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Study of cadherin alleles associated with resistance development to *Bacillus thuringiensis* in pink bollworm population of district Faisalabad, Punjab Pakistan

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Abstract
Although the country has adopted transgenic cotton (Bollguard-I) over the area of 86%. Monitoring the early phases of pest resistance to (BT) crops is crucial, but it has been extremely difficult because bioassays usually cannot detect heterozygotes harbouring one allele for resistance. The pink bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) is one of the most serious lepidopteron pests of cotton in the world. Cotton production in Pakistan has remained stagnant due to many reasons. Transgenic crops producing toxins from the bacterium *Bacillus thuringiensis* (BT) kill insect pests and can reduce reliance on insecticide sprays. Although (BT) cotton (*Gossypium hirsutum* L.) cover a large scale cultivated area in Pakistan but their success could be cut short by evolution of pest resistance. Monitoring of resistance to (BT) cotton with DNA-based screening was performed for the detection of single resistance alleles in heterozygotes. Sampling of cotton bolls were carried out from cotton field grown in different locations of district Faisalabad. For the detection of resistance gene, a simple Polymerase chain reaction (PCR) was performed with gene specific primers that specifically amplify cadherin gene linked with resistance to (Bt) cotton in pink bollworm, *P. gossypiella* later on the quality of the amplified genes (PCR product) were analysed on 1% Agarose gel electrophoresis. This preliminary study would be helpful in devising effective resistance management strategies for the control of PBW.

Keywords: Resistance, pink bollworm, cadherin, bt cotton, Faisalabad, Pakistan

Introduction
Cotton is one of the most important cash crops of Pakistan. Cotton delivers employment to millions of the formers, ginning factories and textile workers and also it earns international exchange. Edible oil and natural fiber is the source of cotton (Aslam *et al.*, 2004) [5]. In agriculture zone cotton value added GDP 1.6% and cotton shares 7.8% significance added (Anonymous, 2013) [4]. In cotton crop producer Pakistan have fourth number in the world (Abro *et al*., 2004) [1]. Cotton is important source of fiber and oil and cotton is the worldwide growing crops. In Pakistan cotton crop was cultivated by the major area. Recently in Punjab and Sindh, pink bollworm has become a real threat to conventional and Bt. varieties of cotton. Pink bollworm attacks to fruiting bolls of cotton and it have ranges 20-30%. The pest (PBW) finishes its fourth generation on cotton crop and the fifth generation of larvae living in the surplus bolls and seed cotton in ginning factories in diapausing stage after final picking (Ahmed, 2013) [2]. For pink bollworm, 53.2% farmers gave opinion that pink bollworm attacks at a medium level thus it can be managed by applying proper control strategies (Jaleel *et al*., 2014) [15].

Cotton *Gossypium hirsutum* L., is major economic crops in Pakistan. It performing key role in Social, financial, political affairs of the country. Cotton called "King of fibers" due to the cultivation commercially for natural fiber and for local usage and its exports needs about 111 countries worldwide. On worldwide India is best grower of cotton. In India 553 kg/ha of average cotton production (Anonymous, 2007) [3]. Insecticidal proteins from the gram-positive bacterium *B. thuringiensis* (BT) are used generally in sprays and transgenic plants to control pest that attack crops and vector disease (Sanahuja *et al.*, 2011) [21]. These *Bt* proteins are particularly significant on the field that they kill some devastating pest, however are not

poisonous to people and most different organisms (Comas *et al.*, 2014; Nicolia *et al.*, 2014; Sanahuja *et al.*, 2011) [7, 20]. Transgenic crops creating *Bacillus thuringiensis* (*Bt*) toxins for pest control are cultivated broadly, with in excess of 98 million hectares of *Bt* cotton, corn, and soybean planted globally in 2016 (James, 2016) [16]. BT crops can suppress pest, reduce insecticide use, and improve Biological control (Hutchison *et al.*, 2010; Lu *et al.*, 2012; Tabashnik *et al.*, 2010) [14, 18]. Although, these advantages have been decreased by increasingly quick evolution of pest resistance from BT crops (Gassmann *et al.*, 2014; Tabashnik and Carriere, 2017) [13, 22]. Insecticidal control is hindered by the larvae being interior feeders; also, resistance from insecticides develop making it frequently more expensive than different strategies. Nonetheless, there is an extensive literature on chemical control, particularly from India. The efficacy of asymethrin has been tested in India by synthetic pyrethroids (Dhawan *et al.*, 1992) [9] that of chlorpyriphos in India by Dhawan *et al.*, (1989) [8] and (Butter *et al.*, 1990) [6]. The insect has been exposed broadly to genetically modified cotton making *Bacillus Thuringinesis* toxin Cry1Ac on the world's best 3 cotton producing country: United States, Republic China, and the India (Tabashnik *et al.*, 2010; Dhurua and Gujar, 2011) [10]. In the United. States, pink bollworm population have not evolved resistance from Cry1Ac following two decades of introduction. (Tabashnik *et al.*, 2010) BT cotton remains the foundation of an effective multi-strategy eradication program. Improvement in resistance from pesticides is commonly viewed as one of the most genuine resistance to viable insect manage nowadays. The pink bollworm is the harmful damage on plant cotton in country like Egypt. (Aswad and Aly, 2007) [11]. the reasons is that a tremendous damage of cotton crop bolls when it ignored. larvae of the *Pectinophora Gossypiella* stabbing cotton toward the start of maturing stage making tremendous damage or damage the cotton green bolls, fiber and seeds and as needs be incredible decrease in the cotton yield. (Khurana, 1990) [17] This pest procured resistance against the greater part of ordinary insecticides. In the present study Cadherin Alleles were studied that developed resistance in Pink bollworm in the region of district Faisalabad Punjab Pakistan. For the detection of resistance gene, a simple Polymerase chain reaction (PCR) was performed with gene specific primers that specifically amplify cadherin gene linked with resistance to (BT) cotton in pink bollworm, *P. gossypiella* later on the quality of the amplified genes (PCR product) were analysed on 1% Agarose gel electrophoresis. This preliminary study would be helpful in devising effective resistance management strategies for the control of PBW

Materials and Methods

Field sampling

Specimen of (BT) cotton from the fields were collected randomly to improve the chance of finding that have comparatively high stages of pink bollworm infestation in the areas of resistance (*r*) alleles from relatively high profusion of (BT*)* cotton. Cotton bolls were sampled/collected from cotton fields from Faisalabad during 2016 to 2018. At each site, bolls was also taken from non (BT) cotton area near (BT) cotton area and brought to Insect Molecular Biology Lab. University of Agriculture Faisalabad from Department of Entomology. Pink bollworm larvae collected by accumulating fourth larval stage that escaped by bolls by germinal and eliminating larval instars present inside. Male pink bollworms were developed from the collected bolls in lab.

DNA extraction and PCR

Insects collected from field were kept in ethanol at -20 ^{0}C. DNAzol reagent was used for the extraction of DNA (Tabashnik *et al.*, 2005b) and performed polymerase chain reaction will be designated by (Morin *et al.*, 2004) [27]. The determined amount of entities verified per five samples. Comprising controls and tests of field appraised PBW for every 3 (*r)* alleles_4,000.

Fig 1: Genomic DNA purification Kit, # K0512

DNA extraction and PCR protocol

DNA of pink boll worm collected from filed collected bolls extracted by following the manufacturer instruction of the kit (Genomic DNA purification Kit, # K0512, Thermo Scientific) and as reported by (Tabashnik *et al.*, 2005). DNA of the experimental insects extracted manually by grinding the wasp and host in RNase and DNase free 1.5 ml Eppendorf tubes where insects were crushed and homogenized inside the Eppendorf tubes or with pestle mortar in the presence of 200µL SDS lysis buffer. After crushing/grinding of insects in SDS lysis buffer, 1.0 µl of RNase A (100mg/ml) were added in the samples and incubated at 37 ºC for 1hour, similarly, protein from samples was removed by incubating samples with 5µl of proteinase K (20mg/ml) at 50 °C for 1 hour. The homogenate was treated with 240 µL of Phenol: chloroform: Isoamyl alcohol (25: 1: 24) and shake gently for 10-20 times for 10 minutes and centrifuged at 12000 rpm. The supernatant was transferred in another 1.5 ml DNase free Eppendorf tube. The phase separation mixture (240 µL) Phenol: Isoamyl alcohol: chloroform, (25:1:24) was mingled again in samples and same process was repeated as described above. Then supernatant/upper phase was transferred into another Eppendorf tube and finally DNA was precipitated with chilled absolute ethanol by centrifugation at 12000 rpm for 15 minutes DNA pellets eroded with ethanol 70% and parched at room temperature and again suspended in TE buffer 1X, finally stored at -20℃ till advance use.

Quantification of DNA

The quantification of the extracted DNA carried out in ng/ul by taking 1 µL of DNA sample using Thermo scientific Nano drop 2000 Spectrophotometer (Thermo Scientific Co., Waltham, MA, USA).

Qualitative analysis of DNA on agarose gel electrophoresis

For extraction of DNA in this way to test the reliability and superiority, 1µl of gel stuffing dye BBF was added with 1µl of DNA (to a final concentration of 1X for bromo phenol blue 10X), electrophoresed by 0.8% Agarose gel, thinned in 8µl double distilled sterile water, equilibrated in successively TBE buffer 1X and run at 90 V perpetual for 30 minutes. Specimens were mixed beside 100 bp/Ikb DNA ladder

(Thermo Scientific). Bands of DNA discolored with ethidium bromide would be visualized by acquaintance to ultraviolet rays. The excellence and reliability of the removed DNA was judged by detecting the strident and strong single band on the gel.

Results and Discussion

PCR based screening for the monitoring of resistance development in BT cotton cultivars grown in district Faisalabad.

A comprehensive survey for the collection of pink Bollworm *P. gossypiella* immature were performed on the experimental field of Department of Entomology and Agronomy, University of Agriculture Faisalabad, Pakistan during August 2017 to December,2017. PCR techniques are present for monitoring the resistance of Pink bollworm and molecular method are used. University has excellent collection because different varieties of BT cotton grow in different fields. The 3rd and 4th instars larvae of pink bollworm *P. gossypiella* were assembled from invasion bolls, preserved in absolute ethanol and stored at -20 ^{0}C. Larvae were picked randomly from stock of already stored samples for diagnostic PCR.DNA of the Pink Bollworm *P. gossypiella* was extracted electrophoresed on 0.8% Agarose gel electrophoresis as shown below. To find out the genetic basis of resistance development a PCR based screening was performed for the monitoring of resistance development in Pink Bollworm *P. gossypiella* against BT cotton grown in District Faisalabad. Experimental procedure involves the collection, preservation, extraction and PCR amplification of Resistance gene (Cadherin) through 3rd and 4th instar PBW larvae.

We are able to find out the resistance genes CADHERIN in Pink bollworm *p.gossypiella* larvae in the FH-142, Lalazar and IUB 2013 through PCR (Polymerase Chain Reaction) amplification these varieties were grown in Entomology field of young wala University of Agriculture Faisalabad Pakistan. Our finding and results related to the previously work done by Indian researcher who performed different splicing and top of the variable CADHERIN transcript Associated PBW to Bt Cotton in India with Field-adapt Resistance (Fabrick *et al,* 2014) [12]. Similarly our finding are in accordance with the pink bollworm resistance to Cry1Ac which monitored by Cadherin DNA (Tabashnik *et al.,* 2010; Tabashnik *et al.,* 2006) [22, 24].

In same way our results related to Indian field selected population of PBW *P. Gossypiella* resistance to Cry1Ac associated with the diversity of CADHERIN mutation. But in China the Resistance to Cry1Ac is not efficiently monitored by the CADHERIN DNA in American Bollworm. In Northern China laboratory and field selected strains involved in resistance to BT toxin. In the same way for both environment field and selected strains CADHERIN resistance alleles play an important role for resistance to Cry1Ac toxin protein (Zhang *et al.,* 2012) [25]. From the previous researchers and finding Arizona State about the *P. gossypiella* resistance it cleared that the CADHERIN protein bind the *Bt* toxin Cry1Ac and cause resistance in the PBW *P. Gossypiella.* In India the following result and finding shows that resistance and mutation occur in the CADHERIN gene which is described in Arizona State associated to the PBW resistance against the Cry1Ac poison. In the same way our finding of DNA screening for the monitoring of resistance development related to field evolved resistance in *P. gossypiella* Pink bollworm to BT toxin Cry1Ac was similar to previous finding in India and Arizona. In 2010 and 2011 from Gujrat (AGJ) and Maharashtra (AMH) PBW Anand population taken, after Bioassays testing it is cleared that 65% population of pink bollworm *P. Gossypiella* in Gujrat (AGJ) is resistant to Cry1Ac. While the other hand in Maharashtra (AMH) 0% Akola population of pink bollworm *P. gossypiella* were resistant to Cry1Ac.These finding support the 2008 and 2009 results in India. Similarly, our results after the DNA screening and PCR amplification of Pink bollworm *P. Gossypiella* larvae were resistant to Cry1Ac because these population were taken from the Bt varieties of Pakistan FH-142, Lalazar and IUB 2013.

5 random sample of pink bollworm *P.gossypiella* have taken from the BT. cotton.fr that purpose 2nd and 3rd instars have selected from India in (KMP) Khandwa Madhya Pradesh for checking the resistance in these 5 larvae. From those 5 larvae only 1 larva has no disrupted CADHERIN alleles (DCA) while other remaining 4 larvae have disrupted CADHERIN alleles (DCA).So from this finding we cannot make any hypothesis about the 1 larvae that is susceptible because no concentration of Cry1Ac is found in those bolls from that, this 1 larva has taken. Another point is that larvae may be feed on the tissue of the plants which has less amount of Cry1Ac toxin protein. Another hypothesis was made that, 1 larva were resistant and this resistant due was not by CADHERIN gene. This resistance may by the other gene not by CADHERIN gene. The resistant occur against the Cry1Ac due to two things, one is the locus mutation occur and second due to Cadherin gene mutation in all bollworms and in particularly in pink bollworm *P. gossypiella*. Our results of this DNA screening for the monitoring of the resistance in Bt fields of District Faisalabad, Pakistan was similar with the above findings by the PCR amplification after DNA extraction of 4 larvae and this resistance is highly due to mutation in CADHERIN gene to Cry1Ac toxin protein.

From the KMP and AGJ almost 8 larvae selected from that eight larvae, 8 disrupted cadherin alleles (DCA) r5 and r12 discovered by the Indian researchers. From that nineteen novel cDNA isomers also discovered.15 isomers have premature stop codon (PMSC) and other 4 isomers cause the deletion of 99 bp in the sequence which the bind the Cry1Ac toxin protein. The (PMSC) cut the top end of the CADHERIN protein not entered in the midget. When CADHERIN protein not entered in the mid than Cry1Ac toxicity not mediate by the CADHERIN protein and this confer the resistance. From the Arizona published literature, it is conformed that little disruption in the CADHERIN protein cause the resistance to Cry1Ac toxin protein (Morin *et al.,* 2003; Fabrick and Tabashnik, 2012) [19, 18, 25]. Our results and finding show that when the disruption occur in the CADHERIN protein his may lead to resistance against the Cry1Ac toxin protein in PBW *P. gossypiella* Pakistan.

The resistance due to mutation in cadherin protein against the Cry1Ac toxin in PBW in laboratory strain of Arizona. But our result similar with India, that field selected strains have also mutation in CADHERIN gene and may cause the resistance against the Cry1Ac. From the India field selected population 19 isomers and 8 cadherin alleles related to resistance found by Indian researchers. According to their opinion more samples size PBW *P. gossypiella* has more CADHERIN locus. From the last one decade researchers results only 22 cadherin alleles has reported for the resistance to Cry1Ac.That data has taken from the many individuals particularly from the main pest of cotton. Genetic variation in

Cadherin not cause the resistance. In India similar findings found for the PBW *P. gossypiella*. It is cleared from the previous studies in China in American bollworm 15 Cadherin resistance alleles are found. From these 15 Cadherin resistance alleles just one r15 alleles detected in many populations and r1 and r8 alleles from these 15 Cadherin resistance alleles found more than one individuals (Zhang *et al*., 2013) [26].

Cadherin mRNA miss-splicing involved in the resistance to Cry1Ac toxin protein in PBW. In the same way resistance also produced by the alternative splicing (Zhang *et al*., 2013) [26] In India 8 Cadherin resistance alleles identified and out of these 8 Cadherin alleles in 7 Cadherin alleles generation of resistance alleles done due to alternative splicing. Resistance monitoring occur due to DNA screening are reduced by the generation resistance and that resistance due to alternative splicing. For the analysing of mRNA two steps are required one is more steps are required than DNA screening and second step is sample preservation for the monitoring of the resistance than genomic DNA. Evolution of resistance accelerated by the alternative splicing. Altered protein confer the high level of resistance by generation by producing the greater diversity of mutations. Our finding are more or less related to above mentioned work cited by different scientist regarding the DNA screening for the monitoring of resistance development in PBW. *P. gossypiella* against BT cotton grown in district Faisalabad, Pakistan. With the previous findings, three Cadherin resistance genes after PCR-based detection suggest that it could be might useful for the monitoring of resistance in the PBW by producing BT cotton.

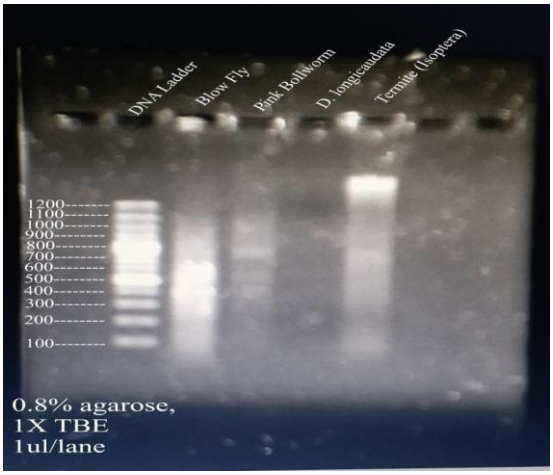

Fig 2: DNA of Pink Bollworm *Pectinophora gossypiella* resolved on 0.8% Agarose gel

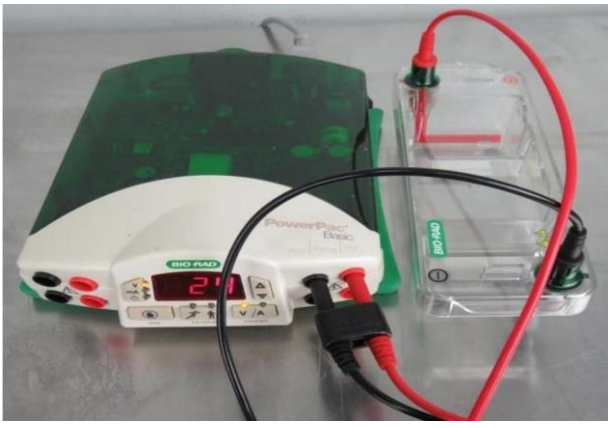

Fig 3: Agarose gel apparatus

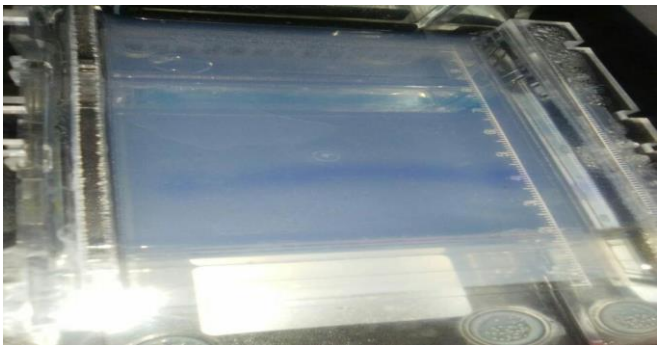

Fig 4: Agarose gel Electrophoresis

Designing of PCR Primers

The success of PCR entirely depends upon the designing of technically good primers. PCR will be performed as defined by (Morin *et al*., 2004) [27].

In the proposed study, primers were designed directly from the sequences of the *Pectinophora gossypiella* cadherin (BtR) gene, BtR-r1 allele, available in gene bank database (ACCESSION AY713482.1) and from already published work reported by (Morin *et al*.,2004) [27]. Different primer designing tools like Primer Blast, Just Bio (Primer3) and Gene Fisher etc. were used for designing of primers.

Table 1: Gene ID, Primer and No. of base pairs

Gene ID	Primer	No. of Base pairs
Pg-cad-1 F	CACCTCACAACCCACACGATA	21
Pg-cad-1 R	ACGGCCACCTTGAATATCCCTA	22
Pg-cad-2 F	CAAGCAGTACGACTCGCAGA	20
Pg-cad-2 R	CAGTCACTCTGGTGAGAGCC	20

PCR screening

The detection is based on the *Pectinophora gossypiella* PBW cadherin (*Bt*R) gene, *Bt*R-r1 allele**.** PCR was performed by using Kit # K017, Thermo Scientific, in a squeaky walled 0.2 ml PCR tube holding 50- µl reaction with primer pairs using 1 µl of DNA and following the profile 94 ℃, 2 min; 94 ℃, 30 s, 58 ℃, 1.5 min, 72 ℃, 2 min (35 cycles); 72 ℃, 5 min. PCR products were detached on 1.2% Agarose gel ethidium bromide discolored bands of DNA were imagined under UV illumination.

Fig 5: T100 Bio Rad PCR Machine

Statistical analysis

The chances of detecting F is the frequency of resistance

alleles and *r* alleles in a sample of *N* individuals was deliberate as (1- [*F*× *D*]) $^{2N\times A,}$ where, *D* is chances of sensing an *r* allele present in separated entities built on records from unsighted controls, 2*N* is the no of alleles partitioned, and *A* is the chance of making many copies of cadherin DNA take place in field sample insect.

Conclusion

Monitoring of resistance to (BT) cotton with DNA-based screening was performed for the detection of single resistance alleles in heterozygotes. Sampling of cotton bolls were carried out from cotton field grown in different locations of district Faisalabad. For the detection of resistance gene, a simple Polymerase chain reaction (PCR) was performed with gene specific primers that specifically amplify cadherin gene linked with resistance to (Bt) cotton in pink bollworm, *P. gossypiella* later on the quality of the amplified genes (PCR product) were analysed on 1% Agarose gel electrophoresis. This preliminary study would be helpful in devising effective resistance management strategies for the control of PBW. It is concluded that Cadherin Alleles are responsible for causing resistance in *Bacillus Thuringiensis* related to Pink Bollworm.

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