

studies further suggest that there existed a low energy depositional environment at this level of sedimentation.

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Multiplex PCR in diagnosis and characterization of bovine viral diarrhoea virus isolates from India

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Bovine viral diarrhoea (BVD) is one of the most economically important infectious diseases in cattle world-wide and the causative agent, BVD Virus (BVDV) is a pestivirus in the family Flaviviridae. Though prevalence of BVDV antibodies in Indian cattle has been established by serology, information on the use of multiplex PCR for diagnosis and characterization of BVDV isolates at genomic level is lacking. In this study, we report development of a multiplex polymerase chain reaction (PCR) assay using primers of 5' untranslated region (UTR) and structural glycoprotein (E1–E2) region, which generated two different amplicons (288 bp and 784 bp) in a single tube when 13 Indian BVDV isolates were tested. Both the amplicons were found specific when restriction enzyme analysis and subsequent nucleotide sequencing of three selected isolates representing different geographic areas of India were performed. The sequence analysis of both the regions grouped them into BVDV 1b genotype. The study demonstrated that multiplex PCR can be used for identification and subsequent genotyping of BVDV isolates.

PESTIVIRUSES are important pathogens of cattle, sheep and pigs and cause significant economic losses throughout the world¹. The genus pestivirus in the family Flaviviridae contains Bovine Viral Diarrhoea Virus (BVDV), Classical Swine Fever Virus (CSFV) and Border Disease Virus

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(BDV)². BVDV has been subdivided into two genotypes, BVDV1 and BVDV2 on the basis of antigenic variation and sequence differences in the 5' UTR³. The economic impact of BVDV infection in dairy and beef cattle is primarily due to decreased reproductive performance besides causing diarrhoea, congenital defects, respiratory disease, mucosal disease, haemorrhagic syndrome and recently reported dermatitis^{4,5}. Persistent life-long infections or carrier status can occur when the foetus is infected in the first trimester of gestation. Because carrier animals are constantly viraemic and continuously shed and maintain the virus, their identification and removal from the herd is essential for control of BVD⁶.

BVDV contains a single-stranded, non-polyadenylated, positive-sense RNA genome of approximately 12.5 kb in length flanked at either end by untranslated regions. The genomic RNA contains one large open reading frame translating a polypeptide, which is cleaved into four structural and eight non-structural proteins by viral and cellular proteases⁷. On the basis of growth characteristics in cell culture, the naturally occurring BVDVs are divided into cytopathic (cp) and non-cytopathic (ncp) biotypes and ncp strains are preponderant in nature⁸.

Though prevalence of BVDV antibodies in Indian cattle showing reproductive and breeding problems has been demonstrated earlier⁹⁻¹¹, we reported¹² isolation and genotyping of BVDV in India. The reverse transcription polymerase chain reaction (RT-PCR) method using primers from highly conserved 5' UTR, has been widely in use for diagnosis and genotyping of BVDV^{3,13}. Multiplex PCR is the simultaneous amplification of multiple products by the use of more than one pair of primers in a single tube and OIE (Office International des Epizooties) also recommends its use in diagnosis, genome amplification and typing of BVDV¹⁴. A nested multiplex PCR was used to detect and type BVDV using primers from *NS5B* gene¹⁵. However, multiplex PCR using primers from two different regions of BVDV has not been tried. Moreover, for detection of BVDV antigen in infected cells we need to import antigen capture ELISA kit and the immunofluorescence test is time-consuming besides being cumbersome. In this study we report the development of a multiplex PCR using primers from conserved 5' UTR region and E1-E2 region responsible for eliciting virus neutralizing antibodies and its usefulness in genetic characterization of BVDV isolates.

Thirteen BVDV isolates originating from cattle of eastern, western and northern parts of India and propagated in bovine turbinate (BT) cell line using medium supplemented with 10% horse serum were used in this study. The cell line and serum were free from contamination with pestiviruses as tested by RT-PCR. All the isolates were of ncp biotype and BVDV antigen was demonstrated by immunofluorescence¹⁶ using an anti NS3 Mab and antimouse FITC conjugate.

The viral RNA was extracted from the frozen and thawed infected BT cell culture supernatant at second passage using a commercially available kit (RNeasy mini kit, Qiagen)

and purified RNA after DNaseI treatment was eluted with 50 µl of nuclease free water. Two sets of oligonucleotide primers were used in the multiplex PCR. The first set¹⁷ consisted of pan pestivirus-specific 5' UTR primers 324 and 326 corresponding to nucleotides 108-128 and 275-395 of NADL strain (a reference strain of BVDV). The second set consisted of a sense primer¹⁸ (C-terminus of *E1* gene) corresponding to nucleotides 2256-2275 and antisense primer¹⁹ (N-terminus of *E2* gene) corresponding to nucleotides 3021-3040 of NADL strain. The first strand cDNA synthesis was performed in a total reaction volume of 20 µl containing 100 ng total RNA, 1X first strand buffer (25 mM Tris-HCl pH 8.3, 72 mM KCl, 3 mM MgCl₂, Gibco BRL), random hexamer primer 0.5 µg, 10 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), RNAase inhibitor 20U, superscript II RT (MMLV) 200 U, 0.1 M DTT and Rnase H 30 U. The cDNA synthesis was carried out at 42°C for 50 min followed by an inactivation step at 70°C for 15 min.

The PCR reaction in a total volume of 25 µl containing 1X multiplex PCR master mix (dNTP mix, 3 mM MgCl₂, hot star Taq DNA polymerase, Qiagen), 2.5 µl of cDNA and 10 pmol each of four primers (standardized by titration) was subjected to amplification in a DNA thermal cycler (Hybaid) with the following cycling parameters: 95°C for 15 min (1 cycle), 94°C for 30 s; 56°C for 90 s; 72°C for 60 s (35 cycles) and 72°C for 10 min (1 cycle). The amplified products were electrophoresed on 1% agarose gel using TAE buffer and ethidium bromide-stained DNA bands were visualized using a UV transilluminator. The amplicons of desired size (288 bp for 5' UTR region and 784 bp for E1-E2 region) were obtained (Figure 1) from all the 13 isolates tested and were well discernible. No amplification was observed with uninfected BT cells. DNA products of three isolates, one each from eastern

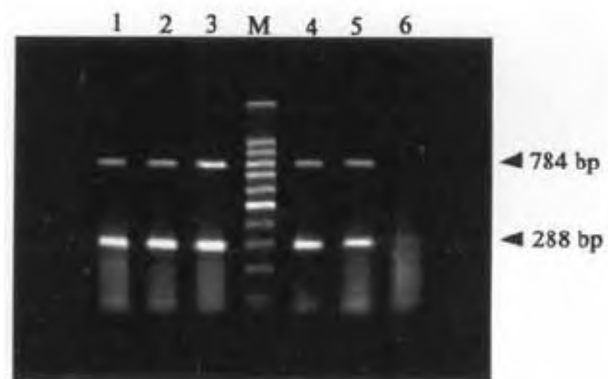


Figure 1. Agarose gel electrophoresis of multiplex PCR-generated amplicons (288 and 784 bp) from BVDV isolates using primers from 5' UTR and E1-E2 region. Lane 1, Isolate Ind S1449, lane 2, Isolate Ind S-1456; lane 3, Isolate Ind S1456, lane M, 100 bp DNA step ladder (Promega); lane 4, Isolate Ind S-1222; lane 5, Isolate Ind S1181, lane 6, Uninfected bovine turbinate cells.

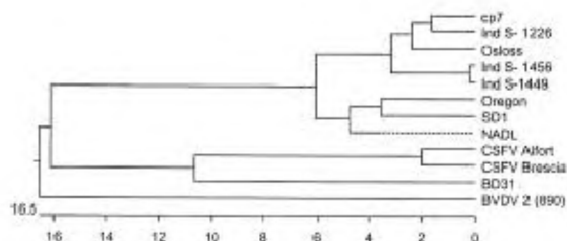


Figure 2. Dendrogram showing relationship of Indian BVDV isolates with other pestivirus isolates in 5' UTR. Sequences were aligned using CLUSTAL method of DNASTAR and phylogenetic tree was obtained by Mega align software package.

(Ind S-1449), western (Ind S-1226) and northern India (Ind S-1456), generated by multiplex PCR were used for further characterization to determine their specificity. The amplicons were extracted from gel, purified and subjected to restriction enzyme analysis. A single *Pst*I site was found in 5' UTR amplicons of the three isolates, which allowed us to classify them as BVDV 1 on the basis of the criteria followed by Harpin and co-workers²⁰. The presence of one *Hinf*I site, two *Dde*I sites and absence of *Msp*I and *Eco*R1 sites in E1–E2 amplicons of three isolates indicated that the amplicons were specific and that the isolates may belong to Osloss-like group (1b) of BVDV.

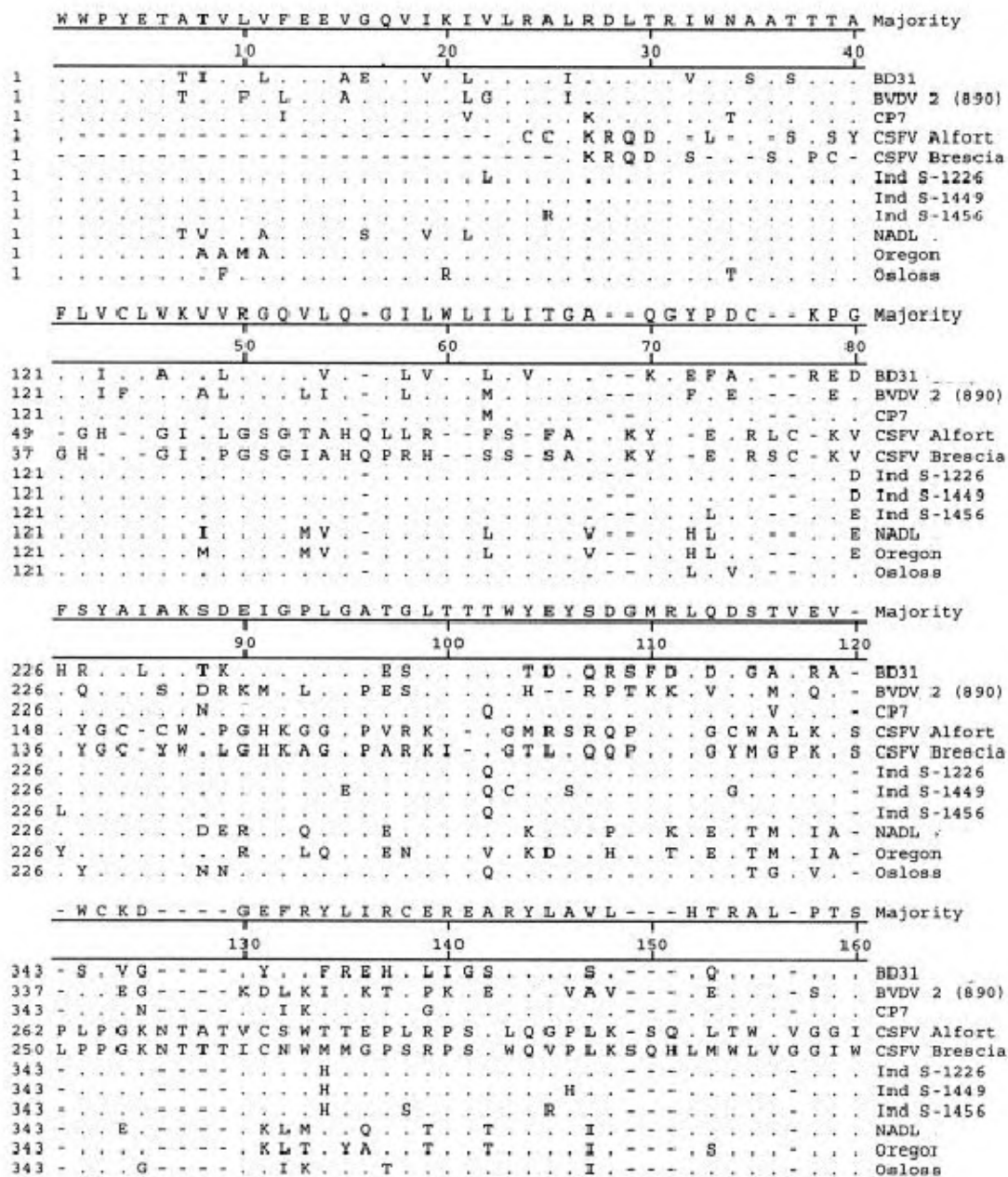


Figure 3. (Continued)

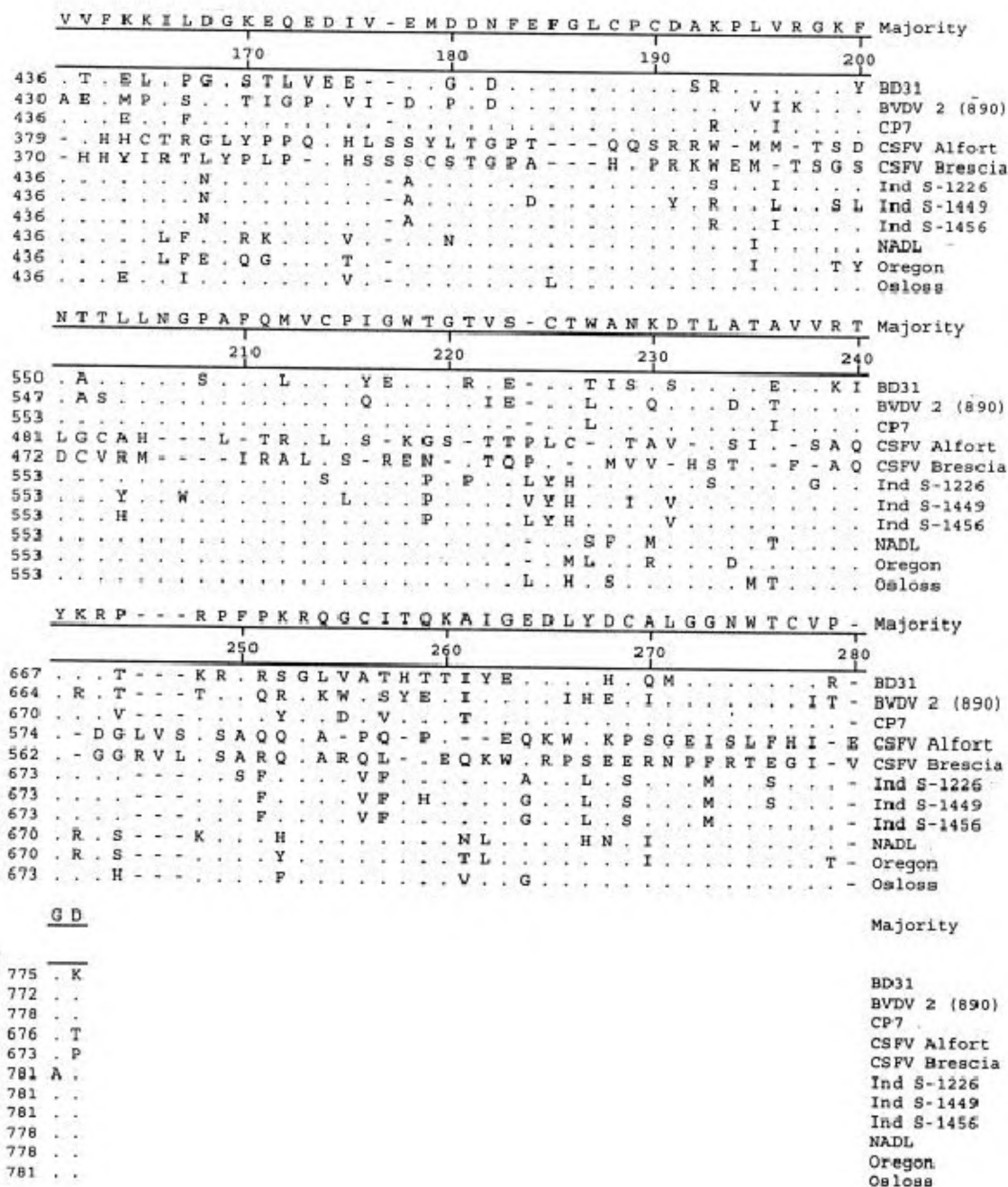


Figure 3. Alignment of deduced amino acid sequences of Indian BVDV isolates and reference pestiviruses in E1-E2 region. Sequences were aligned using CLUSTAL method with PAM250 residue weight table. Residues identical to the consensus are indicated by dots.

The purified 5' UTR and E1-E2 amplicons of three isolates generated by multiplex PCR were cloned into pGEMT Easy vector (Promega), and three recombinant plasmids checked for the presence of insert by *Eco*R1 restriction digestion, from each ligation reaction were sequenced. Nucleotide sequences were determined by using f mol cycle sequencing kit along with γ -³³P labelled M 13 forward

primer by electrophoresing through 6% polyacrylamide gel using a SQ3 sequencer (Hoefer). Individual nucleotide sequences were assembled and proof-read using the Edit Seq program of DNASTAR. The sequences determined for the amplicons generated from three isolates were analysed using Mega Align program (CLUSTAL method) and phylogenetic trees were constructed. Additional sequen-

ces of reference BVDV and other pestiviruses were obtained from NCBI database (NADL-M31182, Osloss-M96687, CP7-U63479, Oregon- AF091605, SD1-M96751, CSFV Alfort-J04358, CSFV Brescia-M31768, BD31-U70263, BVDV 2 (890)-U18059). The GenBank accession numbers of the three Indian 5' UTR sequences are AY273155, AY273156 and AY273157 and of the three E1-E2 sequences are AY707084, AY707085 and AY707086.

The 5' UTR amplicons generated by multiplex PCR were found specific by determination of nucleotide sequences. Highly conserved 5' UTR region makes it an ideal target for PCR-based diagnosis from a wide range of hosts and is frequently used in studying differences between and within pestivirus genotypes^{3,21,22}. The highest per cent identity of 5' UTR sequences of three Indian isolates was observed (89.5–93.0%) with CP7 strain of BVDV. The highest nucleotide identity was observed between Ind S-1449 (originated from Orissa) and Ind S-1456 (originated from Uttar Pradesh). The phylogenetic tree analysis (Figure 2) showed that the three Indian isolates belong to BVDV 1b. Based on the phylogenetic analysis, BVDV 1 genotype is divided broadly into two subgroups, namely 1a (NADL-like) and 1b (Osloss-like), although it has been separated into eleven genetic groups recently^{21,23}.

The nucleotide sequences also showed the specificity of E1-E2 DNA products produced by multiplex PCR. The nucleotide sequences encompassing C-terminus of E1 and N-terminus of E2 were targetted for amplification as *E1* and *E2* genes encode viral structural glycoproteins, and phylogenetic analysis in this region is more useful for identification of sub genotypes. Furthermore, N-terminus of *E2* (*gp 53*) gene has been shown to be associated with the production of neutralizing antibodies²⁴ and is the most variable region in the viral genome²⁵. The deduced amino acid sequence analysis of E1-E2 region of three Indian BVDV isolates showed (Figure 3) high degree of genetic variability like other reference pestiviruses consistent with earlier findings^{26,27}. The presence of hydrophobic amino acids followed by Von Heine's consensus in the *E1-E2* gene junction of Indian isolates (⁶³LITGAQG-YP/LDCKPD/E⁸⁰) is in agreement with the findings of earlier workers^{28,29}, which is required for proteolytic processing to generate E2 glycoprotein. The maximum per cent nucleo-

tide identity (84.3–86.2%) and amino acid identity (83.5%) of Indian isolates was observed with CP7 strain. The phylogenetic tree analysis (Figure 4) grouped them again into BVDV 1b, and Indian isolates were more similar to CP7 strain. The phylogenetic analysis of several genetic regions has already been found useful for genetic characterization of field BVDV isolates³⁰. This study confirmed the prevalence of BVDV 1b genotype in Indian cattle. BVDV 1b isolates have been reported in many countries of Europe besides USA and Australia. Since India had trade links with these countries for many years, the infection might have been introduced through import of animals from these countries.

The multiplex PCR with two useful genetic regions developed in this study was successfully used in diagnosis and genetic characterization of BVDV isolates. The ability to generate two specific amplicons simultaneously was also useful in defining the isolates at genotype level, which has implications for epidemiology and control of BVDV infections. We also envisage studying the usefulness of the multiplex PCR in diagnosis of BVD in clinical samples, specifically because antibodies may interfere with BVDV detection by virus isolation and antigen capture ELISA³¹ and early detection of carrier animals can lead to creation of infection-free herds.

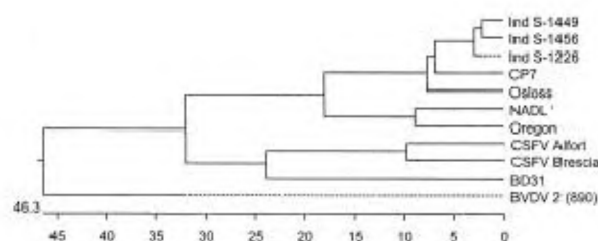


Figure 4. Dendrogram showing relationship of Indian BVDV isolates with other pestiviruses in E1-E2 region. Sequences were aligned using CLUSTAL method of DNASTAR and phylogenetic tree was obtained by Mega align software package.

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Baseline susceptibility of the American bollworm, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* Berl var. *kurstaki* and its endotoxins in India

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Baseline susceptibility of larvae of the American bollworm, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* Berl var. *kurstaki* was studied by a diet incorporation method. Ninety-six hour median lethal concentrations (LC_{50}) of *Bt* var. *kurstaki* strains and parasporal crystal toxins varied widely for neonate larvae of different populations. Insect populations from nine locations in India showed differences in their susceptibility to *Bt* var. *kurstaki* strains and individual Cry toxins, viz. Cry1Aa 10.5, Cry1Ab 12.8, Cry1Ac 16.2, HD-1 14.1 and HD-73 5.7-fold. Insect populations obtained from pigeon pea crops at Navsari from December 2000 to January 2001, and at Delhi from October 1998 to November 2000 showed temporal variation in their susceptibility to *Bt* var. *kurstaki* HD-1 and HD-73. Temporal variation in insect susceptibility was correlated with temperature at these two locations. Insect acclimation to pre-treatment temperature influenced the susceptibility of the F₁ generation to *Bt* var. *kurstaki*. An increase in ambient temperature (about 10°C) increased the susceptibility to *Bt* var. *kurstaki* HD-73 by 7.5-fold. The role of selection pressure, host-plant, xenobiotic and other agroecological conditions on the susceptibility of *H. armigera* is discussed in relation to development of tolerance/resistance and integrated pest management.

BACILLUS thuringiensis (*Bt*) is a spore-forming, Gram-positive bacterium of ubiquitous occurrence, with as many as 50 serotypes or 63 serovars¹. It produces proteinaceous crystal (Cry) toxins, which are activated by proteases in the alkaline conditions of the midgut. These activated toxins bind with receptors on the brush border membrane vesicles of the midgut epithelium and perforate the cell membrane, which leads to ionic imbalance and eventual insect death². The *Bt* Cry toxins are grouped into 45 classes; many possessing insecticidal-specific insecticidal activity, viz. Cry1, Cry9 (Lepidoptera), Cry2 (Lepidoptera and Diptera), Cry3, Cry7, Cry8, Cry14 (Coleoptera), Cry4, Cry10, Cry11 (Diptera) and Cry5, Cry6, Cry12–14 (nematodes) (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/toxins2.html; <http://bgsc.org>). *Bt* is an effective insecticide, relatively harmless to natural enemies, safe to the higher animals; and environmen-

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