different from 0.

As the computed p-value is lower than the significance level $\alpha=0.05$, we can reject the null hypothesis H0, and accept the alternative hypothesis Ha

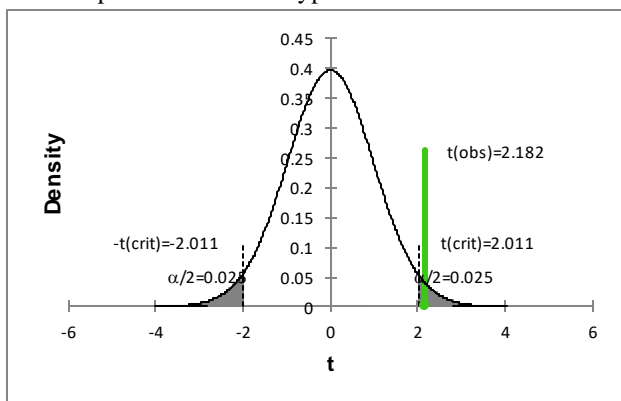


Fig2: t-test for two independent samples / Two-tailed test

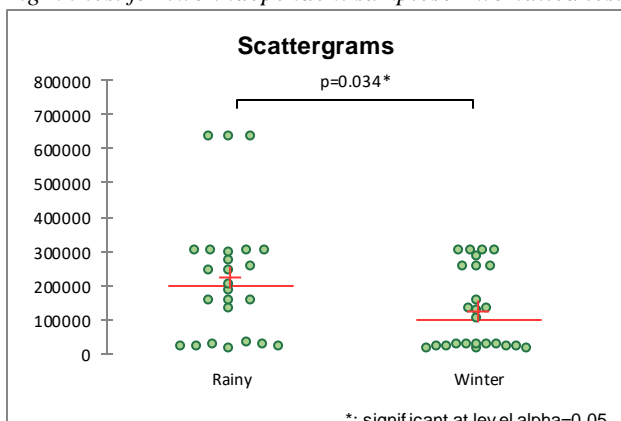


Fig 3: Scatter grams of TVC Count on the basis of Rainy and Winter Season.

Table 3: Summary statistics (Quantitative data): One way ANOVA test

Variable	Variable
Observations	Observations
Obs. with missing data	Obs. with missing data
Obs. without missing data	Obs. without missing data
Minimum	Minimum
Maximum	Maximum
Mean	Mean
Std. deviation	Std. deviation

Table 4: Analysis of variance, ANOVA (TVC)

Source	DF	Sum of Square	Mean square	F	Pr>F	F Crit
Between Groups	4	370940200	927350500	4.542	0.004	2.45
Within Groups	45	918771799	204171511			
Total	49	128971200	261171511			

Our Calculated value, $F(4, 45) = 4.452$, $P=0.004$ is higher than F Critical Value 2.45 so there is a significant difference among the TVC count of different collection Site.

Table 5: Summary of all pair wise comparisons for C.S (Fisher LSD)

Category	LS means	Standard error	Lower bound (95%)	Upper bound (95%)	Groups
Wholeseller	264000	45185.342	172992.050	355007.950	A
Local shop	231100	45185.342	140092.050	322107.950	A
L.S.W.W	198600	45185.342	107592.050	289607.950	A
Transport	144100	45185.342	53092.050	235107.950	A
Field	18200	45185.342	72807.950	109207.950	B

(e.g. *Aspergillus* spp-5.43%, *Fusarium* spp- 4.89%, *Rhizopus* spp-3.84%, *Zygosaccharomyces* spp- 3.26%, *Rhizoctonia* spp-2.71%) were isolated [26] [27].

Out of 184 isolates we found the following percentage of isolated microorganisms 17.9% in betel leaf field, 19.5% in Transport, 19.5% in wholesaler, 28.8% in local shop and 14.3% in betel leaf washing water from local shop.

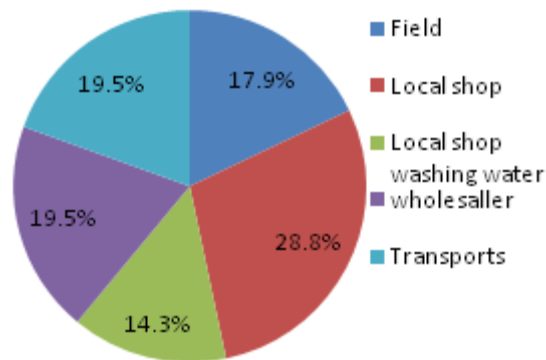


Fig.5: Percentage of isolated pathogen from betel leaf

Antibiotic sensitivity test showed that all of the isolates were resistant to Bacitracin, Penicillin, Vancomycin, Erythromycin and against other 5 antibiotics (Azithromycin, Gentamycin, Cephalexin, Ciprofloxacin and Chloramphenicol) isolates showed Resistant, Moderate and Sensitive Results. [28]

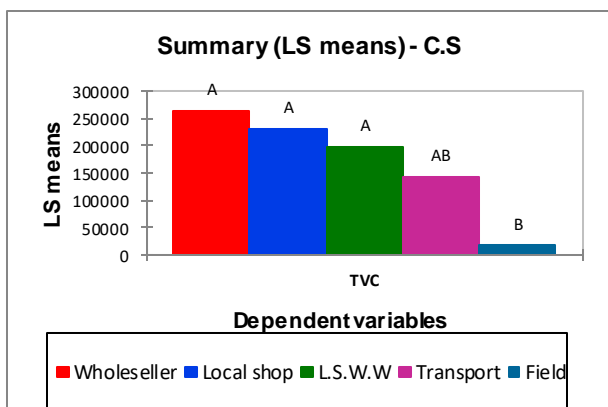


Fig 4: Bar diagram of TVC Count (LS means) on the basis of Collection Site.

2.5 Isolation and Identification of Microorganisms

After Microscopic observation followed by cultural and biochemical test results observation, 10 genera of bacteria (e.g. *E.coli*-21.73%, *Vibrio* spp - 7.6%, *Bacillus* spp -2.7%, *Pseudomonas* spp-3.84%, *Klebsiella* spp - 7.06%, *Salmonella* spp -19.5%, *Shigella* spp - 5.43%, *Staphylococcus* spp-5.43%, *Enterococcus* spp-4.89% and *Proteus* spp-1.63%) and 5 genera of fungus

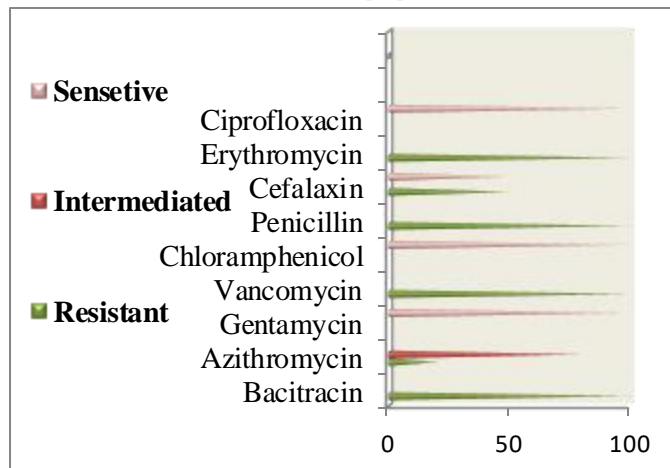


Fig.6: Antibiotic Sensitivity of Bacteria isolated (total 125) from betel leaf

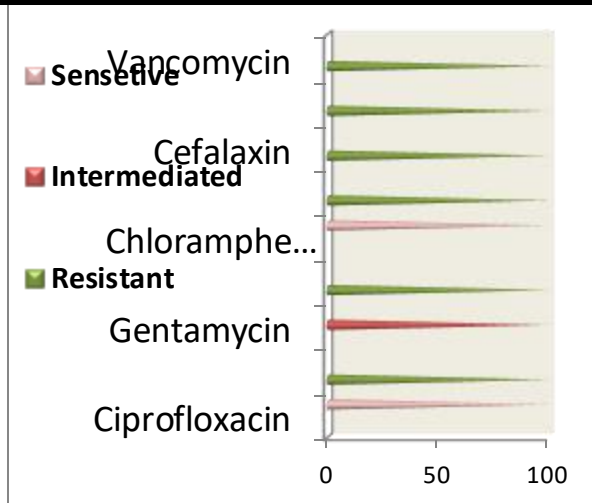


Fig.7: Summary of antibiogram profile of *Salmonella* spp. against 9 antibiotics.

III. DISCUSSION

Highest TVC counted from Whole seller sample (2.64×10^6) and the lowest TVC counted from field sample (1.82×10^4). TVC count from different source vary might be due to unsanitary environment, use of polluted water to wash Betel leaf and unclean utensil used to storage Betel leaf.

This study results showed that during rainy season (July-October) TVC count was higher than winter season (November-February).

We also conducted t Paired test, ANOVA test followed by Fisher (LSD) to find out whether our calculated value had any significance. From the statistical data analysis we have found that our observed data were significant at 95% confidence level.

In the present study, selective media (EMB, TCBS, SS, Macconkey, SDA, MSA) were used for isolation of *E. coli*, *Vibrio* spp., *Salmonella* spp., and *Klebsiella* spp. *Bacillus* spp., *Staphylococcus* spp., *Enterococcus* spp.

In this study, the colony characteristics of *Vibrio* spp. on TCBS agar plate were similar to the findings of Tankeshwar Acharya. In Gram's staining bacteria exhibited curved rod shaped appearance which was also observed by other researchers [29][30][31].

The colonies of *Salmonella* spp. on agar SS plate were opaque, translucent with black centers which were similar to the findings of Cheesbrough. [32]. In Gram's staining *Salmonella* spp. exhibited short rods, Gram negative, single or paired in arrangement. Similar findings were also reported by Buxton and Frase [33][34].

From this study 10 genera of bacteria, were isolated from betel leaf such as *E. coli*-21.73%, *Vibrio* spp -7.6%, *Bacillus* spp -2.7%, *Pseudomonas* spp-3.84%, *Klebsiella* spp-7.06% , *Salmonella* spp-19.5%, *Shigella* spp-5.43%, *Staphylococcus* spp-5.43%, *Enterococcus* spp-4.89% and *Proteus* spp-1.63%).

A study conducted in Bangladesh found that the prevalence of *E. coli* was 17.34% (17 of 98), *Salmonella* spp. was 25.51% (25 of 98), *Vibrio* spp. was 19.39% (19 of 98), *Bacillus* spp. was 18.37% (18 of 98), and *Staphylococcus* spp. was 19.39 (19 of 98) [35].

A total of 5 genera of fungus (e.g. *Aspergillus* spp-5.43%, *Fusarium* spp- 4.89%. *Rhizopus* spp-3.84%, *Zygosaccharomyces* spp- 3.26%, *Rhizoctonia* spp-2.71%) were isolated

A study conducted in India isolated from Betel leaves isolated *Xanthomonas compestris* PV. *Betticola* fungi from diseased Betel leaves. [2]

From our observation out of 184 isolates we found the following percentage of isolated microorganisms: 17.9% in betel leaf field, 19.5% in Transport, 19.5% in wholesaler, 28.8% in local shop and 14.3% in betel leaf washing water from local shop.

In case of *Salmonella* we have found that 38% of betel leaf sample was contaminated with *Salmonella* spp. Among them 7, 6 and 5 no of *Salmonella* spp were isolated from Transport, Whole seller and Local Shop Betel leaf Samples respectively. Our study showed that Transport is the major source of *Salmonella* spp contamination in Betel leaf consumed in Bangladesh.

A study conducted in Bangladesh found that 77% betel leaf sample collected from different markets of Dhaka city was found to be contaminated with *Salmonella* spp. [36].

Antibiotic sensitivity test show most of the isolates were resistant to bacitracin, penicillin. More shocking report is, most of the people in Bangladesh use Erythromycin and Azithromycin antibiotic vigorously but this study show erythromycin were resistant against four isolates and azithromycin show both moderate and resistant result and Ciprofloxacin was sensitive to all tested isolates. On the other hand gentamicin shows sensitive against the isolates. Cephalexin show both sensitive and resistant result [37].

Our isolated 18 isolates of *Salmonella* spp showed completely resistance to Bacitracin, Penicillin, Vancomycin, Erythromycin, Azithromycin, Amoxicillin and sensitive against other 2 antibiotics, Ciprofloxacin and Chloramphenicol. They are intermediately sensitive to Amoxicillin. Indiscriminate use of antibiotic is responsible for emergence of multidrug resistant *Salmonella* spp. [19] [38].

Data of this study suggest that Betel leaves from different source harbor multidrug resistant [39] bacteria which underscore the need of implementation of hygienic practices during production, harvesting, transportation, storage, selling and preparation of Betel leave to safeguard public health. From this study we could suggest that Betel leaves might be contaminated with bacteria not

only due to use of potable water for washing, handling of Betel leave with unclean hands but also use of unclean utensil or cutting board when preparing ready to eat Betel leaves.

IV. CONCLUSION

Data of this study suggest that Betel leaves from different source harbor multidrug resistant bacteria which underscore the need of implementation of hygienic practices during production, harvesting, transportation, storage, selling and preparation of Betel leaves to safeguard public health.

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