

Characterization of *Acinetobacter radioresistance* : A New Bacterial Soft Rot Associated Pathogen of Tomato in India

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ABSTRACT

Bacterial soft rot disease is a severe disease of tomato in fields during heavy rainfall and hot temperature conditions. The bacterium isolated from rotten tomato (isolate TMT2) was a gram-negative rod and produced whitish smooth mucoid colonies on nutrient agar (NA). TMT2 was biochemically distinct to class *gammaproteo bacteria*. Molecular characterization using 16s rRNA sequence analysis revealed that the TMT2 isolate closely matching with *Acinetobacter radioresistance*. In cross infectivity assay, the TMT2 infected carrot, radish, tomato, potato, chilli, cabbage, cauliflower, knol-khol, cucumber and capsicum in laboratory conditions. The TMT2 showed the enzymatic activity of pectin lyase, polygalacturonase, cellulase using *in-vitro* plate assay and produced biofilm (O. D. @ 570nm). The antimicrobial susceptibility assay revealed that the pathogen was susceptible to ampicillin-sulbactam, piperacillin / tazobactam, cefepime, gentamicin, tobramycin and levofloxacin. The isolate showed an intermediate reaction to cefotaxime, ceftriaxone, amikacin ciprofloxacin, while resistant to tetracycline and doxycycline. The present study identified the isolate TMT2 as *Acinetobacter radioresistance*, a new soft rot associated pathogen of tomato in India.

Keywords : *Acinetobacter radioresistance*, Cross infectivity, CWDE'S, Biofilm, Antimicrobial susceptibility

TOMATO (*Solanum lycopersicum*) is one of the most important vegetable grown in India. It is rich in minerals, essential amino acids, sugars, vitamins, antioxidants and is consumed in raw, cooked or processed forms. In field conditions, tomato is affected by various biotic agents such as fungi, bacteria, viruses, nematode and viroid. Among the bacterial diseases, soft rot caused by *Pectobacterium caratovorum* directly impacts fruit loss in India (Maisuria and Nerurkar, 2013). The soft rot bacteria are mainly from the *Enterobacteriaceae* family naturally found in soil, which enter the plant through wounds caused by tools, insects, hail and sometimes natural openings. These produce water-soaked lesions, maceration and rotting symptoms on fruit.

Recently, bacterial genera other than *Pectobacterium* from the class *gammaproteo bacteria* evolved as soft rot pathogens, which include *Acinetobacter* (Patro *et al.*, 2006 and Khan *et al.*, 2014), *Enterobacter* (Nishijima & Couey, 1987

and Schroeder *et al.*, 2009), *Klebsiella* (Chandra shekar *et al.*, 2018 and Ajayasree & Borkar, 2018) and *Pseudomonas* (Godfrey & Marshall, 2002 and Krejzar *et al.*, 2008).

The genus *Acinetobacter* is a gram-negative bacterium that is considered ubiquitous, found in soil, waste, water, sewage, sludge, polluted environment, spoiled food and is also associated with plants and animals (Atrouni *et al.*, 2016). The evolution of different species of *Acinetobacter* is quite interesting. As per the previous reports, *A. antiviralis* and *A. lactucae* were found as endophytes associated with tobacco roots and lettuce plants, respectively (Lee *et al.*, 2009 and Rooney *et al.*, 2016), *A. baumannii* and *A. calcoaceticus* reported in sugarcane (Patro *et al.*, 2006) and farm vegetables, respectively (Dahiru & Enabulele, 2015 and Atrouni *et al.*, 2015), which solubilize the minerals in soil (Zamin *et al.*, 2011). *A. radioresistans* was found to be antagonist to leaf rot disease of *Aloe vera* plant (Nayantara &

Saharan, 2017) and also had plant growth-promoting activity in desert plant *Indigofera argentea* (Lafi *et al.*, 2017). The bacterium *Acinetobacter* plant pathogen opportunistically colonises on *Ralstonia* wilted plants (Kay *et al.*, 2002). *Acinetobacter* species showed resistance to many antibiotics (Odsbu *et al.*, 2018) and also *A. baumannii* and *A. radioresistans* were reported to infect human beings (Wang *et al.*, 2019).

Hence the present study aimed to identify and characterize the causal agent of bacterial soft rot of tomato by fulfilling Koch's postulates, phenotypic, biochemical and molecular characterization, host range, virulence, antibiotic resistance.

MATERIAL AND METHODS

Collection and Isolation

The tomato fruits showing depressed and rotting symptoms (Fig. 1a) were collected from the vegetable field (13°04'51.6"N 77°34'00.9"E) of the Horticulture Department, UAS, GKVK, Bengaluru, Karnataka (India). The fruits were placed in sterile plastic zip lock covers and brought to the laboratory for further analysis. The surface microflora was removed by swabbing the fruit surface with 70 per cent alcohol, the lesion length of ~1 sq. cm was cut and treated with sodium hypochlorite (0.5%) for 30 seconds, serially washed with sterile distilled water (SDW). The tissue was macerated to release the bacteria into SDW, and the same suspension was streaked onto Nutrient Agar (NA) plates, incubated at 28 °C for 48 hr. The single colony of white mucoid convex-shaped was purified for further analysis.

Pathogenicity

Pathogenicity was proved according to a previous report by Chandrashekar *et al.* (2018). Collected healthy tomato fruits were disinfected with alcohol (70%), and the pathogenicity was proved by using whole fruit and slices of tomato. The bacterial suspension of 1×10^8 colony forming units (CFU) mL^{-1} of 200 μL was injected into tomato fruit with a sterile hypodermal syringe. The slices were also inoculated with the same concentration by placing the

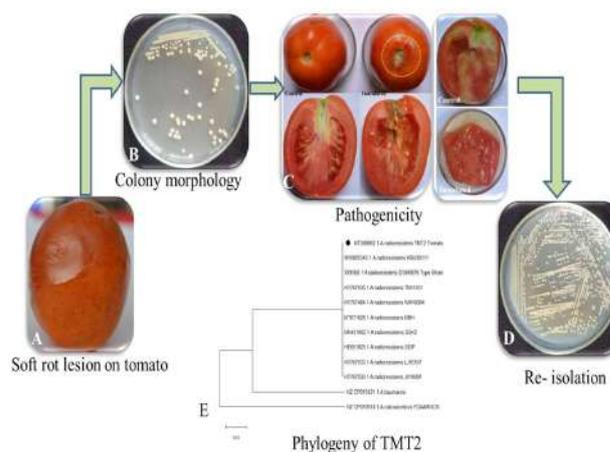


Fig. 1: Koch's postulates of TMT2 isolate infecting tomato

suspension on the cut end surface and water inoculated fruits / slices served as a control. The same fruits / slices were incubated in a plant growth chamber (SANYO Electric Company Ltd., Japan) at 35 °C with 90 per cent relative humidity (RH) for a period of 48-72 hr. The isolates showing rotting symptoms on fruit were re-isolated to fulfil Koch's postulates.

Biochemical Characterization

The biochemical tests *viz.*, gram staining, KOH (3%), growth at high temperature (37 °C), pigmentation on YDCA, muciodness, growth on NaCl (5%) medium, oxidase, catalase, nitrate reductase, oxidative fermentation, phenyl deaminase, acetoin, amylase production, urease production. Utilization of carbohydrates like adonitol, citrate, cellobiose, delucitol, fructose, glucose, lactose, maltose, mannitol, sorbitol, sucrose, xylose was done according to Bergey's Manual of Systematics of Archaea and Bacteria (Whitman and Barnaby, 2015) and Laboratory guide for identification of plant pathogenic bacteria (Schaad *et al.*, 2001).

Molecular Detection through 16s rRNA Gene

Genomic DNA was extracted by a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method, according to Wang *et al.* (2010). The DNA was quantified in nanodrop spectrophotometrically (DeNovix Inc. Wilmington, USA) and confirmed by amplification of 16s rRNA gene by universal primers

Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rp1 3'-ACGGCTACCTTGTTACGACTT-5'). Total of 20 µL PCR reaction containing 10µL of master mix, 2µL each of forward and reverse primers, 2µL of template and rest of 4µL was balanced with nuclease-free water. The PCR conditions include 94 °C for 1min of denaturation, annealing of 60 °C for 1min and extension with 72 °C for 1min 30s with 30 cycles of repetition. The amplified PCR product of 5µL was gel electrophoresed on one per cent agarose and the same product was sequenced (Eurofins Scientific India Pvt. Ltd.). A BLAST search on the NCBI Gene Bank database (www.ncbi.nlm.nih.gov/) was used to identify the isolates.

Cross Infectivity on different Vegetable Slices

A total of ten vegetables viz., carrot, tomato, potato, chilli, cabbage, cauliflower, knol-khol, capsicum, radish and cucumber were used for cross infectivity assay. The disinfected vegetables (70 % alcohol), except chilli, which was used as whole fruit. 200µL of bacterial suspension (10^8 CFU mL^{-1}) was placed on the cut end of the fruit and incubated at 35 °C for 72 hr for symptom expression (Chandra shekar, 2017).

Semi Quantification of Cell Wall Degrading Enzymes and Estimation of Biofilm Formation

The estimation of CWDE's like pectin lyase, polygalacturonase and cellulase was done according to the protocol given by Chatterjee *et al.* (1995) and the zone of inhibition produced on enzyme media was recorded.

The biofilm formation of the isolate was estimated according to Lee *et al.* (2013) and the production of biofilm was measured using a spectrometer at 570nm, compared with un-inoculated control.

Antibiotic Sensitivity Test

The antibiotics mentioned in Table 3 were placed on Mueller-Hinton agar medium (M173, HiMedia, Mumbai, India). The plates were incubated for 24 h and the zone of inhibition was measured in diameter (mm) around antibiotic discs and compared to the

criteria set by the Clinical and Laboratory Standards Institute (CLSI) 2020. The organism was classified as Resistant (R), Intermediate (I) or Susceptible (S).

RESULTS AND DISCUSSION

Isolation of the Pathogen

The tomato fruits showing rotten depressed lesions were collected (Fig. 1a) and the pathogen associated with the disease was isolated. A total of two colonies were recovered after the incubation period. Isolated bacterium showed whitish, non-pigmented, mucoid, smooth, convex, opaque shaped with medium-sized colonies on NA (Fig. 1B). Morphological characters of the isolate were compared with Bergy's manual of systematics of archaea and bacteria (Whitman and Barnaby, 2015). The isolated pathogen was phenotypically similar to *A. radioresistance* which produces smooth, entire, convex, glistening, opaque, and yellowish-white to pale yellow colonies on NA. These colonies were further evaluated for pathogenicity on tomato fruits.

Pathogenicity on Tomato Fruits

Out of two isolates recovered, only one isolate showed symptoms on tomato fruit and slices. In whole fruit inoculation, rotten lesions at the inoculated point formed depression on the surface after 48 h. In cut opened fruit, disintegration and loosening of tissue were recorded around the inoculation area, rotting with a less foul smell (Fig. 1C). However, un-inoculated site tissue was intact and healthy. In slice inoculation assay, rotting started after 24 hr of inoculation. In the time course, the rotting area enlarged (48 hr) and the tissue was macerated with mucous formation followed by watery accumulation after 72 hr. There were no symptoms observed on water inoculated control. The *Acenitobactor radioresistance* is a ubiquitous bacterium (Atrouni *et al.*, 2016). Dahiru and Enabulele (2015) observed *A. baumannii* in bird faeces and many farms irrigated vegetables. *A. baumannii* was associated with the top rot phase of sugarcane red stripe disease in India produced rotting on the top phase due to bud infection (Patro *et al.*, 2006). In our study, the TMT2 isolate was found pathogenic on

tomato fruits causing brown lesions and rotting, leading to tissue maceration.

Biochemical Characterization

The biochemical characteristics of isolate TMT2 were listed in Table 1. The isolate was gram-negative, rod-shaped bacteria and formed a thick mucoid strand with KOH (3%). It was tolerant to high temperatures (37 °C) and salt conditions (5% NaCl).

TABLE 1
Biochemical characteristics of pathogenic isolate TMT2

Biochemical test	TMT2
Gram reaction	-
KOH(3%)	+
Growth at 37!	+
5 % NaCl	+
Colonies on YDCA	whitish
Muciodness	+
Motility	Non-motile
Oxidase	-
Catalase	+
Nitrate reductase	+
Oxidative fermentation	+/+
Acetoin	-
Phenylalanine deaminase	-
Urease	+
Malonate	+
Cellobiose	+
Citrate	+
Glucose	+
Sorbitol	+
Mannitol	-
Lactose	+
Fructose	+
Sucrose	+
Dulcitol	-
Maltose	-
Xylose	+
Adonitol	+

Notes: + positive, - negative

The isolate produced whitish non pigmented mucoid colonies on Luria Bertani agar media. The cells were non-motile, oxidase negative but catalase-positive, produced nitrate reductase enzyme and showed both oxidative and fermentative metabolism. The isolate was negative for acetoin production, phenylalanine utilization and produced urease enzyme to degrade urea. TMT2 utilized many carbohydrates viz., malonate, cellobiose, citrate, glucose, sorbitol, lactose, fructose, sucrose, xylose and adonitol but unutilized mannitol, deucitol and maltose. This isolate was biochemically similar to type strain FO-1, DSM 6976, IAM 13186 of *A. radioresistance*.

Molecular Detection through 16s rRNA Gene Sequencing

The BLAST search from the NCBI Gene Bank database revealed that the isolate was 97.87 per cent identical to *Acinetobacter radioresistance* (accession numbers: KY767535, KY767484, MW805343, CP059684) with a query coverage of 99 per cent. The aligned sequence of the TMT2 isolate was deposited in NCBI GenBank (accession number MT349962). The 16s rRNA gene sequence is one of the gold standards to identify the bacteria. The same gene was used to identify *A. antiviralis* (Lee *et al.* 2009) and *A. radioresistance* (Wang *et al.*, 2019) from tobacco roots and humans. Khan *et al.* (2014) identified the *Acinetobacter* associated with bacterial dieback pathogens through the 16s rRNA gene and Zamin *et al.* (2011) used the same gene to characterize *Acinetobacter* from the rhizosphere. Phylogenetic analysis based on alignment using the maximum likelihood method for 16S rRNA sequences of reference strains and TMT2 isolate indicated closest relative of *A. radioresistance* and *A. baumannii* and *A. calcoaceticus* formed out-group (Fig. 1E).

Cross Infectivity onto Different Vegetable Slices

The isolate TMT2 infected all ten vegetable slices (Table 2). Brown watery rotten areas were observed on carrot and tomato slices were macerated, the capsicum green slice turned into brown colour, the potato slice is macerated, cucumber slice turned

TABLE 2
Host range of TMT2 isolate on different vegetable slices

Method	Carrot	Potato	Tomato	Radish	Chilli	Bell pepper	Knol-khol	cauliflower	cucumber	cabbage
Slice inoculation method	+	+	+	+	+	+	+	+	+	+

Note: + = Infected; - = Not infected;

to brown and rotten, the capsicum vein turned into brown colour but no rotting, cauliflower produced water-soaked lesion the rotten, in knol-khol, the tissue turned brown, radish showed dull whitish rotten lesion, the chilli fruit becomes brown and flaccid. The symptoms started after 24 hr of inoculation and complete rotten observed at 72 hr (Fig. 2).



Fig. 2: Cross infectivity of TMT2 isolate to different vegetable slice

A. baumannii was commonly associated with many farm irrigated vegetables as a source of bird droppings (Dahiru and Enabulele, 2015). *A. calcoaceticus* was found in many vegetables of the Lebanon region (Atrouni *et al.*, 2015), which indicated the broad host range association of *Acinetobacter* spp. In our study, the *A. radioresistance* proved the broad host range association of vegetables.

Semi Quantification of Cell Wall Degrading Enzymes and Estimation of Biofilm Formation

The *A. radioresistance* produced cell wall degrading enzymes in semi quantification plate assay

viz., pectin lyase (9 mm zone), polygalacturonase (9 mm), cellulose (8 mm) which indicated that this pathogen used a type two secretion system to cause infection to vegetables, which is a common feature of soft rot pathogen *Pectobacterium caratovorum* (Lee *et al.*, 2014). The TMT2 also produced a biofilm of 3.19 (O.D. @ 570 nm) which was higher compared to control 0.34 (O.D. @ 570 nm). Biofilm plays an important role in attachment to the tissue surface, protects the bacteria under adverse conditions and promote virulence in *Pectobacterium caratovorum* (Lee *et al.*, 2013).

Antibiotic Sensitivity Test

The isolate TMT2 was susceptible to Ampicillin-sulbactam, Piperacillin / Tazobactam, Cefepime, Gentamicin, Tobramycin and Levofloxacin. The isolate showed intermediate reaction to Cefotaxime, Ceftriaxone, Amikacin, Ciprofloxacin while resistant to Tetracycline and Doxycycline (Table 3). *A. radioresistance* was clinically known to cause bacteremia and pneumonia in humans, which is susceptible to ampicillin-sulbactam, cefepime, ceftazidime, ceftriaxone, gentamicin, levofloxacin, meropenem, piperacillin-tazobactam, tobramycin and trimethoprim-sulfamethoxazole (Wang *et al.*, 2019). The *A. baumannii* associated with farm vegetables showed a resistant reaction to ceporex, ampicillin, amoxicillin-clavulanic acid, ofloxacin, ofloxacin, streptomycin and recorded intermediate reaction to co-trimoxazole, nalidixic acid, gentamycin, and ciproflox (Dahiru and Enabulele, 2015).

Thus the present study proved *A. radioresistance* is a new soft rot associated pathogen of tomato in India and associated with many vegetables. But still, the

TABLE 3
Antibiotic susceptibility tests of TMT2 isolate

Antibiotic class	Antibiotic	Symbol	Disc Conc.	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)			TMT 2 Zonediamer (mm)	TMT 2 Reaction
				S	I	R		
β-lactam/ β-lactamase inhibitor combinations	Ampicillin-sulbactam	A/S	10/10 µg	≥15	12-14	≤11	14.33	S
	Piperacillin/Tazobactam	PIT	100/10 µg	≥21	18-20	≤17	22.66	S
	Cephems (including cephalosporins i, ii, iii & iv)	Cefepime Cefotaxime Ceftriaxone	CPM CTX CTR	30 µg 30 µg 30 µg	≥18 ≥23 ≥21	15-17 15-22 14-20	≤14 ≤14 ≤13	17.66 15.00 15.00
Aminoglycoside	Amikacin	AK	30 µg	≥17	15-16	≤14	14.33	I
	Gentamicin	GEN	10 µg	≥15	13-14	≤12	15.00	S
	Tobramycin	TOB	10 µg	≥15	13-14	≤12	16.00	S
Fluoro-quinolones	Ciprofloxacin	CIP	5 µg	≥21	16-20	≤15	19.33	I
	Levofloxacin	LE	5 µg	≥17	14-16	≤13	20.00	S
Tetracyclines	Tetracycline	TE	30 µg	≥15	12-14	≤11	00.00	R
Tetracyclines	Doxycycline	DO	30 µg	≥13	10-12	≤9	0000	R

Note: S- Susceptible, I-Intermediate, R-Resistant

research is needed to determine the evolution of pathogenicity factors to infect plants, characterization of genes responsible for virulence and confirmation of horizontal gene transfer from another bacterium of the *Enterobacteriaceae* family.

Bacterial soft rot is a devastating disease of tomatoes under high temperatures and relative humidity. The direct fruit loss persisted in storage as well. *Pectobacterium caratovorum* is a well-known soft rot pathogen of various vegetables, but recently, many pathogens are associated with soft rot disease, which can cause direct damage and occasionally take advantage of damage caused by other factors. The *A. radioresistance* reported in this study causes tomato rotting and has a broad host range with a variable response to antibiotics. This is the first report of *A. radioresistance* causing bacterial soft rot of vegetables and in particular tomatoes.

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