Original Article

Remedy of contamination of multidrug resistant *Salmonella* and *Escherichia coli* from betel leaves (*Piper betle*) keeping them fresh for long time
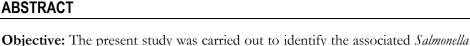
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ABSTRACT

Objective: The present study was carried out to identify the associated *Salmonella* and *Escherichia coli* in betel leaves (*Piper betle*), and to develop an effective method to remove those microbes.

Materials and methods: Betel leaves were collected from local and whole sale markets, and *borouj* (cultivation place). *Salmonella* and *E. coli* were isolated and identified by cultural, morphological, and biochemical tests followed by confirmation by polymerase chain reaction (PCR) targeting the genus specific 16S rRNA genes. Antibiogram of the isolated bacteria was performed by disc diffusion method. Different concentrations of Salmosan-A Soln were used to remediate the contaminating bacteria keeping the quality of betel leaves for longer periods.

Results: Total *Salmonella* counts in the betel leaves were 3.9×10^5, 4.9×10^6, 3.5×10^4, 1.1×10^3 and 1.5×10^3 CFU/mL, while *E. coli* counts were 5.5×10^7, 6.3×10^7, 4.4×10^5, 3.3×10^3 and 3.1×10^3 CFU/mL in the betel leaves collected from K.R. market, Kewatkhali Bazaar, whole sale market, *borouj* in Kushtia and *borouj* in Natore, respectively. Antibiogram study revealed that the isolated bacteria were sensitive to doxycyclline, ciprofloxacin, chloramphenicol and cefotaxime. Application of 0.3% Salmosan-A Soln was found to be the most effective and suitable, where <1 CFU/mL in the betel leaves could be detected after treatment for 10 min.

Conclusion: Results of this study indicated that treatment with 0.3% Salmosan-A Soln for 10 min is capable of removing *Salmonella* and *E. coli* from betel leaves keeping the treated leaves fresh up to 7 days of post-treatment. Method developed in this study can be adapted to reduce bacterial contamination of betel leaves more effectively in Bangladesh and other betel leaf exporting countries.

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KEYWORDS

Betel leaves; Contamination; *Escherichia coli*; *Salmonella*; Treatment

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INTRODUCTION

Salmonella and *Escherichia coli* (*E. coli*) are Gram-negative, rod-shaped bacteria under *Enterobacteriaceae* family. *Salmonella* is the causative agent of typhoid fever, paratyphoid fever, and food poisoning worldwide (Furchtgott et al., 2011). *E. coli* are considered as a part of normal enteric microflora, and most of them are opportunistic pathogens for animal and human; however, this bacterium may cause serious diarrhea and other systemic diseases in healthy humans and animals (Levine, 1984).

The betel leaf ('*Paan*' in Bengali) is the leaf of a vine belonging to the family *Piperaceae*. The betel (*Piper betle*) has no fruit and is cultivated only for its green leaves, which have medicinal properties. The betel plant is an evergreen, perennial and shade loving root climber (Chakraborty, 2011). *Paan* is available in most south and southeast Asian countries including India, Pakistan, Burma, Vietnam, Malaysia, Singapore, Thailand, Philippines and Bangladesh. Besides, *Paan* is consumed in some other countries in the world, particularly by the Asian immigrants.

In Bangladesh, the yield of betel leaf varies from region to region. Betel leaf cultivation is one of the major sources of income generation for the farmers in Bangladesh. About 2,825 hectares of land is dedicated for betel leaf production in Bangladesh (Husna et al., 2015). The average production cost for betel leaf production in Bangladesh is USD 4000 per hectare with an average profit of USD 1334 (Annon, 2011).

In Bangladesh, exportable betel leaf is cultivated in Kushtia, Rajshahi, Natore, Jhenaidah, Khulna, Barishal, Satkhira, Bagerhat, Sylhet and Chittagong districts. Bangladesh exports betel leaves mostly to Europe and Middle Eastern countries. Bangladesh exported betel leaves worthing USD 31.10 million in the fiscal year 2011-12, which was USD 42.6 million in 2010-11 (Annon, 2011). According to the Export Promotion Bureau (EPB), United Kingdom customs in 2011 had examined 60 consignments of *Paan* that were imported to the country and found that 44 were infected with *Salmonella* (Husna et al., 2015). Currently, European Union (EU) has an embargo on the export of *Paan* from Bangladesh to EU countries due to potential *Salmonella* contamination (Chowdhury and Kallol, 2013). Husna et al. (2015) could isolate *Salmonella* from *Paan*, and used 1.5% vinegar to mitigate the *Salmonella* contamination.

However, a detailed study is yet to be done to identify the point(s) from where the contamination is originated. Also, it is crucial to develop an applicable technique to remediate the *Salmonella* and *E. coli* contamination from the betel leaves keeping them fresh and consumable for long time. The present study was designed to detect multidrug resistant *Salmonella* and *E. coli* in betel leaves originated from different places including cultivation place (commonly known as "*barouj*" in Bengali), whole sale markets and local/retail markets. In this study, an applicable method was developed to effectively remove contaminating *Salmonella* and *E. coli*, thus keeping the leaves in fresh and good condition for long time.

MATERIALS AND METHODS

Study locations: The betel leaves were collected from *barouj* in Kushtia and Natore districts, whole sale and local markets (K.R. market and Kewatkhali), and were transported to the Bacteriology Laboratory at the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh for bacteriological analysis.

Bacterial cultural and identification: The betel leaves were washed with phosphate buffer solution (PBS) and then aliquots were spread onto Salmonella Shigella (S-S), Xylose Lysine Deoxycholate (XLD) and Eosin Methylene Blue (EMB) agar plates, and incubated at 37°C overnight. Black color colonies formed on S-S and XLD agars, and green metallic sheen colonies formed on EMB agar were screened, as described by Rehman et al. (2014). The black colonies were subjected to subculture on S-S agar to isolate pure culture, which was used for Gram stain, as described by Tanzin et al. (2016) and Rahman et al. (2016). The organisms were also sub-cultured on XLD and EMB agars (Parvej et al., 2016). Pure cultures were used for further testing.

Bacterial biochemical characterization: The isolated pure cultures were subjected to conventional biochemical tests using sugar fermentation, Methyl-Red Voges-Proskauer (MR-VP), Methyl-Red, Voges-Proskauer (VP) and Indole tests. Motility test was done as per the method described by Ifeanyichukwu et al. (2016) and Rahman et al. (2016).

DNA extraction and molecular identification: Pure and single bacterial colony was used to isolate bacterial DNA. Bacterial colonies were mixed with 200 μL of distilled water and boiled for 10 min. Immediate after

Table 1. Primers used for molecular detection of *E. coli* and *Salmonella*

Primer Name	Targeted Gene (primer)	Primer Sequence (5´-3´)	PCR amplicon size (bp)	Reference
EC16SrRNA F	16S rRNA (*E. coli* genus specific)	GACCTCGGTTTAGTTCACAGA	585	Seidavi et al. (2010), Serena et al. (2010), Hassan et al. (2014).
EC16SrRNA R		CACACGCTGACGCTGACCA		
F strand	*Salmonella* genus specific	ACTGGCGTTATCCCTTTCTCTGGTG	496	Noah et al. (1993)
R strand		ATGTTGTCCTGCCCCTGGTAAGAGA		

Table 2. Total *Salmonella* and *E. coli* counts in betel leaves before and after treatment with 0.3% Salmosan-A solution

Sources betel leaf	TVC/mL before treatment		TVC after treatment	
	Salmonella	*E. coli*	*Salmonella*	*E. coli*
Borouj				
i) Kushtia	1.1×10^3	3.3×10^3	< 1	< 1
ii) Natore	1.5×10^3	3.1×10^3	< 1	< 1
Whole sale market				
Hathiaboro bazaar, Kusthia	3.5×10^4	4.4×10^5	< 1	< 1
Local market				< 1
i) K. R. market, BAU	3.9×10^5	5.5×10^7	< 1	
ii) Kewatkhali, Mymensingh	4.9×10^6	6.3×10^7	< 1	< 1

boiling, the eppendorf tube was kept on ice for another 10 min. Centrifugation at 10,000 rpm for 10 min was done for precipitating the cellular debris. The supernatant was collected and used as DNA template for PCR reactions (Saifullah et al., 2016).

16SrRNA gene amplification: PCR reaction (25 μL scale) contained master mixture (12.5 μL), forward and reverse primers (1 μL each), DNA template (4 μL), and nucleus free water (6.5 μL). The *16SrRNA* gene from *Salmonella* was amplified using the thermocycler (Eppendorf, Germany). The reaction conditions included an initial denaturation at 94°C for 5 min, followed by a 33 cycles of reaction of denaturation at 94°C for 30 Sec, annealing of primers at 65°C for 30 Sec, elongation at 72°C for 45 Sec, and finally an extension at 72°C for 7 min. Similarly, the *16SrRNA* gene of *E. coli* was amplified using an initial denaturation at 95°C for 3 min, followed by a 30 cycles of reaction of denaturation at 94°C for 45 Sec, annealing of primers at 58°C for 45 Sec, elongation at 72°C for 1 min, and a final extension at 72°C for 3 min. All the PCR amplicons were visualized using gel electrophoresis on 2% agar after staining with ethidium bromide under UV transilluminator (UV Solo, Germany).

Treatment of *Salmonella* and *E. coli* contaminations in betel leaves: The betel leaves were washed using different concentration of Salmosan-A Soln, as mentioned in the **Table 4**. Then, the leaves were washed with distilled water. The leaves were then dipped into PBS, and the washings were inoculated onto XLD and EMB agars for evaluating the presence of *Salmonella* and *E. coli*, and the plates were incubated at 37°C overnight. On the other hand, Salmosan-A Soln treated leaves were stored at room temperature and observed daily for 8-10 days.

Antibiotic sensitivity test: The antibiotic sensitivity tests were performed using commercially available antibiotic discs (Oxoid, UK) using disc diffusion method, as described previously (Nazir et al., 2005; Tanzin et al., 2016). The antibiotics were amoxicillin (AML; 10 μg/disc), ampicillin (AMP; 10 μg/disc), cefixime (CFM; 5 μg/disc), cefotaxime (CTX; 30 μg/disc), chloramphenicol (C; 30 μg/disc), ciprofloxacin (CIP; 5 μg/disc) and Doxycycline (DO; 30 μg/disc). The sizes of the zones of inhibition were interpreted as per the method described by CLSI (2010).

RESULTS AND DISCUSSION

Salmonella were detected in the betel leaves. On S-S agar, all of the isolates produced translucent, black, smooth and small round colonies which were suspected for *Salmonella*. All the suspected *Salmonella* isolates produced pink color colony with black center after incubated at 37°C overnight on XLD agar. Gram stain revealed the presence of Gram-negative, pink colored, short rod bacteria, arranged in single and paired. The bacteria were motile seen by hanging drop slide technique.
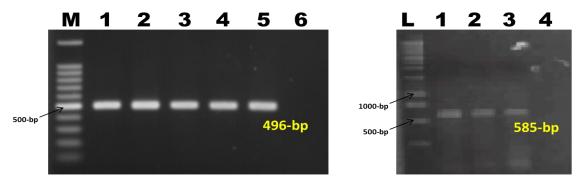
The *E. coli* isolates detected in betel leaves showed greenish-black colonies with metallic sheen on EMB agar and bright pink or red colonies on McConkey agar plates. Gram stain of *E. coli* revealed as Gram-negative, pink color, short rod, arranged in single or paired.

E. coli and *Salmonella* were confirmed by conventional biochemical tests. All *E. coli* isolates fermented the five basic sugars (dextrose, sucrose, lactose, maltose and mannitol) producing both acid and gas. On the other hand, *Salmonella* could not ferment Sucrose and Lactose. All of the test isolates *Salmonella* were indole negative,

Table 4: Treatment of betel leaves with salmosan-A soln and observation of bacterial growth and stability of the leaves

Concentration of Salmosan-A Soln	Time (min)	Non-treated		Treated		Condition of treated leaves
		Salmonella	*E. coli*	*Salmonella*	*E. coli*	
2%	10	HG	HG	NG	NG	Decomposed on 2nd day
1.5%	10	HG	HG	NG	NG	Decomposed on 3rd day
0.5%	5	HG	HG	NG	NG	Decomposed on 3rd day
0.5%	5	HG	HG	few colonies	NG	Decomposed on 4th day
0.5%	12	HG	HG	NG	NG	Decomposed on 3rd day
0.3%	5	HG	HG	Few colonies	NG	Decomposed on 6th day
0.3%	10	HG	HG	NG	NG	Remain fresh up to 7th day

HG= Huge growth, NG=No growth

(A)   (B)

Figure 1. (A) PCR image of *Salmonella* spp. Lane M: 100-bp DNA ladder, Lane 1: Positive control, Lane 2-5: Isolated positive *Salmonella*, Lane 6: Negative control; **(B)** PCR image of *E. coli* Lane L: 1000-bp DNA ladder, Lane 1: Positive control, Lane 2-3,: Isolated positive *E. coli*, Lane 4: Negative control.

Table 3: Antibiotic sensitivity pattern of *Salmonella spp.* and *E. coli* isolated from betel leaves.

Name of the antibiotics	*E. coli*	*Salmonella*
Ciprofloxacin (CIP)	S	S
Amoxycillin (AML)	R	R
Chloramphenicol (C)	S	S
Cefixime (CFM)	R	R
Doxycyclline (DO)	S	S
Ampicillin (AMP)	R	R
Cefotaxime (CTX)	S	S

S=Sensitive, R=Resistance

positive for Methyl-Red (M-R) test and negative for V-P test. *E. coli* isolates were catalase positive, V-P test negative, M-R positive, citrate negative and Indole test positive. The isolated *E. coli* were found to be motile using hanging drop slide technique.

PCR using *Salmonella* genus specific primer for *Salmonella* and 16SrRNA detects primers for *E. coli*, showed positive band at 496-bp and 585-bp, respectively (**Figure 1**).

All the isolated *Salmonella* were found to be sensitive to CIP, C, DO and CTX, whereas resistant to AML, CFM and AMP. On the other hand, the isolated *E. coli* isolates were sensitive to CIP, C, DO and CTX, and resistant to AML, CFM and AMP (**Table 4**).

We detected *Salmonella* contamination in local (**Table 3**) market samples to be more than the *borouj* samples in

non-treated group. In addition, *E. coli* contamination was detected (**Table 3**) in local market sample more than the *borouj* samples in non-treated group.

Experiments to remove *Salmonella* and *E. coli*

Different experiments were conducted to remove *Salmonella* and *E. coli* from betel leaves keeping them in good condition for 7 days. The experiments were shown in **Table 4**.

Various concentrations of Salmosan-A Soln were used to remove *Salmonella* and *E. coli* from betel leaves. We found that 0.3% Salmosan-A Soln was the most effective to protect the leaves. With this concentration of Salmosan-A Soln, not only *Salmonella* and *E. coli* were removed successfully but also the leaves were remained in good condition up to 7 days (**Figure 2**).

In case of large scale experiments, 0.3% Salmosan-A Soln was found as effective. We found that all the treated betel leaves were free from *Salmonella* and *E coli*, whereas presence of *Salmonella* and *E coli* could be detected in untreated betel leaves, as well as all the treated leaves were in good quality as consumable even after 7th day of treatment (**Table 4**).
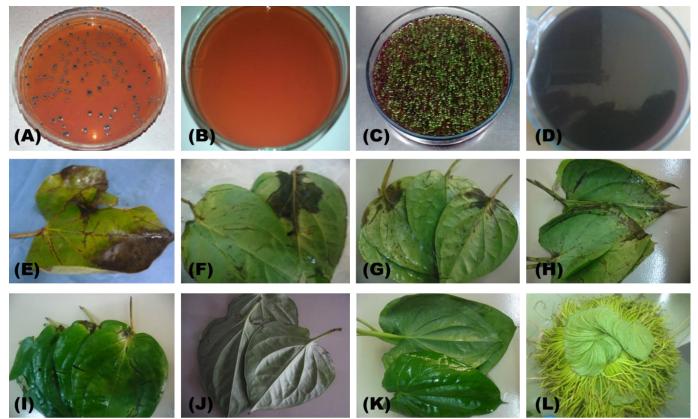
Figure 2. Bacterial count before and after the treatment of betel leaves with Salmosan-A Soln (A) *Salmonella* count in non-treated betel leaf, (B) No *Salmonella* in treated betel leaf, (C) *E. coli* count in non-treated betel leaf, (D) No *E. coli* growth in treated betel leaf, (E) Treated leaf was decomposed on 2nd day, (F) Treated leaves were decomposed on 3rd day, (G) Leaves were decomposed after 4th day, (H) Leaves were decomposed after 5th day, (I) Leaves were decomposed after 6th day, (J) Leaves were fresh up to 7 days but color changed, (K) Betel leaves were remained fresh until 8 days, (L) Large scale application of Salmosan-A sol.

In this study, PCR was used to confirm the *Salmonella* and *E. coli* (Saifullah et al., 2016; Parvej et al., 2016). PCR with *Salmonella* genus specific primer showed positive band at 496-bp confirming that the isolates were *Salmonella spp.* (Noah et al., 1993; Saifullah et al., 2016). PCR with *E. coli* genus specific 16SrRNA primer showed positive band at 585-bp confirming that the isolates were *E. coli* (Seidavi et al., 2010; Serena et al., 2010).

The sugar fermentation test profile of *Salmonella spp.* in the present study was similar to previous reports (Rahman et al., 2016). In motility test, the organism showed swinging movement as reported previously (Merchant and Packer, 1967; Buxton and Fraser, 1977). The genus *Salmonella* usually includes motile rods of peritrichous flagella with the exception of *Salmonella gallinarum* and *S. pullorum* (Blood et al., 2003). Therefore, the result of the motility test of this study indicates that the isolates are belonging to the species of *Salmonella* other than *S. gallinarum* and *S. pullorum*. The isolates might be any of the followings namely *S. typhimurium, S.*

enteritidis, S. heidelberg, S. agona, S. indiana, S. hadar, S. virchow, S. newport etc. (Perilla, 2003; Cheesbrough, 2006).

Antibiogram of the isolated bacteria was determined and antibiotic sensitivity test revealed that the isolated *Salmonella* and *E. coli* showed same resistance pattern where, all of the isolates were sensitive to CIP, C, DO, CTX while resistant to AML, CFM, and AMP.

In the present study, total *Salmonella* count was 3.9×10^5 CFU/mL and total *E. coli* count was 5.5×10^7 CFU/mL in K.R. market betel leaf, whereas total *Salmonella* count was 4.9×10^6 CFU/mL, and *E. coli* was 6.3×10^7 CFU/mL in Kewatkhali betel leaf. Samples collected from the whole sale market from Hathiaboro bazaar, Khusthia had a total *Salmonella* count of 3.5×10^4 CFU/mL in control group and found <1 colony in treated group. While *E. coli* count was 4.4×10^5 CFU/mL in control group. On the other hand, samples which were brought from *borouj* were also examined and the total *Salmonella* count was 1.1×10^3 CFU/mL and total *E. coli* count was 3.3×10^3 CFU/mL in

Kushtia and had a total *Salmonella* count was 1.5×10^3 CFU/mL and total *E. coli* count was 3.1×10^3 CFU/mL in the sample brought from Natore in control group. However, after application of Salmosan A Soln, we found <1 colony in treated group.

Husna et al. (2015) also studied the occurrence of *Salmonella* in betel leaf. It was found that *Salmonella* in the betel leaf sold at local markets and different *barouj* in Mymensingh. In addition, it was found that vinegar was an effective agent to mitigate *Salmonella*. Another study was conducted on fruits and vegetables purchased from street vendors to determine microbial load and the bacterial load ranged from 1.3×10^4 to 1.8×10^6 CFU/gm (Nwachukwu and Chukwu, 2013). Another study was on restaurant salad vegetables in Chittagong city recorded TVC ranged from log 5.20 to 6.87 CFU/gm (Nawas et al., 2012). In Nigeria, TVC of RTE salad vegetables was ranged from log 6.20 to 8.47 CFU/gm (Mercy et al., 2011).

As we found fractional result between local market sample and *borouj* sample that the *Samonella* and *E. coli* load were much higher in local market *Paan* than the *borouj*, and it gradually increased from *borouj* to whole sale market to local markets. It might be because of the unhygienic transportation of the betel leaves after picking from the trees to sell in the market. Though betel leaves cultivators trades are not much aware about producing and selling *Paan* at market in unhygienic condition. They use unhygienic water on *Paan* to keep those fresh. And, the water may cause high level of contamination.

Singla et al. (2009) artificially inoculated betel leaves with *Aeromonas*, *Salmonella* and *Yersinia* followed by the application of Salmosan-A Soln on that leaves and found effective result to remove these bacteria. In the present study, we also used 2% Salmosan-A Soln and it can remove *Salmonella* and *E. coli* but the betel leaves were found to be rotten within 2 days. However, when we used 0.3% Salmosan-A Soln, results were good and the leaves remained in original condition as like non-treated one.

CONCLUSION

The betel leaves examined in this study are contaminated with multidrug resistant *Salmonella* and *E. coli*. Load of these bacteria gradually increases from *borouj* to retail markets. The cultivators and traders use contaminated water on the betel leaves to keep them fresh, from where the contaminating bacteria originate. Here, we optimize the concentration of Salmosan-A Soln suitable not only to keep the betel leaves free from *Salmonella* and *E. coli*, but also to keep the leaves in good condition for long time. The best result was found with 0.3% Salmosan-A Soln for 10 min, in which the leaves were remained as consumable for 7 days. This technique may help in promoting export business for betel leaves. Measures should be taken to improve the general knowledge of hygiene and sanitation of the farmers cultivating *Paan* so that consumers can access contamination free safe *Paan*.

CONFLICT OF INTEREST

The authors declare that there is no conflicting interest with regards to the publication of this manuscript.

AUTHORS' CONTRIBUTION

TK and WKA conducted the research work. SNP and MMA helped in collection of samples. MSP, SA and MLH did the necessary analysis. TK made the primary draft of this manuscript. KHMNHN, MTR, MR and MFRK critically reviewed and improved the manuscript. MBR supervised the total team and finally approved the manuscript for publication. Before submission, all the authors approved the final version having no conflict of itnerest.

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