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Full Length Research Paper

Antibiotic susceptibility testing of isolated *Bacillus thuringiensis* from three soil types around Iligan City, Philippines

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This study was conducted with the aim of isolating and identifying entomopathogenic isolate of *Bacillus thuringiensis* (*Bt*) from the three different kinds of soils and to determine its antibiotic susceptibility. Soil samples were taken from three different uncultivated sites that have no history of treatment with *Bt* products. Isolation of the *Bt* strain was conducted based on the conventional biochemical method and the phenotypic characteristics of the isolated *Bt* were determined by making use of standard methods. 20 presumptively identified strains of *Bt* were subjected to antibiotic testing by using ampicillin, amoxicillin, tetracycline, streptomycin, and ofloxacin. Results show that the isolated strains of *Bt* were resistant to β-lactams (amoxicillin and ampicillin).

Keywords: antibiotic susceptibility, β-lactams, vermicast.

INTRODUCTION

Bacillus thuringiensis (*Bt*) is considered to be the most widely used entomopathogenic microorganism used as a biological agent against insects of agricultural pests (Samsonovet al., 1997). The said bacterium is a member of a group of crystalliferous spore-forming aerobic gram-positive, rod-shaped belonging to the genus *Bacillus* that is uniquely characterized by the ability to form endospores and one or more proteinaceous parasoporal crystals that are resistant to inactivation by heat, desiccation and organic solvents.

B. thuringiensis is largely used in agriculture especially in organic farming, in urban aerial spraying programs, and in transgenic crops. Its proteins have been used in many organic farms for over 50 years as a microbial pest control agent. Previous researchers and investigators have reported that natural environments of tropics and su*bt*ropics of Southeast Asia are a good reservoir of *B. thuringiensis* populations with a great diversity of serological and biological characteristics (Attathom, et al*.,* 1995). Moreover, *B. thuringiensis* can also be found among insect cadavers, stored product dust, leaves of plants, aquatic environments and from the marine sediments (Maeda et al., 2000). *Bt*, like other *Bacillus* species, has been classified on the basis of its cellular, cultural, biochemical and genetic characteristics with a cell width approximately 1 and 5 µm in length (Madigan and Martinko, 2005; Sakai et al*.,* 2007).

The apparent increase of the occurrence of antibiotic resistance among bacteria during the past years and its possible implication in public health has led to an intensified surveillance of bacterial resistance in many countries (Sarker et al., 2010). Also, little interest was shown in antimicrobial susceptibility profiles of *Bacillus* species because of low recognition of the ability of *Bacillus* species (Turnbull et al., 2004). This study was conducted to isolate potential *B. thuringiensis* isolates using standard methods and to test their antibiotic susceptibility to β-lactam antibiotics amoxicillin and

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Table 1. The three (3) soil description based on its physical properties and pH.

Sample number	Soil description	Texture	pH
1	Dark gray loam, high humus content	Loamy	7.06
2	Grayish brown loamy sand, low humus content	Sandy loam	7.64
3	Grayish light-brown sandy loam, low humus content	Sandy loam	7.37

1, Carbide Village; 2, Barangay Luinab; 3, BarangayTibanga.

ampicillin.

MATERIALS AND METHODS

Soil collection and characterization

Soil samples were collected from three different uncultivated sites that have no history of treatment with *B. thuringiensis* products. The sampling sites included: a residential house with a backyard garden at Carbide Village, a corn plantation at Luinab and a vegetable garden at Tibanga, Iligan City.

About 100 g of soil samples were collected by scraping off 2-5 cm below the surface with a sterile spatula. All samples were placed in a sterile autoclavable plastic ware aseptically and were brought to the laboratory immediately for processing.

The soil texture was determined and pH was determined by suspending 50 g of the soil in 100 ml of distilled deionized water. The suspension was stirred for 1 h at 800 rpm on a rotary shaker. The pH of the supernatants was recorded using a pH meter.

Presumptive identification

Isolation of the *Bt* strain was conducted according to the method described by Travers et al. (1987). The samples were processed by acetate selective method in concentrations of sodium acetate (pH= 6.3). In this procedure, acetate inhibits germination of *B. thuringiensis* spores, so other spore germinates and non-spore forming bacteria are eliminated by heat treatment (7 min at 80°C). The surviving spores were plated and grown on a suitable medium and incubated at 30°C for 24 h to o*bt*ain colonies. *Bt*-like colonies, which are usually described as cream-colored and have the appearance of a fried egg on a plate, were purified and cultured. Smears were Gram-stained with malachite green method, and were examined under phase-contrast light microscope for the observation and determination of the presence of spores and parasporal bodies (delta-endotoxin crystals) of the bacterium at 24 h intervals.

Cultural method of characterization of bacterial isolates

The following differential tests were performed following the identification flow charts on Bergey's Manual of Determinative Bacteriology: determination of oxygen requirement, starch hydrolysis, catalase test, Voges-Proskauer Test, and test on Triple Sugar Iron agar.

Antibiotic susceptibility testing

The antibiotic resistance of the isolated *B. thuringiensis* was tested against specified antibiotic discs common against the said species using the disc diffusion method (Bauer et al., 1966). A 16 h broth cultures of the isolated strains grown at 37°C was transferred using sterile cotton swabs into the Mueller Hinton Agar (MHA) plates by aseptically dipping the swabs into the tubes and streaked on the plate. Then, ampicillin (10 µg/disc), amoxicillin (30 µg/disc), tetracycline (30 µg/disc, streptomycin (10 µg/disc), and ofloxacin (30 µg/disc) antibiotics were distributed on the plate. The plates were incubated at room temperature for 16 h and the growth of the bacteria was observed.

RESULTS AND DISCUSSION

Soil characteristics

Soil sample from Carbide Village has a pH of 7.06 and with a dark –loamy description (Table 1); this is because the soil is composed of degraded organic materials such as banana peels, rice, etc. Accordingly, soil pH influences the solubility of nutrients and affects the activities of many microorganisms responsible for breaking down organic matter and most chemical transformations in the soil (USDA, 1998). The type and population densities of these microorganisms change with respect to the pH of the soil. A pH of 6.6 to 7.3 (a neutral soil) is favorable for microbial activities that contribute to availability of nitrogen, sulphur, and phosphorus in soils. Thus, soil sample from a backyard is on its range which may harbor a number of microorganisms unlike the other two samples which has a pH above the neutral line.

Presumptive identification

The isolated colony that was identified as gram-positive was allowed to grow on sporulation medium to induce endospore formation and to identify bacterial isolates belonging to the genus *Bacillus*. The most distinguishing feature of *B. thuringiensis* from closely related bacillus species (*B. cereus, B. anthracis)* is the presence of a parasporal crystal body that is near to the spore, outside the exosporangium during endospore formation (Andrews et al*.,* 1985, 1987; Bulla et al., 1995). *Bt* is a member of the genus *Bacillus* and like the other members of the taxon, it has the ability to form endospores that are resistant to inactivation by heat, desiccation and organic solvents. Another distinguishing feature is the production of endospores, which are highly refractile resting

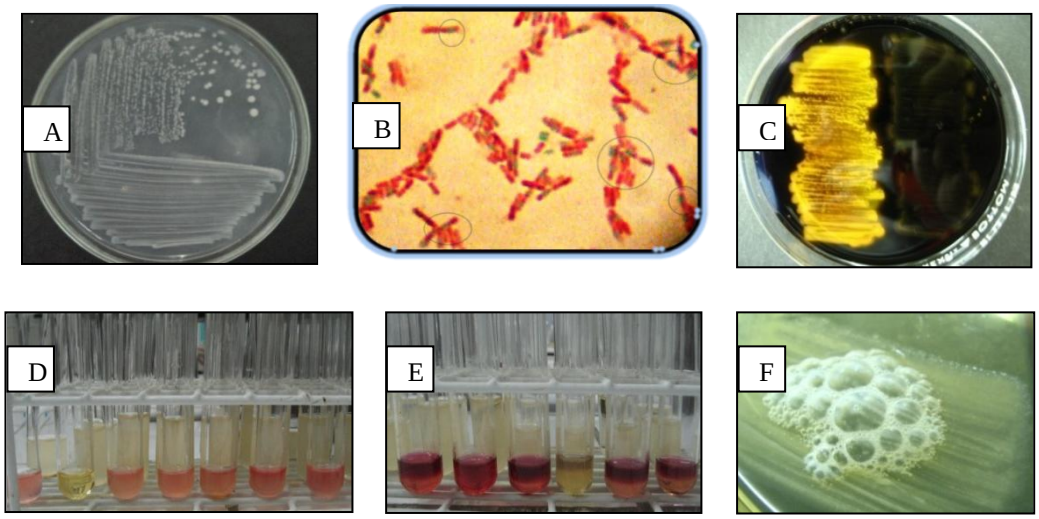

Figure 1. Conventional method of identification of *Bacillus thuringiensis*: (A) Purified culture. (B) Endospore staining. (C) Starch hydrolysis. (D) Methyl Red. (E) Voges- Proskauer.(F) Catalase test.

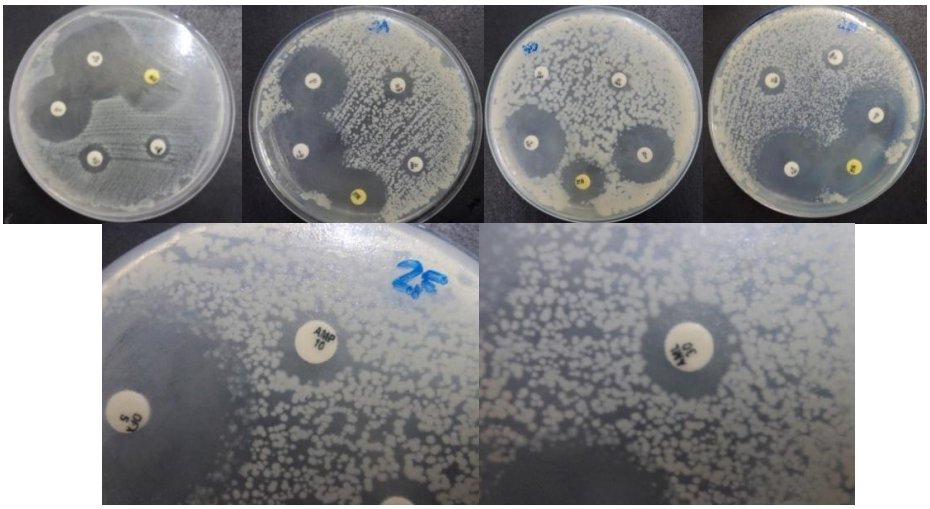

Figure 2. Ampicillin and amoxicillin resistant isolates.

structures formed within the bacterial cells (Todar, 2005).

The three sampling sites harbor a number of microorganisms that are able to tolerate extreme conditions. Out of the 100 isolates, only 20 were randomly chosen and were successfully purified and characterized. The identification of bacteria was based on cultural, cellular, and biochemical characteristics exhibited by each respective species and strain (Figure 1).

Antibiotic susceptibility

The twenty (20) purified isolates were subjected to antibiotic assay to determine the isolates' sensitivity to a set of antibiotics available. The isolates were tested for resistance to amoxicillin, ofloxacin, ampicilin, tetracycline, and streptomycin (Figure 2).

Identified *B. thuringiensis* from verrmicast, a loam soil, was susceptible to three out of five broad spectrum antibiotics (Figure 3). Figure 4 shows the sensitivity of the isolates from the two other soil samples. Six isolates showed amoxicillin and ampicillin resistance (Figure 2). Ampicillin and amoxicillin are closely related belonging to a class of antibiotics called penicillins that are used for treating bacterial infections. These antibiotics all have a similar mechanism of action; stopping bacteria from multiplying by preventing it from forming the walls that

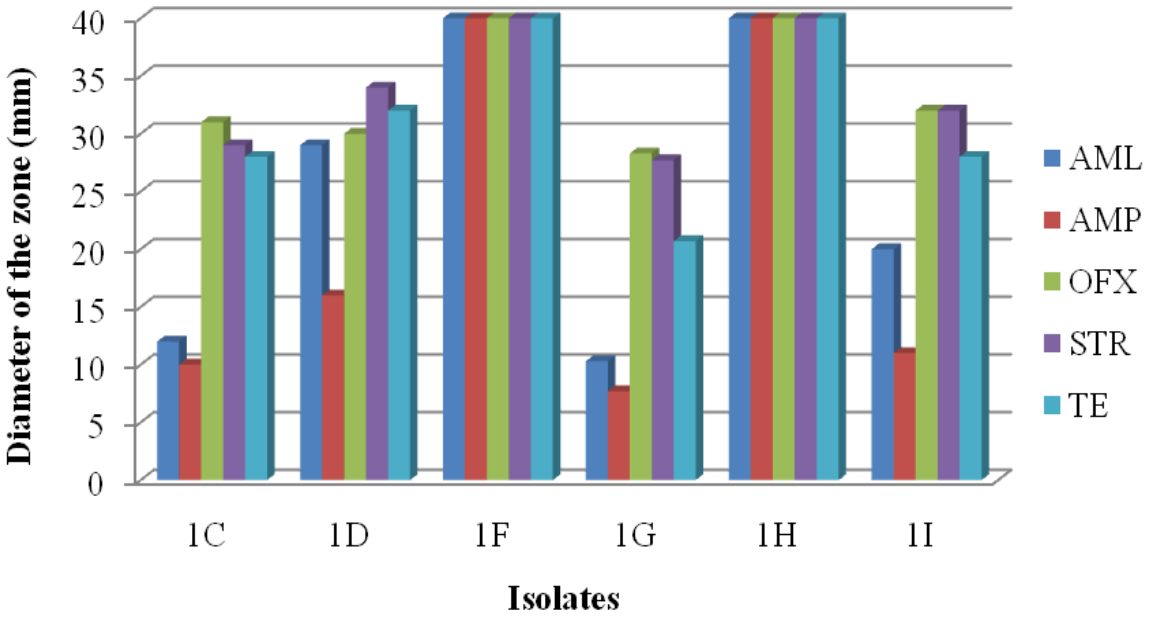

Figure 3. Antibiotic sensitivity of isolates from vermicast type of soil isolated from Carbide Village, Iligan City. AML, Amoxicillin; OFX, Ofloxacin ; STR, Streptomycin; AMP, Ampicillin; TE , tetracycline; BS, *Bacillus subtilis; BT*, *B. thuringiensis*

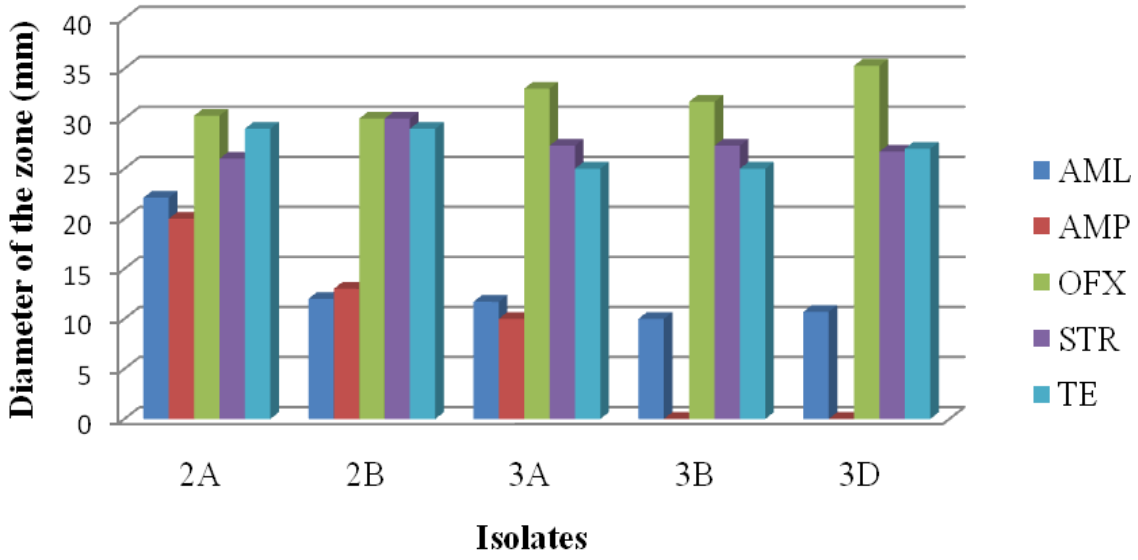

Figure 4. Antibiotic sensitivity of isolates from Tibanga (2A-2B) and Luinab (3A, 3B, and 3D) soil samples. AML, Amoxicillin; OFX, Ofloxacin; STR, Streptomycin; AMP, Ampicillin; TE , tetracycline; BS, *Bacillus subtilis; BT*, *B. thuringiensis*

surround them. *Bt* which has been long considered as non-pathogenic for humans and used extensively for pest control were found to be resistant to the β-lactams (amoxicillin and ampicillin).This characteristic is essential in the identification of *B. thuringiensis* species for most of the selected species that are resistant to the listed antibiotics while susceptible to the remaining antimicrobials (Luna et al., 2007).

Based on the conventional method of characterization and antibiotic resistance of the microorganisms isolated, out of the 20 isolates, only ten (50%) were presumptively identified as *B. thuringiensis* and the ten isolates were

only identified as belonging to the genus *Bacillus* spp.

Conclusion

The purpose of this paper was to isolate *B. thuringiensis* strains that were collected from three soil samples around Iligan City by sodium acetate method in order to select the target organism, particularly mosquitoes that are disease bearing such as *Aedes aegypti*. As a result, there are a number of different *Bacillus* sp. that were isolated. Vermicast type of soil which has a pH of 7.1 harbors the most number of microorganisms. Out of the numerous counts of isolated colony, only 20 colonies were purified and characterized by conventional method of identification according to the Bergey's manual of bacteriology and its antibiotic resistance against β-lactam antibiotics namely ampicillin and amoxicillin. From the 20 characterized colonies, ten were identified as *B. thuringiensis* based on colonial morphology, gram stain, presence of endospore, cell diameter and its resistance to ampicillin and amoxicillin.

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