Bacteriological Studies in Bacillus Thuringiensis and its Use Controlling Insecticidesin Poultry Houses

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Abstract

The microorganisms which present on the clay soils, were studied. The result recorded that bacteria isolated from clay soils were identified ***Bacillus thuringiensis, E. coli, Pseudomonas, Klebsiella and Shigella. The B. thuringiensis*** is the highest bacteria causing mortality reached 100% to house fly and mosquitoes, while other bacteria causing 30%, zero%, zero% and zero% mortality for E. coli, Klebsiella, Pseudomonas and Shigellarespectively. Most strain of ***Bacillus thuringiensis*** isolates produce a typical crystal often heterogenous in size and shape. Extraction of total DNA from ***B. thuringiensis*** isolate for PCR analysis was done. All ***B. thuringiensis*** isolate were characterized by PCR. Parasporal bodies of ***B. thuringiensis*** isolates had biological activity when assayed againsthouse fly and mosquitoes. Field application of ***B. thuringiensis*** as toxic spray on poultry houses by using 2 x 10^3c.f.u/m showed that mortality of mosquitoes reached 100% after 4 days. It can be concluded that ***Bacillus thuringiensis*** (BT) are bacteriocidal causing mortality reached to 100% after 4 days post-treatment with concentration of 2 x 10^3cfu/ml. respectively. Most strain of ***Bacillus thuringiensis*** isolates produce a typical crystal

Introduction

The house fly, Musca domostica L., is a key pest of poultry facilities and a vector of many metaxenic pathogens and can cause serious sanitary problems because of its high reproductive potential, feeding habits , and ability to disperse. Organic wastes from intensive poultry farms provide excellent habitats for the growth and development of this insect (Thomas and Skoda 1993)

Control recommendations for the house fly are currently limited to use of chemical insecticides to kill the house fly adults and larvae. Due to the problems associated with the development of pesticide resistance by the house fly (Keiding, 1999; Scott et al., 2000 and Kaufman et al., 2001), as well as other environmental and regulatory concerns, research toward developing alternative control strategies is warranted .Beauveriabassiana (Balsamo) Vuillemin and Bacillus thuringiensis Berliner (Bt), which occur naturally as pathogens of M domestica, are some of the potential alternatives.

Bacillus thuringiensis has been found to be toxic to the house fly

(Hodgman et al., 1993)

Several natural isolates of Bt have also been found that are active against larvae of the house fly (Johnson et al, 1998)

Thuringiensin -containing preparations have been used to control larvae of M domestica (Mullens et al., 1988 and Mullens and Rodriguez, . 1988)

It has also been reported by Carlberg et al (1991) that nuisance flies m cattle sheds, slaughter houses, and latrines could be successfully controlled by applying Btvarthuringiensis to larval breeding sites.

The entomopathogenic fungus, B. bassiana, is a ubiquitous and important entomopathogen of several insect pests (Feng et al., 1994 and Inglis et al., 2001) and can be used effectively to suppress house fly populations. One approach to controlling house flies with B. bassiana would be to target the adult house flies as they fly around the poultry houses or rest on the walls.

Steinkraus et al. (1990) reported that B. bassiana infected 1% of house fly adults under natural conditions in poultry houses . Despite the lowprevalence of disease, strains collected by Steinkraus were vi1ulent in subsequent laboratory studies (Watson et al., 1995). One strain (P89)،when formulated in water and a surfactant induced 99% mortality in house flies (dose I x I 0^8 conidia/cm2) within 6 days of exposure.Prior research, however, does not indicate great potential for B.baasitwa to control the larval stage of house files (Lecuona et al., 2005).

Geden et al. (1995) and Lecuona et al. (2005) found the virulence ofB. bassiana to be relatively poor against M. domesatim. Combining B. bassiana, with other entomopathogens might result insynergistic . interactions that would enhance the potential for biological control of M. domestica larvae.

The aim of the present study is to evaluate the use of Bacillus thuringiensis as biological control of mosquitoes and house fly.

Material and Methods

Samples: Soil suspended

Culture media: The media used during this study were prepared according to Gams et al. (1998).

Isolation of microorganisms associated with clay soils: according to Berkley and Goodfellow (1968).

Bacterial isolates: were identified according to Bergey's manual of

determinative bacteriology (Holt et al., 1984).

Isolation of B. thuringiensis (B.t) Bioassay: according to Pandua et al., (1980):

Isolation of ***B. thuringiensis*** (B.t) approximately 0.5 g of clay soils was suspended in I 0 ml of sterile distilled water, and the preparations were mixed vigorously by vortexing for 1 min. After mixing, homogenized of clay soils was allowed to settle out for 2 min, and then 1 ml of the • supernatant was stored at 80°C for 3 min in prewanned 20-ml glass universal bottles to kill most non-spore-forming organisms . Samples were plated at two concentrations (undiluted and 10 dilution) on to nutrient agar (Oxoid) containing extra Technical No. 3 agar (Oxoid) so that the final agar concentration_ was 2%. The plates were incubated at 30°C for 48 h and examined ***B. thuringiensis*** (B.t) morphologically for reparation of biocidal isolates as insecticide.

Table (1): Biochemical activity of B. thuringiensis (B.t) strains biotyping according to Martin and Travers, (1989)

Biochemicaltype(describedsubsp)	Biochemicalandphysiologicaltestresult			
	Hydrolysis of		Utilization of	
	Esculin	Lecithin	Sucrose	Salicin
thuringiensis	+	+	+	+
kurstaki	+	+	-	+
indiana	+	-	+	+
galleriae	+	-	-	+
aotto	+	+	+	-
dendrolimus	+	+	-	-
mornson	+	-	+	-
darmstadiensis	+	-	-	-
Ostriniae	-	-	-	+
Israelensis	-	+	-	-

(+) Positive reaction; (-) Negative reaction

Crystal morphology:

During growth of B. thuringiensis (B.t) isolates in Proflo B4 broth and prior

to cell lysis, the isolates were examined by phase-contrast microscopy and, on the basis of parasporal crystal morphology according to Martin and Travers, (1989)

Identification of B. thuringiensis isolates by 16S rDNA:

The DNA of selected bacteria was extracted using GenElute™ Genomic DNA Kit, sigma Aldrich according to Birnboim, (1983)

Growth of isolates for bioassays for in vivo:

Isolates were cultured at 30°C on Proflo B4 broth in 250-ml fluted Erlenmeyer flasks shaken at 250 rpm. During growth, the cultures were periodically examined by phase-contrast microscopy, and the crystal morphology of each culture was determined. Cultures were harvested either when at least 95% of the population had lysed, releasing spores and : crystals, or, in rare cases, when maximum lysis (less than 95%) had been reached, as determined by at least three consecutive microscopic examinations. Cultures were then stored in a 15% (vol/vol) glycerol (AnalaR) solution at -70°C.

Biological control of B. thuringiensis:

It was tested against the same poultry houses mosquitoes by different concentration in the laboratory (in vitro) and in the field (in vivo) according t***o Lokma and Harpy*** (1999)

Poisonous spray technique:

Three concentrations $3x10^5$, $l.5x\ 10^5$ and $0.5x\ 10^5$ of B. thuringiensis (B.t) were prepared by incorporating the appropriate amount of each concentration for spraying. Three plastic boxes (3/4 kg capacity) were used for each concentration. The three concentrations were sprayed into each box. Control treatment was prepared using spray free from any compounds. Five and fifty individual's house fly and adult mosquitoes each were introduced into each box, then covered with muslin cloth and secured with rubber band to prevent escape. Mortality was observed according to ***El- Okda (1981).***

Results and Discussion

The microorganism isolated belonging to 5 bacteria were Bacillus thuringiensis , E. coli, Pseudomonas, Klebsiella and Shigella.

The effect of isolated bacteria on mosquitoes indicated that B. thuringiensis, showed the highest mortality rate 100% of mosquitoes by occurred B. thuringiensis, the second effective bacteria against mosquitoes was E. coli cause 30% mortality while Klebsiella, Pseudomonas and Shegella have no effect mosquitoes mean while the effect of isolate tested bacteria on house fly indicated B. thuringiensis E. coli, Pseudomonas, Klebsiella and Shigella show 100% mortality by B. thuringiensis and

no effect by other isolates.

More efforts have been done for producing new substance instead of chemicals through using microorganism which can produce some activity antimicrobial agents in controlling insects (Cook, 1993). The obtained results showed that 5 bacteria were isolated from the clay soils, this results agreed with Soufiane and Cote, (2009).

Identification of B. thuringiensis:

B. thuringiensis on nutrient agar (2%) media produced flat, dry, white colonies with uneven borders.

B. thuringiensis is gram positive soil-dwelling, spore-forming rod shaped bacteria. It produces a diamond shaped crystal from its crystal protein (Cry proteins).

The main criterion for B. thuringiensis differentiation from other spore-forming bacteria was crystal production by Sporulation culture.

Crystal morphology:

Most strains produce spherical and bipyramidal crystals. Only a low percentage of strain (16%) formed atypical crystals often heterogeneous in size and shape. The protein profiles of spherical and bipyramidal crystals consist of poorly defined component which could be a source of novel insecticidal properties (Fig. 1).

Most strains produced a typical crystals often heterogenous in size and shape. Abundance of heterogenous crystal of B. thuringiensis strain has already been reported by Lecadet et al, (1999) who found more than 50% of B. thuringiensis strains produce irregular or heterogenic crystal. The protein profiles of heterogenic crystals consist of many poorly defined components which could be a source of novel insecticidal properties (Burtseva et al., 1995 and Chauaux et al., 1997).

Bacillus thuringiensis was found effective against mosquitoes. The mortality rate reached 100% according to variety of bacteria and the method of pathogen application and spraying method was find highly effective application.

Isolation and identification of B. thuringiensis have been found to colonize many different habitats (Heimpel, 1967 and Goldberg and Margalit, 1967) but its normal habitat is the soil (Dulmage and Aizawa, 1982).

Biochemical typing:

Using the biochemical typjng method, all the B. thuringiensis strain were divided 4 biochemical types.

Based on biochemical typing, B. thuringiensis Subsp. Kurstaki (Es-r Sa+, Le+ and

Su-) of the whole isolates as result in table (1).

By using the biochemical typing method, all B. thuringiensis isolates were typed as Bacillus Thuringiensis Var. Kurstaki . This system is based on the biochemical tests which have been identified by Debarjac, (1981) and have been used for B. thuringiensis classification in many investigations (Dow and Lone 1999).

Extraction of total DNA from B. thuringiensis strain/or PCR analysis:

A total of 5 randomly selected Bt isolates representative for the different assigned morphology; biochemical type and abundant crystal shape morphology were selected for this experiment. The analysis of PCR products by agarose gel electrophoresis revealed amplified target bands-1550 bp (Fig. 2).

Isolate sequence:

Seq16sequenceexportedfromseq16-Sequencer4a

TTAAAAAAAAGTGGGGGGGGGGCAATCAGCAGGCGAGCGATGGGTT
AGAGTTGCTCTTATGAAGCTAGCGTCGGAATAAGACCAGGAGCGTGTCCC
TGCAATGATACTGGACCACTCTCAGAACAGTAACTATATGGGTACCACTT
AAGCTGAAGAATCCTTCA
TATTAATGCTACATTGACATCTGAGACTCGTGCCGGGAGTGTCTCGAGTCT
TCTACCGCTATTGTCGTTTTTCTGTACGGTGTATAGTCGCCCCTAAGGGATCT
GACCAGAGTTTTTCTGTTGGGCACGACAGCACGGAGAGTCGCTGTTTAAT
GATG

The BLAST analysis of the nucleotide sequence returned confident results and confirmed that the 5 tested local *Bt* isolates was belonged to *B. thuringiensis* as in Table (2).

The PCR assay described in this study to was identified to B. thuringiensis from soils showed high sensitivity and specificity. The specificity of the oligonucleotide primers is important to avoid false positive results when using PCR B. thuringiensis closely related to B. cereus based on l6S rDNA sequencing and share of many of their characters

The high detection rate of n PCR approaches may be reason of concern when non quantitative assays are employed. It has claimed that because PCR can detect a very low number of cells of samples.

Several researchers (Soufiane and Cote, 2009; Poornima et al., 2010) have used l 6S rDNA gene analysis as a molecular identification tool for Bt, while Soufiane and Cote (2009) not only used this tool for the identification of Bt species but also claimed its ability to discriminate Bt different H serotypes. In the present

study, l 6S rDNA gene analysis proved useful in the identity of the tested local Et isolates and the two Bt reference strains as belonged to B. thuringiensis. Hence, it unambiguously confirmed the biochemical phenotypic identity.

Efficacy of different concentration of B. thuringiensis as toxic substances to house fly and adult mosquitoes:

Data presented in table (3) illustrated the efficacy of B. thuringiensis isolated on house fly and adult mosquitoes under laboratory condition. Results revealed that none of the tested B. thuringiensis exhibit any bacteriological activity one day post treatment. Mortality percentage increased gradually to reaches in maximum after 4 days where it gave highest effect 100%.

Field application of B. thuringiensis as toxics pray on house fly and adult mosquitoes at poultry houses:

This field trail was conducted to evaluated B. thuringiensis in dilution (2 x 10^3 c.f.u/ml) as poisonous spray against house fly mosquitoes showed that initial bactericidal was 50% after 2 days post treatment and highest gave mortality percentages 100% was revealed after day 4 post treatment.

This result was conducted to evaluated B. thuringiensis 2 x 10^3 c.f.u/ml as poisonous against house fly and mesquites and cause mortality rate 100% after 4 days post treatment. These result agreed with Ghamry (1997) who detected the B. thuringiensis were found effective against II vestalis, M. cartusiana and Ruminadecollata. The mortality rates differ from 100% to 59% according to variety of bacteria and method of pathogen application. Soaking spray method was found highly effective than toxic baits application (Foster et al., 1991).

Table (2): Sequence similarity of the isolate sequenced 16srDNA genes from *B.t* isolates

Accession	Description	Evalue	Maxident
GU936826.l	UnculturedBacillussp.clone A8DMCS0516SribosomalRNAgene,partial sequence	6e-06	90%
EU201189.1	DS-4 strain *cereus* *Bacillus* 16SribosomalRNAgene,partialsequence	6e-06	90%
JQ289048.l	*Bacillusthuringiensis*strainSP-17-SP-15 16Sribosomal RNA gene,partialsequence	7e-05	86%
JQ289052.1	*Bacillusthuringiens i*sstrainSP20R-SU7l6SribosomalRNAgene,partialsequence	2e-04	88%
JQ289046.1	*Bacillusthuringiensis*strainSP15F-Sp-2016SribosomalRNA gene,partialsequence	2e-04	88%
JN887351.l	CB4 strain *cereus* *Bacillus* 16SribosomalRNAgene,partialsequence	2e-04	88%
JN698960.1	*Bacilluscereus*strainAIMST9MPE116SribosomalRNAgene,partialsequence	2e-04	88%

Table (3):Efficacy of different concentration in vitro of B. thuringiensis as toxic spray to house fly and adult stage of mosquitoes

Concentration	Reduction percentage larvae				Reduction percentage adult mosquitoes			
	1 day	2 days	3 days	4 days	1 day	2 Days	3 days	4 days
2 x 10^2 c.f.u/ml	0	24	48	80	0	24	48	80
2 x 10^3 c.f.u/ml	0	50	70	100	0	50	70	100
2 x 10^5 c.f.u/ml	0	50	90	100	0	50	90	100

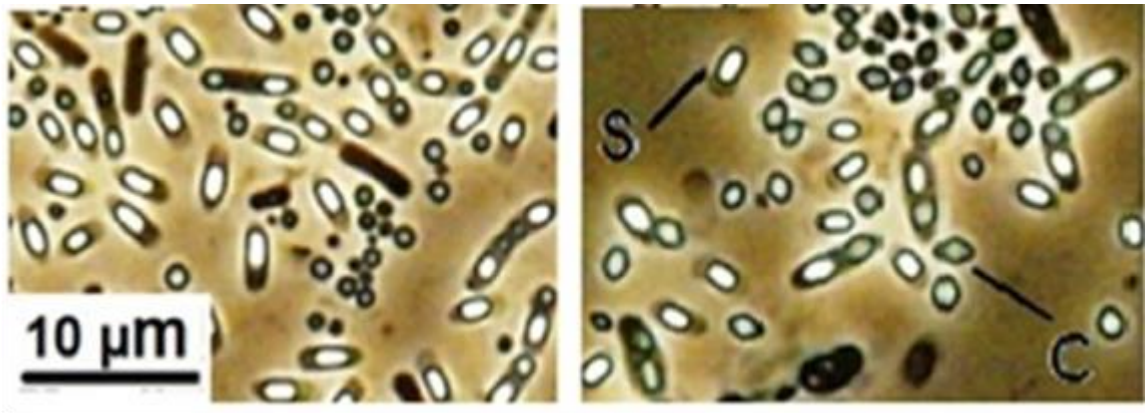

Figure (1): Phase contrast micrographs of BT isolates showing spore (S) and crystal shapes (c) :isolates number 1(spherical), 2 (bipyramidal)

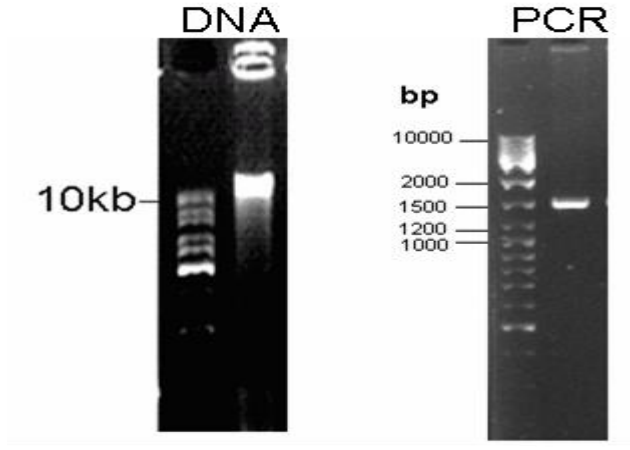

Figure (2): Analysis of PCR products by agarose gel electrophoresis amplified target band ~ 1550bp.

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