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Spectrum of Antibiotic Resistance in *cry4* Positive Local Isolates of *Bacillus thuringiensis*

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Abstract.- The sensitivity of thirteen different antibiotics was tested against *cry4* positive isolates of *Bacillus thuringiensis (Bt)*. All *cry4* positive *Bt* isolates and positive controls of *cry4* gene IPS78 and HD500 were found resistant to ampicillin, amoxicillin and bacitracin and were sensitive to chloramphenicol, erythromycin, kanamycin, neomycin, nalidixic acid, oxytetracyclin, polymixin, streptomycin, tetracyclin and vancomycin. To identify the plasmid borne antibiotic resistance gene, the competent cells of *E. coli* (DH5ά) were transformed with plasmid DNA isolated from the *Bt* cultures. The transformed *E. coli* showed resistance to ampicillin and amoxicillin. It was found that both ampicillin and amoxicillin were plasmid borne and present on 1.5Kb and 1.0Kb plasmids, respectively, while no antibiotic resistance genes were found on 23Kb mega plasmid.

Keywords: Antibiotic sensitivity, plasmid borne antibiotic genes, non-chromosomal antibiotic resistance.

INTRODUCTION

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. It can develop naturally via natural selection through random mutation or it can also be introduced artificially into a microorganism. Resistance to antibiotic can be conferred by chromosomal or mobile genetic element (plasmids) (Jain *et al*., 2009). Most of the known resistance determinants have been discovered in clinical and veterinary bacterial isolates, whereas other environmental reservoirs of antibiotic resistance are not well characterized (Nwosu, 2001; Séveno *et al*., 2002). Cultured microorganisms have been the source of almost all characterized antibiotic resistance genes; therefore, most of the previous studies have ignored the potential reservoir of antibiotic resistance genes in uncultured bacteria. The majority of bacteria are not readily cultured on standard laboratory media (Ward *et al*., 1990; Amann *et al*., 1995; Hugenholtz *et al*., 1998), and the diversity of the uncultured majority is vast (Torsvik *et al*., 1998; Whitman *et al*., 1998; Béjà *et al*., 2002).

The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are (1) drug inactivation or modification: *e.g.* enzymatic deactivation of Penicillin G in some penicillin-resistant bacteria through the production of β-lactamases. (2) alteration of target site: *e.g*. alteration of PBP - the binding target site of penicillins - in MRSA and other penicillin-resistant bacteria. (3) alteration of metabolic pathway: *e.g*. some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA) - an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides. Instead, like mammalian cells, they turn to utilizing preformed folic acid. (4) Reduced drug accumulation: by decreasing drug permeability and/or increasing active efflux on the cell surface. Development of antibiotic resistance is not only confined to the pathogens or disease causing agents but it is also developing very rapidly in few apparently free living soil bacteria for example *Bacillus thuringiensis (Bt).*

In nature plasmids increase bacterial genetic diversity and promote bacterial adaptation by horizontal gene spread (Bergstorm *et al*., 2000; Gogarten *et al*., 2002; Levin and Bergstrom, 2000). Plasmids can be of small and large size. Small size may be of 10 bp and mega plasmids can have a size of 214 to 275 Kbs. The first plasmid was isolated and characterized in 1950s associated with newly acquired antibiotic resistance. Since then plasmids have been studied for both genotypic and phenotypic properties and heavy metal resistance,

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degradation of xenobiotic compound and toxic heavy metal resistance, bacteriocin production, resistance to radiation and increased mutation frequency. These are also called Accessory functions. These facilitate rapid adaptation to new transient environmental selection pressure, are typically located on mobile genetic elements such as genomic islands, conjugative transposons, mobilized transposons and plasmids as well. Evidence from bacterial sequencing projects clearly indicates that bacteria adapt and genomes evolved by rearranging existing DNA and by acquiring new sequences (Bergstorm *et al*., 2000; Gogarten *et al*., 2002; Levin and Bergstrom, 2000).

Belykh *et al*. (1982) reported that *Bt* strains forming colonies of the S and R morphology were found to be susceptible to streptomycin, chloramphenicol, rifampicin, neomycin, lincomycin, monomycin, kanamycin, and resistant to ampicillin and polymyxin. The S strains were shown to be susceptible to tetracycline (Tet^s), whereas the R strains were either susceptible (Tet^s) or resistant (Tet^r). No significant differences were found in the plasmid composition of the Tet^s and Tet^r strains, and no correlation was established between the presence of plasmids and the resistance to tetracycline.

The present study was undertaken with the idea of identifying and characterizing the antibiotic resistances and sensitivity of local isolates of *Bt* against different antibiotics and to screen the location of antibiotic genes either present on chromosome or plasmid DNA.

MATERIALS AND METHODS

Bacterial strains

Twenty two local isolates of *Bt* (SBS-*Bt* 11, 13, 15, 18, 19, 23, 26, 29, 32, 26, 29, 32, 34, 35, 37, 38, 40-48) which were previously characterized for the toxicity against mosquito larvae *Anopheles stephensi* and the presence of *cry4* gene (Bukhari, 2007) were used. The reference strains HD500 and IPS78 of *cry4* gene kindly provided by Prof Dr. Daniel R. Zeigler, Director, Bacillus Genetic Stock Centre,(BGSC) Columbus, Ohio , USA were used as positive controls. *E. coli* DH5α competent cells were used for transformation studies.

Antibiotics

Thirteen antibiotics *i.e.* ampicillin, amoxicillin, bacitracin, chloramphenicol, erythromycin, kanamycin, neomycin, nalidixic acid, oxytetracyclin, polymixin, streptomycine, tetracycline, vancomycin, were used in this research work and each antibiotic disc had a 30µg of antibiotic. Discs were made by Bioanalyse.

Determination of antibiotic sensitivity and resistance of Bt *isolates*

For determination of antibiotic sensitivity and resistance of local *Bt* isolates the 24 hours old isolated *Bt* colony was inoculated in 5ml of LB broth and incubated at 37°C in the shaking incubator for 18 hours. This inoculum was spread on LB agar plates. The antibiotic discs (30µg) were placed on the solid medium and incubated at 37°C for 24 hours. The antibiotic sensitivity against bacteria was checked by measuring zone of inhibition with a millimeter scale.

Isolation of plasmid DNA

Plasmid DNA was isolated according to Jenson *et al*. (1995). *Bt* was grown overnight at 30°C in 2ml LB broth. The culture (2ml) was pelleted at 12000 rpm (5000 x g) for 10 min and then resuspended in 100 µl of E - Buffer (15%w/v sucrose, 40mM Tris-HCl, 2 mM EDTA, pH 7.9) by pipetting up and down. Two hundred µl of lysing solution (3% SDS, 50 mM Tris-HCl, pH 12.5) was added. The lysate was heat shocked at 60°C for 30 min, and then 5 units of proteinase K were added and mixed thoroughly by inverting the tube 20 times. The mixture was incubated at 37°C for 90 min. One ml of phenol-chloroform-isoamyl alcohol (25:24:1) was added, vortexed, and then centrifuged at 5000 x g for 15 min. The supernatant was analyzed by electrophoresis on a horizontal 0.5% agarose gel.

Plasmid DNA of *Bt* was then isolated from the low melting point agarose gels (Sambrook *et al.,* 1989), using Fermentas gene clean kit (#K0513). The required fragment was cut out of the gel, weighed in an eppendorf tube and then 3 volumes of NaI was added and kept at 55°C for 5 min. Then

10µl of silica milk was added and incubated again at 55°C for 5 min. The mixture was centrifuged at 10,000rpm (3500 x g) for 20 seconds. The supernatant was discarded and the pellet was washed with 500µl of wash buffer three times. The pellet was air dried for 10 minutes and dissolved in 30µl of autoclaved distilled water. The supernatant was transferred to new eppendorf tubes after centrifugation for 10 min at 10,000 rpm (3500 x g) and later used for transformation of competent cells of *E. coli* DH5α.

Transformation

The competent cells were prepared by incubating 250 ml LB broth inoculated with 2ml of *E. coli* DH5α culture at 37°C for 2 h or until the OD of culture reached 0.2-0.3. The culture was then placed on ice and then shifted to a precooled sterile Oakridge centrifuged tube. Centrifugation was done at 6000rpm (4500 x g) for 5 minutes at 4°C. The pelleted cells were gently resuspended in 50mM ice-cold $CaCl_2$, left on ice for 40 min, and then centrifuged in precooled rotor at 6000rpm (4500 x g) for 5 min at 4°C. The cells in pellet were gently resuspended in 500 µl of 50mM ice cold $CaCl_2$, and left on ice until used for transformation.

For transformation of DH5α cells, 25 µl (10 ng/µl) of plasmid DNA was added in 200µl of competent cells (DH5α) in sterile precooled microcentrifuge tube, mixed gently and left on ice for 40 minutes. The cells were quickly transferred to 42°C for 2 minutes and then returned to ice for 5 minutes. One ml of LB medium was added in tube and was incubated at 37°C for 2 hours without shaking. The transformed cells (200 µl) were spread on dried LB agar plates containing respective antibiotics (100µg/ml). The plates were then incubated at 37°C for 24 hours to determine the antibiotic activity. Plasmid DNA of transformants was isolated and visualized on 0.8% agarose gel.

RESULTS

Antibiotic resistance of Bt *isolates*

All *cry4* positive *Bt* isolates and positive controls of *cry*4 gene IPS78 and HD500 were found resistant to chloramphenicol, ampicillin, amoxicillin and bacitracin except for *Bt* 32, 37 and 47 which were sensitive to amoxicillin, and all were sensitive to chloramphenicol. All *Bt* isolates were sensitive to erythromycin, kanamycin, neomycin, nalidixic acid, oxytetracyclin, polymixin, streptomycin, tetracyclin and vancomycin except for SBS-*Bt* 11 which was resistant to nalidixic acid, oxytetracyclin, polymixin and vancomycin; SBS-*Bt* 18 which was resistant to nalidixic acid; SBS-*Bt* 23 which was resistant to nalidixic acid, polymixin and tetracycline; SBS-*Bt* 26 which was resistant to erytheromycin, nalidixic acid, polymixin and tetracycline; SBS-*Bt* 29 and 32 were resistant to polymixin and streptomycin; SBS-*Bt* 34 was resistant to oxytetracyclin; SBS-*Bt* 37 was resistant to neomycin, polymixin and streptomycin; SBS-*Bt* 41 was resistant to erytheromycin, polymixin and streptomycin; SBS-*Bt* 43 showed resistance to kanamycin and polymixin; SBS-*Bt* 44 and 48 showed resistance to erytheromycin; SBS-*Bt* 46 and HD500 showed resistance to polymixin, whereas SBS-*Bt* 47 showed resistance to oxytetracyclin and polymixin. SBS-*Bt* 11 and 27 were resistant to six antibiotics, four isolates were resistant to five antibiotics, eight isolates were resistant to four antibiotics, while 9 isolates were resistant to three antibiotics (Table I).

Plasmid DNA of Bt

After primary screening of all the *Bt* isolates for antibiotic resistance secondary screening was carried out to locate the antibiotic resistance genes. The three plasmid DNA bands 23Kb, 1.5 Kb and 1.0 Kb plasmid DNA were isolated in all the *Bt* isolates and IPS 78 positive control (Fig. 1).

Plasmid borne antibiotic resistance gene

E. coli (DH5α) were transformed with the isolated plasmids *viz.* 1kb, 1.5 kb and 23 kb plasmid and grown on media containing different antibiotics. It was concluded that ampicillin and amoxicillin resistant genes were present on 1.5Kb and 1.0Kb plasmids, respectively while no antibiotic resistance genes were found on mega plasmid (23Kb). Figure 2 show the transformants of 1.5Kb and 1.0Kb plasmids in *E. coli* (DH5α).

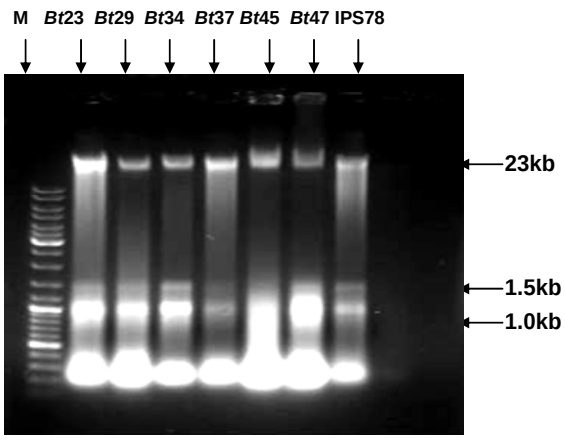
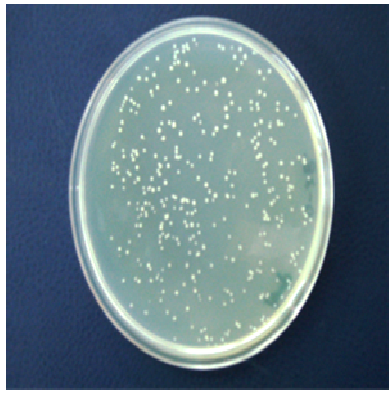


Fig.1. Total plasmid DNA profile of local *Bt* isolates and IPS 78, the positive control.



A

B

Fig. 2. Transformation of *E. coli* DH5α with 1.5kb and 1.0kb plasmid DNA of *Bt* in the medium containing Ampicillin (A) and Amoxicilin (B) plate.

DISCUSSION

The antibacterial activity of certain antibiotics has been attributed to two kinds of mechanisms (i) Cell wall peptidoglycan production is inhibited as a result of the formation of a ternary complex constituted by particular antibiotic a divalent cation (Stone and Strominger, 1971). β-Lactam antibiotics are bactericidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. (ii) The other mechanism is alteration of membrane permeability (Snoke and Cornell, 1965; Storm and Strominger, 1974). The beta-lactam antibiotics bind to and inhibit enzymes needed for the synthesis of the peptidoglycan wall. Ampicillin does not induce the synthesis of proteins in susceptible (*Micrococcus lysodeikticus*) or outer membrane bearing resistant bacteria (*Escherichia coli*) (Snoke and Cornell, 1965; Storm and Strominger, 1974).

Plasmid DNA invades a cell, replicates at the cost of that cell, and stays with the cell ever after. The number of plasmids in *Bt* are variable from one to more than six (Carlson and Kolst, 1993; Carlson *et al.,* 1994). In the present *Bt* isolates three plasmid *viz*., 23kb, 1.5kb and 1.0 kb were detected. Rosado and Seldin (1993) isolated first linear plasmid from the genus *Bacillus* with molecular size estimated between 16-17 kb. Many of pathogenic strains of *B. sphaericus* contain one or several large plasmids. It has been reported that most of the genes coding for endotoxins are present on plasmids (Carlson and Kolsto, 1993; Mahillon *et al*., 1994). The *Bt* strains have different patterns of plasmids and show different toxicities against insects (Li and Li, 1994; Ren *et al.,* 1995). A 127kb mega plasmid is necessary for *Bt* subsp *israelensis* for the toxicity. The *cry* genes are usually located on large plasmids (50kb or larger) (Kronstad *et al.*, 1983; Carlton and Gonzales, 1985) although chromosomal *cry* genes have also been reported in some *Bt* strains (Klier *et al*., 1982; Carlson and Kolsto, 1993). Lopez- Meza and Ibarra (1996) reported three plasmids of *Bt* strain LBIT-113, the pattern of which was considerably different from those of *Bt* subsp. *kurstaki*, *tenebrionis* and *israelensis*. Thomas *et al*. (2001) reported a number of insecticidal protein toxins encoded on a single 72-Mda plasmid in *Bt* subsp *israelensis*. In addition to the plasmids

Table I.- Behaviour of local *Bt* isolates (SBS *Bt* 11-48) and positive controls (IPS78, HD 500) of *cry4* gene against different antibiotics.

Isolates	Amp	Am	Baci	Chl	Ery	Kan	Nal	Neo	Oxy	P.mix	Strep	Tet	Van
SBS *Bt*-11	R	R	R	S	S	S	R	S	R	R	S	S	R
SBS *Bt*-13	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt*-15	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt*-18	R	R	R	S	S	S	R	S	S	S	S	S	S
SBS *Bt*-19	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt* -23	R	R	R	S	S	S	R	S	S	R	S	S	S
SBS *Bt* -26	R	R	R	S	R	S	R	S	S	R	S	R	S
SBS *Bt*- 29	R	R	R	S	S	S	S	S	S	R	R	S	S
SBS *Bt*- 32	R	S	R	S	S	S	S	S	S	R	R	S	S
SBS *Bt*- 34	R	R	R	S	S	S	S	S	R	S	S	S	S
SBS *Bt* -35	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt*- 37	R	S	R	S	S	S	S	R	S	R	R	S	S
SBS *Bt* -38	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt*- 40	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt*- 41	R	R	R	S	R	S	S	S	S	R	R	S	S
SBS *Bt* -42	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt* -43	R	R	R	S	S	R	S	S	S	R	S	S	S
SBS *Bt* -44	R	R	R	S	R	S	S	S	S	S	S	S	S
SBS *Bt* -45	R	R	R	S	S	S	S	S	S	S	S	S	S
SBS *Bt* -46	R	R	R	S	S	S	S	S	S	R	S	S	S
SBS *Bt* -47	R	S	R	S	S	S	S	S	R	R	S	S	S
SBS *Bt* -48	R	R	R	S	R	S	S	S	S	S	S	S	S
IPS 78	R	R	R	S	S	S	S	S	S	S	S	S	S
HD500	R	R	R	S	S	S	S	S	S	R	S	S	S

Abbreviations used; Am, Amoxicillin; Amp, Ampicillin; Baci, Bacitracin; Chl, Chloramphenicol; Ery, Erythromycin; Kan, Kanamycin; Nal, Nalidixic acid, Neo, Neomycin; Oxy, Oxytetracyclin; P.mix, Polymixin; Strep , Streptomycin; Tet, Tetracyclin;Van, Vancomycin.

carrying insecticidal toxin genes many other plasmids such as pX011, pX013, pX014, pX015, pX016 ns pAW63, have been detected in *Bt* and these plasmids have no known function apart from their conjugative ability (Battisti *et al*., 1985; Reddy *et al*., 1987; Wilcks *et al*., 1998).

Plasmid DNA of *cry4* positive local *Bt* strains was isolated to determine the occurrence of plasmid borne antibiotic genes. The three plasmid DNA bands 23Kb, 1.5 Kb and 1.0 Kb plasmid DNA were isolated in all the *Bt* isolates and IPS 78 positive control. To determine the plasmid borne antibiotic resistant gene the *Bt* plasmids were transformed in *E. coli* (DH5α) It was found that ampicillin and amoxicillin resistant genes were present on 1.5Kb and 1.0Kb plasmids, respectively while no antibiotic resistance genes were found on mega plasmid (23Kb). Plasmids contain genes that the cell can benefit from. Instead of being a neutral invader, the plasmid now becomes a profitable extra genetic moiety. These can be antibiotic resistance genes, or virulence genes, but antibiotic resistance genes are not always encoded on plasmids: there are many mechanisms in which antibiotic are encoded on the chromosome. Typically, a plasmid contains an antibiotic resistance gene, sometimes more than one. The gene, in the form of DNA, must be transcribed into messenger RNA, and then translated into the protein that counteracts the effect of the antibiotic. Plasmid-encoded RNA could exist in the absence of the plasmid if the plasmid was transcribed sufficiently prior to the action of the DNase. However, messenger RNA has a relatively short half-life (depending on the gene and developmental state), which itself being degraded by RNase enzymes, and so the amount of time that plasmid mRNA is outlive the plasmid itself is too short. In the absence of the antibiotic resistance gene encoded on the plasmid, a bacterial cell could not survive longer after the destruction of the plasmid.

To conclude, bacteria under antibiotic selective pressure have the ability to acquire and exchange antibiotic resistance genes, developing new proteins and loosing some other proteins

making them unsusceptible to certain antibiotic treatments. The development of multiple antibiotic resistances among bacterial population is probably through horizontal gene transfer.

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