BIBLIOGRAPHY

CUADRA, ILENE C. APRIL 2007. Etiology of the Bacterial Leaf Spot of Lettuce in La Trinidad, Benguet: A First Report. Benguet State University, La Trinidad, Benguet.

Adviser: Jocelyn C. Perez, MSc
Co-Adviser: Lily Ann D. Lando, PhD

ABSTRACT

The bacterium causing leaf spot in lettuce leaves was isolated and produced similar symptoms to the artificially inoculated lettuce leaves.

Cultural characteristic of the isolates are yellow colonies produced in seven (7) different media. The morphological characterization was done through gram staining which is negative and motility test which is motile because of the polar flagella of the bacterium. In the physiological characterization, growth in O/F test was never fermentative and oxidase negative. Thus, the bacterium was not able to produce gelatinase enzymes in the gelatin liquefaction test. A clear zone was observed in the starch hydrolysis test swabbed with *Xanthomonas campestris pv. vitians*.

Sensitivity of the bacterium to streptomycin sulfate and chlortetracycline was very obvious. Cephalexin hydrate and cycloheximide were not effective against the bacterium.

TABLE OF CONTENTS

INTRODUCTION

In 2005, Nagpala *et al.* (PIA, 2006) reported a leaf spot of head lettuce (*Lactuca sativa* L.) collected from Loo and Poblacion in Buguias, Benguet. The lettuce leaves had black lesions that often become papery thin and then eventually tears the leaves to give it a tattered appearance (Villa *et al*., 2005).

The initial survey results of Nagpala *et al.* (2005) showed a 50-90% disease incidence in the sites sampled. Laboratory tests showed the association of a bacterium with the diseased plant tissues. Identification done by the team and continued by Villa *et al.* (2005) pointed to the possibility of the causal bacterium being a *Xanthomonas campestris* pathovar.

Bacterial spot of lettuce has not been reported in the Philippines as revealed in a review of the Host Index of Plant Diseases in the Philippines (Tangonan, 1999). Therefore, this disease may be new or unrecognized and thus unreported.

Studies on the transmission of the organism have been inconclusive but several reports say that it may be both seed- and soilborne. As such, the pathogen presents a new challenge to the vegetable industry in Benguet. To adequately meet this challenge, the identity of the pathogen has to be verified and its pathogenicity determined. The information may then be used to prevent the spread of the pathogen and develop an integrated approach to the management of the disease.

This study was conceived and conducted to:

1. verify the identity of the causal bacterium of the disease;
2. determine the sensitivity of the bacterial isolates to antibiotics (streptomycin sulfate, cycloheximide, cephalexin hydrate, and chlortetracycline).
3. determine the pathogenicity of the isolates to commonly-grown lettuce cultivars; and
4. compare the isolates to common *Xanthomonas* species in the locality.

The study was conducted in the Department of Plant Pathology laboratory from November 2006 to March 2007.

REVIEW OF LITERATURE

The Host Plant

Lettuce (*Lactuca sativa* L.) including cos or romaine, belongs to Asteraceae (compositae) or daisy family. This is the largest dicotyledonous family in the plant kingdom. Notable leafy crop species within the Asteraceae family includes lettuce, chichory and endive as well as other lesser known plants such as fuki and oyster plant (Ryder, 1999).

Lettuce, a cool-season vegetable crop is one of the easiest to grow. High summer temperature usually cause seedstalk formation (bolting) and bitter flavor. Slow-bolting or heat resistant varieties are available and are recommended for extending the lettuce-growing season (Robinson, 2003).

The plant is an annual herb with milky latex in the leaves and stems. Lettuce has a shallow root system up to 1 foot deep (30 cm). The most important characteristics of lettuce are its size, compactness, sweetness and succulence.

Lettuce is popular in salads because of its nutritional content. One cup of raw leaf lettuce (chopped) contains only nine calories. It contains 1.3 g dietary fiber, 1 g protein, 1.34 g carbohydrates, 1456 IU of vitamin A, 13.44 I.U. vitamin C, 20.16 mg calcium, 0.62 mg iron and 162.5 mg potassium (Wolford, 2006).

There are several types of lettuce commonly grown in garden: the crisphead, butterhead, cos, leaf, stem and oil-seed.

Crisphead also known as iceberg (approximately 60-75 days when direct seeded). The most widely available as a fresh market type, it weighs about 1 kg with six or seven

outer leaves. Outer leaves are bright green or dull green, and the interior color may be white to creamy yellow.

Butterhead or Bibb lettuce (approximately 60 days when direct seeded). It is a loose-heading type with dark green leaves that are somewhat thicker than those of iceburg lettuce.

Romaine or cos (approximately 60 days). They are elongated leaves that range in color from yellow to dark green forming upright heads with rather wavy, attractive leaves. They are very nutritious and weigh up to 750g each (Stephen, 1988).

Leaf type lettuce (approximately 50- 60 days). Have green or reddish leaves, this type is fast growing, long-lasting lettuce. It is basically planted, thinned and harvested.

Stem lettuce or stalk or asparagus lettuce (approximately 85 days). Its stem thickens and elongates and its leaves are long and narrow.

Oil-seed cultivars are grown beyond the rosette stage and allowed to bolt. They are 50 % larger than other types of lettuce. Their seed is collected and pressed for oil.

The Pathogen and Symptoms Caused

Xanthomonas campestris pv. vitians causes the bacterial leaf spot (BLS) of lettuce and is known to survive on or in diseased plant debris for short periods of time. It is also known to be seedborne and can survive on dried seeds on extended periods (Sahin *et al.*, 1997). It is a strictly aerobic, Gram-negative, rod-shaped bacterium ranging in size from 0.2-0.8 um (Pernezny *et al*., 1995; Toussaint, 1999). It is non-sporulating with a single polar flagellum (Robinson, 2003).

There are two discrete symptoms associated with BLS. The first include water-soaked, brown lesions that later turn black about 1-2 mm in diameter. These lesions

become V-shaped, translucent and collapses (Toussaint, 1999; Sahin *et al.*, 1997; Sahin and Miller, 1998). Lesions may expand along the veins of the plant (Sahin *et al.*, 1997; Sahin and Miller, 1997; Toussaint, 1999; Wallis and Joubert, 1972). The second type of symptom consists of small black spots scattered along the leaf surface (Sahin and Miller, 1997).

Distribution

Since 1918, BLS has been reported from Canada (Toussaint, 1999), Venezuela (Daboin and Tortolero, 1991), Japan (Tsuchiya *et al*., 1981), and South Africa (Wallis and Joubert, 1972). In the United States, it has been found in California (Schroth *et al*., 1964) and Florida (Pernezny *et al*., 1995).

In 2005, Nagpala *et al*. reported the occurrence of the disease in Benguet, Philippines. These findings result are included in the recently concluded project titled, "Survey, Identification and Mapping of Soilborne Plant Pathogens and Pathogen Vectors and Alternative Host Weeds in Benguet" that was conducted under the Semi-Temperate Vegetables Research and Development Center (STVRDC) of the Benguet Sate University (BSU).

Epidemiology

Epidemiology of this *X. c. pv. vitians* that caused leaf spot on lettuce, that is now present in La Trinidad, Benguet has limited research.

Studies have subsequently demonstrated that the bacterium can be transmitted by infected seeds and planting materials It can be seedborne and may survive in association

with seed for extended periods and in soil associated with plant debris (Barak *et al.*, 2001)

Experiments were established in commercial fields and representative lettuce seeds lots, and it results to the development of bacterial leaf spot (Umesh *et al.*, 1996). One explanation for this was that the lettuce seed was internally infected with *X.c. pv. vitians* and that this inoculum was not detected with seed wash assays, but was able to colonize germinated seedlings and cause outbreaks of BLS (Barak *et al.*, 2002). High populations of *X.c. pv. vitians* were recoverable from filed debris one month after had been plowed in the summer (Robinson, 2003).

X.c. pv. vitians has also been shown to survive on lettuce leaf surfaces as epiphyte using standard dilution planting techniques (Toussaint, 1999). Toussaint *et al.*, (2001) used scanning electrons microscopy to find bacteria on the leaf surface of asymptomatic leaves.

Sahin *et al.*, (1997) studied eight commercial lettuce cultivars. They found high susceptibility in the cos as two of the green leaf types. The most susceptible cultivars were butterhead and cos types according to Carisse *et al.*, (2000) in Florida condition.

Tsuchiya et al. (1981) performed experiments on 99 crop species from 19 families as well as 97 weed species from 31 families in Japan in these studies, plants were artificially inoculated with the pathogen and rated for disease incidence. They found a number of hosts within Cruciferae, Polygonaceae, Tropaeloceae, and Compositae. Sahin *et al.*, (1997) studied eight commercial lettuce cultivars. They found high susceptibility in the cos as well as two of the green types. Pernezny *et al.*, (1995) found that the cos types were the most susceptible in Florida although a plethora of host range studies have been

conducted. Carisse *et al.,* (2000) reported that the most susceptible cultivars were butterhead and cos types with the least susceptible being green leaf types in Canada.

MATERIALS AND METHODS

Verification of the Pathogen Identity

Collection of Disease Specimens, Initial Processing and Description of Symptoms

Samples of symptomatic lettuce were collected from the BSU experimental areas where it is observed. Samples were placed in paper bags to absorb the moisture, labeled and then brought to the Plant Pathology Laboratory for further diagnosis. Symptoms of samples were described and all observations were recorded.

Identification of Isolated Bacteria

Isolation of associated bacteria. Nutrient Glucose Agar (NGA) was used for isolation of the bacterium. Thin sections were cut from the advancing portions of infected leaves and mounted on a drop of sterile distilled water on a slide to induce bacterial ooze. Appearance of the ooze was confirmed under 40x magnification of an ordinary light microscope. The bacterium was allowed to ooze out of the tissues for five minutes. After which, the tissues were removed from the slide and a flame-sterilized wire loop was used to get a sample of bacterial suspension for streaking onto previously prepared NGA plates. Plates thus streaked were incubated at 27-30^{o}C for four days. Three well-separated, isolated single colonies were transferred into NGA slants and stocks cultures were stored at 5^{0}C.

Identification of isolated bacteria. The following tests were performed to determine the genera of the isolated bacteria.

1. Growth on various media [Appendix 1]
    a. Yeast Extract Dextrose $CACO_3$ Agar (YDCA)
    b. King's Medium B Agar (KMBA)
    c. Nutrient Glucose Agar (NGA)
    d. Sucrose Peptone Agar (SPA)
    e. *Xanthomonas* Isolation Medium (XIM)
    f. Casimino Peptone Glucose Agar (CPGA)
    g. Potato Dextrose Peptone Agar (PDPA)
2. Gram Staining

Smears were prepared from bacterial ooze obtained from symptomatic leaves. Cut sections of about 2 to 3 mm were placed in a clean glass slide with a drop of water then removed after 3 to 5 minutes using a teasing needle. The resulting smear was then air-dried prior to the standard staining procedure.

3. Other tests. The procedures for the following tests are as outlined in Schaad (1998).

    a. Growth and O/F metabolism. Ten ml of Hugh and Leifson Agar (basal medium) was poured into test tubes (13 cm diameter). For each replication, the bacterial isolate were stabbed into two test tubes of basal medium after which one test tube was covered using water agar and the other was left uncovered. The set-up was incubated at 30^0C for 24 to 48 hours.

b. Gelatin Liquefaction tests. Ten ml of gelatin medium was dispensed in 13-cm test tubes. Bacteria were stabbed into the medium and inoculated tubes were incubated at 20 to 27^0C for 21 days. After 3,7,14 and 21 days, the test tubes were refrigerated for 30 minutes.

c. Motility test. A loopful of the bacterial suspension taken from the freshly cut disease lesion was placed on the underside of a cover slip, which was then inverted over a depression slide. The slide was viewed under the microscope at 400x magnification

d. Starch hydrolysis test. Bacterium was streaked onto starch agar and the plates were incubated for 2-7 days. The plates were then flooded with iodine solution and any clearing zone was observed.

Determining the Pathogenicity of the Isolates

Preparation of Test Plants

Lettuce seedlings, about two weeks old, were obtained and kept in the greenhouse for pathogenicity test.

Inoculum Preparation and Inoculation

The bacterial suspension was prepared and standardized at 1 x 10^6 cfu/ml using the McFarland's Turbidity Standard. Three inoculation techniques were tested following the procedure of Dayao (2004), as follows:

1. Spraying- Using a low-pressure sprayer, the inoculum was sprayed onto the leaf surface until run-off.

2. Injection- Bacterial suspension was injected into the intercellular spaces at the undersurface of the leaf.

3. Pricking- The leaves was pricked using a sterilize needle and sprayed with the bacterial suspension.

The inoculated plants were covered with a plastic bag and incubated overnight in the laboratory after which the plastic bags were removed and the inoculated plants were brought to the greenhouse. Symptom development was observed and was compared to the initial findings.

Re-isolation of Causal Organism

Lesions from artificially inoculated plants were used as sources of bacterial suspension for re-isolation onto Nutrient Glucose Agar (NGA). Isolates thus obtained were compared to the original isolates.

Testing the Antibiotic Sensitivity of the Isolates

One ml of standardized bacterial suspension of 1 x 10^6 cfu/ml was added to 5 ml water agar and was poured onto previously prepared NGA plates. The test antibiotics were prepared at different test concentrations (50, 75, 100, 200, 300, 400, 500 and 600 ppm).

Sterilized filter paper discs (20 mm diameter) were dipped into the prepared antibiotics and then plated at the center of the medium using sterilized forceps. Plates thus prepared were sealed and incubated for 48 hours at 28°C.

Data Gathered

1.Symptoms of the diseased plants

2. Cultural, morphological and physiological characteristics of the isolates in various media. Specifically the size, shape, color, elevation, margins, and production of pigment in the media.

3. Pathogenicity test. The symptoms produced by the inoculated bacterium compared to the original symptoms observed.

4. Inhibition zones (mm). Inhibition zones around the antibiotic disks will be measured.

5. Photodocumentation. Photographs will be taken of test results and experimental set-up and will be saved as jpeg files.

RESULTS AND DISCUSSION

Collection of Sample and Description of Collection Sites

Collecting of bacterial leaf spot of lettuce was done in the BSU experimental areas: namely, at the Swamp Area, Balili experimental area, and Pomology. Lettuce leaves were collected on May 2006 and were well-formed and firm. The lesions were scattered on the entire surface of the leaves.

Areas from where specimens collected have a flat elevation and it is commonly used to plant vegetables crops than that of the flowers. Almost square meters of the field are for hired. Crop rotation is not employed as seen that the farmers are not strictly monitored on what type of crops they like to plant. Irrigation are through canals, stock waters in drums.

Disease Symptoms

Lettuce leaves collected have brown lesions that are translucent, irregular in shape and water-soaked. Lesions later may turn black. The first symptoms appear at the leaf margin and consist of water-soaked lesions that become necrotic and confluent (Figure 1).

Figure 1. Symptoms of infected lettuce leaves in the field

Cultural Characteristics of the Pathogen on Various Media

Various standard and differential media were used to grow the bacterium. A loopful of bacterial ooze from the lettuce leaves was streaked onto NGA, XIM, SPA, CPGA, KMBA, YDCA, and PDPA and incubated for 4- 5 days at 27-30^{0}C.

Colonies were measured and ranged from approximately from <1mm to 4mm in diameter (Table 2). Colonies are circular and appear shiny and mucoidal. Under light, the colonies appear translucent and are mostly from light to dark yellow (Figure 2). When plated on YDCA and XIM, the bacterium produces whitish to cream colonies. On CPGA, it produces red colonies because of the TTZ in the media.

Table 1. Cultural characteristics of *Xanthomonas* in plate growth

MEDIA	SIZE	CONFIGURATION	DENSITY	ELEVATIION	COLOR
CPGA	< 1mm	Circular	Opaque	Mucoid	Red
PDPA	3-4 mm	Circular	Opaque	Mucoid	Cream
SPA	4-5 mm	Circular	Opaque	Mucoid	Yellow
YDCA	2-4 mm	Circular	Opaque	Mucoid	Yellow
XIM	< 1 mm	Circular	Translucent	Mucoid	White
KMBA	2-4 mm	Circular	Translucent	Mucoid	Orange
NGA	3-4 mm	Circular	Opaque	Mucoid	Yellow

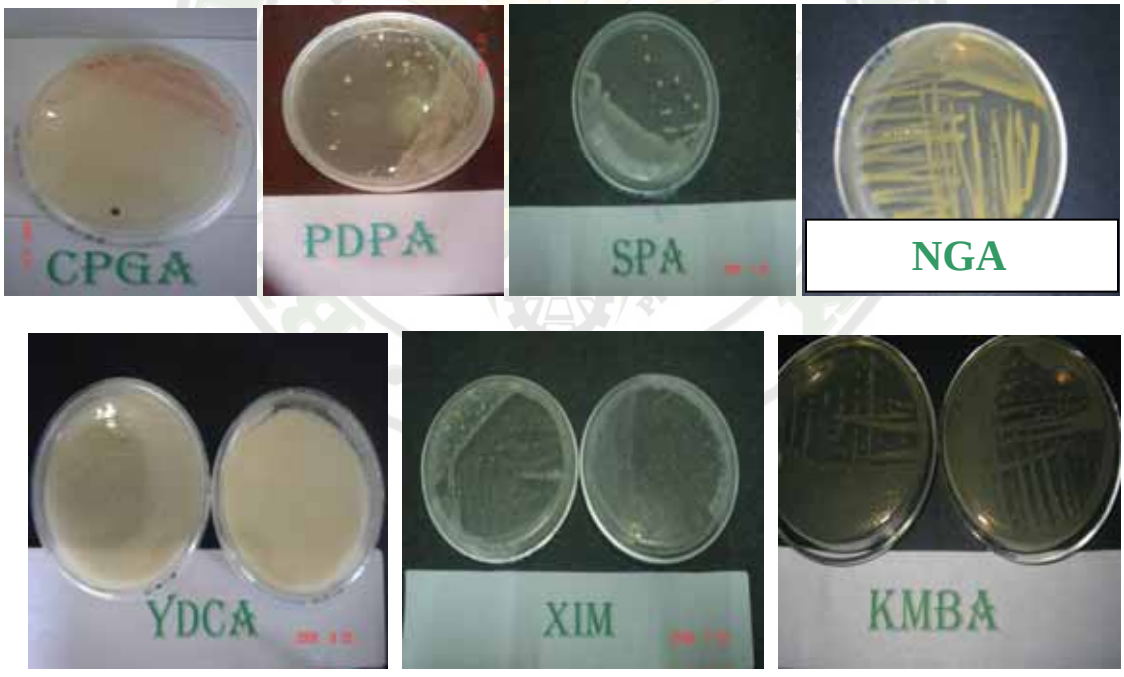


Figure 2**.** Cultural characteristics of the bacterium on various standard and differential media

Morphological Characteristics of the Pathogen

Gram Stain Reaction. Bacterial ooze from the disease specimen (Figure 3a) was preferred for staining rather than organisms cultured in a solid medium. Those coming from a solid medium produce a thick dense surface growth and are not amenable to direct transfer to the glass slide (Cappuccino 2002).

The bacterium was stained pink, which means that the alcohol decolorized the Gram stain (Figure 3b). The bacterium is therefore confirmed to be Gram negative (Frobisher, 1957). The rod-shaped bacteria are less short and cylindrical. These characteristics confirm to those of *Xanthomonas* as cited by Agrios (2002).

Figure 3a. Bacterial ooze from the sectioned lettuce leaves (400x)

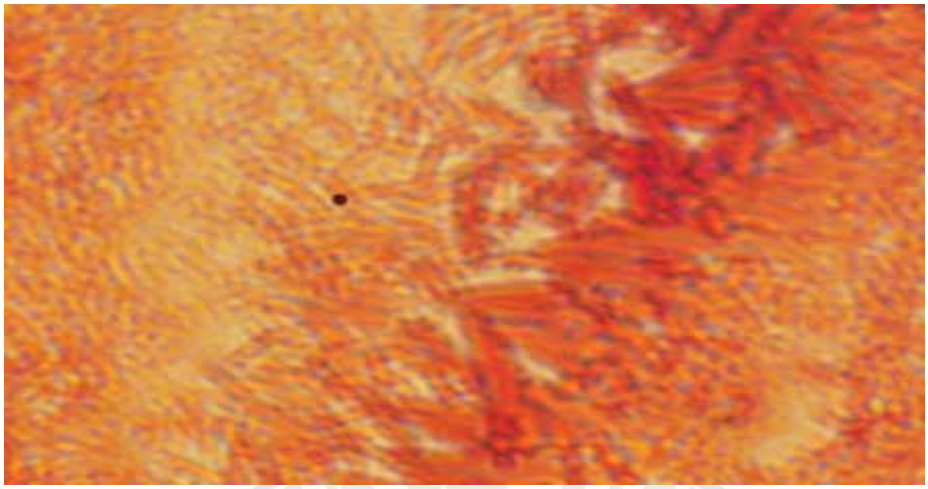


Figure 3b**.** Gram-stained bacterial cells (1000x)

Motility Test A loopful of bacteria were suspended in the center of a clear thin cover slip and inverted to the depression slide. The transparent bacterial cells were motile. Motile bacteria can only move in fluids but it is extremely difficult to steady them since lack of color prevents details from showing clearly. The observation of bacteria in hanging drop preparations, therefore, yields limited though valuable information (Frobisher, 1957).

Physiological Characteristics
of the Pathogen

Growth and O/F Metabolism. Pure culture of *Xanthomonas campestris pv. vitians* was stabbed in the basal medium (Hugh and Leifson Agar). One tube was covered with water agar to prevent the entry of air and the other treatment remained uncovered. Observations were done after 24 to 48 hours at 30 ^{0}C. In comparison with the original

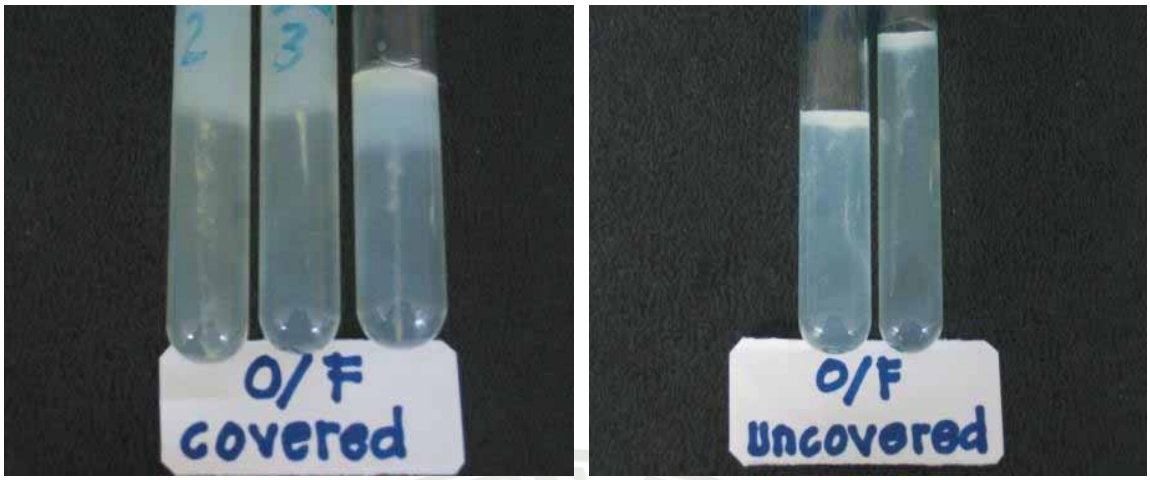


Figure 4. Results of the O/F Test on Hugh and Leifson Agar

medium (control), the color in the covered and uncovered tubes did not change even after 48 hours of incubation. This confirms the description by Robinson (2003) that *X. c. pv. vitians* that is never fermentative and oxidase negative.

Gelatin Liquefaction Test. Gelatin is hydrolyzed by quite a few organisms and liquefies at room temperature (Benson, 1998). The bacterium was not able to liquefy the gelatin even after 3, 7, 14 and 21 days of incubation (Figure 4). Therefore, it can be deduced that the bacteria was not able to produce gelatinase and thus could not hydrolyze the gelatin. This inability to liquefy gelatin is a characteristic of *Xanthomonas* species.

Starch Hydrolysis Test. The bacterium was streaked onto starch agar medium and incubated for 2 to 7 days. The iodine solution was poured over the growth after the incubation period. Starch in the presence of iodine will impart a blue-black color to the medium, indicating the absence of starch-splitting enzymes and representing a negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis will surround the

growth of the organism. This is a positive result (Cappuccino, 2002). If the area immediately adjacent to the growth is clear; amylase has been produced (Benson, 1998).

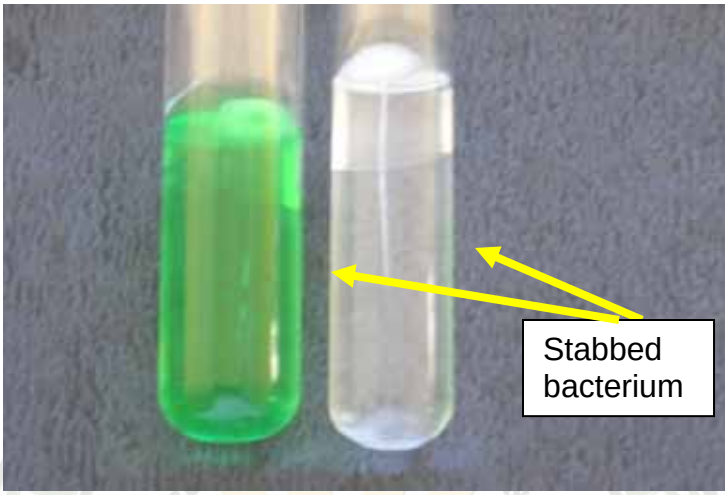


Figure 5. Results of the Gelatin Liquefaction Test on Gelatin Agar (gulaman: green-Flavored and white- unflavored)

Figure 6. Results of the Starch Hydrolysis Test on starch medium (7 days after incubation)

If the starch is undegraded, a blue-black color results when iodine is allowed to react with it. This is because potassium iodide molecules readily occupy a space within the helical coiling of the amylase component of starch. This results in a blue-black complex (Raymundo *et al*., 1991).

Pathogenicity Test

Spraying until run-off of the bacterial suspension effected the symptoms most closely approximating the original specimens. This shows the passage of the pathogen through the hydathodes resulting in initial yellowing at the edges of the infected leaf.

Injection of bacterial suspension into cellular spaces of the leaf using an insulin syringe led to formation of translucent spots. These symptoms are also observed in the field in advanced stages of the disease or when there is a lot of moisture. Pricking and spraying resulted in chlorosis and collapse of the leaf (Figure 7).

Re-isolation of the Bacterium From Artificially-Inoculated Lettuce Leaves

The bacterium was re-isolated from the artificially inoculated lettuce leaves and had the same appearance and characteristics on NGA medium as the original isolate (Figure 8).

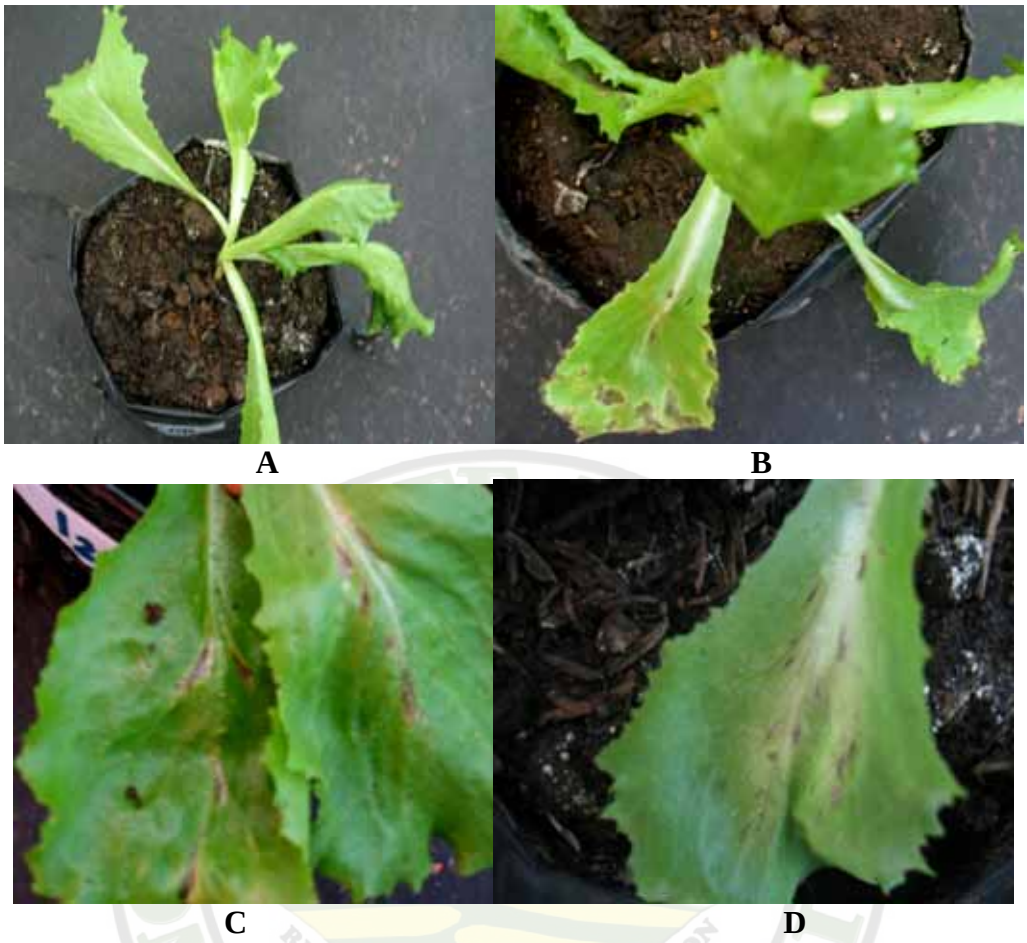


Figure 7. Artificially inoculated lettuce seedlings and its symptoms (A. Uninoculated B. Spraying C. Injection D. Pricking and Spraying)

Figure 8. Bacteria isolated from the artificially inoculated lettuce (NGA, 4 days)

Antibiotic Sensitivity Testing
of the Pathogen

The bacterium was sensitive to both streptomycin and chlortetracycline (Table 3). This means that the bacterium has not been exposed yet to these antibiotics and thus has not been able to develop resistance to the chemical. Expectedly, cephalexin hydrate and cycloheximide were not effective against the bacterium (Figure 9). Cephalexin is highly effective against Gram-positive bacteria but performs poorly against the Gram negatives. On the other hand, cycloheximide was formulated for eukaryotic microorganisms (eMedicineHealth.com, 2005).

Streptomycin comes from *Streptomyces griseus* and is one among the aminoglycoside group of antibiotics. It damages bacterial cell membranes by inhibiting protein synthesis. Specifically, it binds to the 30S ribosome and changes its shape thus causing a misreading of messenger RNA information (Wikipedia 2007).

Chlortetracycline hydrate, also known as aureomycin, comes from *S. aureofaciens*. It is a broad-spectrum bacteriostatic agent that inhibits bacterial protein synthesis. Specifically, it blocks the attachment of the transfer RNA-amino acid to the ribosome. More precisely, it inhibits the codon-anticodon interaction (Wikipedia 2007).

Table 2. The inhibition zones affected by various antibiotics and their concentrations on the growth of *Xanthomonas campestris* pv *vitians*

ANTIBIOTICS	CONCENTRATION (ppm)	MEAN INHIBITION ZONE (mm)
Streptomycin sulfate	50	43.00
	75	39.20
	100	41.00
	200	40.00
	300	39.30
	400	39.80
	500	41.80
	600	43.30
Chlortetracycline	50	49.50
	75	51.00
	100	52.00
	200	52.50
	300	53.20
	400	58.00
	500	57.50
	600	58.50
Cephalexin hydrate	50	23.00
	75	27.20
	100	30.30
	200	26.30
	300	26.50
	400	34.50
	500	26.20
	600	28.00
Cycloheximide	50	35.00
	75	27.20
	100	22.80
	200	20.80
	300	21.50
	400	21.30
	500	20.30
	600	20.30

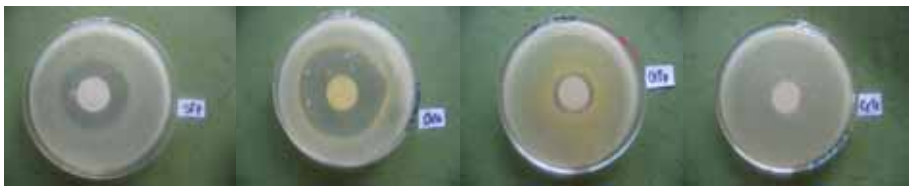
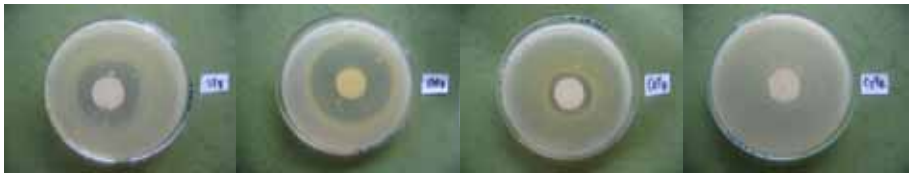
ANTIBIOTIC	Streptomycin Sulfate	Chlortetracycline	Cephalexin hydrate	Cycloheximide
CONCENTRATION (ppm)				
50				
75				
100				
200				
300				
400				
500				
600				

Figure 9. Inhibition zones effected by various antibiotics and their concentrations on the growth of *Xanthomonas campestris* pv *vitians*

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary

The isolated bacterium from symptomatic lettuce leaves exhibited the characteristics of *Xanthomonas campestris pv. vitians*. It produces circular, fried egg-like and mucoid colonies with yellow pigment. Cells are rod-shaped and short. It is oxidase negative and never fermentative. It does not produce the enzyme gelatinase. Artificially inoculated 2 week-old lettuce seedlings show the symptoms of the disease observed in the field. The bacterium was sensitive to both streptomycin and chlortetracycline hydrochloride but was not affected by cycloheximide and cephalexin.

Conclusion

The leaf spot observed in symptomatic lettuce is caused by *Xanthomonas campestris pv. vitians*. The bacterium is sensitive to the standard antibiotics and thus, may not have been exposed to antibiotics. This may indicate that the pathogen is a new pathogen, i.e., newly introduced into the area. Support for this conclusion is the fact that the disease is not yet recorded in the Disease Index (Tangonan, 1999).

Recommendations

Limitations of the study needs firther investigate the fpllowing:

1. pathogenicity test of the bacterium to other crucifer cultivars and other crops likes tomato and bell pepper which have been reported to be affected by the same bacterium;
2. development of integrated disease management measures;
3. survey to determine spread and distribution of the disease in lettuce-

growing areas in Benguet; and

4. possible determination of transmission and spread of the disease as it has been reported abroad to be both soilborne and spread via infected seeds.

LITERATURE CITED

AGRIOS, G. N. 2002. Plant Pathology 3rd ed. Academic Press INC. San Diego, California

BARAK, J. O., S. T. KOIKE and R. L. GILBERTSON. 2002. Movement of *Xanthomonas campestris pv. viitans* in the stems of lettuce and seed contamination. Plant Pathology 51, 506-512.

BARAK, J. D., Koike S. T., and Gilbertson. R. L. 2001. The Role of Crop Debris and Weeds in the Epidemiology of the Bacterial Leaf Spot of Lettuce in Califonia

BENSON, H. J. 1998. Microbiological Application: Laboratory Manual in General Microbiology. 7th ed. The McGraw Hill Companies, INC. United State of America.

CARISSE, O., A. Quimet., V. Toussaint and V. PHILION. 2000. Evaluation of Seed Treatments, Bacteriacides, and Cultivars on Bacterial leaf spot of Lettuce caused by *Xanthomonas campestris pv. vitians*. Plant Dis. 84:295-299

CAPPUCCINO, J. G. and N. Sherman. 2002. Microbiology: A Laboratory Manual 6th ed. State University of New York Rockland Community College. Pearson Education, Inc. San Francisco. P. 49.

DABOIN, C., and O. TORTOLERO. 1991. Mancha bacterial foliar de la lechuga en Algunos campos Andinos de Venezuela. Fitopatol. Venez 6:8-10.

DAYAO, S. A. 2004. Etiology of the Leaf Blight of Calla lily (*Zantedeschia* sp.). BS Thesis. BSU, La Trinidad, Benguet. Pp. 9-10

MEDICINE HEALTH. 2005. Antibiotics. Retrieved 26 March 2007 from http://www.emedicinehealth.com/script/main/hp.asp.

FROBISHER, M. 1957. Fundamentals of Microbiology 6th ed. W.B. Saunders Company. Tokyo, Japan. Pp. 105-106.

NAGPALA, A. L., L. D. LANDO, C. BA-A and J. GALENG. 2005. Progress Report of a Project; Survey, Mapping and Identification of Soilborne Plant Pathogens, Pathogen Vectors and Alternative Host Weeds in Benguet. A report made to the STVRDC, BSU, La Trinidad, Benguet.

PERNEZNY, K., R. N. RAID, R. E. STALL, N. C. HODGE and J. COLLINS. Investigation of seed treatments for management of bacterial leaf spot of lettuce.

PERNEZNY, K., R. N. RAID, R. E. STALL, N. C. HODGE and J. COLLINS. 1995. An Outbreak of bacterial leaf spot of Lettuce in Florida caused by *X.c*.pv. v*itians.* Plant Dis. 79:359-360.

PHILIPPINE INFORMATION AGENCY (PIA). 2006. BSU researchers confirm new crop diseases in Benguet. PIA Press Release dated Jan. 09, 2006. Retrieved 30 November 2006 from http://www.pia.gov.ph.

RAYMUNDO, A. K., A. F. ZAMORA, and I. F. DALMACIO. 1991. Manual on microbiological Techniques. Los Baños, Laguna, Philippines

ROBINSON, P.E. 2003. The Epidemiology of *Xanthomonas campestris pv. vitians*, Causal Organism of Bacterial Leaf Spot of Lettuce. MS thesis. University of Florida. Pp 1-6.

RYDER, E.J. 1999. Lettuce, Endive, and Chicory. Crop Production Science in Horticulture series. Cambridge UK: CAB International.

SAHIN, F. and S. A. MILLER. 1997. Identification of the bacterial leaf spot of lettuce, *Xanthomomas campestris* pv. *vitians* in Ohio, and assessment of cultivar resistance and seed treatments. Plant Dis. 81:1443-1446.

SAHIN, F. and S. A. MILLER. 1998. Two new host of *Xanthomonas campestris* pv. *vitians.* Plant Dis. 81: 1443-1446v.

SAHIN. F., P. A. ABBASI and S. A. MILLER. 1997. Variation among strains of *Xanthomonas campestris* pv. v*itians* in Ohio, and assessment of cultivar resistance and seed treatments. Plant Dis. 81:1443-1446.

SCHAAD, N.W., 1998. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. St. Paul, Minnesota: APS Press.

SCHROTH, M .N., J. P., THOMPSOM, R. BARDIN and A. GREATHEAD. 1964. A new disease of lettuce… bacterial leaf spot of lettuce. Calif. Agric. 2-3.

STEPHENS, J. M. 1988. Manual of Minor Vegetables. Gainesville FL: University of FL.

TANGONAN, N. G. 1999. Host Index of Plant Diseases in the Philippines 3rd. Department of Agriculture Philippine Rice Research Institute (PHILRICE) Munoz, Nueva Ecija, Philippines.

TOUSSAINT, V., C. E. MORRIS and O. CARISSE. 2001. A new semi-selective medium for *Xanthomonas campestris pv. vitians*, the causal agent of bacterial leaf spot of lettuce. Plant dis. 85:131-136.

TOUSSAINT, V. 1999. Bacterial leaf spot, a new disease of lettuce in Quebec caused by *Xanthomonas campestris pv. vitians*. Phytoprotection 80: 121-125.

TSUCHIYA, Y., K. OHATA, K. AZEGAMI and M. MATSUZAKI. 1981. Pathogenicity of *Xanthomonas campestris* pv. *vitians* to various crops and weeds. Nogyo gijutsu kenkyusho hokoku-Bull. Natal Inst. Agric. Sci. 35:57-66.

UMESH, K.C., S. T. KOIKE AND K. L. GILBERTSON. 1996. Association of *Xanthomonas campstris pv.* v*itians* with lettuce seed. Phytopathology 86.S3.

VILLA, A. N. E., C. K. C. JAIME and Y. B. NAVARRO. 2005. Dry Leaf Spot of Lettuce in Benguet: A First Report. A report of Investigatory Project presented during the Regional DOST-INTEL Science Fair and Quiz, held at Tabuk, Kalinga.

WELLMAN-DESBIANS, E. 1999. Dissemination of *Xanthomonas campestris pv. vitians* during lettuce transplant production. Phythopathology 89:S84.

WALLIS, F. M., and J. J. JOUBERT. 1972. Bacterial leafspot of lettuce in Natal. Phytolactica 4:137-138.

WIKIPEDIA. The Free Encyclopedia. 2007. Retrieved March 6, 2007 posted at enwikipedia.org/wiki.html.

WOLFORD, R. 2006. Watch Your Garden-Grown Lettuce. Extension Educator- Urban Horticulture and Gardening. Extension Educator- Nutrition and Wellness, University of Illinois Extension. Retrieved 06 March 2006 from http://www.urbanext.uiuc.edu/veggies/lettuce1.html.

APPENDICES

APPENDIX TABLE 1. Computation of the antibiotic concentration (ppm)

CONCENTRATION (ppm)	ANTIBIOTICS			
	SS	CPH	CY	CH
50	52.63	500.00	53.19	62.50
75	78.95	750.00	79.79	93.75
100	105.26	1000	106.38	125.00
200	210.53	2000	212.77	250.00
300	315.79	3000	319.15	375.00
400	380.00	4000	425.53	500.00
500	526.32	5000	531.91	625.00
600	631.58	6000	638.30	750.00

* 100ml preparation only divided by the amount by 10 ml.

Streptomycin sulfate (SS) – 95%

Cephalexin hydrate (CPH) – 100% (assumed)

Cycloheximide (CY) – 94%

Chlortetracycline/ Aureomycin (CH) – 80%

APPENDIX TABLE 2. Measurement of the inhibition zones (mm)

TREATMENTS	REPLICATION			TOTAL	MEAN
	I	II	III		
SDW	20.0	20.5	20	60.5	20.2
		Streptomycin sulfate			
75 ppm	39.5	39.5	38.5	117.5	39.2
100 ppm	43.5	40.5	39	123	41.0
200 ppm	42.0	40.5	37.5	120	40.0
300 ppm	37.5	39.5	41	118	39.3
400 ppm	41.0	40	38.5	119.5	39.8
500 ppm	40.5	40.5	44.5	125.5	41.8
600 ppm	45.0	41	44	130	43.3
		Chlortetracycline			
50 ppm	55	43	50.5	148.5	49.5
75 ppm	54.5	52	46.5	153	51.0
100 ppm	54	51	51	156	52.0
200 ppm	54.5	51	52	157.5	52.5
300 ppm	54	51.5	54	159.5	53.2
400 ppm	54.5	62.5	57	174	58.0
500 ppm	56.5	60	56	172.5	57.5
600 ppm	56	56.5	63	175.5	58.5
		Cephalexin hydrate			
50 ppm	22	23.5	23.5	69	23.0
75 ppm	29.5	24	28	81.5	27.2
100 ppm	27.5	32	31.5	91	30.3
200 ppm	26.5	25.5	27	79	26.3
300 ppm	25.5	27	27	79.5	26.5
400 ppm	35.5	36	32	103.5	34.5
500 ppm	27	26	25.5	78.5	26.2
600 ppm	29.5	25	29.5	84	28.0
		Cycloheximide			
50 ppm	30.5	35	39.5	105	35.0
75 ppm	25	27	29.5	81.5	27.2
100 ppm	22	25	20	67	22.3
200 ppm	21	21	20	62	20.7
300 ppm	20	23.5	21	64.5	21.5
400 ppm	21	22	21	64	21.3
500 ppm	20	20	21	61	20.3
600 ppm	21	20	20	61	20.3

APPENDIX TABLE 3. Effect of different antibiotics

ANTIBIOTIC	MEAN
Streptomycin sulfate	40.88 b
Chlortetracycline	54.03 a
Cephalexin hydrate	27.75 c
Cycloheximide	23.64 d

* Means with the same letters are not significantly different at 5% DMRT

APPENDIX TABLE 4. Effect of concentration of antibiotic

CONCENTRATION	MEAN
50 ppm	32.933
75 ppm	33.167
100 ppm	31.933
200 pmm	31.133
300 ppm	32.133
400 ppm	34.767
500 ppm	33.200
600 ppm	34.133

APPENDIX TABLE 5. Interaction between antibiotic and *X.c.pv. vitians*

ANTIBIOTIC/ CONCENTRATION	CONCENTRATION (ppm)							
	50	75	100	200	300	400	500	600
Streptomycin sulfate	43.0c	39.2c	41.0c	40.0c	39.3c	39.8c	41.8c	43.3c
Chlortetracycline	49.5b	51.0b	52.0b	52.5b	53.2b	58.0a	57.5a	58.5a
Cephalexin hydrate	23.0fgh	27.2e	30.3e	26.3ef	26.5ef	34.5d	26.2efg	28.0e
Cycloheximide	35.0d	27.2e	22.8gh	20.7h	21.5h	21.3h	20.3h	20.3h

APPENDIX TABLE 6. Analysis of variance

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F VALUE	COMPUTED F 0.05	0.01
Factor A	4	18,829.846	4,707.461	926.4377**	2.48	3.56
Factor B	7	103.967	14.852	2.9230 ns		
AB	28	994.887	35.532	6.9927**		
Error	80	406.500	5.081			
TOTAL	119					

Ns – not significant Coefficient of variation = 6.77%
** -

APPENDIX A

APPENDIX 1. Standard and Differential Media

Yeast Extract Dextrose Agar- $CaCo_3$ (YDCA)

Component	Amount
Yeast	10.0 g
Dextrose (Glucose)	20.0 g
Calcium carbonate, USP light powder	20.0 g
Agar	15.0 g
Distilled Water	1000 ml

Prepare 10 g of yeast, 20 g of dextrose (glucose), and 20 g of calcium carbonate, USP light powder and 15 g of agar. Dissolve in 1 liter of distilled water except dextrose.

Prepare it separately into the flask at 100 ml distilled water. Sterilize in autoclave at 15 psi for 15-20 mins. After sterilizing, mix the dextrose in a separate flask with yeast, $CaCo_3$ and agar. Mix thoroughly before pouring into the petri plates.

King Medium B Agar (KMBA)

Component	Amount
Protease peptone/ peptone	20.0 g
Potassium phosphate (K_2HPO_4)	1.5 g
Magnesium sulfate ($MgSO_4.7H_20$)	1.5 g
Agar	15.0 g
Glycerol	15.0 ml

Dispensed individual 250 ml erlenmeyer flask and sterilized in autoclave for 30 minutes at 15 psi before plating in sterilized petri plates.

Nutrient Glucose Agar (NGA)

Component	Amount
Beef Extract	3.0 g
Peptone	5.0 g
Glucose	2.5 g
Agar	15.0–20.0 g
Distilled water	1000 ml

Sucrose Peptone Agar (SPA)

Component	Amount
Sucrose	20.0 g

Peptone	5.0 g
K2HPO4	0.5 g
MgSO47H2O	0.25 g
Agar	15.0 g

Adjust pH to 7.2

40 % NaOH

40 % 100m distilled H2O

Xanthomonas Isolation Medium (XIM)

Component	Amount
Cellobiose	10.0 g
Sodium phosphate (NaH2PO4)	0.5 g
MgSO4. 7H20	0.3 g
K2HPO4	3.0 g
Ammonium Chloride (NH4Cl)	1.0 g
Agar	15.0 g

Potato Dextrose Peptone Agar (PDPA)

Component	Amount
Potato Dextrose Agar	8.0 g
Peptone	5.0 g
Distilled water	1000 ml

Casamino Acid Peptone Glucose Agar (CPGA)

Component	Amount
Peptone	2.0 g
Casamino acid	0.2 g
Glucose	1.0 g
Agar	3.0 g
Distilled water	140-200 ml
TZC	should be 1g/li or 1ml/100ml CPG

APPENDIX 2. Basal Medium for Anaerobic Growth

Hugh and Leifson Agar

Component	Amount
Peptone	2.0 g
NaCl	5.0 g
KH2PO4	0.3 g
Agar	3.0 g
Bromthymol blue	3.0 ml (1% aqueous solution)
Distilled water	1000 ml

Dissolve ingredients and adjust pH to 7.1. Add 10ml of basal medium to 13 cm diameter test tubes and sterilize at 121º C for 20 minutes. Prepare a 10% aqueous solution of glucose and sterilize by filtration. Add 0.5 ml of sterile glucose aseptically to each tube of basal medium. Inoculate two tubes with the organism to be tested. Cover one tube with a

layer of water agar or paraffin to a depth of 5mm. A color change from blue to yellow in both tubes is recorded as positive for anaerobic growth.

APPENDIX 3. Procedure of McFarland's Turbidity Standard

The 0.5 McFarland may be prepared by adding 0.5 ml of 1.175% (wt/vol) barium chloride dehydrate ($BaCl2.2H2O$) solution to 99.5 ml of 1% (vol/vol) sulfuric acid. The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland standards may be stored for up to 6 months in the dark at room temperature (22ºC to 25ºC). Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube. The accuracy of the density of a prepared McFarland standard should be checked using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standards, the absorbance at a wavelength of 625 nm should be 0.08 to 0.1.