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# First Report of Soft Rot Disease of Papaya Caused by *Klebsiella variicola* in Bangladesh

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Twelve bacteria were isolated from soft rotten papaya collected from markets of different locations of Gazipur district, Bangladesh. Among these, five isolates (CP01–CP05) cased soft rot symptoms on potato slices. The isolates also produced dark brown to blackish characteristics soft rot symptoms on papaya fruit by artificial inoculation. The isolates were Gram–negative and also negative in oxidase, methyl red, arginine dihydrolase, gelatin liquefaction and indole tests. All of the isolates were positive in catalase, oxidative fermentative, nitrate reduction, acetoin and urease production tests. The isolates grew well at 41°C and 5% of salt concentration. They utilized lactose, glucose, rhamnose, sucrose, melibiose, arabinose, mannitol, inositol, sorbitol and citrate but not adonitol as sole sources of carbon. Phylogenetic analysis based on 16 S rRNA gene sequence indicated that an isolate CP03 was closely related with *K. variicola*. These results suggested that the isolates from the diseased papaya were *K. variicola*. This study reports for the first time *K. variicola* causing soft rot of papaya in Bangladesh.

**Key words**: Soft rot, papaya, *Klebsiella variicola*, Bangladesh

## INTRODUCTION

Papaya (*Carica papaya*) is very nutritious and commonly used as fruits and vegetables. It is infected by various pathogens including fungi, bacteria and virus. Among these infectious diseases, soft rot caused by bacterial pathogen is the one of the most common and destructive disease causing severe economic loss.

Several bacterial species are known to macerate tissues of a wide range fruits and vegetables enzymatically. Species of *Erwinia* belonging to the "*carotovora*" group including *Erwinia carotovora* (synonym: *Pectobacterium carotovorum*), *E. chrysanthemi* (synonym: *Dickeya dadantii*), are usually referred as the soft rot bacteria. Moreover, pseudomonads such as *Pseudomonas viridiflava* and *P. marginalis* were reported to cause soft rot of different fruits and vegetables (Perombelon and Kelman, 1980; Kim *et al*., 2002). Although *Klebsiella* species are isolated from clinical samples, the bacteria are associated with several plants such as banana, rice, sugarcane and maize (Rosenblueth *et al*., 2004). *K. variicola* was reported to cause soft rot of banana in China (Fan *et al*., 2016) and *K. pneumoniae* induces soft rot of onion (Liu *et al*., 2015).

In Bangladesh, *P. carotovorum*, *D. dadantii* and *Pseudomonas* species were reported in various fruits and vegetables as soft rot bacteria (Meah and Khan, 1987; Hossain, 2016).

In this study, several bacteria were isolated from soft rotted papaya in Bangladesh. Although *P. carotovorum* was reported to cause soft rot in papaya (Himel *et al*., 2016), the isolates were different from *P. carotovorum* in the preliminary tests. There is no report showing other bacterial pathogens cause soft rot in papaya in the country. Therefore, identification of the bacterial pathogen was carried out in this study.

## MATERIALS AND METHODS

### Isolation of bacterial isolates from soft rotted papaya

Bacteria were isolated from the diseased papaya fruits collected from markets of different locations of Gazipur district, Bangladesh by the previously described method (Mortensen, 1997). In brief, small part from the margin of rotted tissues was cut and surface sterilized with 1% sodium hypochlorite (NaOCl) for 2–3 min. Sterilized samples were washed several times with sterilized distilled water to remove the residual sodium hypochlorite. The samples were placed in Petri dishes containing sterilized distilled water and were crushed with a sterile scalpel. After crushing, the Petri dishes were kept undisturbed for 10–15 min to release the bacteria associated with rotted tissues. One loopful of the resulting suspension was streaked on YPDA (yeast extract peptone dextrose agar medium: yeast extract 3 g, peptone 0.6 g, dextrose 3 g, agar 15 g, distilled water 1 litter, pH 7.2) plate. The plates were incubated at 30°C for 48 h. Preferential individual bacterial colonies that appeared on plate were picked and re–streaked on another fresh plate to obtain pure culture. The isolates were preserved in 30% glycerol and stored at –20°C for further study.

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**Potato tissue maceration test**

All of the bacterial isolates originated from single colonies were tested for their ability to macerate tissue using potato (De Boer and Kelman, 2001), since ability of tissue maceration correlates with the pectolytic characteristic and pathogenicity. Potato tubers were sterilized with 70% ethyl alcohol, rinsed in sterilized distilled water and aseptically cut into slices. The potato slices were put in Petri dishes containing sterilized filter paper impregnated with 2 ml of sterilized distilled water. The potato slices were inoculated with the isolates by the needle pricking method. The inoculated slices were maintained in moistened Petri dishes and incubated at 30˚C for 2 days. The bacterial isolates caused soft rot on potato slices were selected for further studies.

**Pathogenicity test of bacterial isolates on papaya**

Healthy papaya fruit was used for pathogenicity test. Papaya fruit was sterilized with 70% ethyl alcohol, rinsed in sterilized distilled water and aseptically punctured with multiple needles. Fresh culture of bacteria was inoculated at the injured site. Then, the inoculated papaya fruit was covered with a polyethylene bag to maintain moisture and incubated at 30˚C for 3 days.

**Physiological and biochemical tests to characterize pathogenic bacterial isolates**

A series of physiological and biochemical tests were performed to characterize the bacterial isolates. The tests were Gram reaction (Suslow *et al*., 1982), catalase production (Hayward, 1992), oxidative fermentative test (Hugh and Leifson, 1953), oxidase test (Kovacs, 1956), gelatin liquefaction test (Schaad, 1988), urease production (Schaad, 1988), nitrate reduction test (Lelliot and Dickey, 1984), indole test (Lelliot and Dickey, 1984), acetoin production (Dye, 1968), methyl red test (Schaad, 1988), arginine dihydrolase (Thornley, 1960), growth at 41˚C temperature (Schaad, 1988), growth in 5% NaCl (Schaad, 1988), and utilization of carbon sources (Ayers *et al*., 1919).

**16S rRNA gene sequencing**

A representative bacterial isolate CP03 was selected for 16S rRNA gene sequencing. The bacterial isolate was cultured in nutrient broth at 28˚C in a shaker incubator. Approximately 24 h–old culture was subjected for genomic DNA isolation. Genomic DNA was extracted by using Gene JET Genomic DNA Purification Kit (Thermo Scientific Ltd.) following the manufacturer's protocol. Amplification of the targeted 16S rRNA gene was performed with the universal primer sets 27F (5'–AGAGTTTGATCCTGGCTCAG–3') and 1492R (5'–GGATACCTTGTTACGACTT–3'). Individual PCR mixture contained 6 $\mu$l of 25 mM $MgCl_2$, 10 $\mu$l of 10 × PCR buffer, 2.0 $\mu$l of 10 mM dNTP mix, 5.0 $\mu$l of 20 $\mu$M each primer, 5.0 $\mu$l (150 ng) of DNA template, 1 $\mu$l (5 U/$\mu$l) of *Taq* DNA polymerase and 66 $\mu$l of sterile double–distilled water thus the total volume of 100 $\mu$l. A negative control (without template DNA) was also included in the PCR run. The PCR amplification was performed in a PCR Thermocycler (Eppendrof Ltd.). The condition for PCR was set as follows: an initial denaturation step at 94˚C for 5 min; 35 cycles of a denaturation step at 94˚C for 1 min, an annealing at 55˚C for 40 sec and an extension at 72˚C for 1 min and a final extension step at 72˚C for 5 min. The PCR product was purified by using a commercial Gene JET PCR Purification Kit (Thermo Scientific Ltd.) following the manufacturer's protocol. The purified PCR product was sequenced from the Centre for Advanced Research in Sciences, University of Dhaka, Bangladesh.

**Phylogenetic analysis**

Nucleotide sequences were aligned by the Clustal X (Thompson *et al*., 1997), and the root phylogenetic tree was drawn using the software njplot (Saitou and Nei, 1987). The statistical confidence of the nodes was estimated by bootstrapping using 1,000 resample.

## RESULTS AND DISCUSSION

**Isolation of bacterial pathogens from soft rotted papaya fruits**

A total of 12 bacteria were isolated from soft rotted papaya fruits collected from markets of different locations of Gazipur district, Bangladesh. Among 12 isolates, five isolates designated as CP01, CP02, CP03, CP04 and CP05 induced characteristic soft rot symptoms on potato slices. The rotted tissues were yellow in the center surrounded by dark brown edge (Fig. 1A). The results suggested the papaya isolates had pectolytic ability. These five isolates were tested for pathogenicity on their original host papaya by artificial inoculation. All isolates caused soft rot on papaya (Table 1). The dark brown to blackish rotted symptoms developed on papaya fruits within 2–3 days (Fig. 1B), which were similar to those observed in the natural infected fruits. The same bacterium was re–isolated from the inoculated fruits (data not shown). Colony morphology of the isolates on YPDA was white to grayish white, smooth, round, glistening, convex raised (Fig. 2).


**Fig. 1.** Rotted symptoms developed on potato slice (A) and papaya fruit (B) by a papaya isolate, CP03.

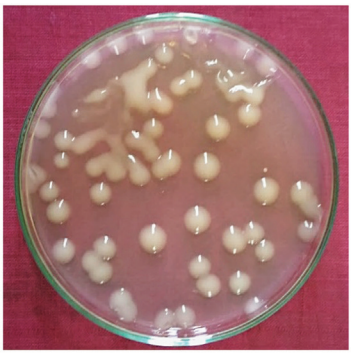

**Fig. 2.** Colony morphology of a papaya isolate, CP03 on YPDA medium.

**Physiological and biochemical characteristics of the isolates**

The isolates were Gram–negative and also negative in oxidase, gelatin liquefaction, indole, methyl red and arginine dihydrolase tests. All of the isolates were positive in catalase, oxidative fermentative, nitrate reduction, acetoin and urease production tests. The isolates were grown well at 41˚C and 5% of salt concentration. They utilized lactose, glucose, melibiose, sucrose, rhamnose, arabinose, mannitol, inositol, sorbitol and citrate but not adonitol as sole sources of carbon (Table. 1).

The results of physiological and biochemical tests of the papaya isolates were consistent with descriptions of Holt *et al.* (1994), Alves *et al.* (2006) and Zheng *et al.* (2014) for *K. variicola*. Bacteriological characteristics

**Table 1.** Physiological and biochemical characteristics of papaya isolates

| Characteristics | Present isolates [1] ($n$=5) | Reference species[2] | | | |
|---|---|---|---|---|---|
| | | *Klebsiella variicola* | *Klebsiella pneumoniae* | *Pectobacterium carotovorum* subsp. *carotovorum* | *Dickeya dadantii* |
| Potato soft rot test | + | nt | nt | + | + |
| Pathogenicity to papaya | + | nt | nt | nt | nt |
| Gram reaction | – | – | – | – | – |
| Catalase | + | + | + | + | + |
| Oxidative fermentative | + | + | + | + | + |
| Oxidase | – | – | – | – | – |
| Gelatin liquefaction | – | – | – | + | + |
| Nitrate reduction | + | + | + | + | + |
| Indole | – | – | – | – | + |
| Methyl red | – | – | – | + | – |
| Acetoin | + | + | + | + | + |
| Urease | + | + | + | – | – |
| Arginine dihydrolase | – | – | – | – | – |
| Growth at 41˚C | + | + | + | + | + |
| Growth in 5% NaCl | + | + | + | + | + |
| Utilization of: | | | | | |
| lactose | + | + | + | + | – |
| glucose | + | + | + | + | + |
| melibiose | + | + | + | + | + |
| sucrose | + | + | + | + | + |
| rhamnose | + | + | + | + | + |
| arabinose | + | + | + | + | + |
| adonitol | – | – | + | – | – |
| mannitol | + | + | + | + | + |
| inositol | + | + | + | + | – |
| sorbitol | + | + | + | – | – |
| citrate | + | + | + | + | + |

+, positive; – , negativ; nt, not tested
1) CP01–CP05
2) Results from Holt *et al.* (1994), Khan *et al.* (2000), Alves *et al.* (2006) and Zheng *et al.* (2014)

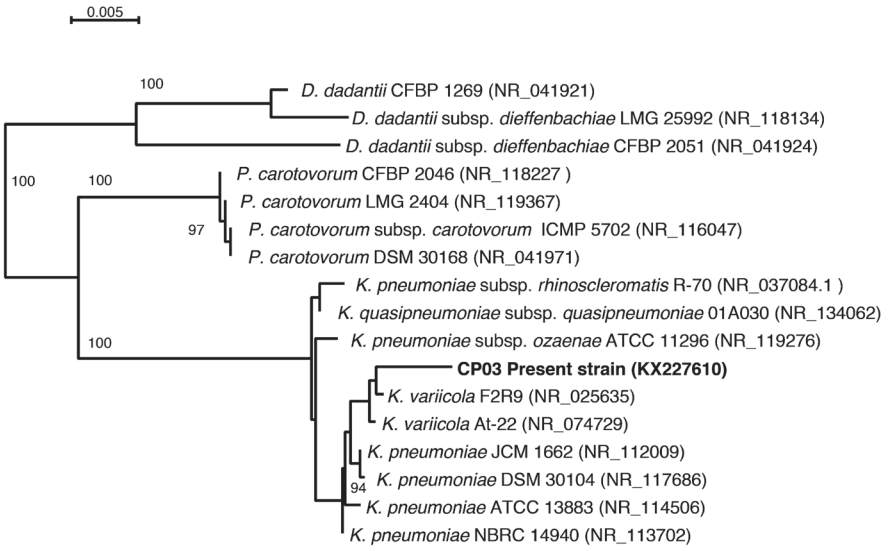


**Fig. 3.** Phylogenetic tree based on 16S rRNA gene sequences. Percentage bootstrap values only higher than 80% of 1,000 replicates are indicated at branching nodes.

of the papaya isolates were different from the characteristics of common bacterial soft rot pathogens *P. carotovorum* and *D. dadantii*; but very close to those of *K. variicola* and *K. pneumoniae*. Papaya isolates did not use adonitol like *K. variicola* but *K. pneumoniae* utilized adonitol as sole source of carbon. Rosenblueth *et al.* (2004) demonstrated that *K. variicola* is a very close species of *K. pneumoniae* but isolated genetically.

**Identification and phylogenetic analysis of bacterial isolate based on 16S rRNA gene sequences**

Analysis of the 16S rRNA gene sequence of the papaya isolate CP03 was performed to identify the bacterium. The partial sequence was deposited in the GenBank with accession number KX227610. The partial sequence of present isolate CP03 showed 99% similarity with 16S rRNA gene sequences of *Klebsiella* species containing *K. pneumoniae*, *K. variicola*.

The related sequences were retrieved from the NCBI and used for phylogenetic analysis.

The papaya isolate CP03 formed a cluster with *Klebsiella variicola* F2R9 and At–22 in the phylogenetic tree based on 16S rRNA gene sequence, but not supported by high bootstrap value (Fig. 3). Based on the results of the physiological and biochemical tests and phylogenetic analysis suggested that papaya soft rot causing isolates were *K. variicola*. For further detailed identification, multi locus sequence typing will be useful (Maatallah *et al.*, 2014)

*K. variicola* and *K. pneumoniae* are reported to cause soft rot in onion and banana (Liu *et al.*, 2015; Fan *et al.*, 2016). Although *Klebsiella* spp. are well recognized as clinical pathogens, previously they were isolated from different plant hosts such as rice, maize, sugarcane, banana, onion etc. (Rosenblueth *et al.*, 2004; Lin *et al.*, 2015; Liu *et al.*, 2015; Fan *et al.*, 2016). The results of this study also revealed the plant pathogenic nature of *K. variicola*. To our knowledge, bacterial soft rot of papaya caused by *K. variicola* is a new disease in Bangladesh. Management of the disease at postharvest level will be the subject for future study.

## AUTHOR CONTRIBUTIONS

S. Hossain conducted experiments and drafted the manuscript. A. A. Khan planed and initiated the research works and edited the manuscript. M. M. Rahman contributed the materials and provide support during molecular works. K. Iiyama performed bioinformatics analysis and edited the manuscript. N. Furuya provided valuable suggestions and edited the manuscript.

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