ISOLATION AND IDENTIFICATION OF BACTERIA FROM BETEL LEAF WITH THEIR ANTIBIOTIC RESISTANCE PATTERN

A THESIS

BY

MUNA OMAR MOHAMED

REGISTRATION NO. 1605580

SEMESTER: JULY-DECEMBER, 2017

SESSION: 2016-2017

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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DECEMBER, 2017

DEDICATED

TO

MY BELOVED PARENTS, SISTER AND BROTHERS

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ABSTRACT

The experiment was carried out to isolate and identify bacteria as well as to detect antibiotic sensitivity pattern of isolated bacteria from infected betel leaf sold at local markets of Dinajpur city, Bangladesh. For this study, a total 20 infected betel vine sample were collected from five different local markets (Leili morr, Terminal road, Doshmile, Gopalgong bazaar, and Basharat) of Dinajpur. A series of test were conducted for isolation, identification and frequency distribution of different bacteria of betel leaf. A total of 20 bacterial isolates belong to five genera (*staphylococcus spp., Bacillus, spp., Escherichia coli, klebsiella spp and Entrobacter spp*.) were identified. Out of 20 samples, 6 *were staphylococcus spp* (30%), 4 were *Bacillus spp. (*20%), 1 were *Escherichia coli (*5%), 5 were *klebsiella spp*. (25%) and 4 were *Entrobacter spp*. (20%). The identified isolation were subjected to antibiogram study in which staphylococcus *spp.* (6) were sensitive to Gentamicin (100%), followed by ciprofloxacin (83.33%), Vancomycin (66.66%), Erythromycin (33.33%), and resistant to Kanamycin (83.33%), *Bacillus spp*. (4) were found sensitive to Erythromycin (100%), were sensitive to followed by ciprofloxacin (75%), Neomycin (75%), Co-trimoxazole (50%), and resistant to Amoxicillin (100%). *E.coli spp*. (1), were found sensitive to Ciprofloxacin (100%), Co-trimoxazole (100%), Neomycin (100%). and resistant to Erythromycin (100%), and Amoxicillin (100%). *klebsiella spp*. (5), were found sensitive to Ciprofloxacin (100%), were sensitive to followed by Co-trimoxazole (80%), Neomycin (80%), and resistant to Amoxicillin (100%), Erythromycin (100%). *Entrobacter spp*. (4), were found sensitive to ciprofloxacin (100%), were sensitive to followed by kanamycin (75%), and resistant to Amoxicillin (100%), Ampicillin (100%), and Cefuroxime Sodium (100%). So Antibiogram result indicated the ciprofloxacin, Co-trimoxazole and Gentamycin, in optimum doses would be the drug of choice to treat the most cases of human infection caused by consumption of infected betel leafs.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	: Negative
+	: Positive
µg	: Microgram
µl	: Microliter
^{0}C	: Degree of Celsius
CFM	: Cefixime
CIP	: Ciprofloxacin
CLSI	: Clinical and Laboratory Standards Institute
CN	: Cefalexin
Cont.	: Control
CXM	: Cefuroxime sodium
E	: Erythromycin
E. coli	: *Escherichia coli*
EMB	: Eosin Methylene Blue
et al.	: Associated
etc	: Etcetera
FAO	: Food and Agricultural Organization
Fig.	: Figure
g	: Gram
GEN	: Gentamycin
Gm	: Gram
H_20_2	: Hydrogen peroxide
H_2S	: Hydrogen sulfide
HSTU	: Hajee Mohammad Danesh Science and Technology University
i.e.	: That is
K	: Kanamycin
Lid	: Limited
M. S	: Master of Science
MC	: MacConkey Agar
MIU	: Motility Indole Urease

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

Ml	: Milliliter
mm	: Millimeter
MR	: Methyi Red
MSA	: Manito Salt Agar
N	: Neomycin
NA	: Nutrient Agar
NB	: Nutrient Broth
No.	: Number
PBS	: Phosphate Buffer Saline
PM	: Post Mortem
Prof	: Professor
PSS	: Physiological Saline Solution
SL	: Serial number
Spp	: Species
VP	: Voges-Proskauer
w/v	: Weight by volume

CHAPTER I

INTRODUCTION

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INTRODUCTION

The deep green heart shaped leaves of betel vine are popularly known as Paan in Bangladesh. It is an important cash crop in Bangladesh. It is also known as Nagaballi, Nagurvel, Saptaseera, Sompatra, Tamalapaku, Tambul, Tambuli, Vaksha Patra, Vettilai, Voojangalata etc in different parts of India (CSIR, 1969; Guha and Jain, 1997).

All classes of people in Bangladesh chew betel vine not only as a habit but also as an item of rituals, etiquette and manners. The scientific name of betel vine is *Piper betle* L. It belongs to the family Piperaceae. It is a climbing plant with shiny, green heart-shaped leaves. The stem initiates many short adventitious roots (Hassan and Shahzad, 2005).

The vine is a dioecious (male and female plants are different), shade loving perennial root climber. There are about 100 varieties of betel vine in the world, of which about 40 are found in India and 30 in West Bengal (Guha, 1997; Maity, 1989; Samanta, 1994).

Desi Bangla, Bangla, Kali Bangla, Jhali, Sanchi, Bhabna, Mitha, Geso, Bonhoogly etc. betel vine cultivars are found in Bangladesh. The most probable place of origin of betel vine is Malaysia (Chattopadhyay and Maity, 1967).

Inspite of its alienness, the plant is much more popular in Bangladesh as well as in India than in any other country of the world since the antiquity. This would be evident from the numerous citations laid down in the ancient literature, particularly the Indian scriptures. In these citations, significance of the leaves has been explained in relation to every sphere of human life including social, cultural, religious and even day-to-day life, which is very much relevant even these days. For example, a well-prepared betel quid is still regarded as an excellent mouth freshener and mild vitalizer, routinely served on the social, cultural and religious occasions like marriage, Puja (religious festivals), Sraddha ceremony (religious function performed after cremation) etc. It is also used as a special item offered to the guests in order to show respect and for such traditional use of betel leaf in the Indian society, the leaf really stands alone without any parallel even today (Guha, 1997; Mehrotra, 1981).

In fact, this edible leaf has achieved an esteemed position in the human society right from the dawn of civilization, particularly in the countries like Bangladesh, Myanmar,

China, India, Indonesia, Malaysia, Nepal, Pakistan, Philippines, South Africa, Sri Lanka, Thailand etc. (Jana, 1996; Khoshoo, 1981; Samanta, 1994; Sharma *et al.,* 1996).

where leaves are traditionally used for chewing in their natural raw condition along with many other ingredients like sliced areca nut, slaked lime, coriander, aniseed, clove, cardamon, sweetener, coconut scrapings, ashes of diamond, pearl, gold and silver (Ayurvedic preparations), jelly, pepper mint, flavouring agent, fruit pulp etc. (CSIR, 1969).

Bangladesh is the second largest grower of betel vine on about 14,000 hectare. Total annual production of the crop in Bangladesh is about 72,500 tons. The average yield is 2.27 tons per acre (Anonymous, 2006).

But the acreage of betel vine is decreasing fast because of some physical and socioeconomic barriers like unavailability of credit facilities, uncontrolled marketing system and infestation of diseases and pest (Islam 2005).

Disease damage to the crop is one of several known limiting factors. The betel vine is highly susceptible to diseases, pests and some natural climates (Sayeeduzzaman, 1988).

A number of leaf spot diseases have been reported of which that due to *Colletotriehum* is most important (Maiti and Sen, 1979).

It causes both leaf spot and stem anthracnose. Although bacterial pathogens have been reported, bacterial leaf spot is now considered to be caused by *Xanthomonas campestris pv. betlieola*. Although much emphasis has been given to foot rot diseases, leaf spot diseases are also important as leaves are the commercial produce and a small spot on the leaf can reduce its marketability to a great extent. Leaf spot caused by *Xanthomonas campestris pv. betlicola* has much similarity to that caused by *Colletotrichum.* In leaf spot caused by *Colletotrichum,* lesions are brownish black surrounded by yellow halo. On stem, elongated dark brown lesions are formed. The only difference with the bacterial disease is the water soaked slimy band on the advancing margin of lesion detected on lower surface of leaf. In stem lesion no apparent difference except that surface of stem lesion caused by bacterial pathogen is somewhat slimy. Because of this similarity, earlier also the bacterial leaf spot was 1U existence but was ignored due to similarity of symptom with that of *Colletotrichum* leaf spot. Wilting of betelvine plants ill different states of India is considered to be caused by *Phytophthora* or *Sclerotium sp*. but as the

leaf spot bacteria also infect stem, it might have a role in wilt complex of betelvine. At present farmer's view in West Bengal is that wilt of vine is more severe than earlier days and symptom of diseases is different from that previously observed (Bidhan Chandra Krishi Viswavidyalaya 2002).

Considering the importance of betel vine as a commercial crop in Bangladesh and also considering the public health importance the present study was undertaken with the following objectives:

1. To isolate and identify some important bacteria from betel leaf
2. To detect the best antibiotic sensitivity pattern of the isolated bacteria from betel leaf

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Md. Mazedul Haque *et al.* (2017) studied the present study was undertaken to determine bacterial load as well as characterize bacterial flora of ready to eat (RTE) betel leaf sold at local markets in Mymensingh city. results and Total viable count of bacteria in betel leaf (log10 mean colony forming unit±standard deviation/ml) was 7.58±0.04 for KR market, 7.72±0.06 for Shesh more, 7.62±0.04 for Kewatkhali, 7.40±0.03 for Jobber more, and 7.60±0.06 for Ganginar par. A total of 98 bacterial isolates belong to five genera (*Escherichia coli*, *Salmonella spp*., *Vibrio spp.*, *Bacillus spp*., and *Staphylococcus spp*.) were identified. The prevalence of *E. coli* was 17.34%, *Salmonella spp*. was 25.51%, *Vibrio spp*. was 19.39%, *Bacillus spp*. was 18.37%, and *Staphylococcus spp*. was 19.39%. Antibiotic sensitivity test showed that all isolates were sensitive to two antibiotics such as ciprofloxacin and gentamicin. Four isolates *(E. coli, Salmonella spp*., *Vibrio spp*., and *Staphylococcus spp*.) were resistant to two antibiotics (ampicillin and cep studhalexin). Data of this study indicate that betel leaf sold at local market harbors multidrug resistance food-borne bacteria which might cause public health hazards if these antibiotic resistant transfer to human through food chain.

Md. Fakruddin *et al.* (2017) identified *Salmonella spp*. is one of the most common pathogens associated with fresh produce related foodborne illness. This study aimed to determine *Salmonella spp.* contamination level in betel leaf, internalization potential and possible decontamination process. Results: A total of 77% betel leaf sample collected from local market was found to be contaminated with *Salmonella spp*. Of all the Salmonella spp. isolated and identified, 28.5% belong to *Salmonella* enterica subsp. enterica serovar Enteritidis, 19.5% belong to *Salmonella* Typhimurium, 15.6% to Salmonella Paratyphi, 10.4% to Salmonella Schottmuelleri, 9.1% to *Salmonella Gallinarum,* 10.4% to *Salmonella Choleraesuis* and 6.5% belong to *Salmonella Bongori.* Internalized *Salmonella spp*. showed moderate resistance to commonly used antibiotics. Treatment with common surface food disinfectants could not remove *Salmonella spp*. completely from betel leaf indicating the possibility that the bacteria may be in internal tissue of the leaf. Assessment of internalization potential showed that *Salmonella spp*.

Manabendra Nayak *et al.* (2016) studied Pathogens present in the mouth are responsible for oral and dental infections. Dental caries is a common disease that results in tooth loss and cavitations. The purpose of the study was to determine the activity of P. betel leaf extracts against the bacteria isolated from oral region (Teeth). The teeth swabs were collected from patients suffering from dental caries. The bacteria present in the samples were isolated and identified by morphological and biochemical characteristics. Extracts preparation was carried out in different solvents (Ethanol, methanol and aqueous). And the isolated bacteria were subjected to antibacterial sensitivity test by agar well diffusion method against different concentrations of ethanol, methanol and aqueous extract. All the extracts showed inhibitory effect against the tested bacteria.

Nison Bhutia *et al.* (2015) Studied the present field trial was conducted in Himalayan Tract of West Bengal with three different light intensities (25%, 50% and 65%) and nine cultivars to standardize the optimum light requirement of different bangla cultivars and its effect on their growth, leaf yield and essential oil yield. The experiment was laid out in Split Plot Design. Lower light intensity (25%) recorded higher statistically significant linear growth during both active and dormant growth phase with increased leaf area (212.70 cm2), maximum vine elongation (26.15 cm), inter nodal distance (10.28 cm) and highest chlorophyll content (49.32). Cultivars like Simurali Gole Bhabna and Local recorded highest monthly yield (2.81no) under 25% light intensity over all cultivars. Interaction effect showed that for all the above characters the Bangla varieties under study gave maximum values in lowest light intensity. On the contrary due to more production of photosynthates higher light intensity gave significantly higher essential oil content (58.47 mg/100 g) of the leaves. Growing of betel vine under 25% light intensity may be considered optimum for better growth and higher leaf yield under closed type system of cultivation for all the cultivars under trial except Utkal Sudam and Bagerhat Local.

Hembram S *et al.* (2015) studied betel vine (*Piper betle* L.) is the most important cash crop grown almost all over the state of West Bengal, India. The most important betel vine growing districts in West Bengal are Midnapore (East), Howrah, Hooghly, 24-Parganas (South) and Nadia. Besides the above districts, cultivation has now been extended to 24-Parganas (North), *Birbhum, Bankura,* West Dinajpur and Murshidabad districts too. It is affected by a large number of diseases, which reduces yield and quality of betelvine leaves. Bacterial leaf spot and stem rot disease caused by *Xanthomonas*

axonopodis pv. betlicola and Pseudomonas betel respectively are gradually become important in West Bengal. Phoma piperis-betle is also prevalent in the state. When X.a.pv. betlicola and Ps. Betel jointly inoculated in same leaf, the size of the lesion become larger as compare to the lesion produced by individual bacterium. Simultaneous inoculation of X. a. pv. betlicola, Ps. betel and P. piperis-betle did not have pronounced effect in respect to lesion size in leaves. But inoculation of bacteria, 48 hours after inoculation of P. piperis-betle greatly increased lesion size.

Bholanath Mondal *et al.* (2014) identified Bacterial wilt caused by Ralstonia solanacearum is widespread in West Bengal (India). Surveys were conducted throughout West Bengal during 2004-2007 and incidence of some economically important crop plants namely; brinjal, tomato, potato, chilli, marigold, ginger, banana, elephant foot yam, Jute, tobacco, water melon, bottle gourd, large cardamom, hasnuhana, bougainvillea and twelve wild plants namely; *Amaranthus spinosus* L., *Amaranthus viridis* L., *Croton sparsiflorus* Mor., *Pennisetum purpurium* Schum., *Malachra capitata* L., *Cestrum diurnum* L., *Datura metel* L., *Solanum indicum* L., *Solanum sisymbriifolium* Lam., *Physalis minima* L., *Melochia corchorifolia* L. and *Costus speciosus* (J. Koenig) Sm. were recorded from diverse location of West Bengal with medium to high disease intensity. During survey, bacterial wilt disease was confirmed by ooze test in the field and biochemical tests in the laboratory in every case. A sharp relationship was observed between disease intensity and different meteorological factors during the experimental period. Crop plants transplanted during summer months were found infected more with this disease than those transplanted in the relatively cooler part of the year. In most of the cases, wilting process of wild plants started from the month of March (Av. Tmax. 32°C and Tmin. 20°C) when temperature gradually rises.

Nutankumar S. Jane *et al.* (2014) reported the vast economic potentiality of the crop can be adequately established by the fact that about 20-30 million people consume betel leaves in India on a regular basis besides those in other countries of the world which may include over 2 billion consumers. Its cultivation is highly labor intensive and offers employment to about 2.0 million families engaged in cultivation, trading and commerce in betel leaf throughout India. Betel vine leaf is used as medicine for certain diseases and also used as an antiseptic. During cultivation betel vine is very much affected by diseases such as Leaf spot, Leaf rot diseases and powdery mildew that result in great loss for the farmers. It occurs in a very virulent form and if not controlled, causes widespread

damage and even total destruction of the entire betel vine plantations without any early indications of the diseases. The aim of this paper is to study and identify various diseases in the betel vine plants.

R Salam *et al.* (2014) identified the antimicrobial effect of neem (*Azadirachta indica*) and betel (Piper betel) leaf on oral bacteria was examined. The saliva samples were collected from 50 patients suffering from dental caries and gingivitis. The bacteria present in the samples were isolated and identified by morphological and biochemical characteristics. The isolated bacteria were subjected to antimicrobial assay by disc diffusion method against different concentrations of aqueous and methanol extract. Both aqueous and methanol extract showed inhibitory effect against the tested bacteria even at lower concentration. Methanol extract exhibited larger zones of inhibition against all the isolated organisms compared to aqueous extract. Betel leaves extracts showed more activity compared to neem leaves extracts. In conclusion it can be said that both neem and betel leaves have potent antimicrobial effect on oral bacteria that lead to infection in mouth or normally present in mouth. Thus these plant materials can be used for treatment of dental caries and gingivitis after detailed pharmacological investigation which will be safe and economically viable.

Dinesh Chandra *et al.* (2013) identified *Xanthomonas axonopodis* pv. betlicola (Patel et al.) Vauterin et al. causes severe damage of betelvine (*Piper betle* L.) in West Bengal by producing different types of leaf spots (small to large, circular to irregular, angular), marginal leaf blight, stem lesion and wilting of vines. A selective medium was developed for isolation of this bacterium from diseased tissues and detection of this bacterium from the leaf surface, in soil and water. This bacterium grew best in Potato Sucrose Peptone Agar (PSPA) medium (Peeled potato 200 g, Sucrose 20 g, Peptone 5 g, Agar agar 20 g, and Water 1000 ml) and this medium was used as basal medium. Some fungicides and antibiotics (Carbendazim 25 mg, Copper oxychloride 25 mg, Metalaxyl 21 mg, Cycloheximide 50 mg, Pentid-200 100 mg, Nitrofurantoin 100 mg) were incorporated into the basal medium before use. Bavistin was used as source of Carbendazim, Blitox 50 as Copper oxychloride, Krilaxyl 35 WS as Metalaxyl, Furadantin capsule (human drug) as Nitrofurantoin. Pentid-200 is a human drug and it contains Penicillin-G potassium 2,00,000 units.

MD. Monirul (2012) discovered an investigation on the diseases of betel vine was done in six upazillas of Satkhira district viz. Kalaroa, Tala, Satkhira Sadar, Assasuni, Debhata and Kaligonj. In Kalaroa, disease incidence and severity of leaf rot of betel vine were ranged from 4.17 to 34.49% and 6 to 30.67%, respectively. In case of foot and root rot of betel vine, disease incidence ranged from 0 to 22.92% where the highest and the lowest count were recorded in August and December for both the diseases. In Tala, disease incidence and severity of leaf rot of betel vine ranged from 12.67 to 46.94% and 11.83 to 34.17%, respectively. In case of foot and root rot of betel vine, disease incidence ranged from 5.21 to 40.75% where maximum and minimum diseases were recorded in August and December for both the diseases. In Satkhira Sadar, disease incidence and severity of leaf rot of betel vine ranged from 3.17 to 27.38% and 2.05 to 15.33%, respectively. In case of foot and root rot of betel vine in Satkhira Sadar, disease incidence ranged from 0 to 15.63%, respectively where maximum diseases were recorded in August and minimum diseases were recorded in November and December. In Assasuni, disease incidence and severity of leaf rot of betel vine ranged from 3.67 to 35.5% and 7.34 to 26.33%. In case of foot and root rot of betel vine, disease incidence ranged from 0% to 23.96% where maximum and minimum diseases were recorded in August and December for both diseases. In Debhata, disease incidence and severity of leaf rot of betel vine ranged from 3.83 to 33.33% and 3.67 to 19.67%, respectively.

Bhattacharya *et al.* (2012) discovered that Betelvine (*Piper betle* L.) is an important cash crop in West Bengal. This crop is commonly affected by stem rot and leaf spot disease caused by two different genera of bacteria- *Xanthomonas axonopodis* pv. betlicola and Pseudomonas betle. Two bacterial pathogens enter into the host through stomata, hydathode and injury. Both the bacteria, at any portion, of the vine stem form prominent dark brown lesions. Surface of such lesion becomes sticky in humid condition. Occasionally small brown cracked lesions are found on the stem. Such lesion is formed due to infection of X. a. pv. betlicola. On the leaf, small to large, circular to irregular and/or angular brown colored spots and marginal leaf blight symptoms are produced by both the bacteria. All types of spots are surrounded by yellow halo or the halo is present in between brown and green tissue. At the underside of the leaf, the brown lesion is encircled by a water soaked zone or water soaked area, which is found in between brown lesion and green tissue in marginal blight. In addition, yellow colony forming bacterium, X. a. pv. betlicola produces very small brown spot surrounded by

prominent yellow halo but without water soaked zone. Frequently both the bacteria have been detected from same leaf spot or stem lesion. Association of these bacteria increases disease severity. Initially the bacteria colonize in parenchyma tissue and later move into vascular tissue. After entry into the vascular tissue X. a. pv. betlicola becomes systemic, produces no further lesion but causes sudden wilting of vine. Such situation causes much damage of the plantation in West Bengal. Pre-inoculation treatment with streptomycin and oxy-tetracycline hydrochloride greatly reduces lesion expansion.

Arani Datta *et al.* (2011) observed the leaves of Piper betel (locally known as Paan) have long been in use in the Indian local system of medicine for its antioxidant and antimicrobial properties. In the present work, the antimicrobial activity of ethanol extract of Piper betel leaves was evaluated against human pathogenic bacteria (both gram-positive and gram-negative). The leaf powder was subjected to phytochemical screening and was found to contain carbohydrate, protein, polyphenolic compounds, flavonoid, alkaloids and total antioxidant. The ethanol extract showed strong free radical scavenging activity as seen by DPPH model. The extract confirmed significant antimicrobial activity against all bacterial strains tested. The effect of the extract was almost proportional to the concentration of the extract tested. The MIC for the bacterial strains was in the range of 25 µg to 40 µg. Concurring with the disc diffusion results, the MIC of the Proteus vulgaris was found to be least 25 µg while for Staphylococcus aureus it was approximately 40 µg. Time-kill kinetics of the ethanol extract treated bacterial strains demonstrated similar results, showing decline in the growth curve after six hour in most of the strains. Crude ethanol extract of Piper betel showed strong antimicrobial activity against the tested pathogenic bacterial strains.

Aparna Priyadars *et al.* (2011) detected In Orissa Betel vine (*Piper betle* L., family Piperaceae) is an important asexually propagated cash crop comprising of several cultivars. There are many cultivares but they are not well demarcated due to similarities in the morphological characters and in certain places same cultivars are cultivated under different local name. Therefore, in the present study DNA fingerprinting technique has been used to differentiate cultivars of betel vine for crop improvement programme. So Comparative study of both RAPD and ISSR markers analysis were used to establish genetic identities and evaluate genetic diversityamong fifteen cultivars of betel vine grown in different parts of Orissa. Thirty RAPD and 25 ISSR primers were tested to resolve the genetic diversity among the cultivars. Twenty RAPD and 18 ISSR primers

resulted in 523 amplicons. Out of these 504 were polymorphic loci and 54 were found to be unique. The extent of genetic diversity and relatedness among 15 cultivars were computed through Jaccard's similarity coefficient. Maximum similarity (0.68) was observed between Balipana and Birkoli and minimum (0.114) for Banglamandesore chitalpudi and Halisahar Sanchi. All the cultivars were related with each other with an average similarity of 0.2913. Dendrogram showed Godibangala was separated from rest of the species into isolated clade in both the analysis. Correlation between RAPD and ISSR marker was very low (r = 0.17). RAPD showed high correlation with all the primers.

Sengupta *et al.* (2011) discovered the experiment was carried out during 2006 and 2007 to study the incidence of foot rot of betel vine caused by Phytophthora parasitica and growth, yield, and keeping quality of betel vine by applying *Trichoderma harzianum* (bio-agent). *T. harzianum* inoculated in 500 kg oil cake ha-1 was applied once at pre-monsoon, two times during pre and post monsoon and four times at quarterly intervals. Bordeaux mixture was used to compare the treatments in preventing the intensity of foot rot. The results revealed that the minimum foot rot disease was recorded where four drenching and eight sprayings of Bordeaux mixture at monthly and fortnightly intervals respectively were applied in 2006, 2007 and pooled analysis of two years data. The maximum foot rot disease was recorded in control treatment in 2006, 2007 and pooled analysis of two years data. The maximum growth, yield parameters like vine elongation, fresh weight of JOO leaves, leaf yield and keeping quality was recorded in treatment where four drenching and eight sprayings of Bordeaux mixture at monthly and fortnightly intervals respectively were applied during 2006, 2007 and pooled analysis of two years data. Highest cost: benefit ratio was recorded where *T. harzianum* were applied at pre monsoon and lowest cost: benefit was recorded where Bordeaux mixture were applied**.**

Rars *et al.* (2011) studied Betel vine (*Piper betle* L.) having the heart shaped deep green leaves is an important horticultural crop of aesthetic and commercial values. The perennial climber is grown throughout the country. There are about 100 varieties of betel leaf (pan) across the world of which 40 are encountered in India and 30 in west Bengal and Bangladesh (Guha 1997; Maity, 1989; Samanta, 1994). The most probable place of origin of Pan is Malayasia (Chattopadhyay and Maity, 1967). Pan contains some vitamins, enzymes, thiamine, riboflavin, tannin, iodine, iron, calcium, minerals, protein,

essential oil and medicine for liver, brain and heart diseases (Chopra et al.1956; Khanra 1997). Apart from the serological aspects the leaves are generally offered to the guests as the symbol of hospitality. It is grown in the rainfall about 2250-4750 mm, relative humidity and temperature ranging from 4080% and 15-90oC respectively (Guha and Jain, 1997). This perennial crop is found to be infected by various diseases of which leaf rot and leaf spot caused by pathogens, Phytophthora parasitica and Colletotrichum capsici are the major constraints for cultivation of the crop across the country (Goswami et al. 2002). Leaf rot can damage the crop within a week when it attacks the vine (Chaurasia 1994). Leaf rot and foot rot have been reported to be caused by Phytophthpra palmivora and leaf rot may cause 30-100% leaf yield loss (Maiti and Sen 1997).

Das *et al.* (2010) observed Betel vine (*Piper betle* L.) is a perennial dioecious evergreen creeper, grown in shady condition with high humidity. Betelvine whitefly, Singhiella (Dialeurodes) pallida (Singh) (Hemiptera : Aleyrodidae) is an important and common pest of betelvine causing severe damage to betel leaves. Screening of betelvine cultivars for resistance to betelvine whitefly, S. pallida was carried out in a conservatory (boroja) located at the Research Farm of Bidhan Chandra Krishi Viswavidyalaya, Nadia, West Bengal for two consecutive years (2005 and 2006). A total of twenty-three cultivars were evaluated. Suitability of three species of Piper as the hosts of betelvine whitefly was also tested. None of the tested cultivars of betelvine was completely free from whitefly infestation, though Simurali Sanchi and Kalipatti (both are of Sanchi type) were found to be moderately resistant in both the years. Here, three species of the genus Piper (*P. longum, P. chaba and P. hamiltonii*) have been recorded as the new hosts of S. pallida.

Bijan Kumar Das (2010) discovered Betel vine (*Piper betle* L.) is a perennial evergreen shade loving creeper belonging to the family Piperaceae. In India, it is commercially cultivated over an area of 50,000 ha as an important and potential cash crop. In spite of the tremendous potentially of the crop, cultivation of betelvine is highly risky and returns are uncertain because of its susceptibility to several pests and diseases, aggravated by the nature of the plantation. A good number of hemipteran insect pests occur in betelvine ecosystem which dwindle betelvine yield potentiality. Among these, the polyphagous betelvine blackfly, *Aleurocanthus rugosa* Singh (Aleyrodidae: Hemiptera) is a major pest causing severe damage to the foliage in the conservatories (borojas) of West Bengal. The seasonal incidence of A. rugosa in boroja was recorded during 20032004. A. rugosa adults were active in the boroja through out the year with two peaks, one in pre-monsoon

and another in post-monsoon i.e., period prior initiation of winter season. During the faunastic survey in betelvine, a new species of Aleurocanthus (description under process) was recorded from West Bengal. This had also been found to occur on Piper longum L. Another species, A. nubilance (Buckton) which was recorded on betelvine in Bangladesh during 1900, was not recorded in this area. Detection of host resistance against insect pests is very relevant for genetic improvement programmes.

Mahesha N. Sigera *et al.* (2009) discovered bacterial leaf blight, *Piper betle* L., Xanthomonas. Betel vine (*Piper betle* L.) is subjected to attack by several pathogens. Damage caused due to diseases is a major constraint in production, as it causes direct damage to the consumable product. Out of bacterial diseases, the most problematic disease is the bacterial leaf blight (BLB) caused by Xanthomonas campestris pv. betlicola1. It was first reported in Sri Lanka in 1928 by Ragunathan as a serious leaf spot in betel. Symptoms are characterized by angular leaf spots surrounded by a conspicuous chlorotic halo. These symptoms later spread to the stem. When the disease becomes serious the leaves and internodes fall off and finally the vine dies. It is reported that most of the betel varieties that are cultivated at commercial level are susceptible to X. campestris pv. Betlicola Up to 60% incidence of this disease has been reported from major betel cultivating areas, and it can increase to about 75% during the rainy seasons2. The conditions that are favourable for the growth of the betel crop are also congenial for the development of the pathogen. Even though this is a very serious disease causing heavy crop losses, no work is reported from Sri Lanka giving information on the spread, biology and management of the bacterial leaf blight2. The only practical method available to control the disease is the use of disease free planting material obtained after indexing the propagating stock.

Guha (2006) discovered the fresh leaves of betel vine are popularly known as Paan in India, which are consumed by about 15-20 million people in the country. It is cultivated following the traditional methods in India on about 55,000 ha with an annual production worth about Rs 9000 million. On an average about 66% of such production is contributed by the state of West Bengal where it is cultivated on about 20,000 ha encompassing about 4-5 lakh Boroj employing about the same number of agricultural families. There is a menacing wastage of the leaves during storage, transportation and the glut season. Moreover, the surplus leaves, if not disposed of properly may cause environmental pollution and health hazards. Such wastage may be minimized by various ways and

means including extraction of essential oil from the surplus betel leaves. This oil may be used as an industrial raw material for manufacturing medicines, perfumes, mouth fresheners, tonics, food additives etc. The leaves are nutritive and contain anti carcinogens showing promise for manufacturing of a blood cancer drug. Some disputed reports also claim that chewing betel leaves excessively may cause oral cancer. The agricultural, industrial, economic, medicinal and allied potentialities of the crop are discussed.

Bidhan (2002) Betel vine (Piper betle) is a perennial dioecious creeper cultivated in India for its leaf since time immemorial. The cultivated betel in India is usually the male plant selected from certain races and consequently does not fruit. This crop belongs to the family Piperaceae and is probably a native of Malaysia. In India, West Bengal, Assam, Karnataka and Tamil Nadu have the highest acreage in terms of cultivation of betel vine. Other important states are Maharastra, Kerala, Andhra Pradesh, Madhya Pradesh, Bihar and Uttar Pradesh. Besides India, betelvine is also cultivated in Malaysia, Indonesia, Mayanmar, Philippines, Bangladesh, Nepal, Sri Lanka etc. The most important betelvine growing districts in West Bengal are Midnapore (East), Howrah, Hooghly, 24-Parganas (South) and Nadia. Besides the above districts, cultivation has now been extended to 24-Parganas (North), Birbhum, Bankura, West Dinajpur and Murshidabad districts. Common varieties cultivated in India are BangIa, Mitha, Sanchi, Kapoori, Desawari, Khasi and Ghanagnete. Cultivation of betelvine is highly specialized and is carried out with intensive care in terms of land preparation, choice of varieties, propagation, aftercare, control of pests and diseases and harvesting and marketing. Hence it is mostly confined to small holdings distributed in almost all states in India except dry north western parts.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present research work was conducted during the period from July to December 2017, in the Bacteriology Laboratory of Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur. The detailed outline of materials and methods are given below:

3.1 Materials

3.1.1 Study area and study population

Total 20 number of the betel leaf samples were collected from different markets located in, Sadar Upozila, Dinajpur, (Leili morr, Terminal road, Doshmile, Gopalgong bazaar, and Basharat) for the isolation and identification of bacteria by staining, cultural and biochemical reactions and for detection of antibiotic resistance patterns of the isolated bacteria.

Table: 1 Study area and study population

SI. No	Name of the place	No. of samples
1	Basharat	4
2	Gopalgong bazaar	4
3	Doshmile	4
4	Terminal road	4
5	Leili morr	4

3.1.2 Laboratory preparation

All items of glass were including test tube pipettes cylinder flask ,conical flasks glass plate slides vials and agglutination test tube soaked in a household dishwashing detergent solution (Trix , Recket and Colman Bangladesh ltd) for overnight contaminated glassware's were disinfected 2% sodium solution hydrochloride solution period cleaning. The glassware were then clean by brushing washed thoroughly and finally sterilized

either by dry heat at 37C for 2 hours or by autoclaving for 15minites at 121C under 15 Ibs pressure per square inch. Autoclaved items were dried in hot air oven over at 50C disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept over at 50C for future use.

3.1.3 Instruments and Appliances

At research laboratory, Department of microbiology, Hajee Mohammed Danesh Science and Technology University. We use various kinds of apparatus, which are given below Test tube, Petri dishes, Conical flask, Pipette, Slides, Micro pipette Microscope, Sterile cotton, Immersion oil, Toothpick, Autoclave, Inoculating loop (Straight and coiled), Aluminum foil roll, Beaker, Cylinder, Refrigerator, Making pen, Bacteriological incubator, Hof air oven, Stop Watch,

3.1.4 Media for culture

Different bacteriological culture media used for isolation bacteria from betel leaf in this experiment are as follows.

3.1.4.1. Liquid culture media

Nutrient Broth (HI-MEDIA, India)

3.1.4.2 Solid culture media

Nutrient Agar media (HI-MEDIA, India)

MacConkey agar media (HI-MEDIA, India)

Eosin Methyl Blue Agar media (HI-MEDIA, India).

Manitol Salt Agar media (HI-MEDIA, India)

Blood agar media (HI-MEDIA, India)

Muller Hinton Agar (HI-MEDIA, India)

3.1.5 Media Used for Biochemical Test

In order to identify bacteria the following media were used for biochemical tests:

- Triple sugar iron (TSI) agar slant (HI-MEDIA, India).

- MTU agar base media. (HI-MEDIA, India).
- Methyl Red (MR) media.
- Voges Proskauer medium base (VP) media.
- Simmons Citrate Agar.

3.1.6 Reagents

- Crystal violet
- Gram's iodine
- Acetone alcohol
- Safranine
- Methyl-Red solution
- Alpha-naphthol
- Potassium hydroxide solution
- Kovac's reagent
- Phenol red solution
- Phosphate buffered saline solution(PBS)
- Distilled water
- Indian Ink

3.1.7 Antibiotic Sensitivity Discs

To determine the drug Sensitivity and resistance patterns of isolated organisms with different types of antimicrobial discs, commercially available antimicrobial discs (Oxoid Ltd., UK,) were used .The method allowed for the raped determination of the efficacy of the by measuring the diameter to the zone of inhibition that resulted from different diffusion of the agent into medium surrounding the disc. The list of commercially available antimicrobial disc used in this study with their concentration is given below

Table: 2 Antimicrobial agents with their disc concentration

S1No	Name of the antibiotics	Disc concentration (µg/disc)
1	Ciprofloxacin (CIP)	5 µg/disc
2	Co-trimoxazole (COT)	25 µg/disc
3	Kanamycin (K)	30 µg/disc
4	Neomycin (N)	30 µg/disc
5	Gentamycin (GEN)	10 µg/disc
6	Erythromycin (E)	15 µg/disc
7	Amoxicillin (AMX)	30 µg/disc
8	Vancomycin (VA)	30 µg/disc
9	Cefuroxime sodium (CMX)	30µg/disc
10	Ampicillin (AMP)	10µg/disc

Note: Sl. No. = Serial Number, jig = Microgram,

3.2 Methods

The following methods were used for the isolation and identification of bacteria.

3.2.1 Experimental Layout

The experimental work was divided into two steps: the first step was performed for the isolation and identification of the organisms of the collected sample using cultural, staining and biochemical techniques. The second step was conducted for the determination of antibiotic sensitivity and resistance pattern of isolated organisms of various samples by using different antibiotic discs available in the market. The layout the diagrammatic illustration of present study is shows in the following figure:

Experimental layout

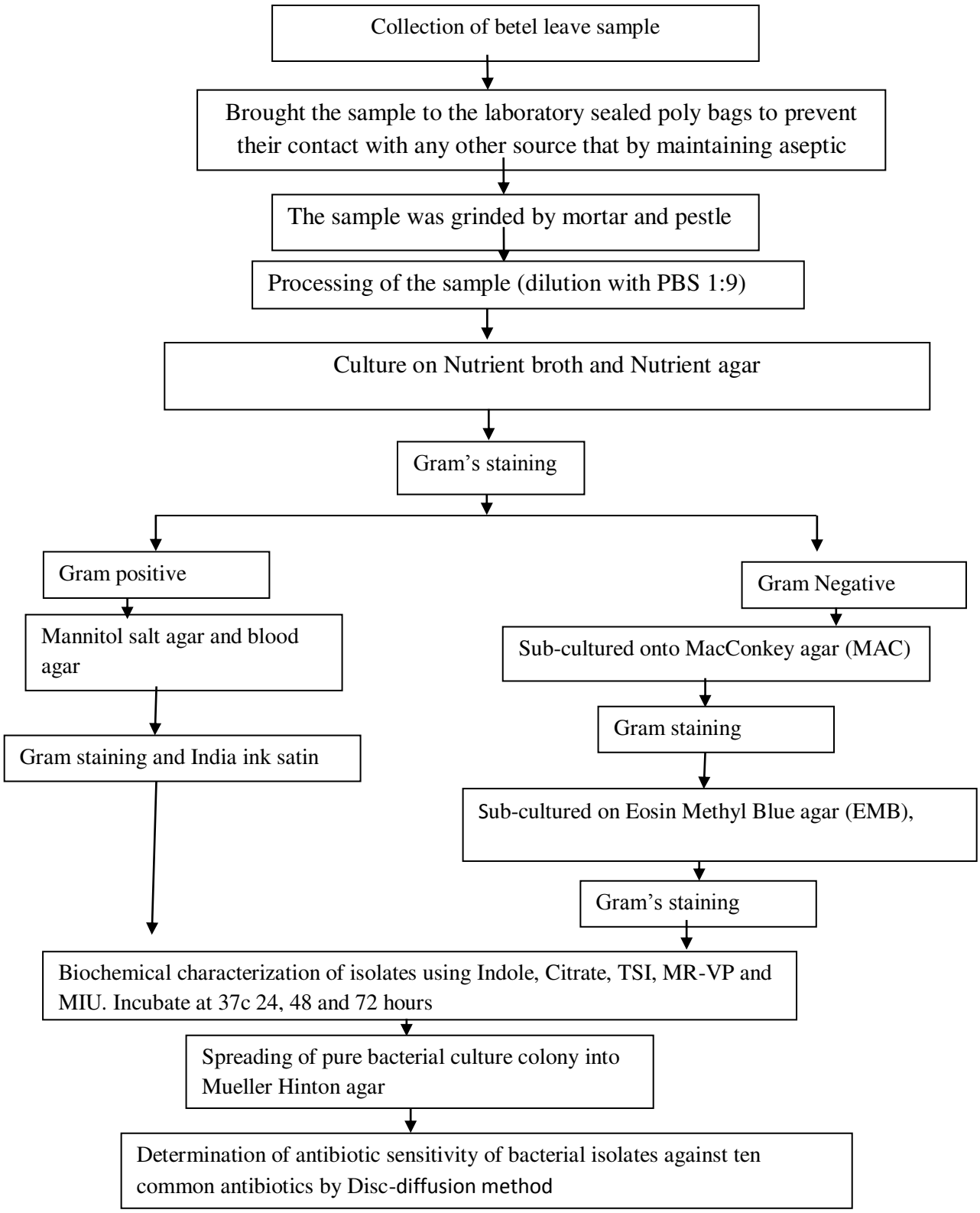

Fig: 1 Schematic illustration of experimental layout

3.2.2 Preparation of culture media

All media broth and reagent use in this experiment were prepared according to instruction.

3.2.2.1 Nutrient broth

Nutrient broth (NB) was use to grow the organisms from the sample collected from the study areas before performing biochemical test (Cheesbrough, 1984).

13 gram of Bacto-nutrient broth (Difco) was discovered in 100 ml of cold distilled water and heated up to boiling to dissolve it completely. The solution was then distributed in tubes stopper with cotton plugs and sterilized in the autoclave machine at the 121C and 15 pounds pressure per square inch for minutes. The Sterility of the medium was judged by incubating overnight at 37C and used for culture characterization or stored at 4C in refrigerator for future use (Carter, 1979).

3.2.2.2 Nutrient Agar

28.0 gram of Nutrient agar power (Hi-media India) was suspended in 100 ml of cold distilled water in flask and heated to boiling for dissolving the medium completely. The medium was the sterilized by autoclaving. After autoclave the medium was poured into each sterile Petridish and allowed to solidify. After solidification of the medium in the pertridishes these were incubated at 37C for overnight to check their sterility and use for culture characterization (Carter, 1979).

3.2.2.3 Eosin methylene blue agar

Eosin methylene blue (EMB) Agar medium was used to observe the growth of Escherichia coli (Cheesebrough, 1984).

36 gram of EMB agar base (Hi-media, India) was added to 1000 ml of distilled water in conical flask and heated until boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in to sterile glass. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the pertridishes partially removed. The sterility of the medium was judged and or stored at 4C in refrigerator for future use (Carter, 1979).

3.2.2.4 MacConkey agar

51.50 grams of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000ml of cold distilled water take in conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured sterile glass pertridishes. To accomplish the surface be quite dry, the medium was allowed to solidify about 2hours with the cover of the pertridishes partially removed. The sterility of the medium was judged and use for culture characterization or stored at 4C in refrigerator future use (Carter, 1979).

3.2.2.5 Blood agar

40 gram of blood agar base (HI-MEDIA, India) power was suspended in 1000 ml of distilled water and boil to dissolve the medium completely the medium was sterilized by autoclave at 1.2kg cm pressure and 121Cfor 15min and 45 C .then 5-10% sterile defibrinated blood was added to the medium and distributed to sterile pertridishes and allowed to solidify.

3.2.2.6 Muller Hilton Agar

Muller Hilton agar is used in antimicrobial susceptibility testing by the disk diffusion method 38 gram of Muller Hilton agar powder was suspended in 1000 ml of distilled water.it was mixed well.it was heated agitating frequently and boiled for about one minite.it was dispensed and sterilized in autoclave at 16-121C (15 Ibs.sp) for minutes, it was cooled to 45C or 50C (Carter, 1979).

3.2.2.7 MIU media

18 gram of MIU agar (Difco) was suspended in 950 ml of cold destined water taken in a conical flask and heated up to boiling to dissolve the medium completely. 95 ml was dispensed into flask and sterilized by autoclave at 15 Ibs pressure (121C) FOR 15, minutes the was cooled to about 50-55C and aseptically add 5 ml was added of sterile 40% basal medium after mixing were dispensed into sterile test tube. Allow to cool in an upright positive. The sterility of the medium was judged and use for cultural characterization or stored at 4C in refrigerator for future use (Carter, 1979).

3.2.3 Reagents preparation

3.2.3.1 Methyl Red – Voges prokauer broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml distilled water dispensed in 2ml amount in each test tube and then the test tube were autoclaved. After autoclave the tube containing medium were incubated at 37C for overnight to check their sterility and use for biochemical characterization or stored at 4c in refrigerator for future use (Cheesbrough, 1984).

3.2.3.2 Methyl-Red solution

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300ml of 95% alcohol and diluting this 500 m with the addition 200ml od distilled water.

3.2.3.3 Voges-proskauer solution

3.2.3.3.1 Alpha-naphthol solution

Alpha-naphthol solution was dissolving 5gram of 1-naphthol in 100ml of 95% Ethyl alcohol.

Potassium hydroxide solution potassium hydroxide (KOH) solution was prepared by adding 40 gram of potassium hydroxide crystals in 100ml of cold distilled water.

3.2.3.4 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40gram of potassium hydroxide Crystals in 100 ml of cold distilled water.

3.2.3.5 Kovac's reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of Amyl alcohol and to this mixing 5 gram of paradimthyl-aminohenyldehide crystal were adding this was then kept in flask equipped with rubber cork for future use (Merchant and packer, 1967).

3.2.3.6 Phosphate buffer solution

For preparation of phosphate buffer saline (PBS) solution, 8gram of solution of sodium chloride (NaCl) 2.89 gram of disodium hydrogen phosphate (Na_2HPO_4, $12H_2O$), 0.2 gram of potassium chloride (KCl) and 0.2 gram of potassium hydrogen phosphate were suspended in 1000ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121C maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The PH the solution was measured by a PH meter and maintained at 7.0 – 7.2 (Cheesbrough, 1984).

3.2.4 Sample collection

20 Betel Leaf samples were collected from five different markets of the Dinajpur (Leili mall (4), Terminal road (4), Doshmile (4), Gopalgong bazaar (4), and Basharat (4), Bangladesh.

3.2.5 Preparation of sample

Adequate amount of different betel leaf samples were uniformly homogenized in mortar and pestle using a sterile diluent as per recommendation of ISO (1995). A homogenized suspension was made with the help of mortar and pestle. A quantity of 1 gm. homogenate sample of each different marked was taken aseptically with a sterile spoon and transferred carefully into a sterile pestle containing 9 ml of PBS. Thus 1:9 dilutions of the samples were obtained.

Fig: 2 Pestle and Mortar with betel leaf

Fig: 3 Infected betel leaf samples

3.2.6 Isolation and identification of bacteria

The cultural examination of betel leaf samples for bacteriological study was done according to the standard method (ICMSF, 1985). Identification of bacteria was performed on the basis of colony morphology; Gram's staining reaction and biochemical test and a cell capsulated for India ink staining

3.2.6.1 Culture of organisms

Diluted betel leaf samples were enriched into nutrient broth and nutrient agar by overnight incubation at 37°C. enriched culture was streaked duplicate into, MacConkey agar, Eosin Methylene Blue agar (EMB), Mannitol salt agar(MSA), blood agar (BA) and incubated at 37 ºC for 24 hrs.

3.2.6.2 Culture on ordinary media

Sample were inoculated separately into media like nutrient agar, nutrient broth ware incubated at 37C for overnight. The colonies on primary culture were repeatedly sub-cultured by streak plate method (Cheesbrough, 1984) until the pure culture with homogenous colonies were obtained

3.2.6.3 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed to obtain isolated colonies as per:

Step1: An inoculum was picked up with a sterile loop and spread on an area of the medium in the Petridish.

Step 2: the loop was sterilized by being heated as red hot in a flame.

Step 3: The inoculum was spread over the reminder of the plate by drawing the cooled parallel line.

This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more time to ensure purity.

3.2.6.4 Morphological characterization of organisms by Gram's staining method

The gram staining was followed to study the morphological and staining characterization of bacteria and provide information about the presumptive bacterial identification as per recommendation of (Cowan and Steel 1979)

Procedure:

- A loop full of sterile distilled water was placed in the center of a clean sterile slide.
- A small colony was picked up with a bacteriological loop and mixed with a distal water of water slide.
- The colony was made to thin smear on slide.
- The smear were fixed by air drying
- 0.5% crystal violet solution was then applied on the smear for one minute.
- Lugols iodine was then added to act as mordant for one minutes.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was then washed with water.
- Safranine was added as counter stain and allowed for one minutes.
- Then the slide was then washed with water.
- Then the slide was blotted with blot paper and was allowed to dry. The slide was examined under microscope with high power.

Gram positive (violate colour) organisms are discarded and gram negative (pink coloured), small rod shaped, single or paired arranged organisms were selected.

3.2.7 Subculture into different media

3.2.7.1 MacConkey agar

Samples were subculture on MacConkey agar media and incubated at 37 C for overnight. After that both lactose fermenter bacteria (rose pink color colony) and lactose non fermenter bacteria (pale color colony) were selected.

3.2.7.2 Culture on selective media

3.2.7.2.1 Eosin methylene Blue (EMB) agar

Sample of positive lactose fermenter were taken and sub-culture on Eosin methylene Blue Agar media and incubate at 37 C for overnight.

Same EMB agar plate showed slightly circular colony with dark center metallic sheet. Also in same EMB agar, the growth was indicated by smooth, characteristics mucoid and pink colored colonies which are a consequence of the organism's abundant polysaccharide capsule.

3.2.7.2.2 Manitol Salt Agar (MSA)

Gram positive cultures were streaked separated MSA agar plate and incubated at 37°C for overnight. Next day demonstrated morphological characteristics of the bacterial colonies.

3.2.7.2.3 Blood agar

Yellow colony from Mannitol salt agar was strike on blood agar

3.2.8 Microscopically study for identification of staphylococcus spp, bacillus app, E.coli spp, klebsiella spp, suspected colonies by gram staining, Indian Ink staining.

3.2.8.1 Gram's staining

Gram's staining was performed by taking colony for selected media to determine the size shape and arrangement of bacteria according to the method described by merchant and packer (1967). Stained slides were examined under light microscope at 100x magnification.

3.2.8.2 Indian ink staining

India ink stain was used to determine if a Cell was gelatinous capsule

- Place a drop of Indian ink onto a clean glass slide
- Using a sterile loop, obtain cultural sample and place it onto the slide and mix it with the drop of India ink

- Obtain another sterile glass slide and have laid it at angle on one end of the first slide, spread out the drop into a film to have a thin layer of smear-ink mixture
- Allow the slide to stand for about 5 mints (air dry)
- Saturate the slide with crystal violet for mint and then tilt the slide to drain excess dye
- Allow the slide to dry (air –dry)
- Place one drop of oil emersion on the dry slide
- View the slide using 100xs

3.2.9 Identification of isolation bacteria by different biochemical test

Isolation organisms with supported growth characteristics of staphylococcus spp, bacillus spp E.coli spp, and klebsiella spp. were maintained in pure culture and subjected to biochemical tests.

3.2.9.1 Procedure of Indole test

2ml of peptone water solution was inoculation with 5ml of culture of each of the isolation bacteria and incubation for 48 hours. 0.5ml Kovac's reagent was added well and examined after 1minute. A red color ring at the top of the reagent indicated production of the indole by the organisms (Cowan**,** 1985)**.**

3.2.9.2 Procedure of MR test

The test performed by inoculating separately a colony of the each of the isolation test organisms in 0.5ml sterile glucose phosphate broth. After overnight incubation at 37C, A drop of methyl red solution was added. A positive methyl red test was shown by the appearance of a bright red color. A yellow or orange color was a negative test (Cowan, 1985).

3.2.9.3 Procedure of VP test

2ml of sterile glucose phosphate peptone water were inoculated separately with 5ml of each of the isolation organisms and incubated at 37C for 48 hours. Avery small amount (knife point) of creatine was added and mixed .3ml of 40% potassium hydroxide were added and shocked well. The bottle cap was removed and left for an hour at room

temperature. It was observed closely for the slow development of pink color for positive cases. In negative cases there no development of pink color (Cowan, 1985).

3.2.9.4 Procedure of motility of indole urease test

MIU media were prepared in test tubes. Then the isolation organisms were inoculation separately into the media by stabbing method with the help of sterile straight wire. Then the test tube was incubation 37C overnight. Single stick that is no turbidity throughout the medium indicates gram negative organisms (non-motile) and turbidity throughout the medium indicate gram positive case (Cowan, 1985).

3.2.9.5 Procedure of Triple Sugar Iron Test

Triple Sugar Iron contains three sugars (glucose, sucrose and lactose). At first TSI agar slant were prepared in test tube. Then the isolation organisms were inoculation separately into the tube butt with a sterilized wire and in the slant with wire loop producing zigzag streaking. The tubes were incubated for 24 hour at 37. Yellow color of butt and slant of the test tube indicates fermentation of glucose, sucrose and lactose fermentation and butt shows blacking indicate H_2S Production (Cowan, 1985).

3.2.9.6 Procedure Citrate Utilization Test

The test organism was cultured in a medium containing sodium citrate, an ammonium salt and bromothymol blue indicator. The organisms use citrate (the only source of nitrogen). The citrate utilization is followed by alkaline reaction (change of the color form light to blue) and growth in the medium was indicated by appearance of turbidity. This test was performed in the identification of *Enterobacteria*. By (Cheesbrough, 1984).

3.2.10 Antibiotic susceptibility test

Bacterial susceptibility to anti-microbial agent was determined in vitro by using the standardized agar disc-diffusion method known as the *Kirby Bauer*, Labeled the covers of each of the agar plates with name of the test organisms was inoculated.

- Using sterile technique, inoculated all agar plates with their respective test organisms as follow:

- Dipped a sterile cotton swab into a well-mixed saline test culture and removed excess inoculums by pressing the saturated swab against the inner wall of the culture tube.
- Using the swab streaked the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface.

- Allowed all culture plates to dry for about 5 minutes.
- Distributed the individual antibiotic discs at equal distance with forceps dipped in alcohol and flamed.
- Gently pressed each disc down with the wooden end of the cotton swab or sterile forceps to ensure that the discs adhered to the surface of the agar.
- The plates were then inverted and incubated at 37^0 C for 24 hours.
- After incubation, the plates were examined and the diameter of the zones of complete inhibition was measured in mm.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

Result of culture test, staining, biochemical test and antibiotic sensitivity test, including percentage of isolated bacteria are presented in different tables and described below under the following heading:-

4.1 Bacteriological investigation

Total 20 numbers of infected betel leaf samples were collected for this study. Out of 20 samples 20 bacterial isolates were found, among them 6 were *staphylococcus spp*, 4 were *bacillus spp*., 4 were *Entrobacter spp*., and 5 were *klebshella spp.* And 1 *were E. coli spp.*

Table-3: Prevalence of bacterial isolates in betel leafs

Total number of sample	Bacterial isolates		Prevalence (%)
20	Staphylococcus	6	30 %
	Bacillus	4	20%
	E.coli	1	5%
	Klebsiella	5	25%
	Entrobacter	4	20%
	Total no. of isolates	20	

4.2 Isolation and Identification of *staphylococcus spp.* by different bacteriological method

4.2.1 Result of cultural examination

4.2.1.1 Nutrients both

Nutrient broth was inoculated with betel leaf samples and incubated at 37 for 24 hours. The growth of bacteria was indicates by the presence of turbidity.

4.2.1.2 Nutrient Agar

Nutrient Agar plates streaked with the organisms separately revealed the growth of bacteria after 24 hours of incubation at 37 aerobically and were indicated by the growth of circular small yellowish colonies **(Plate-1).**

4.2.1.3 Manitol salt Agar

Manitol salt Agar plates streaked with the organisms separately revealed the growth of bacteria after 24 hours of incubates at 37aerobically and were indicated by the growth of small yellowish colonies **(Plate-2).**

4.2.1.5 Blood agar

Blood agar plate streaked separately with the organism and incubates at 37 aerobically for 24 hours and B- hemolysis were produced **(Plate-3).**

4.2.2 Result of gram's staining

Gam's staining smear for Nutrient agar, Mannitol salt agar, blood agar was examined and revealed Gram-positive cocci arranged in grape like cluster **(Plate-4).**

Table-4: Cultural, morphological and biochemical properties of isolated *staphylococcus spp.*

Culture characteristics			**Biochemical Characteristics**		**Staining Characteristics**
Nutrient Agar	**Manitol Salt Agar**	**Blood agar**	**Test**	**Result**	**Staining properties**
Small Yellow colonies	Yellow colonies	B-type of Hemolytic Were produced	Catalase Indole MIU MR VP TSI Citrate utilization test	+ _ + + + + _	Gram positive cocci arranged in grape like cluster

Legends

MR=Methyl-red test, VP=Voges-prokauer test, TSI=Triple Sugar Iron, MIU=motility Indole urease test, citrate, $_+$ positive,**-** negative

4.2.3 Result of biochemical test

4.2.3.1 Methyl-red and voges-proskauer test

The isolates were MR positive **(Plate -21)** and VP positive **(Plate -22).**

4.2.3.2 Indole test

The isolates were Indole negative **(Plate -23)**

4.2.3.3 Motility Indole urease test

The isolates were MIU positive **(Plate -43)**

4.2.3.4 Citrate utilization test

The isolates were citrate Positive **(Plate -25)**

4.2.3.5 Triple sugar iron test

The isolates were TSI Negative **(Plate -26)**

4.2.3.6 Catalase test

The isolates were catalase positive **(Plate -27)**

4.3 Isolation and Identification of *Bacillus spp* by different bacteriological methods

4.3.1 Result of culture examination

4.3.1.1 Nutrient broth

Nutrient broth was inoculated with betel leaf samples and incubated 37 for 24 hours. The growth of bacteria was indicates by the presence of turbidity.

4.3.1.2 Nutrient Agar

Nutrient Agar plates streaked with the organisms revealed the growth of bacteria after 24 hours of incubates at 37 aerobically and were indicated by the growth of thick grayish – white, or cream coloured colonies with irregular ends **(Plate-5)**.

4.3.1.3 Manitol salt Agar

Manitol salt Agar plates streaked with the organisms separately revealed the growth of bacteria after 24 hours of incubates at 37aerobically and were indicated by the growth of colonies media Grayish white colored **(Plate-6).**

4.3.1.4 Blood Agar

Blood agar plates streaked separately with the organism and incubate at 37 aerobically for 24 hours. Large cream colonies with hemolysis were produced **(Plate-7).**

4.3.2 Result of gram's staining

4.3.2.1 Gram's staining

Gram's staining smear for Nutrient agar and blood agar were examined microscopically which revealed Gram-positive rod shaped organisms **(Plate-8).**

4.3.2.2 Result of Indian ink staining

India ink staining smear for Nutrient agar and Blood agar were examined microscopically which revealed capsulated bacteria **(Plate-9).**

Table-5: Cultural, Morphological and Biochemical Properties of Isolated *Bacillus ssp.*

Culture characteristics			**Biochemical Characteristics**		**Staining and morphological**	**India ink staining**
Nutrient Agar	**Manitol salt agar**	**Blood agar**	**Test**	**Result**	**Staining properties**	**Staining properties**
Thick, grayish white or, cream colored colonies	Grayish-white colored	B-type of Hemolytic were production	Catalase Indole MIU MR VP TSI Citrate utilization	**+** **–** **–** **–** **–** **+** **–**	Gram-positive, rod-shaped organism	Capsulated

Legends

MR=Methyl-red test, VP=Voges-prokauer test, TSI=Triple Sugar Iron, MIU=motility Indole urease test, citrate, $_+$ positive,**-** negative

4.3.3 Result of biochemical test

4.3.3.1 Methyl-red and voges-proskauer test

The isolates were MR negative **(Plate -21)** and VP Negative **(Plate -22)**

4.3.3.2 Indole test

The isolates were Indole negative **(Plate -23)**

4.3.3.4 Motility Indole urease test

The isolates were MIU negative **(Plate -24)**

4.3.3.5 Citrate test

The isolates were Citrate negative **(Plate -25)**

4.3.3.6 Triple sugar iron test

The isolates were TSI positive **(Plate -26)**

4.2.3.7 Catalase test

The isolates were catalase positive **(Plate -27)**

4.4 Isolation and Identification of *Escherichia Coli spp* by different bacteriological methods

4.4.1 Result of culture examination

4.4.1.1 Nutrient broth

Nutrient broth was inoculated with betel leaf sample and incubated 37 for 24 hours the growth of bacteria was indicated by the presence of turbidity.

4.4.1.2 Nutrient Agar

Nutrient Agar plates streaked with the organisms revealed the growth of bacteria after 24 hours of incubation at 37 aerobically by the growth circular, smooth opaque and colorless colonies **(Plate-10).**

4.4.1.3 MacConkey Agar

MacConkey Agar plates streaked separately with the organism revealed the growth of bacteria after 24 house of incubation at 37 aerobically and were indicated by the growth of pink colored colonies **(Plate-11).**

4.4.1.4 Eosin Methylene Blue (EMB) Agar

EMB streaked separately with the organism from MacConkey agar revealed the growth of bacteria after 24 hours of incubates at 37 aerobically and were indicated by the growth of metallic sheen colonies **(Plate-12).**

4.4.2 Result of gram's staining

The microscopic examination of gram's stained smear from MacConkey Agar and EMB agar revealed gram-negative, small rod shaped organism arranged in single pairs or short chain **(Plate-13).**

Table-6: Cultural, Morphological and Biochemical Properties of Isolated *Escherichia coli*

Culture characteristics		**Biochemical Characteristics**		**Staining and morphological**
MacConkey Agar	**EMB Agar**	**Test**	**Result**	**Staining properties**
Pink colored colonies	Metallic-sheen color colonies	Indole MIU MR VP TSI Citrate utilization	**+** **_** **+** **_** **_** **_**	gram-negative, pink-colored, small rod shaped organism arranged in single pairs or short chain

Legends

MC= MacConkey, EMB=Eosin Methyl Blue, MR=Methyl-red test, VP=Voges-prokauer test, TSI=Triple Sugar Iron, MIU=motility Indole urease test, citrate, positive,**-** negative

4.4.3 Result of biochemical test

4.4.3.1 Methyl-red and voges-proskauer test

The isolates were MR positive **(Plate -21)** and VP negative **(Plate -22)**

4.4.3.2 Indole test

The isolates were Indole positive **(Plate -23)**

4.4.3.3 Motility Indole urease test

The isolates were MIU negative **(Plate -24)**

4.4.3.4 Citrate utilization test

The isolates were citrate negative **(Plate -25)**

4.4.3.5 Triple sugar iron test

The isolates were TSI Negative **(Plate -26)**

4.5 Isolation and Identification of *klebsiella spp*. by different bacteriological method

4.5.1 Results of cultural test

4.5.1.1 Nutrient broth

Nutrient broth was inoculated in betel leave sample and incubated 37 for 24 hours the growth of bacteria was indicates by the presence of turbidity.

4.5.1.2 Nutrient Agar

Nutrient Agar plates streaked with the organisms revealed the growth of bacteria after 24 hours of incubation at 37 aerobically **(Plate-14).**

4.5.1.3 MacConkey (MC) Agar

MacConkey Agar plates streaked separately with the organism revealed the growth of bacteria after 24 house of incubates at 37 aerobically and were indicated by the growth of rod shaped pink colored mucoid colony **(Plate-15).**

4.5.1.4 Eosin Methylene Blue (EMB) Agar

EMB streaked separately with the organisms from MacConkey agar revealed the growth of bacteria after 24 hours of incubation at 37 aerobically and were indicated by the growth of white pink mucoid large colonies **(Plate-16).**

4.5.2 Result of gram's staining

The microscopic examination of gram's stained smear from MacConkey and EMB agar revealed gram-negative, pink colored, small rod shaped organism arranged in single pairs or short chain **(Plate-17).**

Table-7: Cultural, Morphological and Biochemical Properties of Isolated *klebsiella.*

Culture characteristics		**Biochemical Characteristics**		**Staining and morphological**
MacConkey Agar	**EMB Agar**	**Test**	**Result**	**Staining properties**
Pink colored colonies	Smooth pink characteristic mucoid colonies	Indole MIU MR VP TSI SC	_ + _ _ + +	gram-negative, colored,rod shaped organism

Legends MC= MacConkey, EMB=Eosin Methyl Blue, MR=Methyl-red test, VP=Voges-prokauer test, TSI=Triple Sugar Iron, MIU=motility Indole urease test, citrate, $_+$ positive,**-** negative

4.5.3 Result of biochemical test

4.5.3.1 Methyl-red and voges-proskauer test

The isolates were MR negative **(Plate -21)** and VP Positive **(Plate -22)**

4.5.3.2 Indole test

The isolates were Indole negative **(Plate -23)**

4.5.3.3Motility Indole urease test

The isolates were MIU positive **(Plate -24)**

4.5.3.4 Citrate utilization test

The isolates were Citrate positive **(Plate -25)**

4.5.3.5 Triple sugar iron test

The isolates were TSI positive **(Plate -26)**

4.6 Isolation and identification of *Entrobacter spp.* By different bacteriological methods

4.6.1 Result of culture examination

4.6.1.1 Nutrient broth

Nutrient broth was inoculated in betel leave sample and incubated 37 for 24 hours the growth of bacteria was indicated by the presence of turbidity.

4.6.1.2 Nutrient Agar

Nutrient Agar plates streaked with the organisms revealed the growth of bacteria after 24 hours of incubated at 37 aerobically by the growth white dry colonies. **(Plate -18)**

4.6.1.3 MacConkey (MC) Agar

MacConkey Agar plates streaked separated with the organism revealed the growth of bacteria after 24 house of incubation at 37 aerobically and were indicated by the growth of pink colonies. **(Plate -19)**

4.6.1.4 Eosin Methylene Blue (EMB) Agar

EMB streaked separately with the organism revealed the growth of bacteria after 24 hours of incubation at 37 aerobically and were indicated by the growth of purple blue colonies. **(Plate -20)**

4.6.2 Result of gram's staining

The microscopic examination of gram's stained smear from MacConkey and EMB agar revealed gram-negative, small rod shaped organisms. **(Plate -21)**

Table-8: Cultural, morphological and biochemical properties of isolated *Entrobacter spp.*

Culture characteristics		Biochemical Characteristics		Staining and morphological
MC Agar	**EMB Agar**	**Test**	**Result**	**Staining properties**
red dry colored colonies	Large mucoid purple colonies	**Indole** **MIU** **MR** **VP** **TSI** **SC**	– – – + + +	gram-negative, colored,rod shaped organism

Legends

MC= MacConkey, EMB=Eosin Methyl Blue, MR=Methyl-red test, VP=Voges-prokauer test, TSI=Triple Sugar Iron, MIU=motility Indole urease test, SC=Simon citrate, + = positive, **-** = negative

4.6.3 Result of biochemical test

4.6.3.2Methyl-red and voges-proskauer test

The isolates were MR **(Plate -21)** negative and VP positive **(Plate -22)**

4.6.3.1Indole test

The isolates were Indole negative **(Plate -23)**

4.6.3.3Motility Indole urease test

The isolates were MIU negative **(Plate -24)**

4.6.3.5 Citrate utilization

The isolates were SC positive **(Plate -25)**

4.6.3.4 Triple sugar iron test

The isolates were TSI positive **(Plate -26)**

4.7 Result of antibiotic sensitivity pattern of isolated bacteria

4.7.1 Antibiotic sensitivity pattern of Staphylococcus spp. (n=6)

The antibiotic study revealed that all of isolates (6) were sensitive to Gentamicin (100%), followed by ciprofloxacin (83.33%), Vancomycin (66.66%), Erythromycin (33.33%) and the isolates were found resistant Kanamycin (83.33%).

Table: 9 Antibiotic sensitivity pattern of Staphylococcus spp.

Antibiotic agent	Disc. concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Kanamycin (K)	30 µg	1	5	16.66	83.33
Erythromycin (E)	5 µg	2	4	33.33	66.66
Vancomycin (VA)	30 µg	4	2	66.66	33.33
Ciprofloxacin (CIP)	30 µg	5	1	83.33	16.66
Gentamycin (GEN)	25 µg	6	0	100	0

4.7.2 Antibiotic sensitivity pattern of *Bacillus spp.* (n=4)

The antibiotic study revealed that all of isolates (4) were sensitive to Erythromycin 100% were sensitive to followed by ciprofloxacin (75%), Neomycin (75%), Co-trimoxazole (50%) and the isolates were found resistant Amoxicillin (100%).

Table: 10 Antibiotic sensitivity pattern of *Bacillus spp*

Antibiotic agent	Disc. Concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Amoxicillin (AMX)	10 µg	1	4	25	100
Co-trimoxazole (COT)	15 µg	2	2	50	50
Neomycin (N)	30 µg	3	1	75	25
Ciprofloxacin(CIP)	30 µg	3	1	75	25
Erythromycin (E)	5 µg	4	0	100	0

4.7.3 Antibiotic sensitivity pattern of *E.coli spp.* (n=1)

The antibiotic study revealed that all of isolates (1) were sensitive to Ciprofloxacin, Co-trimoxazole (100%), Neomycin. (100%). And the isolates were found resistant Erythromycin (100%). and Amoxicillin (100%).

Table 11: Antibiotic sensitivity pattern of *E.coli spp*

Antibiotic agent	Disc. Concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Amoxicillin (AMX)	10 µg	0	1	0	100
Erythromycin (E)	5 µg	0	1	0	100
Neomycin (N)	30 µg	1	0	100	0
Co-trimoxazole (COT)	15µg	1	0	100	0
Ciprofloxacin (CIP)	30µg	1	0	100	0

4.7.4 Antibiotic sensitivity pattern *of Klebsiella spp.* (n=5)

The antibiotic study revealed that all of isolates (5), were found sensitive to Ciprofloxacin (100%) were sensitive to followed by Co-trimoxazole (80%), Neomycin (80%). And the isolates were found resistant Amoxicillin (100%), Erythromycin (100%).

Table: 12 Antibiotic sensitivity pattern *of Klebsiella spp.*

Antibiotic agent	Disc. Concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Amoxicillin (AMX)	10 µg	0	5	0	100
Erythromycin (E)	5 µg	0	5	0	100
Neomycin (N)	30 µg	4	1	80	20
Co-trimoxazole (COT)	15 µg	4	1	80	20
Ciprofloxacin(CIP)	30 µg	5	0	100	0

4.7.5 Antibiotic sensitivity pattern of *Entrobacter spp.* (n=4)

The antibiotic study revealed that all isolates (4) were sensitive to ciprofloxacin (100%) were sensitive to followed by kanamycin (75%). and the isolates were found resistant Amoxicillin (100%), Ampicillin (100%), Cefuroxime Sodium (100%).

Table: 13 Antibiotic sensitivity pattern of *Entrobacter spp.*

Antibiotic agent	Disc. Concentration (µg/disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistance	Sensitive	Resistance
Amoxicillin (AMX)	10 µg	0	4	0	100
Cefuroxime sodium (CXM)	30µg	0	4	0	100
Ampicillin	10 µg	0	4	0	100
Kanamycin	30µg	3	1	75	25
Ciprofloxacin(CIP)	30 µg	4	0	100	0

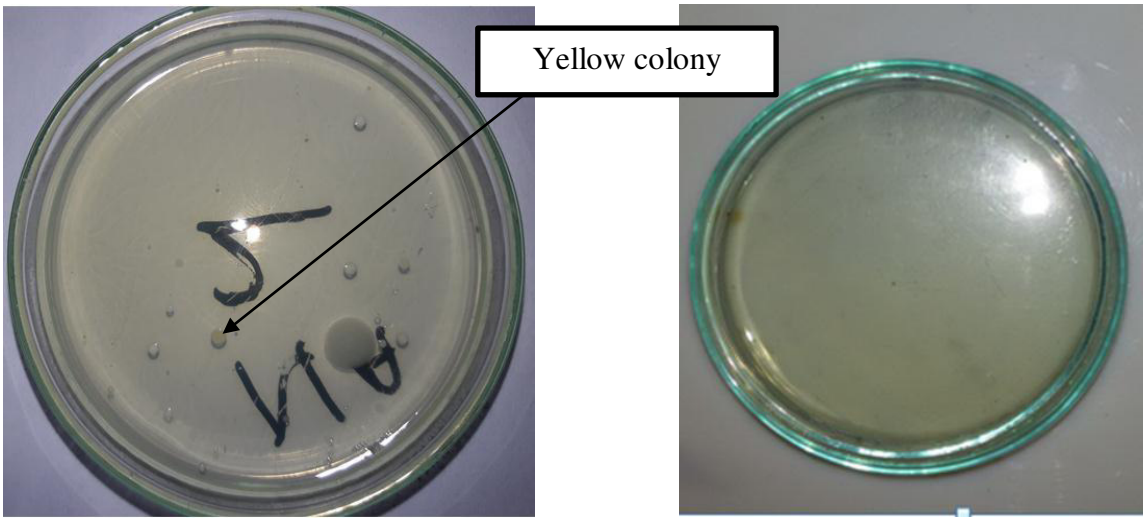

Plate 1: Nutrient agar media showing small yellow colony for *staphylococcus spp.*

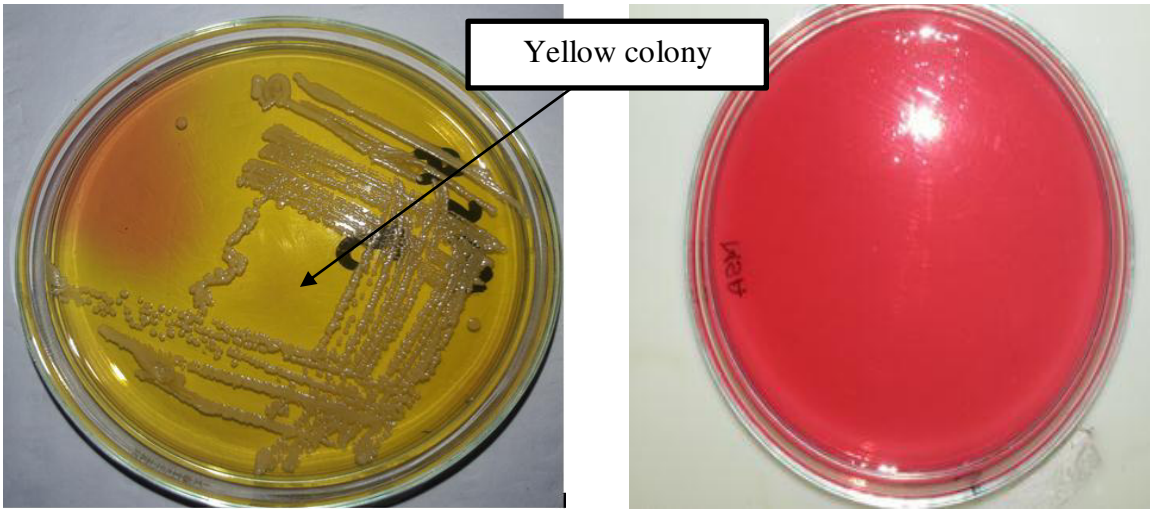

Plate 2: Manitol salt agar media yellow colony for *staphylococcus spp*

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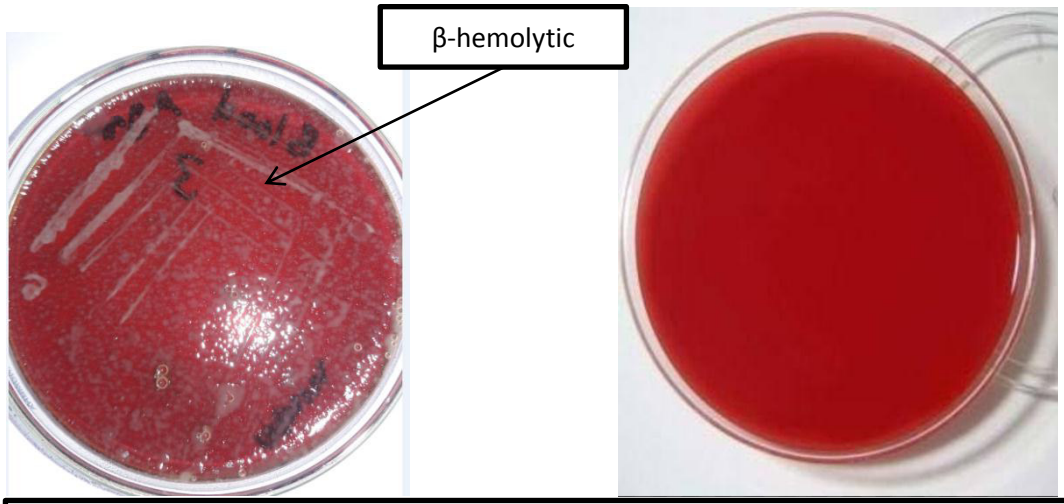

Plate 3: Blood agar media showing β-hemolytic reaction for staphylococcus

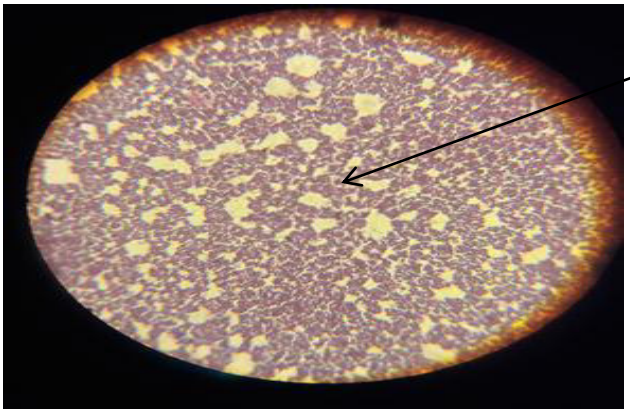

Plate 4: Gram's staining staphylococcus spp. Showing Gram positive cocci arranged in grape like cluster

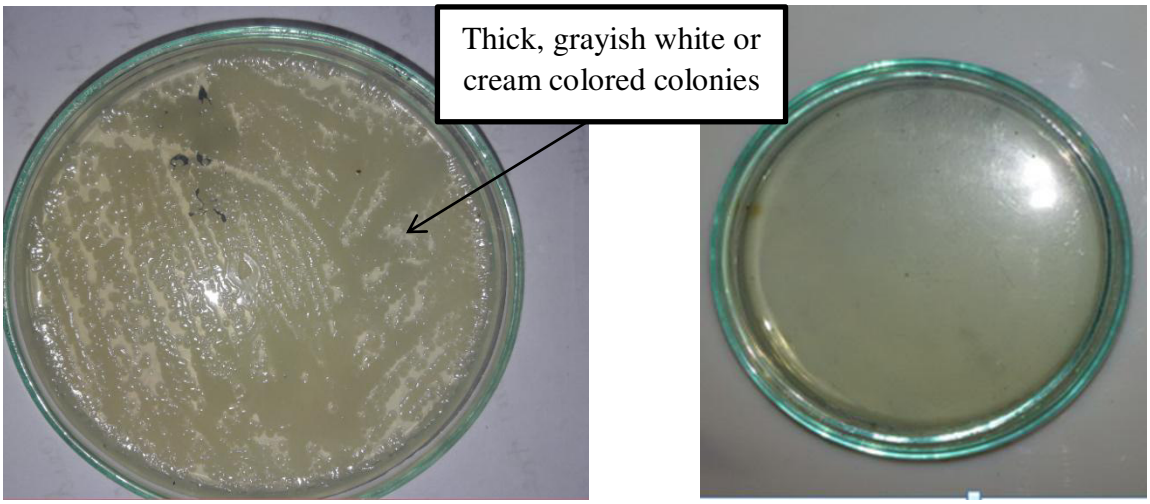

Plate 5: Nutrient agar media showing for thick, grayish white or cream colored colonies *Bacillus spp.*

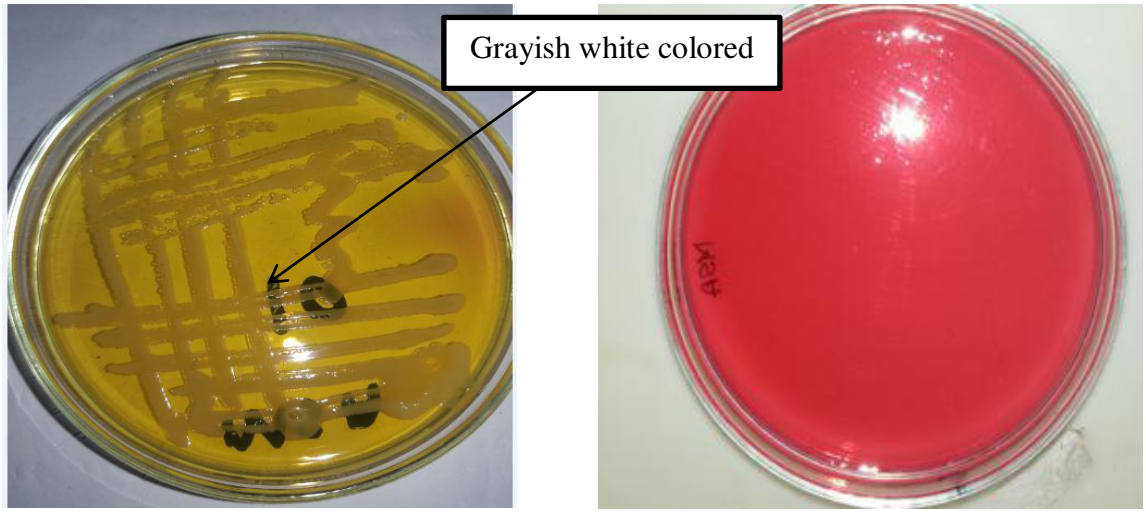

Plate 6: Manitol salt agar media Grayish white colored for *Bacillus spp.*

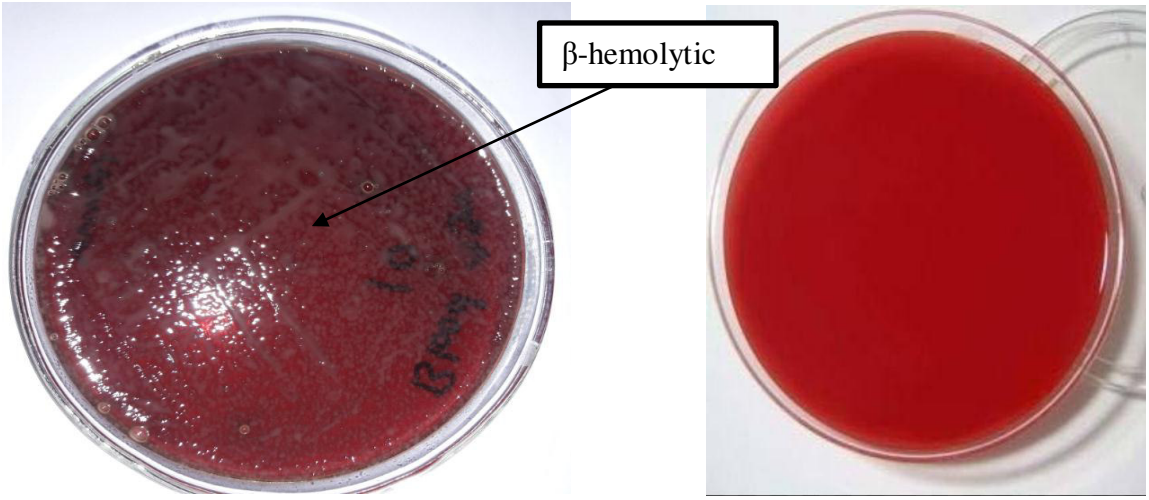

Plate 7: Blood agar media β-hemolytic for *Bacillus spp*

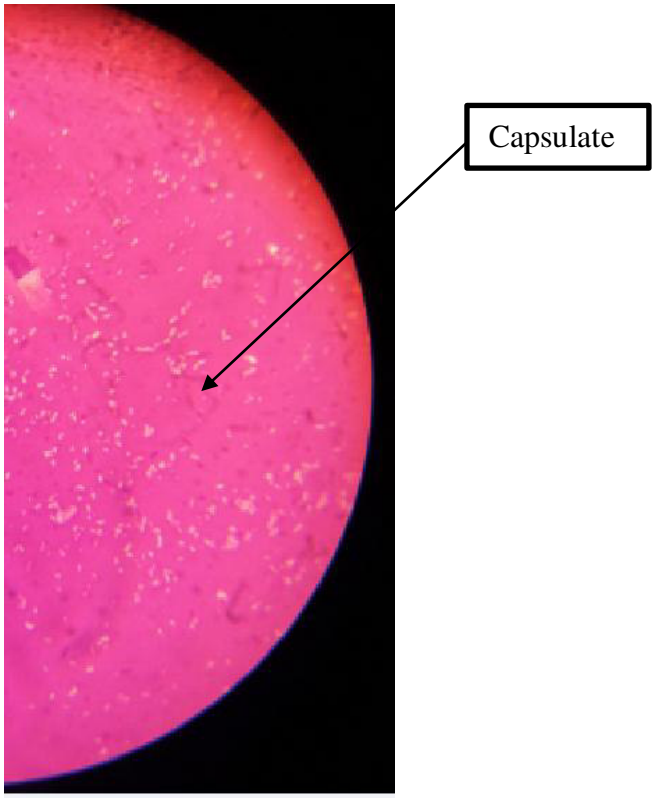

Plate 8: Indian ink staining showing capsulated for *Bacillus spp.*

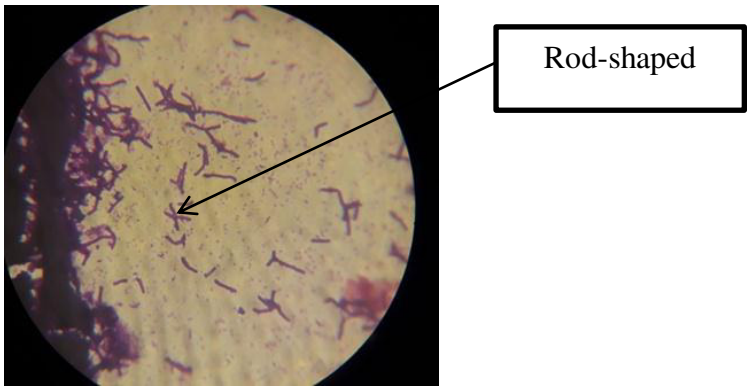

Plate 9: Gram's staining *Bacillus spp* Showing Gram positive Rod-shaped

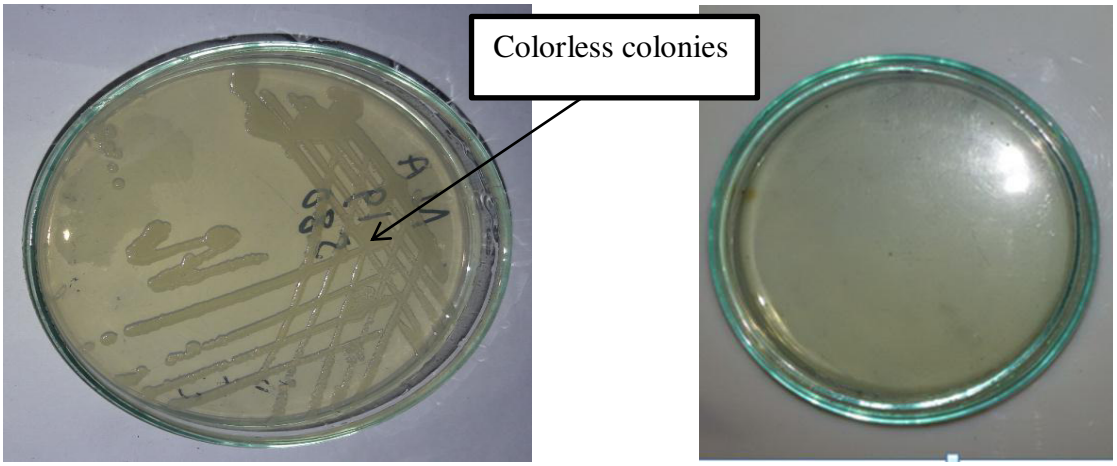

Plate 10: Nutrient agar media for Colorless colonies *Escherichia Coli spp.*

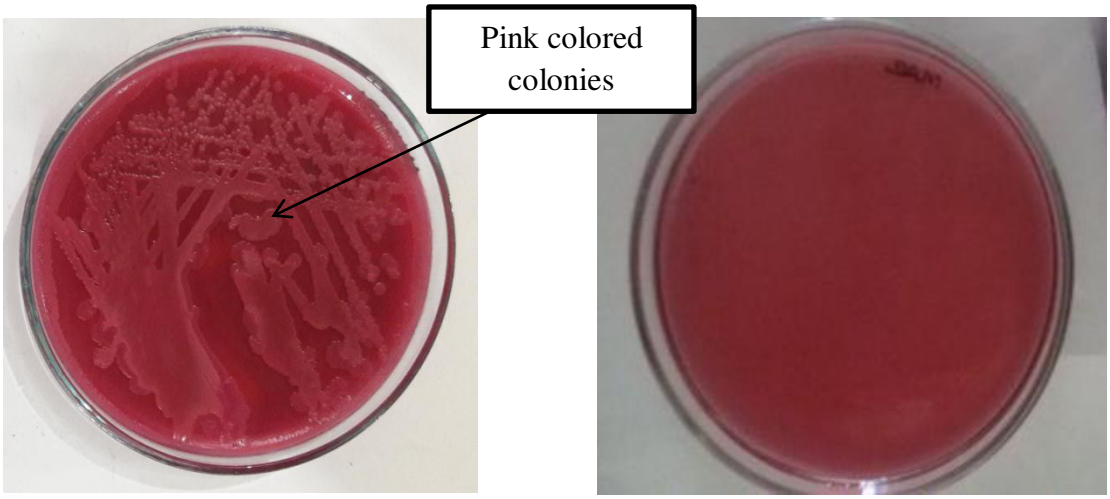

Plate 11: MacConkey agar media for Pink colored colonies *Escherichia Coli spp*

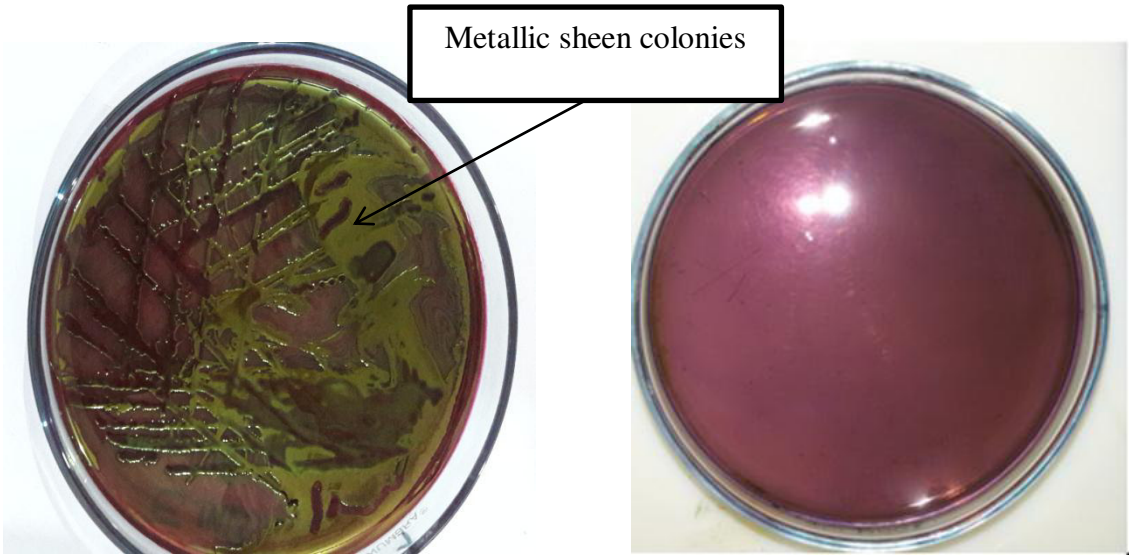

Plate 12: Eosin Methylene Blue (EMB) Agar media for metallic sheen colonies *Escherichia Coli spp.*

Plate 13: Gram's staining *Escherichia Coli spp*

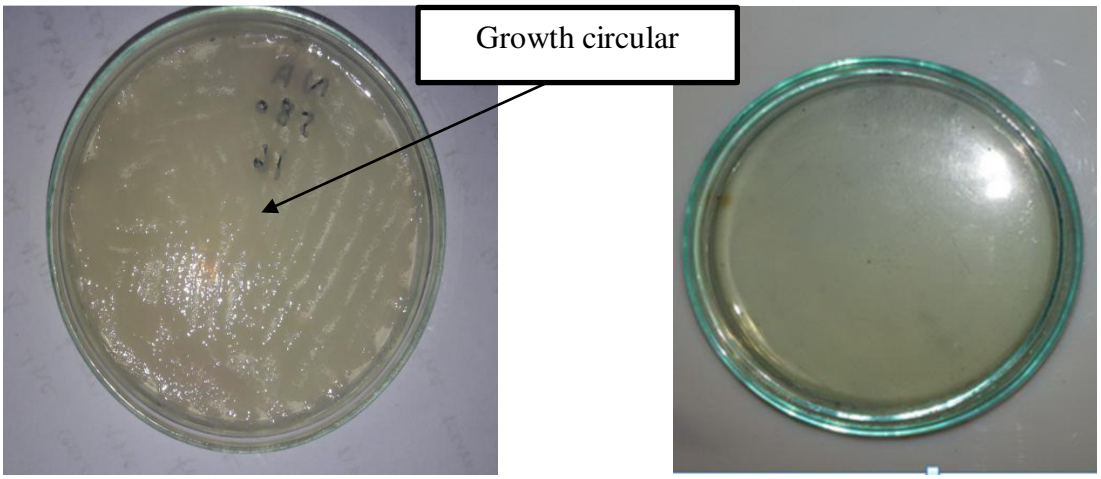

Plate 14: Nutrient agar media for Colorless colonies *klebsiella spp*

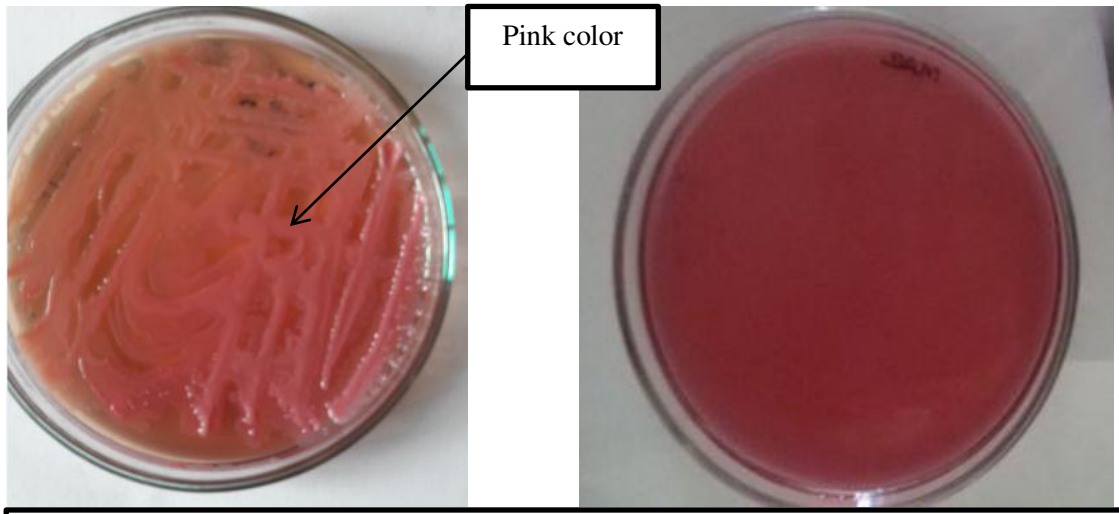

Plate 15: MacConkey agar media pink colored colonies

Plate16: Eosin Methylene Blue (EMB) Agar media for Pink mucose large colonies *klebsiella spp.*

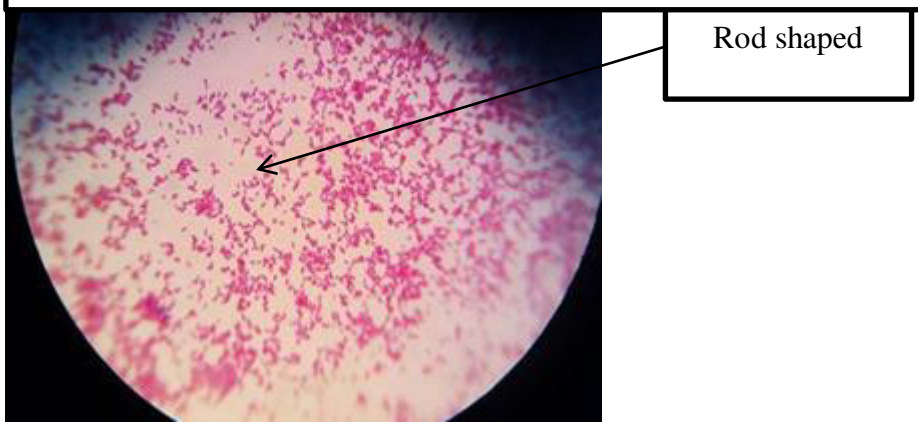

Plate 17: Gram's staining klebsiella *spp* Showing Gram Negative colored, Rod-shaped organism

Plate 18: Nutrient agar media for Colorless colonies *Entrobacter spp*

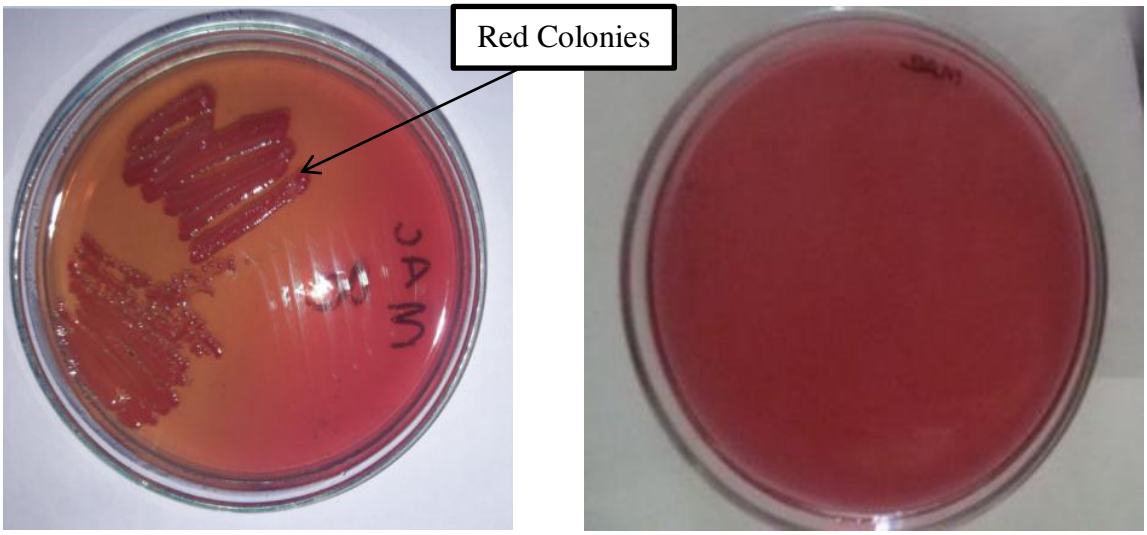

Plate19: MacConkey agar media Red Colored colonies for *Entrobacter spp*

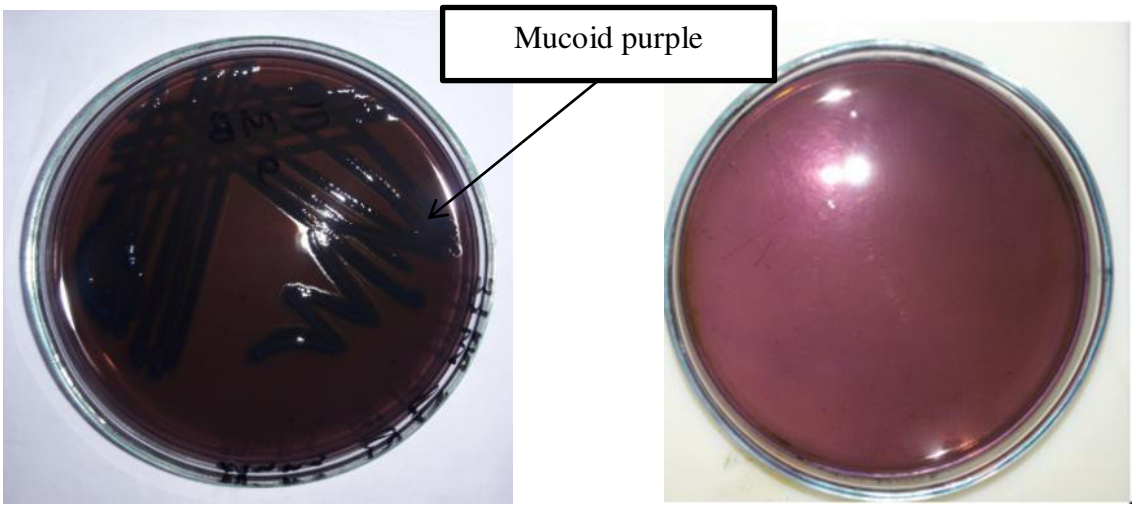

Plate20: Eosin Methylene Blue (EMB) Agar media for blue colonies *Entrobacter spp*

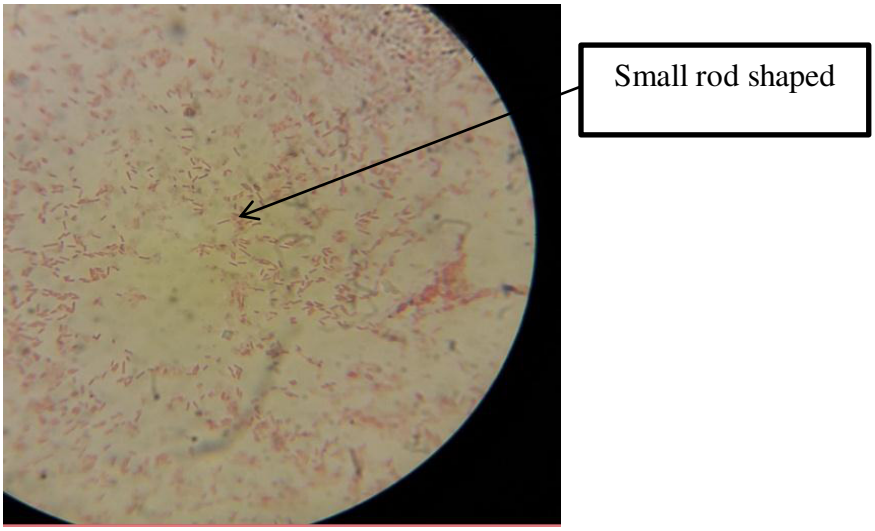

Plate 21: Gram's staining *Entrobacter spp* Showing Gram Negative colored, small Rod-shaped organism

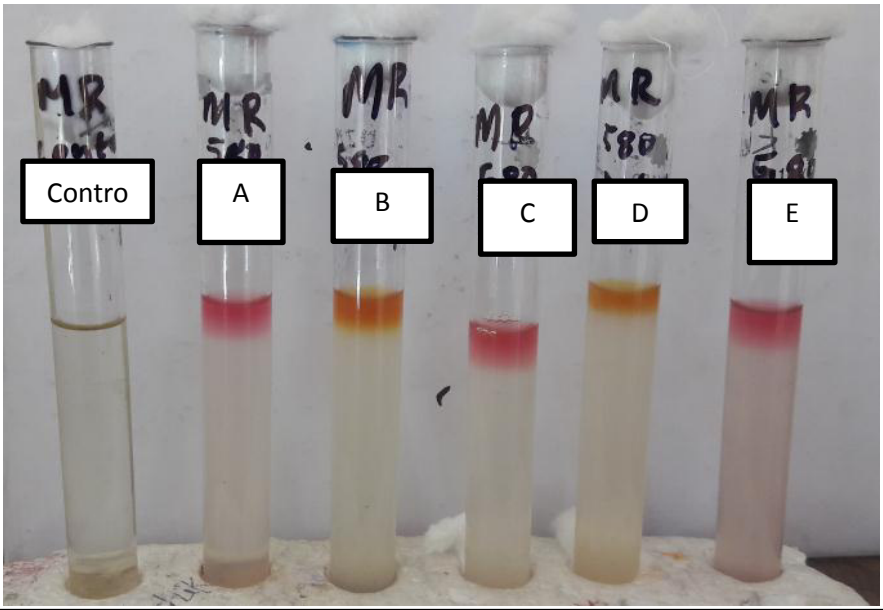

Plate 22: MR test result A=Klebsiella (positive), B= Entrobacter (negative), C= Staphylococcus (positive), D=Bacillus (Negative), E=E.coli(positive)

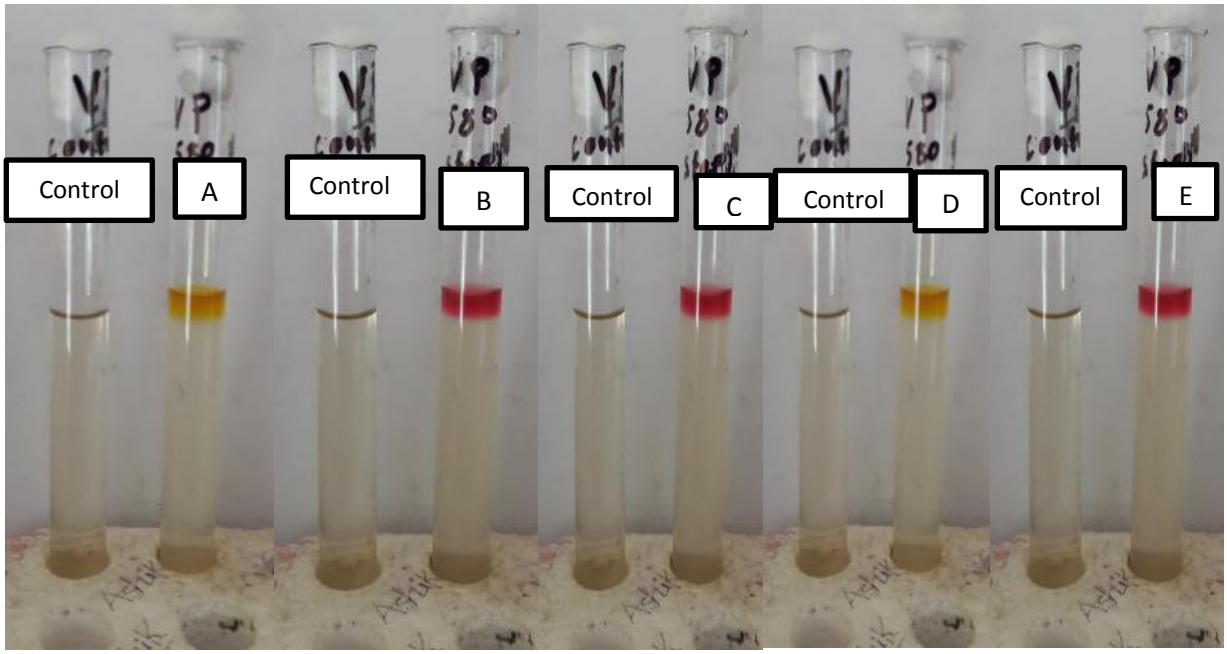

Plate 23: VP test result A=E.coli (negative), B= klebsiella (positive), C= Staphylococcus (positive), D=Bacillus (negative), E= Entrobacter (positive)

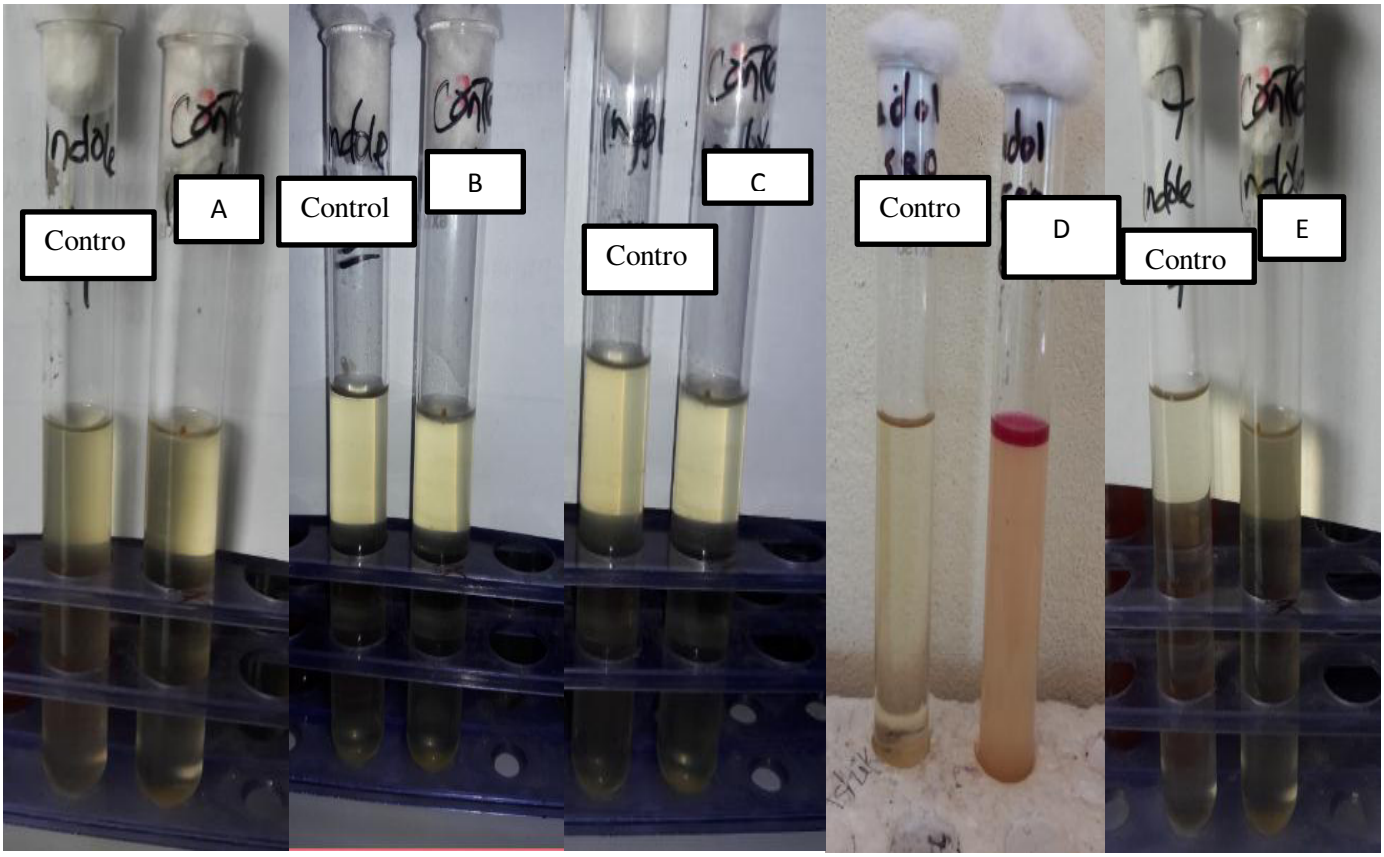

Plate 24: Indole test A=Entrobacter (negative), B=Staphylococcus (positive), C=Klebsiella (positive), D=E.coli (positive), E=Bacillus (positive)

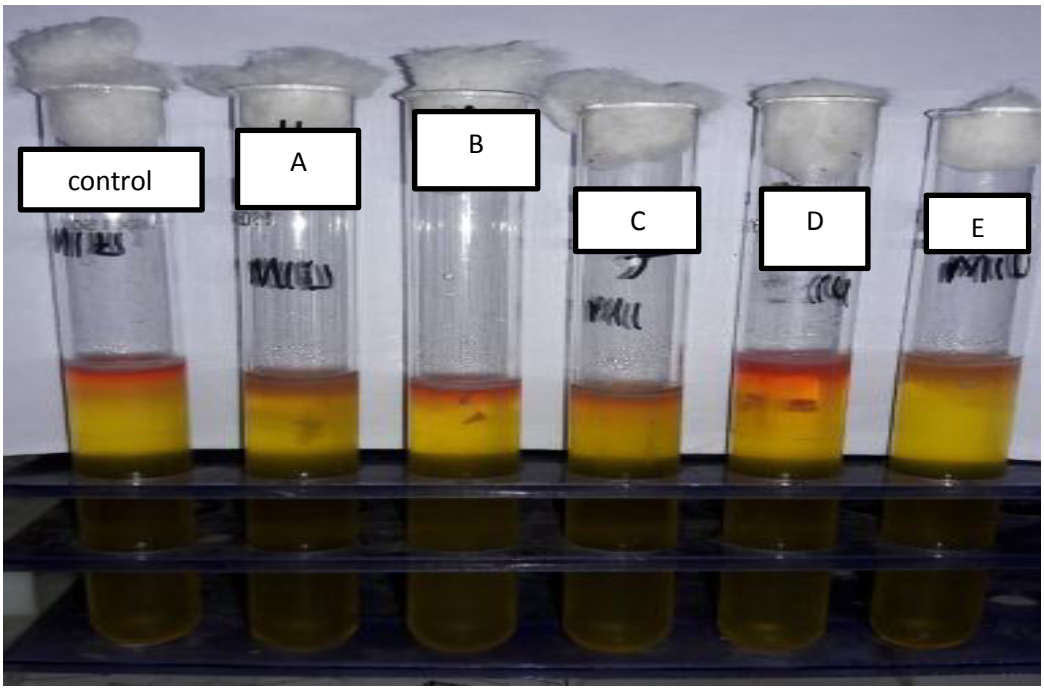

Plate 25: MIU test showing A=E.coli (negative), B=Staphylococcus (positive), C=Bacillus (negative), D=Klebsiella (positive), E=Entrobacter (positive)

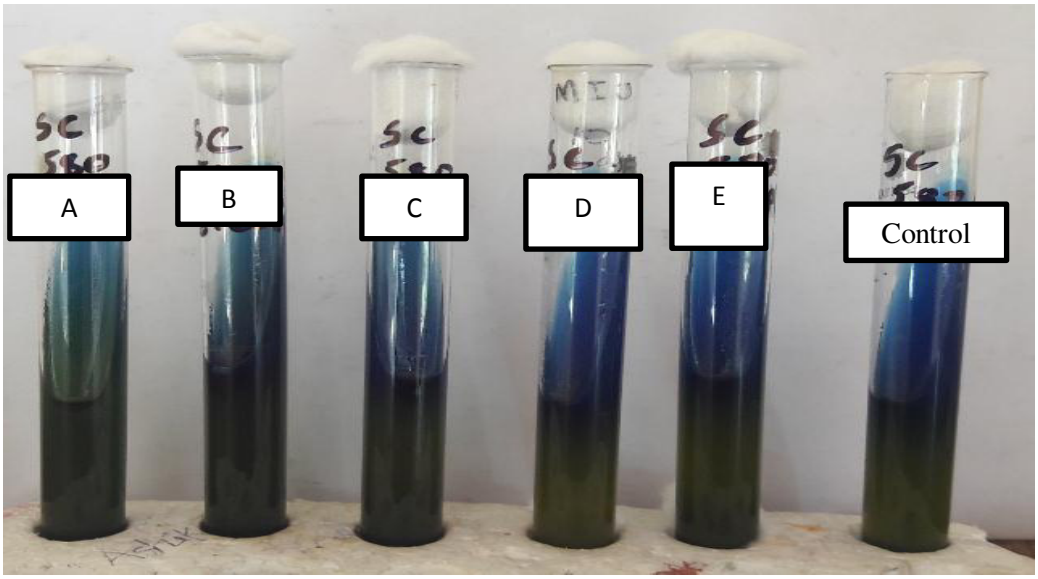

Plate 26: Simon's Citrate test result (right)A=E.coli(positive), B=Bacillus (positive), C=Entrobacter (positive), D=Staphylococcus (positive), E=Klebsiella (positive)

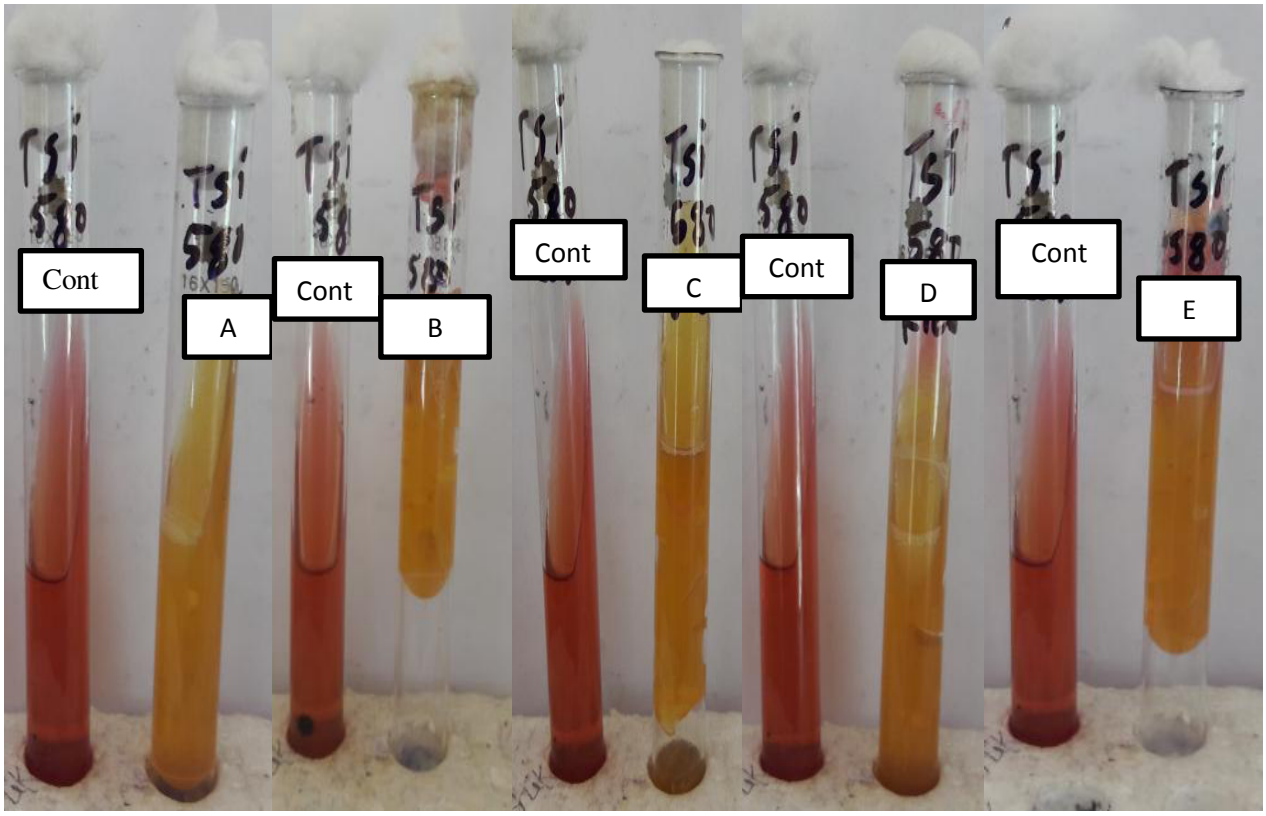

Plate 27: TSI test result A=Staphylococcus (positive and gas), B=Entrobacter (Negative and gas). C=E.coli (Positive and gas), E=Bacillus (Negative and gas)

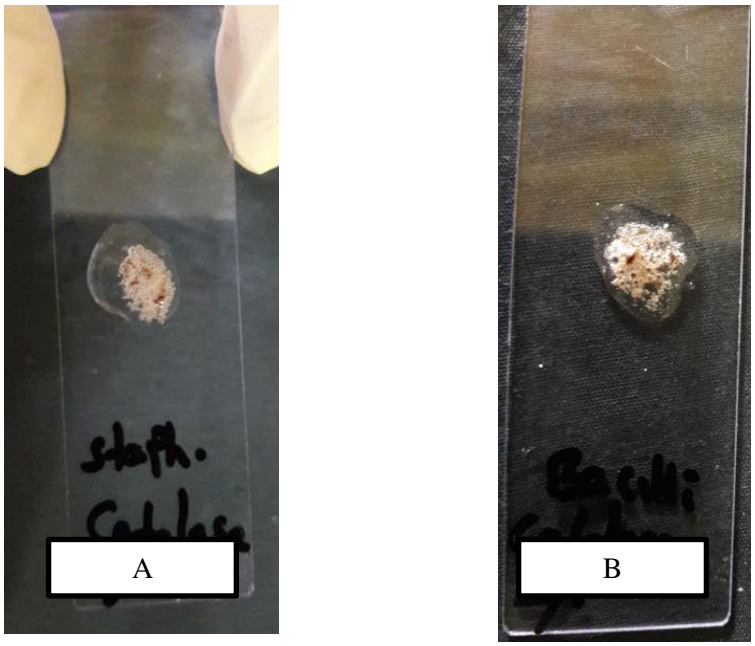

Plate 28: Catalase test A=*Staphylococcus spp.* (positive), B=*Bacillus spp.* (positive)

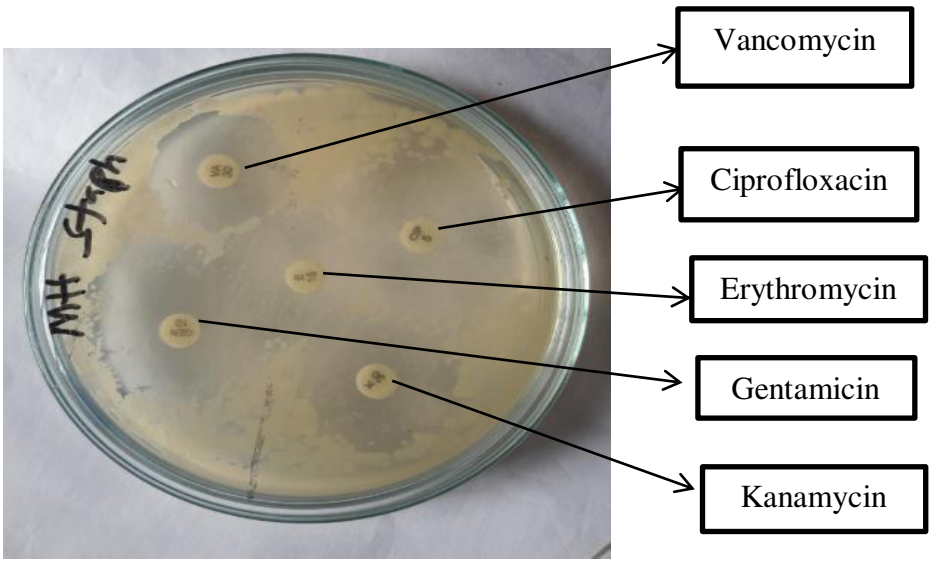

Plate 29: Antibiotic sensitivity test result *of Staphylococcus spp.* on Mueller Hinton agar

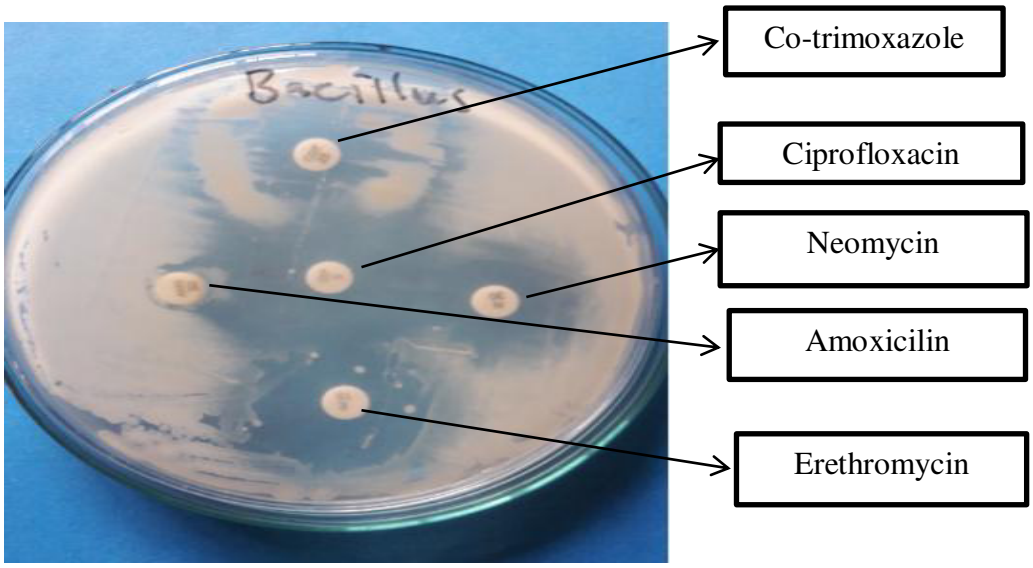

Plate 30: Antibiotic sensitivity test result of *Bacillus spp*. on Mueller Hinton agar

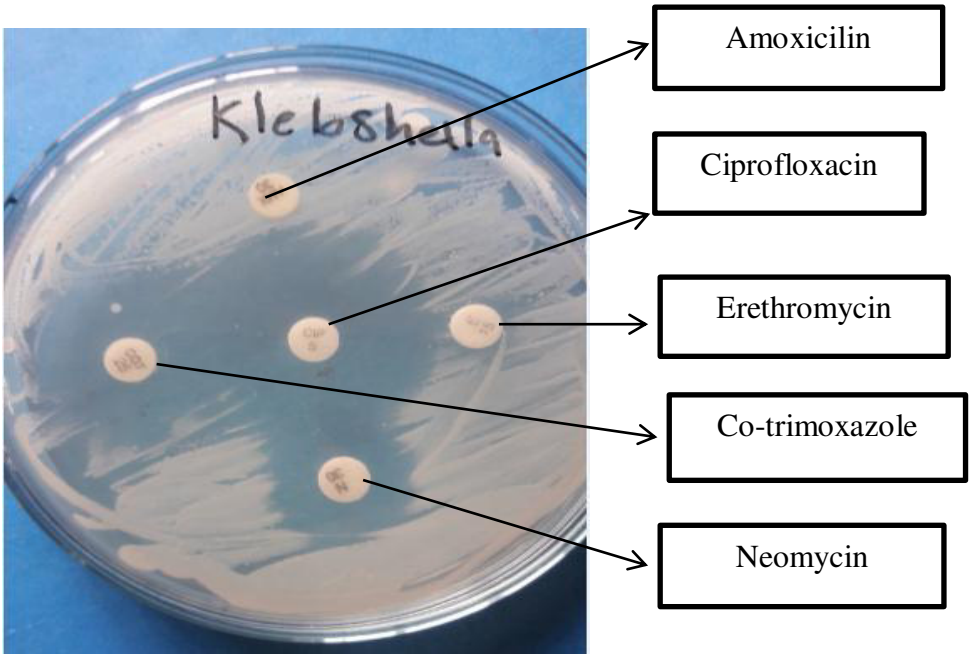

Plate 31: Antibiotic sensitivity test result of *Klebsiella spp*. on Mueller Hinton agar

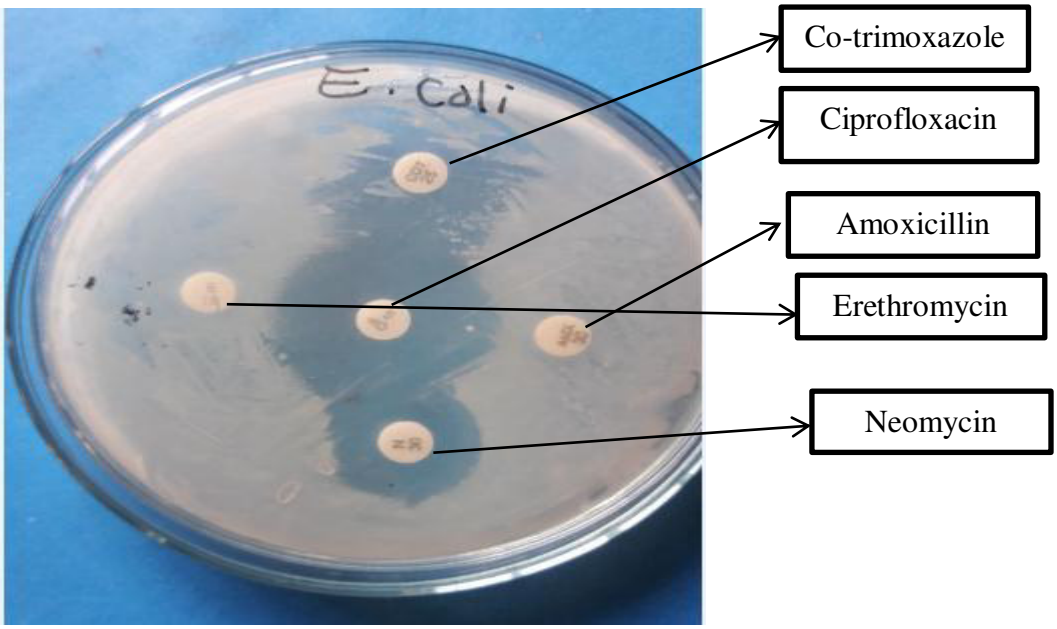

Plate 32: Antibiotic sensitivity test result of *E.coli* on Mueller Hinton agar

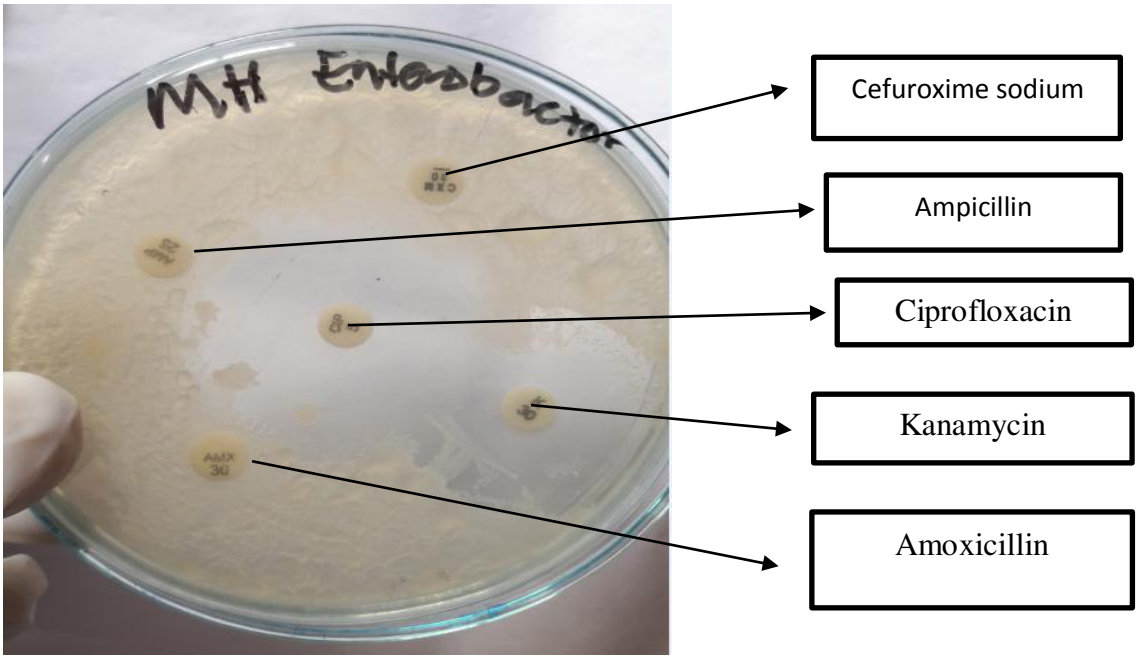

Plate 33: Antibiotic sensitivity test result of Entrobacter on Mueller Hinton agar

CHAPTER V

DISCUSSION

CHAPTER V

DICUSION

The experiment was carried out to isolate and identify bacteria as well as to detect antibiotic sensitivity pattern of isolated bacteria from infected betel leaf sold at local markets of Dinajpur city, Bangladesh. For this study, a total 20 of infected betel vine sample were collected from five different local markets (Leili mall, Terminal road, Doshmile, Gopalgong bazaar, and Basharat) of Dinajpur. A series of test were conducted for isolation, identification and frequency distribution of different bacteria of betel leaf. A total of 20 bacterial isolates belong to five genera (*staphylococcus spp., Bacillus, spp., Escherichia coli, klebsiella spp and Entrobacter spp*.) were identified. The prevalence of *staphylococcus spp. was* 30%, *Bacillus spp.* was 20%, *Escherichia coli* was 5%, *klebsiella spp*. was 25% and *Entrobacter spp*. was 20%. This findings were more or less similar to the findings made by Md. Mazedul Haque *et al.* (2017), in which they reported the prevalence of *Escherichia coli, Bacillus spp*. and *Staphylococcus spp.* in betel leaf samples was 17.34%, 18.37%, and 19.39% respectively. Many infectious agents could be implicated as causes of betel leaf contamination but *Staphylococcus* spp, *Bacillus* spp, *E. coli spp*, *Klebsiella spp*, *Entrobacter spp*, were isolated from infected betel leafs in this study. The frequency distribution of different species bacteria isolates in different betel leafs sample were found variable. Result of this study indicated that all the five different types of bacteria were not present in the same betel leaf sample collected from the different markets. *Staphylococcus spp* have been reported as the main pathogen of infected betel leafs. The *staphylococcus spp., bacillus spp., E.coli, klebsiella spp.* and *Entrobacter spp.* showed identical result in different biochemical test of including catalase test, indole test, methyl-red, voges-Proskauer, motility indole urease test, triple sugar iron test citrate utilization test.

The in vitro antibiotic sensitivity test of isolated bacteria 10 different antibiotics such as Gentamycin, Ciprofloxacin, Cefuroxime sodium, Co-trimoxazole, Kanamycin, Neomycin, Erythromycin, Amoxicillin, Vancomycin, and Ampicillin ware used. Staphylococcus was study a major variation was noticed in the result of sensitivity of isolation agent's 5 different antibiotics use.

From the antibiotic sensitivity test of *staphylococcus spp.*, it was found that 100% of the isolated *staphylococcus spp.* was sensitive to Gentamicin followed by ciprofloxacin (83.33%) Vancomycin (66.66%), Erythromycin (33.33%) and Amoxicillin (16.66%).These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

From the antibiotic sensitivity test *Bacillus spp*., it was found that 100% of the isolated *Bacillus spp.* was sensitive to Erythromycin followed by ciprofloxacin (75%), Neomycin (75%) Co-trimoxazole (50%) and Amoxicillin (25%). These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

From the antibiotic sensitivity test *E.coli,* it was found that 100% of the isolated *E.coli* was sensitive to Ciprofloxacin, Co-trimoxazole, Neomycin. 100% resistant against Erythromycin and Amoxicillin. These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

From the antibiotic sensitivity test *Klebsiella sp,,* it was found that 100% of the isolated *Klebsiella spp.* was sensitive to Ciprofloxacin followed by Co-trimoxazole (80%), Neomycin (80%), Erythromycin (0%), and Amoxicillin (0%). These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

From the antibiotic sensitivity test *Entrobacter spp.,* it was found that 100% of the isolated *Entrobacter spp*. was sensitive to Ciprofloxacin followed by kanamycin (75%), Ampicillin (0%), Cefuroxime Sodium (0%), and Amoxicillin (0%). These findings were more or less similar to the findings of Md. Mazedul Haque *et al.* (2017).

Overall more sensitive antibiotics against the isolated organisms was ciprofloxacin followed by Co-trimoxazole, Gentamycin, neomycin, kanamycin and Vancomycin but the isolated organisms were found to be resistant against Amoxicillin, Erethromycin, Ampicillin and Cefuroxime sodium. The variation in the sensitivity of commonly used antibiotic could be a result of extensive and indiscriminate use of these antibiotics, maximum sensitivity to ciprofloxacin, Co-trimoxazole and Gentamycin might probably be due it is rarely used.

There for effective treatment of infection caused by consumption of contaminated betel leaf medicinal formulation should preferably contain antibiotics that have good spectrum

of inhibition against the isolated bacteria in this context. It is interesting to note ciprofloxacin, Co-trimoxazole Gentamycin, Vancomycin, Neomycin and kanamycin should be the antibiotic of choice. In deed ciprofloxacin, Co-trimoxazole and Gentamycin could cover most of the infection by prevalent bacteria. Therefore these antibiotics appear to be promising for the treatment of the infection caused by consumption of infected betel leafs in Bangladesh.

CHAPTER VI

SUMMARY AND CONCLUSION

CHAPTER VI

SUMMARY AND CONCLUTION

The recent study was conducted for isolation and identification, determination of biochemical properties and antibiotic sensitivity pattern of the bacteria isolated from infected betel leaf samples. A total of 20 betel leaf samples were collected from Sadar Upozila Dinajpur district in Bangladesh for this study.

A series of test were conducted for isolation, identification and frequency distribution of different bacteria of betel leaf. A total of 20 bacterial isolates belong to five genera (*staphylococcus spp., Bacillus, spp., Escherichia coli, klebsiella spp and Entrobacter spp*.) were identified. The prevalence of *staphylococcus spp. was* 30%, *Bacillus spp.* was 20%, *Escherichia coli* was 5%, *klebsiella spp*. was 25% and *Entrobacter spp*. was 20%. The result of antibiotic sensitivity tests revealed that staphylococcus spp were sensitive to Gentamicin, ciprofloxacin, Vancomycin, Erythromycin, and Kanamycin in various degrees. *Bacillus spp* were sensitive to Erythromycin, Ciprofloxacin, Neomycin, Co-trimoxazole, in various degrees. *E. coli* was sensitive to Ciprofloxacin, Co-trimoxazole, Neomycin in various degrees. *Klebsiella spp* were sensitive to Ciprofloxacin, Co-trimoxazole, Neomycin in various degrees. *Entrobacter spp* was sensitive to Ciprofloxacin, Co-trimoxazole, Neomycin in various degrees. Overall sensitivity revealed that ciprofloxacin, Co-trimoxazole and Gentamycin were most efficacious drugs. Therefore; it may be recommended that ciprofloxacin, Co-trimoxazole and Gentamycin in optimum doses to treat most case of infection caused by consumption of betel leaf at Sadar Upazila in Dinajpur district in Bangladesh.

From the result of the present study, it may be concluded that.

1. Staphylococcus spp, bacillus spp, E.coli spp, klebsiella spp, Entrobacter spp were the major etiological agent isolated from betel leafs.
2. Antibiogram result indicated the ciprofloxacin, Co-trimoxazole and Gentamycin, in optimum doses would be the drug of choice to treat the most cases of human infection caused by consumption of infected betel leafs.
3. Antibiotic sensitivity test revealed that Ampicillin, Cefuroxime Sodium, Amoxicillin and Erythromycin would not be recommended because isolated bacteria were resistant to these drugs.

REFERENCES

REFERENCES

Anonymous, (2006). Asiatic society of Bangladesh.pdf

Aparna Priyadarshini Patra, Arup Kumar Mukherjee and Laxmikant Acharya (2011). Comparative Study of RAPsD and ISSR Markers to Assess the Genetic Diversity of Betel Vine (*Piper betle* L.) in Orissa, India *American Journal of Biochemistry and Molecular Biology,* 1: 200-211.

Arani Datta, Shreya Ghoshdastidar and Mukesh , Singh (2011). Singh Antimicrobial Property of Piper betel Leaf against Clinical Isolates of Bacteria International Journal of Pharma Sciences and Research (IJPSR) 2(3): 104-109.

B.K. Das and S.K. Mallick (2010). Screening of betel vine cultivars for resistance to betel vine whitefly, *Singhiella pallida* (Singh) (hemiptera : aleyrodidae) and new host plant records

Bholanath Mondal, Indrabrata Bhattacharya and Dinesh Chandra Khatua (2014). Incidence of bacterial wilt disease in West Bengal, India Academia Journal of Agricultural Research 2(6): 139-146

Bidhan Chandra Krishi Viswavidyalaya Mohanpur, Nadia, West Bengal (2002). Bacterial Diseases of Betelvine pdf 4-5.

Bijan Kumar Das (2010). Incidence of Aleurocanthus spp. (Aleyrodidae: Hemiptera) on betelvine (*Piper betle* L.) and their interaction with host plants The Journal of Plant Protection Sciences, 2(2): 53-59,

Carter, G.R. (1979). Diagnosis procedures in veterinary bacteriology and mycology 3rd end. Charles C, Thomas publisher U.S.A.398-417

Chattopadhyay, S. B. and Maity, S. (1967). Diseases of Betelvine and Spices. ICAR, New Delhi.

Cheesbrough, M, (1984). Medical laboratory manual for tropical countries. 1st end.vol.02 microbiology, English language Book Socity, London, 35: 40-57.

Cowan, S.T. and steel, K.T, (1979) manual for identification of medical bacteria. 2and Education, Cambridge university press, London. 22-122

CSIR (Council of Scientific and Industrial Research, New Delhi) (1969). The Wealth of India, CSIR, New Delhi. 8: 84-94.

D. K. Sengupta B. Dasgupta and P, Datta (2011). Management of foot rot of betel vine (*Piper betle* L.) caused by *Phytophthora parasitica* Dastur. Journal of Crop and Weed 7(2): 179-183

Dinesh Chandra Khatua, Bholanath Mondal and Rana Bhattachayya (2013) A selective medium for *Xanthomonas axonopodis* pv. Betlicola, bacterial pathogen of betelvine Dinesh. African Journal of Agricultural Research. 8(49): 6388-6393.

Guha, P. and Jain, R.K. (1997). Status Report on Production, Processing and Marketing of Betel Leaf (*Piper betle* L.). Agricultural and Food Engineering Department, IIT, Kharagpur, India.15-22 pp.

Hassan, S. A. and Shahazad, S. (2005). Disease affecting betel vine. 3(2): 4- 5.

Hembram S and Baskey S (2015) interaction of different pathogens on the extent of lesion development of betel vine (piperis betel). International Journal of Recent Scientific Research Vol. 6, Issue, 11, pp.7162-7165,

International Commission on Microbiology Specification for foods (ICMSF), (1986). Microorganisms in food 2-sampling for microbiology analysis: principle and specific.

Islam, M. (2005). Country news, Holiday Publication Limited. 8: 3-4.

Jana, B. L. (1996). 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.

Khoshoo, T. N. (1981). Welcome address. In: Proc. Of Group Discussion on Improvement of Betel vine Cultivation. S. D. Khanduja and V. R. Balasubrahmanyam (Eds.). National Botanical Research Institute, Lucknow, India. 17-20pp.

Mahesha N. Sigera Nadugala and B.H.R.R. Amarasinghe (2009). diversity among different isolates of Xanthomonas campestris pv. betlicola on the basis of phenotypic and virulence characteristics Journal of the National Science Foundation of Sri Lanka 37 (1):77-80

Maiti, S. (1989): Extension Bulletin: The Betelvine. All India Coordinated Research Project on Betel vine, Indian Institute of Horticultural Research, Hessarghatta, Bangalore, India.

Maiti, S. and Sen, C. (1977). Leaf and foot rot of *Piper betle* caused by Phytophthora palmivora. Indian Phytopath., 30: 438-439.

Manabendra Nayak, Rahul Nayak, Lukpu Ngulom (2016) activity of betel leaf extract of against bacteria causing dental caries; 5(87): 6454-6460.,

Marchat I.A and Packer, R.A. (1967) veterinary bacteriology and virology 07th edition. The lowa state university press USA.

Md. Fakruddin, Razia Sultana, Md. Nur Hossain, Md. Mizanur Rahaman, Md. Kamrul Islam and Monzur Morshed Ahmed (2017) Detection of food-borne bacteria in ready to eat betel leaf sold at local markets in Mymensingh International Journal of Food Contamination 4:6 DOI 10.1186/s40550-017-0051-0.

Md. Mazedul Haque, Md. Atiqur Rahman Sarker, Rafia Afroze Rifa, Md. Ariful Islam and Mst. Minara Khatun (2017) Detection of food-borne bacteria in ready to eat betel leaf sold at local markets in Mymensingh 10(9): 1040-1045.

MD.Monirul Islam Mollah,2012 Investigation on the leaf rot and food and root of betel vine (piper betel L.) in satkhira district of Bangladesh pdf 1-2.

Mehrotra, R. S. (1981): Fungal diseases of betelvine and their control, In: Proc. of Group Discussion on Improvement of Betel vine Cultivation. S.D.

Nison Bhutia , Parthasarathi Medda, P. choudhuri, A.chosh and G.pandit (2015) Effect of light intensity on different betel vine germplasmunder terairegion of west bangal 10(3): 1163-1168.

Nutankumar S. Jane, Prof. Mrs. Anupama P.Deshmukh, Prof. Mrs. Madhavi S. Joshi (2014) Review Of Study Of Different Diseases On Betelvine Plant and Control Measure International Journal of Application or Innovation in Engineering & Management (IJAIEM) Volume 3, Issue 3.

P. Guha (2006) Betel Leaf: The Neglected Green Gold of India 19(2): 87-93.

R Salam, JU Khokon, S Baidya MT Mussa (2014) Effect of neem and betel leaf against oral bacteria International Journal of Natural and Social Sciences 1 52-57.

R. Bhattacharya, B. Mondal, S. K. Ray1 and D. C. Khatua (2012) A Study on Bacterial Disease of Betelvine in West Bengal International Journal of Bio-resource and Stress Management 3(2):211-216.

Samanta, C. (1994). 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.

Sayeeduzzaman, M. (1988). An economic geographical study of betel leaf cultivation in Bangladesh. A M.Sc. thesis submitted to Geography, University of Dhaka. 45-47pp.

Sharma, M. L., Rawat, A. K. S., Khanna, R. K., Chowdhury, A. R. and Raina, R. M. (1996). Flavour characteristics of betel leaves. Euro cosmetics, 5: 22-24.