

Characterization of New Bacterial Leaf Blight of Rice Caused by *Pantoea stewartii* subsp. *indologenes* in Southern Districts of Tamil Nadu

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Abstract— A survey was conducted in the rice fields of Tirunelveli, Tuticorin, Kanyakumari and Madurai districts of Tamil Nadu during 2016 to assess the importance of bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae*. Bacterial Leaf Blight affected leaf samples showing yellowing symptoms or orange to brown stripes on one or both halves of the leaf blade were collected from seventeen places and maintained as isolates. Upon isolation, symptomatic leaf pieces were surface sterilized and placed in wakimoto semi-synthetic medium. The yellow pigmented, raised and translucent colonies with smooth margin were obtained after incubation at 28°C for 2 days. The biochemical characterization revealed that the bacteria belong to gram negative facultative anaerobes with small rods either arranged singly or in chains. Thirteen isolates show positive results in biochemical tests viz., Gram staining, KOH test, starch hydrolysis, anaerobic growth test, tween 80 hydrolysis test, catalase test, citrate utilization test and production of yellow pigment on Yeast Dextrose Chalk agar medium. In virulence test, Isolate 1, Isolate 3 and Isolate 4 were considered virulent as they have caused severe blight symptoms both in TN1 and ADT 43, the susceptible check varieties. Based on 16S rRNA sequence analysis, the causal agent was identified as *Pantoea stewartii* subsp. *indologenes* (Accession No. SUB2733370; MF163273; MF163274; MF16327). The biochemical and molecular analysis revealed that the causal agent was not *Xanthomonas oryzae* pv. *oryzae*, but a new species of bacterium namely *Pantoea stewartii* subsp. *indologenes*. This is the first report of new bacterial leaf blight disease of rice caused by *Pantoea stewartii* subsp. *indologenes* in southern districts of Tamil Nadu.

Keywords— Bacterial leaf blight, *Xanthomonas oryzae* pv. *oryzae*, Wakimoto semi-synthetic medium, virulence test, 16S rRNA sequence analysis, *Pantoea stewartii* subsp. *indologenes*.

I. INTRODUCTION

Rice is one of the cereal crops of great significance in India and primary staple food for huge population in Asia, Africa and Latin America. Consumption of rice accounts for over 90 per cent of the world's population in Asia and China, India and Indonesia producing 30.85 per cent, 20.12 per cent and 8.21 per cent respectively of total global rice production (USDA, 2012; Kadu, *et al.*, 2015). Global rice utilization is projected over around 501.2 million tonnes (milled basis) in 2016-17, which is just one percent more than the 2015-16 estimates. The increase would be sustained by a 5.0 million tonnes expansion in food use to 402.5 million tonnes, much of which concentrating in Asia and Africa (FAOSTAT, 2016). In India, rice is being grown in 44.10 Mha area with production of 106.5 million tonnes and productivity of 3.52 MT/ha (USDA, 2016). In Tamil Nadu, rice is grown in an area of 20.16 lakh hectares with the production of 62.53 lakh million tonnes with the average productivity of 3,102 kg/ha (INDIASTAT, 2015). The highly valuable crop is pressurized by diverse fungal and bacterial attacks (Khan, 2009). Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the most important and oldest known bacterial disease of rice in Asia (Hasan Naqvi *et al.*, 2014) and the most serious bacterial diseases in many of the rice growing regions of the world (Xu *et al.*, 2010). The bacterial leaf blight of rice is caused by *Xanthomonas oryzae* pv. *oryzae* and also known to be caused by *Pantoea* (Lee *et al.* 2010; Mondal *et al.* 2011). *Pantoea* spp. are opportunistic pathogens

documented to cause different diseases in economically important crop plants including grain discoloration of rice in China (Yan *et al.* 2010), leaf blight and bulb decay of onion in the United States (Schwartz and Otto, 2000) and leaf blight of rice reported in Korea (Lee *et al.* 2010), India (Mondal *et al.*, 2011), Venezuela (Gonzalez *et al.* 2015), Benin and Togo (Kini *et al.* 2017). *Pantoea ananatis* was reported as the causal agent of the newly emerged rice leaf blight disease reported in northern India (Mondal *et al.*, 2011).

II. MATERIALS AND METHODS

2.1. ISOLATION

The diseased leaves of rice showing typical bacterial blight (BLB) symptoms were collected in brown paper bags from seventeen places of Tirunelveli, Tuticorin, Madurai and Kanyakumari districts and maintained as different isolates.

The diseased portion along with adjacent healthy tissues were cut into 1.5 to 2 cm pieces separately. These diseased pieces of each isolate were surface sterilized separately for 30 seconds in 0.1 per cent mercuric chloride (HgCl₂) solution followed by three subsequent washing with sterilized distilled water in aseptic condition to remove the traces of HgCl₂. Then the pieces were kept on microscopic slide and were further cut with the help of sterilized blade. Then the cut pieces were placed on wakimoto's potato semi synthetic medium. The inoculated plates were incubated at room temperature (27 ± 2°C) for 48 hours. The bacterial colonies with typical straw or yellow colored with smooth margin and raised nature were transferred to the nutrient agar slant and maintained as isolates.

To prove the Koch's postulate and to confirm the pathogenic nature of isolated bacterium, pathogenicity test was carried out. The isolates were multiplied in nutrient broth medium and the pathogenicity test was carried out on rice plants in net house by employing clip inoculation technique.

2.2. BIOCHEMICAL CHARACTERIZATION

In order to characterize the bacteria, the following biochemical tests were carried out.

2.2.1. Gram staining

Bacteria were heat fixed on a glass slide treated with (0.5%) crystal violet for 30 seconds then washed with tap water and treated with iodine solution for 1 min, washed again and decolorized with 95 per cent ethanol for 30 seconds. Then washed again and counter stained with safranin for 1 min. While observing under microscope, gram negative bacteria stained red whereas gram positive bacteria retained color of crystal violet (Jonit *et al.*, 2016).

2.2.2. KOH test

The bacterial culture taken in test tube was vigorously stirred in drop of 3% KOH solution. The thread like slime formation will be indicated by the presence of gram negative bacterium (Jonit *et al.*, 2016).

2.2.3. Catalase test

One colony from pure culture was taken out and put on the slide. One drop of 3 per cent hydrogen peroxide was placed on the colony. The production of bubble gives positive result (Jonit *et al.*, 2016).

2.2.4. Citrate utilization test

Basal medium [Sodium chloride: 5g; Sodium citrate: 2g; Agar: 15g; Ammonium dihydrogen phosphate: 1g; Dipotassium phosphate: 1g; Magnesium sulfate: 0.2g; Bromothymol blue: 0.08g] was prepared and sterilized. Bacterial inoculum was taken from the center of the well isolated colony and placed on the medium and incubated aerobically at 35 to 37°C for 4-7 days. Color change from green to blue will indicate the nature of bacterium (Hafiz Muhammad *et al.*, 2015).

2.2.5. Production of yellow pigment on YDC medium

YDC agar medium was prepared and poured into petridish. The bacterium was inoculated on the plate and then incubated at 28°C for 24 h. The production of yellow pigment on the plate gives positive result (Haliatur Rahma *et al.* 2014).

2.2.6. Anaerobic growth test

Basal medium [Peptone: 2g; NaCl: 5g; Agar: 0.3g; KH₂PO₄: 0.3g; bromothymol blue: (3g) in 1% aqueous solution, 5ml] was prepared. An amount 0.5 ml 10% glucose suspension was added to each tube aseptically. For each isolate two test tubes were inoculated and incubated at 28°C. Color change occurred from blue to yellow indicates the anaerobic growth of the bacteria (Jonit *et al.*, 2016).

2.2.7. Starch hydrolysis test

Starch is an insoluble polymer of glucose, some bacteria possess the ability to produce amylase that breaks starch into maltose and the amylase is an extra cellular enzyme which is released from microorganism. Starch agar plates (soluble starch 2 g/l; peptone 5 g/l; beefs extract 3 g/l; agar 20 g/l dissolve the nutrient agar powder in water by heating and dissolve the starch in 10ml distilled water and add to molten agar) were inoculated by streaking the bacterial isolates and incubated for 4 days at 27±2°C. Plates were flooded with Lugol's iodine solution and observed for appearance of clear zone of hydrolysis around the bacterial growth which indicates that the starch has been hydrolyzed (Lelliot and Stead, 1987).

2.2.8. Tween 80 hydrolysis

The hydrolytic activity of bacterial isolates were done on Tween 80 media. This media has been prepared by adding peptone, NaCl₂.2H₂O, agar in distilled water and pH was maintained at 7.2-7.4 then autoclaved at 121°C for 15

minutes, Tween 80 was mixed in sterilized media. Media was poured into autoclaved Petri plates. After 24 hours these plates were inoculated with fresh bacterial culture and incubated at 28°C for 2 days. Positive reaction of milky white precipitation was formed around the colonies (Ishaq Ahmad *et al.*, 2015).

2.3. VIRULENCE TEST

Among 17 isolates, five isolates were tested to know their virulence and the isolates were multiplied on nutrient broth medium. They were inoculated to test their virulence on highly susceptible var. TN-1 and ADT 43 by employing standard clip inoculation method. The appearance of bacterial blight symptoms and their development was recorded till the end of the experiment.

2.4. MOLECULAR CHARACTERIZATION

The isolates which show higher level of virulence have been selected for characterization based on the earlier symptom expression and per cent leaf area blighted.

2.4.1. Preparation of template DNA: It is important to use a pure cultured bacterium for the identification. The colonies were picked up using a sterilized toothpick and suspended in 0.5 µl of sterilized saline in a 1.5 ml centrifuge tube and which was centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). Incubated at 56°C for 30 min and then heated up to 100°C for 10 min. After heating, supernatant can be used for PCR.

2.4.2. PCR: 1 µl of template DNA was added into 20 µl of PCR reaction solution. 518F/800R primers used for bacteria and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec and 72°C for 60 sec was performed. DNA fragments were amplified about 1,400 bp in the case of bacteria. It includes a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

2.4.3. Purification of PCR products: Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

2.4.4. Sequencing: The purified PCR products of approximately 1,400 bp were sequenced by using the primers (785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

III. RESULTS AND DISCUSSION

The diseased leaves of rice showing typical bacterial blight (BB) symptoms were visually observed and collected in brown paper bags from Tirunelveli, Tuticorin, Madurai and Kanyakumari districts. The typical

symptoms of BLB such as yellowing symptoms or one to two orange or brown stripes on one or both halves of the leafblade were visually observed and critically recorded. Similarly, Mondal *et al.*, (2011) revealed that the symptom exhibited as water soaked lesions at the tip of rice leaves and turned light brown, exhibiting a blighted appearance. Upon isolation, symptomatic leaf pieces were surface-sterilized and macerated in sterile water and maintained as 17 isolates. Upon plating on semi selective peptone-sucrose-agar (PSA) medium, yellow pigmented straw to yellow colored, raised and translucent with smooth margin colonies were obtained after incubation at 28°C for 1 or 2 days. The bacteria are gram-negative, facultative anaerobes with small rods either arranged singly or in chains.

The pathogenicity test revealed that inoculated rice leaves exhibited bacterial blight symptoms similar to those produced under natural field condition. Thus, isolated bacteria proved pathogenic to rice beyond doubt satisfying Koch's postulate. Similarly, Kini *et al.*, (2017) also reported that the leaves inoculated with bacterial suspension showed typical BLB-like lesions and the reisolated bacteria from diseased leaves also yielded colonies.

All the isolates showed positive results in gram staining, KOH test, starchhydrolysis, anaerobic growth test, citrate utilization test and production of yellow pigment on yeast dextrose chalk agar medium except isolates numbering 5,10,11,12,14 and 17 (Table 1). Similar results were obtained by Mondal *et al.* (2011) and Gonzalez *et al.* (2015). Anaerobic growth test is a key test for the identification of the bacterial genera *Erwinia* and *Pantoea*. Most of the isolates exhibit anaerobic growth which indicated clearly that the organism belongs to Enterobacteriaceae (i.e. facultative anaerobes) not belongs to Xanthomonadaceae family (i.e. True aerobes). The production of yellow pigmentation on YDC medium indicates that the isolate belongs to *Pantoea* species. Similar results were observed by Pérez-y-Terrón *et al.* (2009) and Haliatur Rahma *et al.* (2014).

The results of virulence test indicated that hundred per cent infection was caused by all the isolates in TN 1 and ADT 43, the susceptible check varieties. Maximum of 80 per cent leaf blight was observed in Isolate 1, Isolate 3 and Isolate 4 in TN1 where as it was 70, 80 and 60 per cent respectively in ADT 43. (Table 2).

The present results are in confirmation with the findings of Gopinathan *et al.* (1991) who reported that the pathogen shows variable virulence on different cultivars. Some of the biochemical tests gave overlapping results regarding the identity of the causal organism of bacterial blight. For these reasons, PCR was performed for three virulent isolates, Based on 16S rRNA sequence analysis,

the causal agent was identified as *Pantoea stewartii* subsp. *indologenes*. The sequencing data obtained has been deposited in NCBI gene bank with accession no. SUB2733370: MF163273 (ASD 16), MF163274 (TN 1), MF163275 (CO 43). The alignment showed maximum (99%) homology with the related sequence in the data bank.

These sequences were further confirmed by constructing the phylogenetic tree to correlate with the family tree of those species. The culture sequence obtained were subjected to BLAST analysis, the phylogenetically similar type strains sequence and other phylogenetic related sequence were selected from the GenBank and they were subjected to multiple sequence alignment and then align sequences were trimmed to similar length in nucleotides and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6. In the tree, the numbers at the nodes indicate the levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support] based on a neighbor-joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated and bar 0.005 substitutions per site which are depicted in Fig.1.

It is concluded that the pathological investigations of the BLB of rice were undertaken by recording the natural symptoms appeared in the field. The microscopic examination and repeated isolation from BLB samples revealed the presence of bacteria and the disease was thought to be caused by *Xanthomonas oryzae* pv. *oryzae*, rice bacterial blight causing pathogen. The pathogenicity test was proved to be positive on rice and satisfied Koch's postulate. The biochemical and molecular analysis revealed that the causal agent was not *Xanthomonas oryzae* pv. *oryzae*, but it was *Pantoea stewartii* subsp. *indologenes*. Similar results were reported by Lee *et al.* (2010), Mondal *et al.* (2011), Gonzalez *et al.* (2015) and Kini *et al.* (2017). The present finding tallied with the report of Kini *et al.* (2017) who published a paper on 'New bacterial leaf blight of rice caused by *Pantoea ananatis* and *Pantoea stewartii* in Benin'. Earlier, Mondal *et al.* (2011) also submitted a first report on 'New leaf blight of rice caused by *Pantoea ananatis* in India'. Likewise, 'First report of leaf blight caused by *Pantoea agglomerans* on rice was published in Korea by Lee *et al.* (2010). The present study proved that the leaf blight of rice can also be caused by species of *Pantoea*. To our knowledge this is the first report of new bacterial leaf blight of rice caused by *Pantoea stewartii* subsp. *indologenes* in Tamil Nadu, India.

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Table.1: Biochemical characterization of the isolates

Isolates	Gram staining	KOH test	Starch hydrolysis	Anaerobic growth test	Tween 80 hydrolysis test	Catalase test	Citrate utilization test	Production of yellow pigment on YDC
1	-	+	+	+	+	+	+	+
2	-	+	+	+	+	+	+	+
3	-	+	+	+	+	+	+	+
4	-	+	+	+	+	+	+	+
5	+	-	-	-	-	-	-	-
6	-	+	+	+	+	+	+	+
7	-	+	+	+	+	+	+	-
8	-	+	+	+	+	+	+	-
9	-	+	+	+	+	+	-	-
10	+	-	+	-	-	-	-	-
11	+	-	-	-	-	-	-	-
12	+	-	-	-	-	-	-	-
13	-	+	+	+	+	+	-	-
14	+	-	-	-	-	-	-	-
15	-	+	+	+	+	+	-	-
16	-	+	+	+	+	+	-	-
17	+	-	-	-	-	-	-	-

Table.2: Disease severity due to virulence of the isolates

Treatment. No	Isolates from different varieties	Per cent plant infected		Per cent leaf area blighted	
		TN 1	ADT 43	TN 1	ADT 43
1	TN 1	100	100	80(9*)	70(9*)
2	Navarai	100	100	20(5)	20(5)
3	ASD 16	100	100	80(9)	80(9)
4	CO 43	100	100	80(9)	60(9)
5	ADT 43	100	100	40(7)	50(7)
6	Control	0	0	0(0)	0(0)

*Data in parenthesis are disease grades

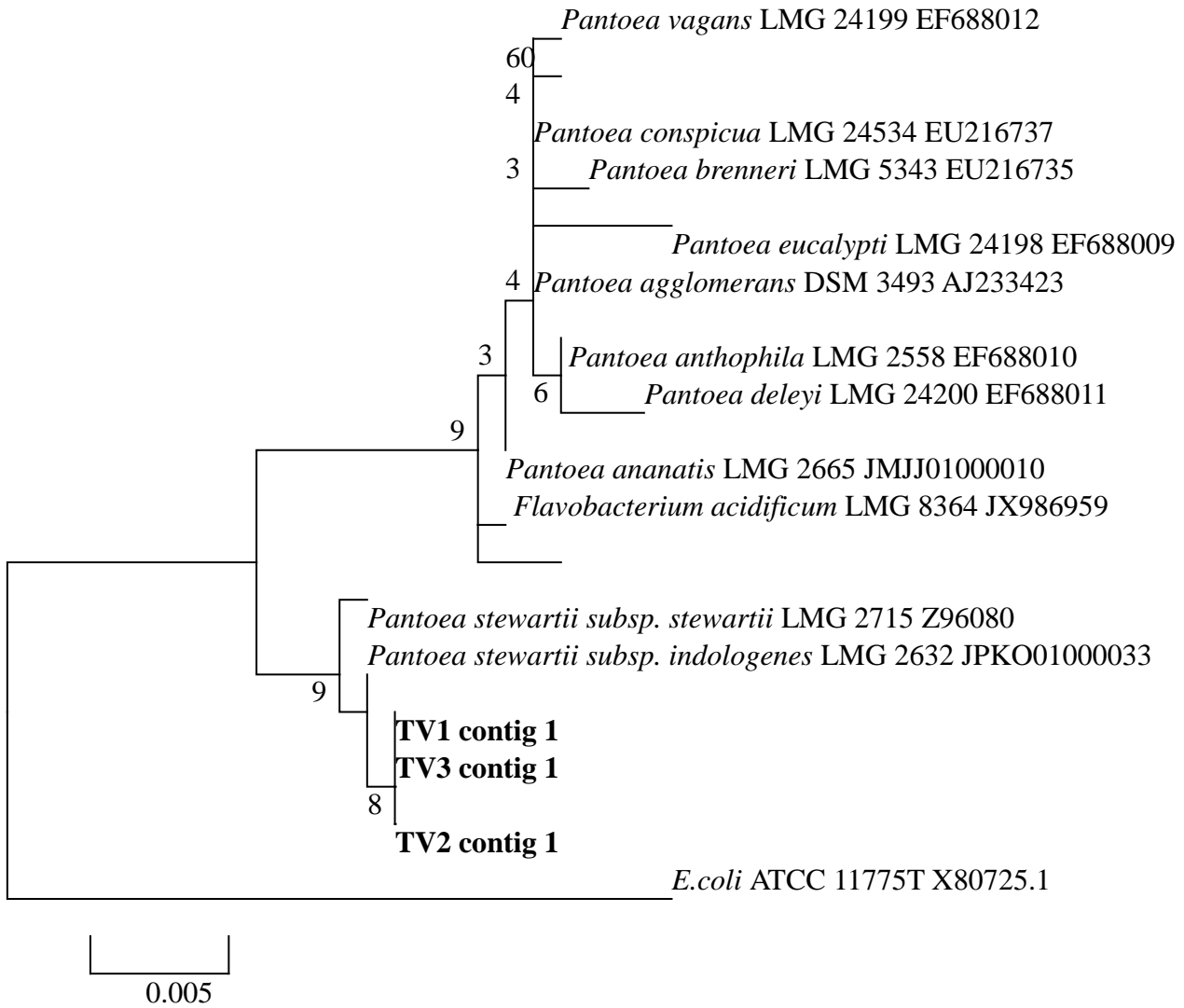


Fig.1: Phylogenetic tree of *Pantoea* genus alongwith new accessions