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PCR BASED IDENTIFICATION OF CRY1AC GENE CONTAINING *BACILLUS THURINGIENSIS* ISOLATED FROM SOIL SAMPLES IN NANDED DISTRICT

Kunal P. Deshmukh[1] & Vikram B.Patil[2]
[1,2]Department of Biotechnology,Yeshwant Mahavidyalaya, Nanded.431602, Maharashtra.
*Corresponding Author email: kunal.deshmukh11@gmail.com

ABSTRACT
About 78 colonies showing the typical *Bacillus*-like characteristics were isolated from different agricultural and non-agricultural soils from different locations in Nanded district. These colonies possessed white to off white colour, slightly raised elevation and regular margins. Since the genus *Bacillus* contains rod-shaped and Gram-positive verified bacteria, so Gram's staining was performed to eliminate all other bacteria that do not have these two features. Total of 35 colonies were selected from Gram's staining result and subjected to light microscopy to screen for the presence of endospore and parasporal body in order to distinguish *B. thuringiensis* from other *Bacillus* groups. Presence of endospore and parasporal body were screened by using Schaeffer & Fulton's spore staining and Coomassie Brilliant Blue staining method. In total 15 colonies were recognized as *B. thuringiensis* by using these methods. According to morphological, physiological and biochemical characteristics, all these 15 bacterial strains were identified as *Bacillus thuringiensis* (*Bt*). Polymerase Chain reaction (PCR) is a molecular appliance extensively used to characterize the insecticidal bacterium *B. thuringiensis*. This method can be used to amplify specific DNA fragments and thus to impel the presence or absence of a target gene. By using cry 1Ac gene specific primer, PCR analysis showed that out of fifteen isolates seven strains harbored cry1Ac gene.

KEY WORDS: Gram positive, spore forming, parasporal crystal, *Bacillus thuringiensis*, PCR, cry 1Ac , gene specific primer.

INTRODUCTION

In several parts of the world the gram positive bacterium *Bacillus thuringiensis* has been identified and used as a biopesticide in the control of both agricultural and medically important insects. Strains of this bacterium have been recognized to be pathogenic to specific orders of insect, namely lepidoptera, diptera, coleoptera and recently spodoptera (Hofte and Whiteley, 1989). This broad spectrum of activity has given it a extensive use in the control of these orders of insect, with new strains being recognized having capacity to control some members of these orders that are otherwise not known to be susceptible to the pathogenic substance, which are crystal proteins produced by formerly identified *B. thuringiensis* strains. Identification of these bacterial strains had formerly been done via the conventional microbiological culture techniques and serological assays, processes that are cumbersome and long. The use of the PCR technique offers a vigorous, systematic and faster alternative method to identify *B. thuringiensis* from other *Bacillus* isolated and even differentiates strains and subspecies of this bacterium (Bourque *et al*., 1993; Brousseau *et al.,* 1993). This paper reports the PCR-based identification of cry 1 Ac gene containing strains of *B. thuringiensis*

MATERIALS & METHODS

a) Soil sample collection

Soil samples were collected from Nanded district. It was attempted to collect soil from locations that were as diverse as possible. Soil samples were collected by scraping off surface material with a sterile spatula and then obtaining approximately 100-gram samples from 2-5 cm below the surface. All samples were stored in sterile plastic bags at ambient temperature.

b) Isolation of *Bacillus* from soil samples

Isolation of *Bacillus thuringiensis* was conducted according to the method of Ohba and Aizawa(1986) and Travers et al (1987).One gram of each sample was suspended in 10 ml sterile distilled water and pasteurized at 80^0C for 30 min.The isolates were cultured onto T3 sporulating plates (3gm Trypton , 0.05 M Sodium Phosphate pH 6.8 , 0.005 gm $MnCl_2$ per liter).After 48 hours of incubation at 28^0C colonies with typical *Bacillus thuringiensis* morphology were picked.

c) Microscopic tests:

Microscopic test like Gram's staining, endospore staining by Schaeffer & Fulton's spore stain, Coomassie Brilliant Blue staining and motility test were performed for initial screening of *Bacillus thuringiensis*

d) Primer selection:

TABLE 1 : Primer used in this study was selected according to Aly *et al* 2007.

Primers	Gene recognized	Product size (bp)	Sequence	Reference
cry 1Ac specific primer	*cry* 1Ac	238	(F) 5´ CTCAATGGGACGCATTTCTT 3' (R) 5´ CGGTTGTAAGGGCACTGTTC 3'	Aly *et al.*, 2007

Genomic DNA extraction

Genomic DNA was extracted by combining two methods (Ausubel et al. 1994; Cardinal et al. 1997). Cells grown in 10 ml of nutrient broth (NB) at 37^0C overnight were harvested and resuspended in 200 µl sucrose solution (25% sucrose and 30 mg lysozyme per ml in TE (Tris-EDTA) buffer (10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 8.0). The samples were incubated for 1 h at 37^0C. After lysis, 370 µl TE containing proteinase K (1 mg ml^{-1}) and 30 µl 10% SDS (w/v) were added and incubated for 1 h at 37^0C. Then, 100 µl NaCl (5 mol l^{-1}) and 80 µl CTAB / NaCl solution (10% cetyltrimethylammonium bromide, 0.7 mol l^{-1} NaCl) were added and incubated for 10 min at 65^0C. The solution was extracted twice with one volume of chloroform: isoamyl alcohol (24:1). DNA in the aqueous phase was precipitated by adding one volume of isopropanol. Following this, the precipitate was washed in 500 µl of 70% v / v ethanol. The pellet was dried and dissolved in 100 µl TE supplemented with 100µg ml^{1} RNase A and kept for 1 h at 37^0C. The sample volume was adjusted to 400 µl with TE. The solubilization of DNA was achieved by alternating heat shocks (10 min at 80^0C, and 20 min at 20^0C). The lysate was extracted with phenol: chloroform: isoamylalcohol (25: 24: 1) to remove impurities. Then, NaCl (0.5 mol l^{-1} of final concentration) and two volumes of 99% v / v ethanol were added to precipitate the DNA. The pellets were washed again in 500 µl 70% v / v ethanol. Finally, the DNA pellets were dried and resuspended in 50–200 µl TE, depending on the amount of the DNA pellet. The samples were stored at -20^0C until further use.

f) PCR amplification and identification of crystal protein gene:

PCR was performed in a 0.2 ml thin walled PCR tube. The PCR was performed in duplicates.

TABLE 2 : The master mix was prepared according to the following composition.

Components	Volume
Nuclease free water	32 µl
Template DNA	2.0 µl
Forward Primer	2.0 µl
Reverse Primer	2.0 µl
10X Reaction Buffer	10 µl
dNTP Mix (10 mM)	1 µl
Taq DNA polymerase (2.5U/µl)	1µl
Total volume	50 µl

The amplification was carried out in a Master cycler® Thermocycler (DNAmp Bhat Biotech).

TABLE 3 : PCR program for *Bacillus thuringiensis* Cry1Ac gene.

Step			
1	Initial denaturation	95°C	5 min
2	Denaturation	94°C	1 min
3	Annealing	51°C	1 min
4	Extension	72°C	1 min
5	Final extension	72°C	10 min

Repeat steps 2 to 4 for 35 cycles.

Molecular characterization was performed to identify the cry 1AC toxin-encoding genes through PCR by using specific primer for cry 1AC. PCR was performed in a 0.2 ml thin walled PCR tube. The PCR was performed in duplicates. The master mix was prepared according to the following composition. Nuclease free water 32µl, Template DNA 2.0µl, Forward Primer 2.0µl, Reverse Primer 2.0 µl , 10X Reaction Buffer 10 µl , dNTP Mix (10 mM) 1 µl , Taq DNA polymerase (2.5U/µl) 1µl , Total volume 50 µl . The amplification was carried out in a Master cycler® Thermocycler (DNAmp Bhat Biotech). PCR conditions were as the following: initial denaturation step for 5 min at 95^0C, followed by a 35 amplification cycles including repetition of denaturation at 94^0C for 1 min, annealing at 51 ^{0}C for 1 min and extension at 72^0C for 1 min. Final extension was carried out at 72^0C for 10 min. After electrophoresis of 10 µl of each PCR product on 1.0% actagarose- EtBr gel, DNA bands were visualized in a gel documentation system.(Morteza Salekjalali *et al.*, 2012).

RESULTS & DISCUSSION

The isolates used in this study were isolated from soil samples in Nanded district. Soil is the chief natural reservoir of Bt spores and is currently the favor substrate for the isolation of Bacillus species (El-kersh *et al*., 2016; Hossain *et al*., 1997; Meadows, 1993; Polanczyk andAlves, 2003; Silva *et al*., 2012; Soares-da-Silva *et al*., 2015). About 78 colonies showing the typical *Bacillus*-like characteristics were selected. These colonies possessed white to off white colour, slightly raised elevation and regular margins. (Mohammedi *et al*., 2006). The morphological characteristics of *Bacillus* can be seen in figure 1.a. These colonies were then isolated and sub-cultured into fresh plates. Since the genus *Bacillus* contains rod-shaped and Gram-positive verified bacteria, so Gram staining was performed to eliminate all other bacteria that do not have these two features. All rod-shaped, Gram positive bacteria, which visualized blue or violet under the light microscope (figure 2.a), were selected, and the remaining isolates that did not possess such description were discarded. Total of 35 colonies were selected and subjected to light microscopy to screen for the presence of endospore and parasporal body (Figure 2.b and 2.c). in order to distinguish *B. thuringiensis* from other *Bacillus* groups. Light microscopy was done after 90 hour to ensure the presence of parasporal bodies. The colonies

were microscopically inspected and those having visible parasporal inclusions were classified as *B. thuringiensis* (Bernhard *et al*., 1997), and selected for further characterization. In total 15 colonies were recognized as *B. thuringiensis* by using this method. All the 35 isolates were further stained with Coomassie Brilliant Blue and viewed under Light microscope. About 15 samples were selected and these isolates were confirmed as Bt as the presence of parasporal bodies was instant and noticeable evident by the presence of numerous dark-blue staining objects (Figures 2.c). The use of Coomassie Brilliant Blue stain relatively allowed a swift and efficient assessment of the 15 isolates. Biochemical studies of these 15 isolates indicated that the isolates had general biochemical characteristics like positive results for Gram's staining (Figure 1.a) , spore staining (Figure 2.b and 2.c), motility test, catalase test (Figure 3.d), Voges Proskauer test (Figure3.e), Asculin hydrolysis (Figure 3.f), Nitrate reduction (Figure 3.h), urease production (Figure 3.i), glucose fermentation (Figure 3.j), starch hydrolysis (Figure 3.l), growth at 5 % (Figure 3.m) and 10% NaCl (Figure3.n) and negative results for indole production (Figure 3.g) and mannitol fermentation test (Figure 3.k) with minor differences similar to those of *Bacillus thuringiensis* as has already reported. (Lecadet M M *et al*., 1999)

A technique such as the PCR is useful because of its specificity and ability to identify strains of bacteria within a very short time (Carozzi N B *et al.,* 1991). Primers selected for crylAc gene yielded amplicons that ranged ~ 238 bp (Figure 4, 5 and 6), were considered as positive. PCR amplification using cry1Ac gene specific primer with DNA from isolates as template suggested the presence of cry 1Ac gene containing strain of *Bacillus thuringiensis*. Out of 15 , isolates *Bacillus thuringiensis kn, Bacillus thuringiensis hd, Bacillus thuringiensis bl, Bacillus thuringiensis nd , Bacillus thuringiensis lh and Bacillus thuringiensis dm* and *Bacillus thuringiensis dg* showed the presence of cry1Ac amplicon of ranged ~238 bp, indicating the cry1Ac gene containing strains of *Bacillus thuringiensis* however for further confirmation of genus and species 16s gene sequencing require.

Biochemical Test:

TABLE 4: The morphological, physiological and biochemical characteristics of *Bacillus thuringiensis* strains.

Sr. No.	Character and /or reaction [1] of *Bacillus thuringiensis*															
	Test	B.t mh	B.t hm	B.t ad	B.t bk	B.t kn	B.t md	B.t um	B.t kd	B.t hd	B.t ng	B.t bl	B.t nd	B.t lh	B.t dm	B.t dg
	Microscopic Test															
1	Shape	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
2	Gram staining	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+	G^+
3	Endospore staining	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+
4	Coomassie Brilliant Blue Staining	C+	C+	C+	C+	C+	C+	C+	C+	C+	C+	C+	C+	C+	C+	C+
5	Motility Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Biochemical Test															
6	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Voges Proskauer Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Asculin Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	Indole Production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	Urease Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	Glucose Fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	Mannitol Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	Growth at NaCl 5 %	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	Growth at NaCl 10%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

G+ = Gram positive , S+ = Presence of spore , + = positive , - = negative

FIGURE 1. a) Pure Culture

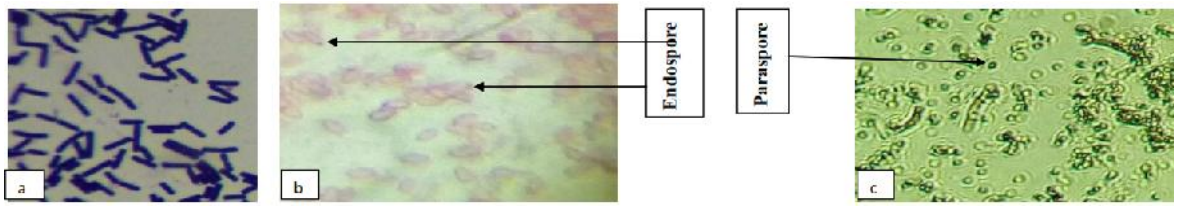

FIGURE 2. Microscopic Test : a) Gram's Staining , b) Endospore staining , c) Coomassiae Brilliant Blue staining

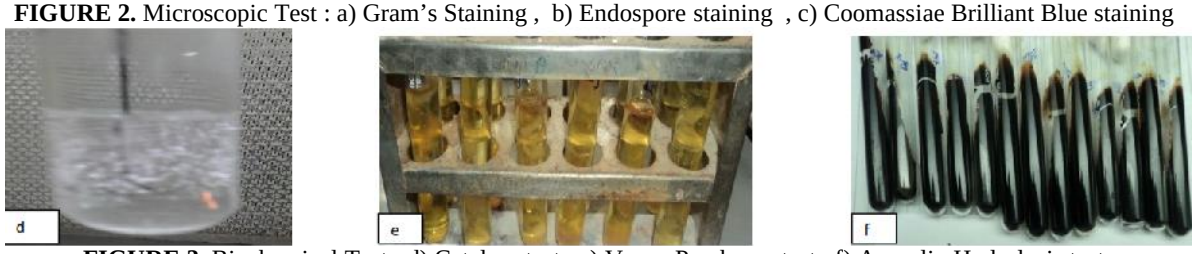

FIGURE 3. Biochemical Test : d) Catalase test, e) Voges Proskauer test, f) Aesculin Hydrolysis test

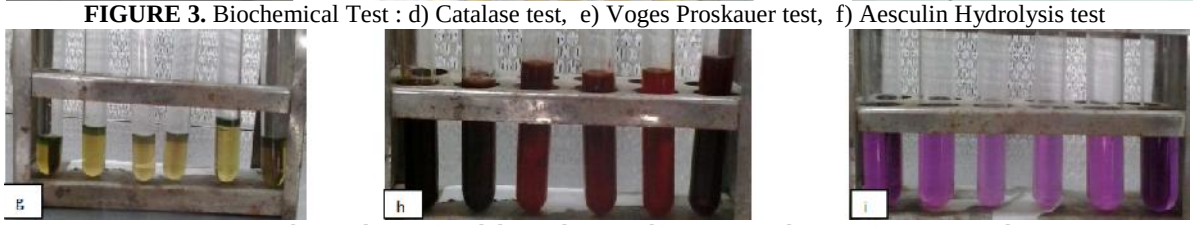

FIGURE 3. Biochemical test: g) Indole production, h) Nitrate Reduction, i) Urease production

FIGURE 3. Biochemical test: j) Glucose fermentation k) Mannitol fermentation l) Starch hydrolysis

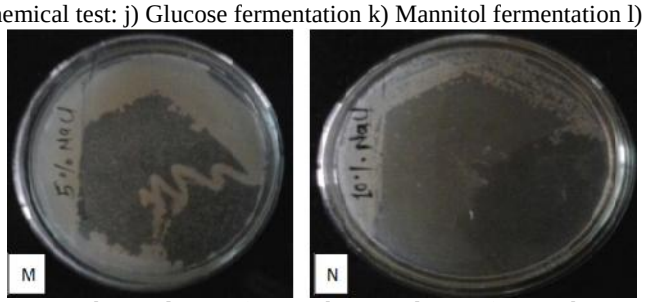

FIGURE 3. Biochemical test: m) Growth at NaCl 5% n) Growth at NaCl 10%

TABLE 5: PCR amplification profiles of cry1Ac-gene found in isolates

PCR amplification profiles of cry 1Ac-gene found in isolates	
Isolates	*Cry* 1Ac
B. thuringiensis mh	-
B. thuringiensis hm	-
B. thuringiensis ad	-
B. thuringiensis bk	-
B. thuringiensis kn	+
B. thuringiensis md	-
B. thuringiensis um	-
B. thuringiensis kd	-
B. thuringiensis hd	+
B. thuringiensis ng	-
B. thuringiensis bl	+
B. thuringiensis nd	+
B. thuringiensis lh	+
B. thuringiensis dm	+
B. thuringiensis dg	+

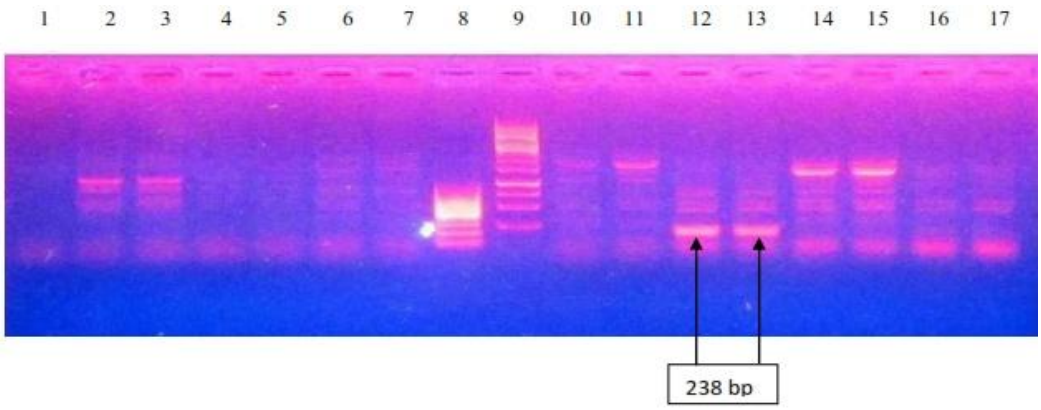

FIGURE 4 : Amplification of specific region of *B. thuringiensis* isolates crystal protein genes using crylAc primer. (a) Photograph of TAE-Agarose gel (1%) electrophoresis of nucleic acid amplification products of Cry IAc primers. Lane 1: Blank , Lane 2 and 3 : *B. thuringiensis* mh , Lane 4 and 5 : *B. thuringiensis* hm, Lane 6 and 7: *B. thuringiensis* ad, Lane 8 : 100bp ladder , Lane 9 : 1 kb ladder , Lane 10 and 11 : *B. thuringiensis* bk , Lane 12 and 13: *B. thuringiensis* kn , Lane 14 and 15 : *B. thuringiensis* md , Lane 16 and 17: *B. thuringiensis* um.

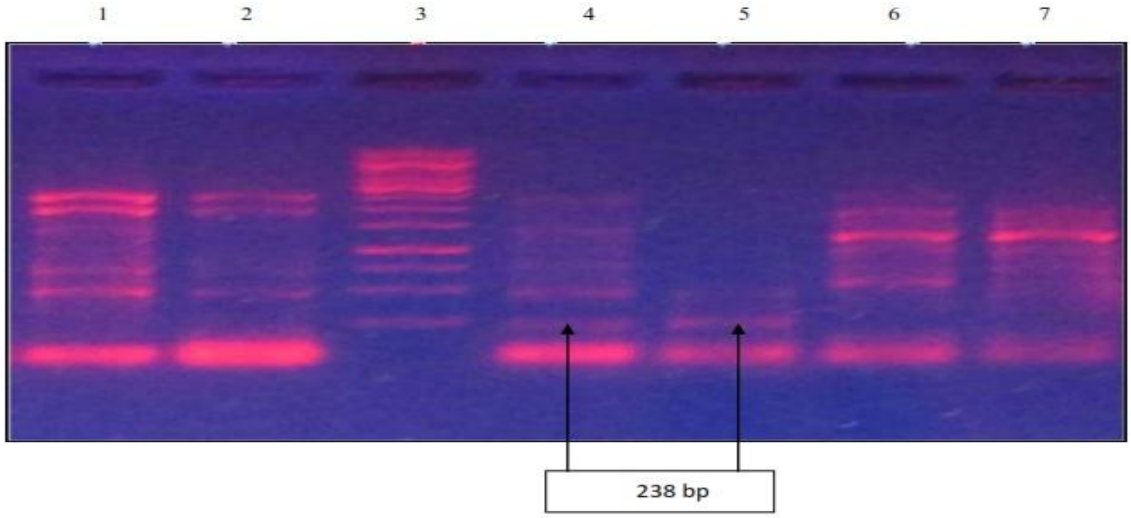

FIGURE 5 : Amplification of specific region of *B. thuringiensis* isolates crystal protein genes using crylAc primer. (a) Photograph of TAE-Agarose gel (1%) electrophoresis of nucleic acid amplification products of Cry IAc primers.Lane 1 and 2 : *B. thuringiensis kd* , Lane 3 : 1 kb ladder , Lane 4 and 5 *: B. thuringiensis* hd , Lane 6 and 7 : *B. thuringiensis ng* .

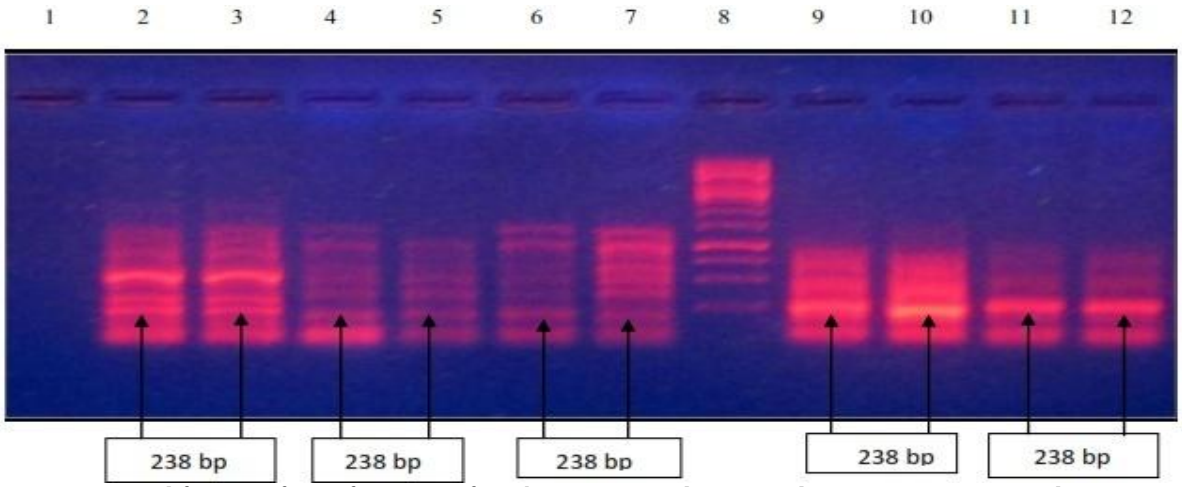

FIGURE 6: Amplification of specific region of *B. thuringiensis* isolates crystal protein genes using crylAc primer. (a) Photograph of TAE-Agarose gel (1%) electrophoresis of nucleic acid amplification products of Cry IAc primers.Lane 1: Blank, Lane 1 : Blank , Lane 2 and 3 **:** *B. thuringiensis bl* , Lane 4 and 5 : *B. thuringiensis nd* , Lane 6 and 7 : *B. thuringiensis lh* , Lane 8 : 1 kb ladder , Lane 9 and 10 : *B. thuringiensis dm* , Lane 11 and 12 : *B. thuringiensis dg.*

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