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ORIGINAL ARTICLES

Isolation, characterization and molecular identification of seed-borne bacterial pathogens of rice in Bangladesh

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ABSTRACT

Rice is attacked by many pathogens including seed-borne bacteria resulting considerable amount of yield loss. The current experiment was conducted to isolate, characterize and molecularly identify the seed-borne bacteria from the infected rice grain. A total of 60 bacterial isolates was isolated from the 60 rice samples collected from Gazipur, Tangail and Mymensingh district during 2018-19 and 2019-20 rice growing seasons (Aman and Boro). Out of 60 bacterial isolates from rice, 20 isolates namely B1, B2, B8, B9, B10, B13, B14, K3, K8, T4, T5, T6, T10, T14, T16, M3, M5, M9, M10, and M13 produced grain rot symptoms. Among 20 isolates, 9 isolates namely B9, B10, B13, K8, T5, T16, M3, M10 and M13 produced blight symptoms along with grain rot. They showed yellow in colony colour and positive in KOH solubility test, catalase test, growth at 36 °C, gelatin liquefaction test and mucoid growth on Yeast extract-dextrose-$CaCO_3$ (YDC) medium whereas negative in oxidative test, urease production, and fermentation of glucose tests. Based on results, nine isolates were identified as *Xanthomonas oryzae*. Rest 11 isolates namely B1, B2, B8, B14, K3, T4, T6, T10, T14, M5 and M9 showed white or greyish white in culture condition, positive in KOH solubility test, catalase test, growth at 36 °C, urease production and gelatin liquefaction test, but negative in oxidative test, fermentation of glucose and mucoid growth on YDC tests, identified as *Burkholderia glumae*. To verify the results of physiological and biochemical tests, representative five yellow and five white or grayish white colony colour isolates then characterized by 16S rDNA sequencing. Five yellow colony colour isolates confirmed as *X. oryzae* and the other five white colony coloured as *B. glumae*. Therefore, this finding will help to explore the effective management against the seed-borne bacterial pathogens of rice.

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Introduction

Rice (*Oryza sativa* L.) is the staple food of Bangladesh grown as a major cereal crop. It constitutes about 90% of the total food grain production and occupies 27184038 acres of land in 2016-17 (BBS, 2018). The yield of rice in Bangladesh is about 2.9 ton/ha (BBS, 2018) which is lower than some other rice-growing countries of the world. There are many causes of low yield of rice in Bangladesh of which disease and pest plays a vital role (Fakir, 1982). Rice suffers from more than 60 different diseases worldwide (Ou, 1985; Hossain et al., 2013). In Bangladesh, 32 diseases are known to occur in rice. Among them, 10 are of major importance (BRRI, 2016). Among them, seed-borne diseases are more destructive. Rice seeds are known to harbor of a wide range of fungi and bacteria (Neergaard et al., 1977). Seed-borne fungal pathogens of rice are well studied in Bangladesh (Basak and Mridha, 1988; Bhuiyan, 1990). But reports on seed-borne bacteria are very scanty. Bacterial diseases such as bacterial leaf blight (BLB) and kreshek (*Xanthomonas oryzae* pv. *oryzae*), bacterial leaf streak (*X. oryzae* pv. *oryzicola*), bacterial grain rot (*Burkholderia glumae*), halo blight *(Pseudomonas syringae* pv. *oryzae),* bacterial foot rot (*Erwinia chrysanthemi*), bacterial palea browning (*E. herbicola* and *P. fascovaginae*), glume blotch (*P. syringae* pv. *syringae*) and leaf stripe (*Acidovorax avenae*) are very devastating and can reduce yield of rice at variable extent (Ou, 1985; Agarwal et al., 1990). The major bacterial diseases causing economic losses in Bangladesh are bacterial leaf blight (*X. oryzae* pv. *oryzae*) and bacterial leaf streak (*X. oryzae* pv. *oryzicola*). Bacterial leaf blight under mild infection causes yield reduction ranging from 10-12% (Mew et al., 1993), whereas under severe conditions, it can be as high as 50% (Ou, 1985). Bacterial leaf blight (*X. oryzae* pv. *oryzae*) and leaf streak (*X. oryzae* pv. *oryzicola*) disease appear severely most of the years in Bangladesh. Seed-borne disease causes seed rot, germination failure and seedling mortality as well as disease of adult plants and thus reduce rice production. Therefore, proper characterization and molecular identification of the seed-borne bacteria of rice are needed to explore their adequate management. The present study is conducted to characterize and identify the seed-borne bacteria which ultimately pave the way to find out proper control measures against the seed-borne bacterial diseases.

Materials and Methods

Collection of rice seed samples

Sixty rice seed samples were collected from the farmers of Gazipur, Tangail and Mymensingh district during the 2018-19 and 2019-2020 rice growing seasons (Aman and Boro).

Isolation of bacteria

Bacterial isolates were isolated from the rice seed samples by streak-plate method as described by Schaad (1988). Collected seed samples were macerated in sterilized distilled water and then streaked onto Yeast extract potato dextrose agar (YPDA) media and incubated at 28±2 °C for 48 hours. Well-separated bacterial colonies with distinct colours were picked up and re-cultured on nutrient agar (NA) plates by repeated streaking for pure culture. Isolated bacteria were preserved in sterilized distilled water in screw-capped test tubes containing 50% glycerol for the further study.

Pathogenicity test

Rice plants (BRRI dhan 29) were inoculated at the flowering stage. The bacterial cells from the pure culture of each isolate were suspended properly in sterilized distilled water at a concentration of 10^8 cfu/ml and then sprayed over the young panicles of rice plants grown in a pot. Leaves were also inoculated by cutting the leaf below 2-3 cm from the tip and dipping the cut end into the bacterial suspension. Sterilized distilled water was used in case of control. Inoculated plants were covered with polyethylene bags to maintain high humidity for 48 hours. Then inoculated pots were transferred to a net house at 28 °C. Bacterial isolates were re-isolated from the symptoms to satisfy the Koch postulates.

Physiological and biochemical characterization of pathogenic bacterial isolates

A series of physiological and biochemical tests were conducted to characterize and identify the isolates following standard methods (Schaad 1988). Ayers' medium was used in carbon utilization test (Ayers et al., 1919). Two strains *Xanthomonas oryzae* pv. *oryzae* FG6 and *Burkholderia glumae* 2 were used as reference.

KOH solubility test

This test was performed as an alternative to the Gram reaction test. One loopful of bacteria from a fresh culture (24 hours old) grown on YPDA media was mixed homogenously in 2 drops of 3% KOH solution on a clean glass slide with a wire loop. Gram negative bacterial suspension produces a fine thread of slime. However, the Gram positive bacterial suspensions don't produce slimy thread and remain watery (Schaad, 1988).

Catalase test

One loopful of young bacterial culture and a drop of 3% H_2O_2 were taken on a clear glass slide. The solution was then mixed with the culture. According to the description of Hayward (1992), the production of air bubbles indicated a positive reaction.

Oxidase test

One loopful of fresh bacterial cells was rubbed on a filter paper soaked with freshly prepared 1% (w/v) aqueous tetramethyle-p-phenylenediamine solution. Oxidase positive microorganisms change the colour to dark purple within 5 to 10 seconds, delayed oxidase positive when the colour changes to purple within 60 seconds and negative if the colour develops after 60 seconds (Schaad, 1988).

Oxidative-Fermentative (O-F) test

The O-F medium (Hugh and Leifson, 1953) was stab inoculated in two tubes for each isolate with a loopful of freshly cultured (24 hours) bacterial cells. In one tube, inoculated medium was covered with liquid paraffin to a depth of 8-10 mm. Tubes were then incubated at 28 ^oC for 48 hours and observed for the production of acid demonstrated by yellow colour. Aerobic organisms produced the acid reactions in the uncovered medium only.

Gelatin liquefaction test

The medium was prepared using beef extract 3.0 g, peptone 5.0 g, and gelatin 120 g in 1L distilled water. Test tubes containing medium were stab inoculated with fresh culture (24 hours old) grown on nutrient agar and incubated at 30 ^oC. After 3, 7 and 14 days the tubes were placed at 4 ^oC for 30 min before the recording of the result. Test tubes when gently tipped the gelatin flowed easily which indicates hydrolysis had taken place by the bacterium, denoted as the positive reaction for the test (Schaad, 1988).

Growth at 36 ^oC

Freshly cultured (24 hours old) bacterial suspension was inoculated into the tubes containing 5 ml yeast solution (YS) broth along with wire loop. Then the tubes were placed in shaking hot water bath for maintaining a temperature of 36 ^oC. Bacterial growth was observed for 3-7 days (Schaad, 1988).

Urease production

A modified YS broth was prepared by adding 0.5 g NH_4PO_4, 0.5 g K_2PO_4, 0.2 g $MgSO_4.7H_2O$, 5.0 g NaCl, 1.0 g yeast extract, and cresol red 0.016 g dissolved in 1 L distilled water. The medium was autoclaved and then filter sterilized urea solution was added to that medium to give it a 2% final concentration. Five milliliter of that medium was poured into each sterile test tubes and were inoculated with bacterial cells of fresh culture and incubated at 30 ^oC. An increase in alkalinity indicated by magenta red colour was the evidence of urease activity (Schaad, 1988).

Mucoid growth on YDC

Suspension of 24 hour bacterial cells were streaked onto the Yeast extract Dextrose Calcium Carbonate

(YDC) medium and incubated at 30 °C for two days to observe the growth (Schaad, 1988).

Carbon source utilization test

Carbon source utilization test was performed by adding a carbon source to mineral based medium of Ayers et al. (1919). To prepare the medium $NH_4H_2PO_4$ 1 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.2 g, agar 12 g, and bromocresol blue (1.5% alcohol solution) 1 ml was dissolved in 1 L distilled water. The pH of the medium was adjusted at 7.0. Utilization of carbon sources was determined by adding agar to medium incorporating 1% (w/v) of each carbon source. Carbon stock solutions were added by filter sterilization through millipore injection. Suspension of 24 hours grown bacterial cells on YPDA was streaked onto each medium, incubated at 30 °C and observed at 3, 7 and 14 days after inoculation. The base medium without addition of any carbon source was considered as a control (Schaad, 1988).

Analysis of 16S rRNA gene sequencing

Extraction of genomic DNA

Selected five yellow (B9, B10, K8, T16, and M10) and five white/grayish white (B1, K3, T6, T14 and M9) colony coloured isolates were used for 16S rRNA gene sequencing**.** The overnight broth culture (max. 2×10^9 cells) was harvested in a microcentrifuge tube by centrifuging for 10 min at 5000 rpm, and the supernatant was discarded. The bacterial pellets were used for the extraction of DNA. DNA was extracted by CTAB method. The extracted DNA was then run into the PCR.

Amplification of the 16S rRNA gene

Amplification of the 16S rRNA region was carried out using universal primers, including forward primer 27F (AGAGTTTGATCCTGGCTCAG) and reverse primer 1492R (GTTACCTTGTTACGACTT) (Sukara et al., 2014). PCR amplification was performed in a 20 µL reaction mixture containing 10 µL of 1X PCR Master-Mix (Promega, USA), 1 µL of 27F primer mix, 1 µL of 1492R primer mix, 1 µL of gDNA template and 7 µL ultra-pure water. PCR amplification was carried out using a thermal cycler (Gen Atlas, G2, Astec, Japan) following PCR amplification conditions: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 48 °C for 30 seconds, extension at 72 °C for 90 seconds, and final extension at 72 °C for 5 min. PCR products were purified using the Gene Jet PCR Purification Kit following the manufacturer's protocol.

Sequencing and phylogenetic tree development

The amplified 16S rRNA PCR products were sequenced by First BASE laboratories, Malaysia (First BASE, Kuala Lumpur, Malaysia) Sequencing was conducted using Applied Biosystems' BigDye v3.1 kit and by following the method of Sanger et al. (1997). The sequences of the isolates were edited using BioEdit 7.2 software. Sequence homologies of the isolates were examined by comparing the sequences obtained with 16S rRNA and sequences deposited in the nucleotide databases of the GenBank (NCBI) using the basic local alignment search tool (BLAST) program, and gene accession numbers were obtained. The representative sequences of the yellow colored isolates (B9, B10, K8, T16, and M10) and white/grayish white isolates (B1, K3, T6, T14 and M9) were then aligned using ClustalW of the MEGA 11 package, and the phylogenetic tree was built using the neighbour-joining method (Hall, 2013). The stability of the tree was assessed by the bootstrap method using 1000 replications.

Results and Discussion

Isolation of bacteria and their pathogenicity test

Altogether 60 bacterial isolates were isolated during 2018-19 and 2019-2020 from rice seed samples collected from the farmers of Gazipur, Tangail and Mymensingh districts of Bangladesh. Bacterial isolates were yellow and white/grayish white in colony colour (Fig. 1). In the pathogenicity test, out of 60 bacterial isolates from rice, 20 isolates namely B1, B2, B8, B9, B10, B13, B14, K3, K8, T4, T5, T6, T10, T14,

T16, M3, M5, M9, M10, and M13 produced grain rot symptoms. Among them, nine isolates namely B9, B10, B13, K8, T5, T16, M3, M10, and M13 also produced blight symptoms on leaves (Fig. 2). These nine isolates had yellow colony whereas the rest 11 isolates (B1, B2, B8, B14, K3, T4, T6, T10, T14, M5, and M9) were in white/grayish white (Table 1).

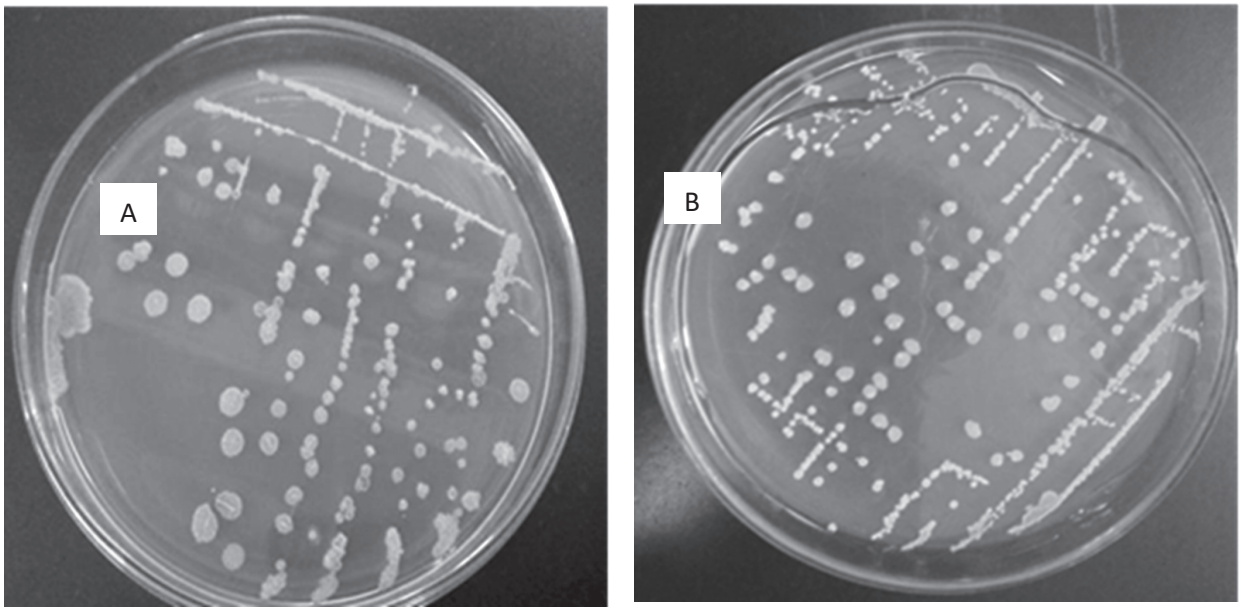

Fig. 1. Bacterial isolates showing Yellow (A) and White/Grayish white (B) colour colonies.

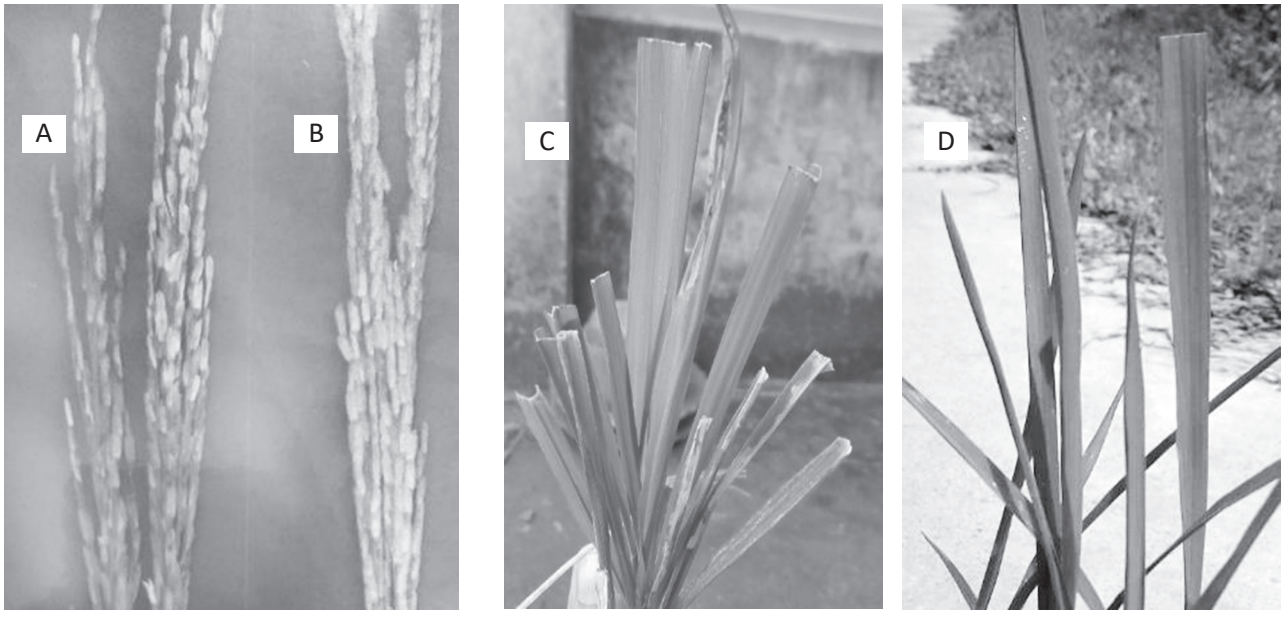

Fig. 2. Pathogenicity test of bacterial isolates. (A) grain rot in inoculated panicle; (B) control, no grain rot symptoms; (C) leaf blight symptom in inoculated leaves, and (D) control (no blight symptoms).

Table 1. List of bacterial isolates produced grain rot and bacterial leaf blight disease symptoms

Isolate no.	Colony colour	Grain rot	Bacterial leaf blight
B1	White/Grayish white	+	-
B2	White/Grayish white	+	-
B8	White/Grayish white	+	-
B9	Yellow	+	+
B10	Yellow	+	+
B13	Yellow	+	+
B14	White/Grayish white	+	-
K3	White/Grayish white	+	-
K8	Yellow	+	+
T4	White/Grayish white	+	-
T5	Yellow	+	+
T6	White/Grayish white	+	-
T10	White/Grayish white	+	-
T14	White/Grayish white	+	-
T16	Yellow	+	+
M3	Yellow	+	+
M5	White/Grayish white	+	-
M9	White/Grayish white	+	-
M10	Yellow	+	+
M13	Yellow	+	+
Control (no bacteria)		-	-

+ = positive in reaction, - = negative in reaction

Physiological and biochemical characteristics of the bacterial isolates

Nine yellow colony color bacterial isolates B9, B10, B13, K8, T5, T16, M3, M10 and M13 showed positive reaction in KOH solubility, catalase test, growth at 36 °C, gelatin liquefaction test and mucoid growth on YDC. Negative reaction was observed for oxidative test, urease production and fermentation of glucose that were similar to reference strain (*X. oryzae* pv. *oryzae* FG6) (Table 2).

Eleven white/grayish white isolates B1, B2, B8, B14, K3, T4, T6, T10, T14, M5, and M9 were positive in KOH solubility, catalase test, growth at 36 °C, urease production, and gelatin liquefaction test. The isolates showed negative reaction in oxidase test, fermentation of glucose and mucoid growth on YDC medium. Reference strain *B. glumae* 2 also showed the similar results (Table 2).

Utilization of carbon sources

Nine yellow colony isolates B9, B10, B13, K8, T5, T16, M3, M10, and M13 and reference strain *X. oryzae* pv. *oryzae* FG6 showed positive results in utilizing glucose, maltose, sucrose, arabinose and ribose as a carbon source. But they were negative in case of utilizing lactose and xylose. In case of control, there was no growth. On the other hand, all the eleven white/grayish white isolates (B1, B2, B8, B14, K3, T4, T6, T10, T14, M5, and M9), and the reference strain *B. glumae* 2 were positive in utilizing glucose, arabinose, xylose and ribose as a carbon source. But were negative in utilizing maltose, sucrose and lactose. Also there was no growth in case of control (Table 3).

Table 2. Physiological and biochemical characteristics of pathogenic bacterial isolates isolated from rice seed samples

Isolates	Diagnostic tests							
	KOH solubility	Oxidase test	Catalase test	Growth at 36 °C	Urease production	Gelatin Liquefaction test	Fermentation of glucose test	Mucoid growth on YDC
Yellow isolates								
B9	+	-	+	+	-	+	-	+
B10	+	-	+	+	-	+	-	+
B13	+	-	+	+	-	+	-	+
K8	+	-	+	+	-	+	-	+
T5	+	-	+	+	-	+	-	+
T16	+	-	+	+	-	+	-	+
M3	+	-	+	+	-	+	-	+
M10	+	-	+	+	-	+	-	+
M13	+	-	+	+	-	+	-	+
X. oryzae pv. *oryzae* FG6	+	-	+	+	-	+	-	+
White/Grayish white isolates								
B1	+	-	+	+	+	+	-	-
B2	+	-	+	+	+	+	-	-
B8	+	-	+	+	+	+	-	-
B14	+	-	+	+	+	+	-	-
K3	+	-	+	+	+	+	-	-
T4	+	-	+	+	+	+	-	-
T6	+	-	+	+	+	+	-	-
T10	+	-	+	+	+	+	-	-
T14		+ - +		+	+	+	-	-
M5	+	-	+	+	+	+	-	-
M9	+	-	+	+	+	+	-	-
B. glumae 2	+	-	+	+	+	+	-	-

+= positive, - = negative.

Physiological and biochemical characteristics and colony color of the bacterial isolates suggested that two kinds of bacterial organism were associated with rice seed samples. Yellow colony color nine isolates B9, B10, B13, K8, T5, T16, M3, M10 and M13 were identified as member of *X. oryzae*. On the other hand, white or grayish-white colored colony of 11 isolates namely B1, B2, B8, B14, K3, T4, T6, T10, T14, M5, and M9 were identified as *B. glumae*.

Molecular identification

Five selected pathogenic yellow colony coloured isolates B9, B10, K8, T16, and M10 and five white/ grayish white colony isolates B1, K3, T6, T14 and M9 were subjected to 16S rRNA gene sequence analyses. The sequence data of 16S rRNA of the isolates were deposited in the Gene Bank (NCBI) and gene accession numbers were obtained. Based on the

Table 3. Utilization of carbon sources by bacterial isolates isolated from rice seed samples

Isolate no.	Carbon Sources							
	Glucose	Maltose	Sucrose	Lactose	Arabinose	Xylose	Ribose	Control
Yellow isolates								
B9	+	+	+	-	+	-	+	-
B10	+	+	+	-	+	-	+	-
B13	+	+	+	-	+	-	+	-
K8	+	+	+	-	+	-	+	-
T5	+	+	+	-	+	-	+	-
T16	+	+	+	-	+	-	+	-
M3	+	+	+	-	+	-	+	-
M10	+	+	+	-	+	-	+	-
M13	+	+	+	-	+	-	+	-
X. oryzae pv. *oryzae* FG6	+	+	+	-	+	-	+	-
White/Grayish white isolates								
B1	+	-	-	-	+	+	+	-
B2	+	-	-	-	+	+	+	-
B8	+	-	-	-	+	+	+	-
B14	+	-	-	-	+	+	+	-
K3	+	-	-	-	+	+	+	-
T4	+	-	-	-	+	+	+	-
T6	+	-	-	-	+	+	+	-
T10	+	-	-	-	+	+	+	-
T14	+	-	-	-	+	+	+	-
M5	+	-	-	-	+	+	+	-
M9	+	-	-	-	+	+	+	-
B. glumae 2	+	-	-	-	+	+	+	-

+= positive, - = negative.

closely related strain from BLAST, bacterial isolates were identified to the species level (Table 4). Yellow colony colour isolates B9, B10, K8, T16, and M10 showed 100 percent identity with the reference strain of *Xanthomonas oryzae* strain LMG 5047 (Accession number: NR_026319.1:1-1494). All the yellow colony colour isolates form a distinct clade with the type species *Xanthomonas oryzae* strain LMG 5047, meaning that all the yellow-coloured isolates were the same species (Fig. 3). On the other hand, white/grayish white coloured isolates B1, K3, T6, T14 and M9 showed identity with the range of 99.00-99.79% with type species *Burkholderia glumae* strain P 1-22-1 (NR_029211.1:3-1418). These white /grayish white colony colour isolates form the sister group relationship with the type species *Burkholderia glumae* strain P 1-22-1 (NR_029211.1:3-1418) (Fig. 4). Thus, the 16S rRNA gene sequence analysis of the isolates confirmed that yellow colony color isolates were the member of *X. oryzae* whereas white/grayish white colony colour isolates were *B. glumae.*

Physiological and biochemical characteristics and colony colour of the bacterial isolates suggested that there were two kinds of bacterial organisms associated with the rice grain samples. Among them nine yellow colony bacterial isolates namely B9, B10, B13, K8, T5, T16, M3, M10, and M13 produced grain discolouration as well as bacterial leaf blight symptom in rice when conducting pathogenicity test. These nine isolates gave similar results as reference strain of *X. oryzae* pv. *oryzae* FG6 causing bacterial leaf blight of rice. Physiological and biochemical results suggested that the isolates B9, B10, B13, K8, T5, T16, M3, M10 and M13 were the member of *X. oryzae* pv. *oryzae*. The bacteriological characteristics of these isolates was consistent with those previously described by Krieg and Holt (1984) and Schaad (1988) for *X. oryzae* pv. *oryzae.*

Eleven bacterial isolates namely B1, B2, B8, B14, K3, T4, T6, T10, T14, M5, and M9, and the reference strain of *B. glumae* 2 were white/grayish white in colour. They showed positive result in catalase test, gelatin liquefaction test, urease production test, protein digestion test, and growth on 36 °C whereas negative in gram reaction test, oxidase test and fermentation of glucose test. These eleven isolates and the reference strain of *B. glumae* 2 showed similar results in other tests also. This study suggested that B1, B2, B8, B14, K3, T4, T6, T10, T14, M5, and M9 isolates were the member of *B. glumae*. The results of this study was similar with those described by Hu et al. (1991) and Khan et al. (2005). Biochemical and physiological techniques were used to characterize the pathogenic strains. Since the molecular characterization tools are more reliable than those of the biochemical and physiological ones (Baghee-Ravari et al., 2011), the results of the present study were validated by the molecular techniques.

Table 4. Identification of pathogenic bacterial isolates using 16S rRNA gene sequences

Isolate name	Microorganism	Accession number	Sequence length	Identity percentage
B1	*Burkholderia glumae*	PP830017	1413	99.79
B9	*Xanthomonas oryzae*	PP830008	1494	100
B10	*X. oryzae*	PP830009	1468	100
K3	*B. glumae*	PP830018	1406	99.00
K8	*X. oryzae*	PP830010	1475	100
T6	*B. glumae*	PP830019	1400	99.00
T14	*B. glumae*	PP830020	1418	99.77
T16	*X. oryzae*	PP830011	1481	100
M9	*B. glumae*	PP830021	1381	99.78
M10	*X. oryzae*	PP830012	1485	100

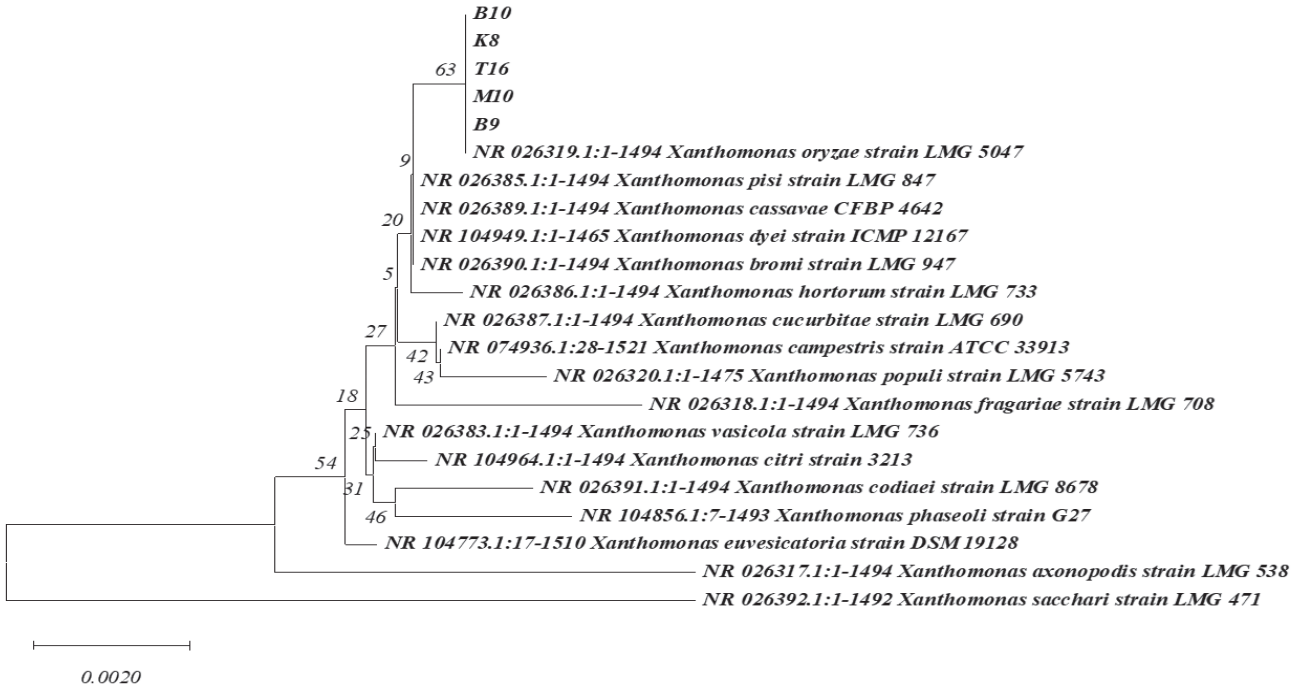

Fig. 3. The phylogenetic tree of five pathogenic yellow isolates B9, B10, K8, T16, and M10 which were constructed using the neighbour-joining method in MEGA 11. The gene sequences of mostly related stains from Gene Bank (NCBI) were used to construct this tree.

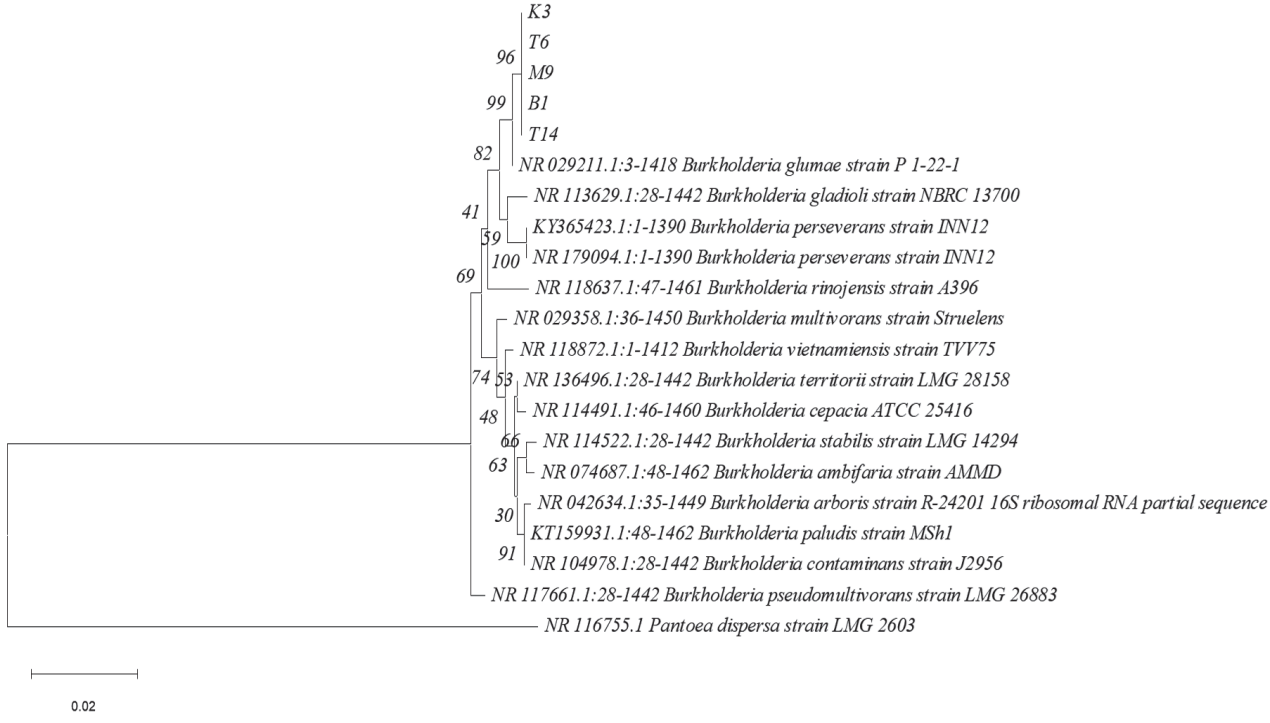

Fig. 4. The phylogenetic tree of five pathogenic yellow isolates B1, K3, T6, T14 and M9 which were constructed using the neighbour-joining method in MEGA 11. The gene sequences of mostly related stains from Gene Bank (NCBI) were used to construct this tree.

Five selected representative yellow colony colour isolates (B9, B10, K8, T16, and M10), and five white/ grayish white colony isolates (B1, K3, T6, T14 and M9) were analyzed by 16S rRNA gene sequence. BLAST search results and phylogenetic tree analysis of the isolates confirmed that the yellow colony colour isolates were the member of *X. oryzae* whereas white/ grayish white colony colour isolates as *B. glumae.*

However, it is challenging to separate pathovars using 16S rRNA gene analysis (Adachi and oku, 2000). For identification of *X. oryzae* at pathovar level Real Time Bio PCR using pathovar specific primer is needed (Cho et al., 2011; Huai et al., 2009; Zhao et al., 2007).

Conclusion

Sixty bacterial isolates were isolated from the rice samples. Among them, 20 isolates were pathogenic, showed grain rot symptoms. Among them, nine isolates namely B9, B10, B13, K8, T5, T16, M3, M10 and M13 were yellow colony colour and caused blight symptoms along with grain rot. Physiological and biochemical results suggested that these isolates were the members of *X. oryzae*. On the other hand, rest 11 isolates namely B1, B2, B8, B14, K3, T4, T6, T10, T14, M5, and M9 causing only grain rot and white/grayish white in colony colour, were identified as *B. glumae*. Based on 16S rRNA gene sequence, representative five yellow-colored isolates B9, B10, K8, T16 and M10 were confirmed as *X. oryzae* whereas white/grayish-white isolates B1, K3, T6, T14 and M9 were as *B. glumae*.

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References

Adachi, N. and T. Oku. 2000. PCR-mediated detection of *Xanthomonas oryzae* pv. *oryzae* by amplification of the 16S–23S rDNA spacer region sequence. *J. Gen. Plant Pathol*. 66 : 303-309.

Agarwal, P. C., C. N. Mortensen and S. B. Mathur. 1990. Seed-borne disease and seed health testing of rice. *Phytopathology* 30: 106

Ayers, S. H., P. Rupp and W. T. Johnson. 1919. A study of the alkali forming bacteria in milk. *US Dept. Agri. Bull*. 782.

Baghaee-Ravari, S., H. Rahimian, M. Shams-Bakhsh, E. Lopez-Solanilla, M. Antúnez-Lamas and P. Rodríguez-Palenzuela. 2011. Characterization of *Pectobacterium* species from Iran using biochemical and molecular methods. *European J. Plant Pathol*. 129(3): 413-425.

Basak, A. B. and A. U. Mridha. 1988. Microflora associated with different varieties of Aman rice collected from Chittagong and Chittagong Hill tracts district of Bangladesh. *Seed Res.* 13: 78-89.

BBS, 2018. Yearbook of Agricultural Statistics 2017. Bangladesh Bureau of Statistics. Ministry of Planning, GoB. 588pp

Bhuiyan, S. A. 1990. Seed-borne nature of *Pyricularia oryza*e Cav. M. Sc. Ag Thesis. Bangladesh Agricultural University. Pp 59.

BRRI, 2016. *Dhan chaser somossa (Bengali).* Bangladesh Rice Research Institute. Pp. 78.

Cho, M. S., M. J. Kang, C. K. Kim, Y. J. Seol, J. H. Hahn, S. C. Park and D. S. Park. 2011. Sensitive and specific detection of *Xanthomonas oryzae* pv. *oryzae* by real-time bio-PCR using pathovar-specific primers based on an rhs family gene. *Plant Dis.* 95 : 589–594.

Fakir, G. A. 1982. An annotated list of seed borne diseases in Bangladesh. Agricultural Information Service. Dhaka, Bangladesh. Pp. 15.

Hall, B. G. 2013. Building phylogenetic trees from molecular data with MEGA. *Mol. Biol. Evol*. 30: 1229-1235.

Hayward, A. C. 1992. Identification of *Pseudomonas solanacearum*. In SAVERANT bacterial wilt training course held on October 5 to November 16 AVRCD. Pp. 101.

Hossain, K. S., M. A. T. Mia and M. A. Bashar. 2013. New method for screening of rice varieties against bakanae disease. *Bangladesh J. Bot*. 42: 315-320.

Hu, F. P., J. M. Young and C. M. Triggs. 1991. Numerical analysis and determinative tests for nonfluorescent plant-pathogenic *Pseudomonas* spp. and genomic analysis and reclassification of species related to *Pseudomonas avenae*. *Int. J. Syste. Evol. Microbiol*. 41: 516-525.

Huai, Y., L. H. Xu, S. H. Yu and G. L. Xie. 2009. Real-time fluorescence PCR method for detection of *Burkholderia glumae* from rice. *Chin. J. Rice Sci.* 23: 107-110

Hugh, R. and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *J. Bacteriol.* 66: 24.

Khan, A. A., M. W. Amin and I. H. Mian 2005. Grain rot (*Burkholderia glumae*) of rice in Bangladesh. *Bangladesh J. Seed Sci. Tech.* 9: 125- 128.

Kreig, N. R. and J. G. Holt. 1984. *Burgeey's manual of systemic bacteriology*. Vol I Williams and Wilkins, London.

Mew, T. W., A. M. Alvarez, J. E. Leach and J. Swings. 1993. Focus on bacterial blight of rice. *Plant Dis*. 77: 5-12.

Neergaard, P. L., A. K. Ambat and S. B. Marthur. 1977. Seed health testing of rice. Testing procedures for detection of *Pyricularia oryzae*. *Proc. Int. Seed Test. Assoc*. 35: 157-163.

Ou, S. H. 1985. *Rice Diseases*. 2nd ed. Commonwealth Mycological Institute, Kew, Surrey.

Sanger, F., S. Nicklen and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences.* 74: 5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Schaad, N. W. 1988. *Laboratory guide for identification of plant pathogenic bacteria.* 2nd edition. APS press, St. Paul, MN, USA.

Sukara, E., A. Salamah, A. Dinoto, and W. Mangunwardoyo. 2014. Identification of lactic acid bacteria in sayur asin from Central Java (Indonesia) based on 16S rDNA sequence. *Intern. Food Res. J*. 21: 527-532.

Zhao, W. J., S. Zhu, X. L. Liao, H. Chen and T. W. Tan. 2007. Detection of *Xanthomonas oryzae* pv. *oryzae* in seeds using a specific TaqMan probe. *Mol. Biotechnol*. 35 : 119-127.