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Full Length Article

Identification and Phylogeny Determination of Pathogens-Associated with Leaf Blight of Cotton

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Abstract

Bacterial blight of cotton (BBC) is known to be a destructive bacterial disease affecting all cotton growing regions of the world including Pakistan. Infected cotton leaves with typical BBC symptoms were collected from three districts (Multan, Burewala and Bahwalpur) of Punjab, Pakistan. Morphological and basic biochemical tests (Gram staining, KOH Test, Tween 80 hydrolysis Test, Fluorescent Pigment Test and Kovacs' Oxidase Test) confirmed isolated bacteria to be gram negative. Genome of four bacterial isolates M5, M8, Bo7 and B10 were sequenced using next generation sequencing (NGS) protocol Illumina HiSeq 4000. Multigene phylogenetic and Average Nucleotide Identity (ANI) analysis has revealed three different *Pantoea* species; *Pantoea anthophila, P. eucrina* and *P. dispersa* to be responsible for BBC in Punjab, Pakistan. Inoculation of four bacterial isolates (M5, M8, Bo7 and B10) on susceptible cotton variety Acala Maxxa in greenhouse conditions has confirmed these isolates to be responsible for BBC. Bacterial isolate from Bahwalpur region (B10) produced highest number of lesions on cotton leaves followed by Bo7 and M8. Discovery of these *Pantoea* species instead of *Xanthomonas citri* pv. *malvacearum* (*Xcm*) as a pathogen of BBC in Pakistan emphasize the fact that we need to understand etiology of this disease to develop better management practices. © 2020 Friends Science Publishers

Keywords: Pathogenicity assay; Foliar spray; Housekeeping genes; Genome sequence

Introduction

Upland cotton (*Gossypium hirsutum* L.) is one of the most leading fiber crop. In Pakistan cotton is a major crop after wheat which occupies the largest cultivated area as compared to other crops (Rehman *et al*. 2016). Pakistan is ranked among 4th largest cotton producing countries worldwide (Shuli *et al*. 2018). Cotton crop is affected by many foliar diseases in field conditions. Historically, bacterial blight of cotton is one of the most significant cotton foliar disease caused by *Xanthomonas citri* pv. *malvacearum* (*Xcm*). The bacterium infect all above portion of cotton plant but cause greatest loss when the bolls are attacked. BBC produce several symptoms include water soaking lesions, leaf and fruit spots, leaf blight, angular shaped necrotic spots and boll rot (Mhedbi-Hajri *et al*. 2011). In favorable environmental conditions BBC can cause 20 to 50% disease losses (Arabsalmani *et al*. 2002; Razaghi *et al*. 2012). In Pakistan, during the past few years 20–37% BBC disease incidence has been reported (Hamid *et al*. 2012).

Pathogenic bacterial identification and classification is always complex, which requires a series of tests for conclusive identification (Alvarez 2004). Misidentification of pathogens has slowed down the efforts to combat many bacterial diseases of Pakistan. However, sequence-based techniques have replaced traditional identification methods due to their high sensitivity and reliability (Brady *et al.* 2013; Meng *et al.* 2015). DNA sequencing methods are useful for identification of both new and established pathogens (Aritua *et al*. 2015). Partial sequence of protein coding genes is useful for bacterial specie identification of family *Enterobacteriaceae*. 16SrRNA genes sequence are useful for the determination of phylogenetic relationship between distantly related species (Dauga 2002). Bacterial housekeeping genes and whole genome sequence provide reliable information for bacterial taxonomic nomenclature (Kim *et al*. 2014). Average nucleotide identity (ANI) is considered as most widely used next generation standard for species identification. The ANI shows identical values between homologous regions of two or more genomes (Haley *et al*. 2010; Chan *et al*. 2012; Yi *et al*.

2012; Grim *et al*. 2013). Using multi-genic and genomic analysis, many researchers have identified and characterized the BBC pathogen in several cotton growing countries of the world (Delannoy *et al*. 2005; Razaghi *et al*. 2012; Pritchard 2016), but in Pakistan the importance of this bacterial diseases on cotton crop was undervalued and not studied well. Some drastic changes in climatic conditions; increase in temperature, humidity and erratic rainfall pattern since last decade have made the BBC more prevalent every year in Pakistan.

The objective of this study was to evaluate and identify the pathogen responsible for bacterial blight of cotton in cotton growing areas of Punjab, Pakistan. Both conventional and molecular approaches were used for correct pathogen identification and species delineation. Bacterial isolates were tested on cotton plants in glasshouse under control conditions to check their ability to produce BBC symptoms.

Materials and Methods

Collection of samples from cotton growing areas

In 2016–2017 infected cotton leaf samples showing typical bacterial blight of cotton symptoms (leaf blight, angular shaped necrotic lesions) were collected from three districts of Punjab, Pakistan that included Burewala, Multan and Bahawalpur. After collection, infected samples were placed in airtight polyethylene bags with other supporting data such as sample location, host variety and sample number.

Isolation and identification of bacterial pathogen

The cotton leaf samples showing BBC symptoms were cut into pieces of 2–3 mm size. These small pieces were dipped into 70% ethanol for 30 sec followed by 2–3 dipping in sterile dd/H_2O for surface sterilization. Samples were dried on autoclave filter paper disc and placed aseptically onto nutrient agar (NA) plates. The petri dishes were incubated for 24h at 28°C to examine for bacterial growth. Different biochemical tests including Gram staining, KOH Test, Tween 80 hydrolysis Test, Fluorescent Pigment Test and Kovacs' Oxidase Test were carried out for the confirmation of bacterial pathogen on genus level.

DNA extraction and normalization

Single bacterial colonies were obtained from overnight growth of bacteria on NA media by using pure stock culture. Pure colonies were suspended in nutrient broth and incubated for 24 h on shaker in growth chamber at 28°C. Total DNA was extracted by using Qiagen DNeasy Blood & Tissue Kit. The isolated dsDNA concentration was quantified by Nano-drop spectrophotometer. The concentration of extracted DNA was visually assessed as above on a 1% agarose gel by loading DNA sample (5 μL DNA + 2 μL loading dye) on gel electrophoresis at 100 V for 30 min. Qubit fluorometer was used for the process of dsDNA normalization. Qubit reagents and standards were prepared according to Kit protocol, which helped to calculate the actual amount of dsDNA in original samples. The samples were normalized up to 6 ng/μL for genomic library preparation.

Genomic library preparation

The normalized DNA (6 ng/μL) of four bacterial isolates (B10, Bo7, M5 and M8) were used for the preparation of genomic library. QIAGEN QIAseq FX DNA library kit protocol was used for genomic library preparation. The quality of DNA libraries were assessed with the help of Agilent Bioanalyzer 2100. The genomic libraries were quantified and normalized with the help of Pico-green and Qubit fluorometer. Next generation sequencing (NGS) protocol Illumina HiSeq 4000 was performed at the Genome Center, University of California, Davis for sequencing of genomic libraries.

Phylogenetic analysis of sequencing data

The quality of sequenced libraries was assessed. SPAdes was used for the assembly of sequenced genomes (Bankevich *et al*. 2012). Perl script was used to extract 16S rRNA and other housekeeping genes (gyrB, atpD, L2, leuS and rpoB) from genome sequence and phylogenetic tree was constructed using Mega 7 (Brady *et al.* 2013). PyANI software was used for specie assignment based on average nucleotide identity (ANI) >95% (Pritchard 2016).

Pathogenicity assay

Seed of BBC susceptible cotton variety (Acala Maxxa) were collected from U.S. National Plant Germplasm System. Cotton seeds were sown in plastic pots under greenhouse controlled conditions (26°C with >85% relative humidity and 16 h photoperiod). Bacterial isolates (B10, Bo7, M5 and M8) were revived from -40 °C by streaking on NA media and cultured for 24 h at 28°C. They were sub-cultured on nutrient broth and harvested by centrifuge after 12 h. The bacterial pellet was suspended in sterile dd/H_2O. The concentration of culture was maintained at 10^6 cfu/mL. Spray technique was used to inoculate cotton plants at 5–7 leaf stage. Sterile ddH_2O was used as control. After inoculation plants were closely monitored for the appearance of bacterial blight symptoms. Analysis of variance (ANOVA) was used to check the variation among bacterial strains with respect to number of lesions. Least significant difference (LSD) test was used to all possible mean comparisons between the bacterial strains (Steel *at al*. 1996).

Results

Sampling for bacterial isolation and identification

Typical bacterial blight of cotton symptoms (water-soaked spots, necrotic lesions, yellowing and defoliation of cotton leaves) were observed during BBC samples collection (Fig. 1a). Creamy or yellow growth was observed around infected samples on nutrient agar media. Pure single colonies of bacterial pathogen were successfully grown by sub-culturing on nutrient agar media (Fig. 2a). Physiological and conventional biochemical tests including Gram staining, KOH Test, Tween 80 hydrolysis Test, Fluorescent Pigment Test and Kovacs' Oxidase Test confirmed isolated bacteria to be Gram negative (Fig. 2b–c).

Genome statistics

High quality genome sequences were obtain using next generation sequence (Table 1). The BLAST searches based on whole genome sequence of four isolates B10, Bo7, M5 and M8 using NCBI (http://www.ncbi.nlm.nih.gov) revealed similarities to *Pantoea* species. The average genome size of four isolates B10, Bo7, M5 and M8 was ~ 4.7 Mbp with 56% genomic GC content. Moreover, average 81.75% tRNA-coding genes along with 7.25% rRNA genes were predicted in the sequence of these four B10, Bo7, M5 and M8 isolates (Table 1). All sequences were submitted to NCBI GenBank for the assignment of accession numbers (Table 1).

Phylogenetic analysis

Phylogenetic trees were constructed using 16SrRNA genes sequence and five common housekeeping genes (gyrB, atpD, L2, leuS and rpoB), which were extracted from the genome sequence of bacterial isolates B10, Bo7, M5 and M8 (Fig. 3 and 4). The phylogenetic tree of 16SrRNA made two clusters M8, Bo7 and B10 were close to *P. agglomerans* while M5 showed resemblance with *P. dispersa* (Fig. 3). As 16sRNA gene was not enough to distinguish the species, different housekeeping genes were used to identify the *Pantoea* species. Three clusters were formed when tree was constructed based on concatenated sequence of five housekeeping genes (Fig. 4). Isolates from Multan (M5 and M8) showed close resemblance with *Pantoea anthophila* and supported by 100% bootstrap values (Fig. 4). Isolates from Burewala (Bo7) and Bahawalpur (B10) were closely related to *P. eucrina* and *P. dispersa* respectively (Fig. 4). Based on initial BLAST searches on NCBI reference genomes of different *Pantoea* species along with four isolates (B10, Bo7, M5 and M8) from current experiment were subjected to construct heatmap based on ANI data (Fig. 5). The ANI_{95} values revealed three groups supporting the clades from multigene analysis.

Fig. 1: (a) field symptoms of BBC during samples collection. (b) Control cotton leaves inoculated with water. (c) Infected cotton leaves showing BBC symptoms in greenhouse

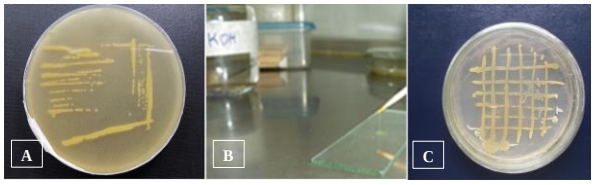


Fig. 2: Image (a) showing single colonies of bacterial isolates on NA medium. (b) representing the viscous thread made during KOH test to confirm gram staining. (c) Milky white precipitation appear when bacterial colonies were subjected to Tween 80 hydrolysis test

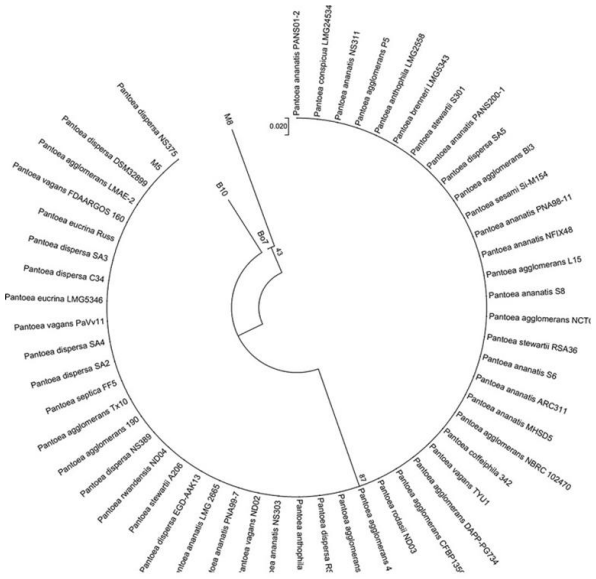


Fig. 3: Maximum likelihood phylogenetic tree based on 16S rRNA sequences of Bo7, B10, M5 and M8 bacterial isolates showing *Pantoea* species and their closest phylogenetic neighbors

Isolates from Multan (M5 and M8) had >98% ANI to *P. anthophila* and some unidentified *Pantoea* species while isolates from Burewala (Bo7) and Bahawalpur (B10) districts showed >99% ANI to *P. eucrina*-LMG-5346 and >98% ANI *P. dispersa*-NS375, respectively (Fig. 5).

Pathogenicity assay

Bacterial blight disease symptoms (water-soaked spots, necrotic lesions with yellow halo and defoliation) were noticed after 2 weeks of cotton leaves inoculation (Fig. 1c).

Table 1: Showing samples name, genome statistics and their accession numbers

Sample	Genome size	Scaffold N50	GC (%)	CDS	Proteins	tRNA	rRNA	Accession Number
Bo7	3878409	488816	0.5647	3699	3608	82	9	VWUX00000000
B10	4986677	189135	0.5746	4892	4796	87	9	VWVO00000000
M8	4647578	341114	0.5680	4374	4300	70	4	VWUI00000000
M5	5397540	55261	0.5676	5514	5419	88	7	VWUG00000000

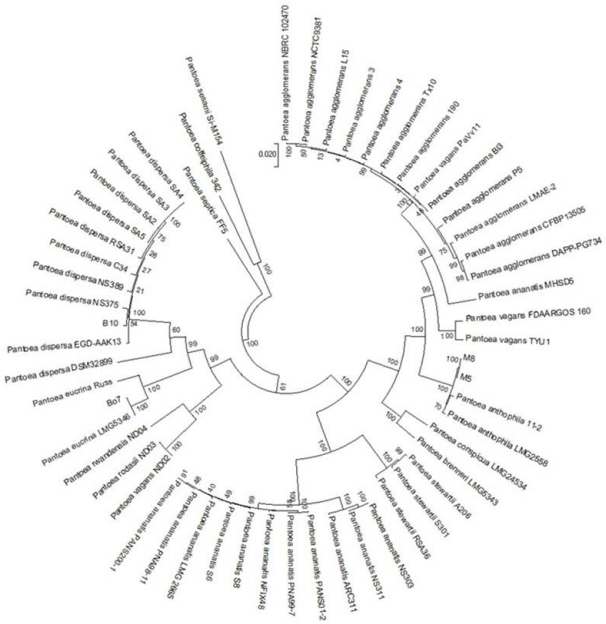
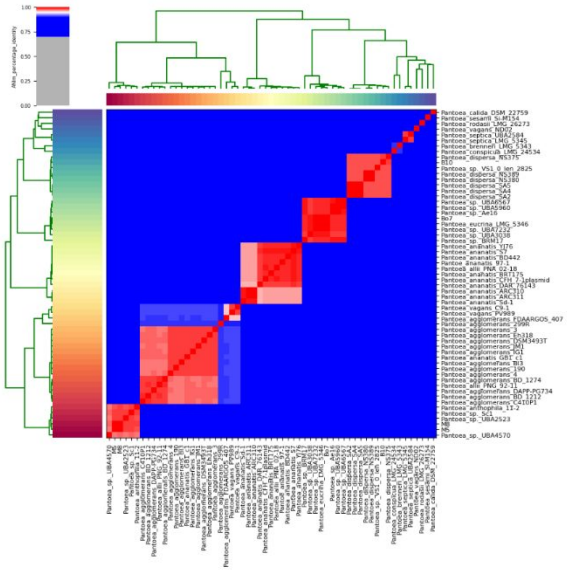


Fig. 4: Phylogenetic tree made by concatenated nucleotide sequences of five housekeeping genes gyrB, atpD, L2, leuS and rpoB. Maximum likelihood tree having bootstrap values after 1000 replicates are shown in percentage



Symptoms observed in the green house were identical to those observed at the farmer fields (Fig. 1a). The bacterial pathogen was re-isolated from the inoculated cotton leaves, which fulfilled the Koch's postulates.

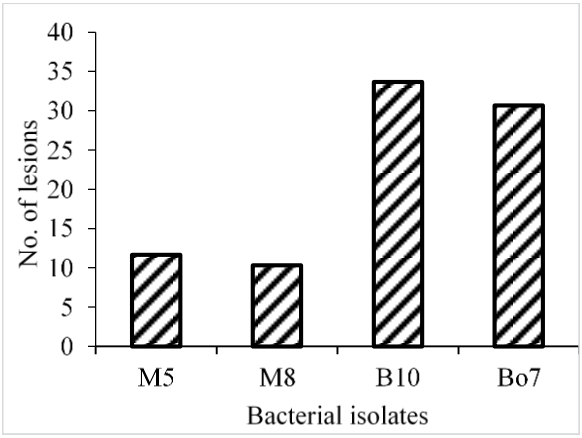


Fig. 6: Comparative effect of four bacterial isolates (B10, B07, M8, M5) on no. of lesions on cotton variety Acala Maxxa

A significant difference was observed among bacterial strains in terms of lesions production on susceptible cotton leaves (Fig. 6). Among four bacterial isolates (B10, Bo7, M5 and M8) the isolate B10 produced highest number of lesions while isolate M5 produced least number of lesions on cotton leaves (Fig. 6).

Discussion

In Pakistan, the pathogen of bacterial blight of cotton was previously diagnosed by using classical methods based on morphology and appearance of symptoms (Hamid *et al*. 2012). In the present study, the samples were collected from core cotton areas of Punjab, Pakistan. For an accurate identification, the BBC pathogen was diagnosed using morphological, molecular and pathogenicity assays. Basic morphological studies provided the preliminary validation of the pathogen based on the bacterial growth pattern, cell shape and color of bacterial colonies (Schaad *et al.* 2001). The growth pattern and colony color formation of BBC isolates found in this study was similar to those previously reported (Delannoy *et al.* 2005). The bacterial morphological features, including colonies color, cell shape are not always remain constant and can be easily influenced by environmental conditions (Brady *et al.* 2013). Therefore, biochemical and molecular techniques are indispensable for correct identification of bacterial genus and species. Phenotypic and biochemical properties of BBC bacterial isolates of our study were almost constant and similar to described earlier (Schaad *et al.* 2001; Medrano and Bell 2007).

The phylogenetic tree of 16SrRNA made two clusters M8, Bo7 and B10 were close to *P. agglomerans* while M5 showed resemblance with *P. dispersa* (Fig. 3). Sequence data of multiple genes are useful for determination of phylogenetic relationships which reduce ambiguities caused by genetic recombination (Nancy *et al*. 2005; Brady *et al.* 2013). Following housekeeping genes (gyrB, atpD, L2, leuS and rpoB) of B10, Bo7, M5 and M8 isolates were used to create phylogenetic tree which formed three clusters with reference strains. Bacterial isolates M5 and M8 showed cluster with *P. anthophila* while other two isolates Bo7, B10 were closely related to *P. eucrina* and *P. dispersa*, respectively (Fig. 4).

The 16SrRNA and housekeeping genes (gyrB, atpD, L2, leuS and rpoB) results of Pakistani isolates were closely related to the findings of Brady *et al.* (2013). The 16SrRNA phylogenetic results were quite different from five housekeeping genes results. Isolates M8, Bo7 and B10 in 16SrRNA phylogenetic tree were close to *P. agglomerans* while in housekeeping phylogenetic tree Bo7 and B10 were close to *P. eucrina* and *P. dispersa*. Similarly, M5 in 16SrRNA phylogenetic tree was close to *P. dispersa* while in five housekeeping genes phylogenetic tree M5 and M8 showed resemblance with *P. anthophila* (Fig. 3 and 4). Sequence of 16S rRNA and other housekeeping genes is indeed a popular traditional method for species delineation. However, these methods based on single or set of conserved genes does not provide sufficient resolution at species level due to genetic discontinuities among closely related taxa (Mende *et al.* 2013). In recent years, the average nucleotide identity (ANI) has emerged as a robust method for bacterial species delineation (Goris *et al*. 2007; Richter and Rossello 2009). The finding of ANI_{95} data of four isolates B10, Bo7, M5 and M8 were similar to housekeeping genes results (Fig. 5). Instead of *Xanthomonas citri* pv. *malvacearum* (*Xcm*) three *Pantoea* spp (*P. eucrina, P. anthophila* and *P. dispersa*) were reported from infected BBC samples (Fig. 5). There are many *Pantoea* species has been identified causing blight symptoms on different hosts (Kini *et al.* 2017; Filho *et al.* 2018). In order to study the isolated bacterial genomes many *in silco* studies were carried out. We have reported single circular chromosome with average of ~ 4.7 Mbp genome size and 56% genomic GC content (Table 1). Our genome statistical results were almost constant and similar to describe by (Palmer *et al.* 2016). Matsuzawa *et al.* (2012) found 4.8 Mbp genome size for *P. agglomerans* strain IG1. Similarly, *P. ananatis* isolated from infected onion have genome size range from 4.8 to 5.1 Mbp (De Maayer *et al*. 2014). Greenhouse experiment confirmed these isolates (M5, M8, Bo7 and B10) to be causal agent of BBC. The isolates from Bahwalpur region B10 produced highest number of lesions on susceptible cotton variety Acala Maxxa followed by Bo7, M8 and M5 (Fig. 6). The greenhouse BBC symptoms were similar to those reported by Sambamurty (2006).

Conclusion

This experimental study specified the importance of advance molecular techniques for pathogen identification and specie delineation. The pathogen was confirmed three *Pantoea* spp (*Pantoea anthophila, P. eucrina* and *P. dispersa*) instead of *Xanthomonas citri* pv. *malvacearum* (*Xcm*). The genome sequence of bacterial isolates (B10, Bo7, M5 and M8) has provided insight into the genomic features of these *Pantoea* species. Greenhouse experiment has confirmed *P. anthophila, P. eucrina* and *P. dispersa* to be responsible for BBC symptoms production.

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