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SOIL AND AGRICULTURAL MICROBIOLOGY - RESEARCH PAPER

Development of antibody to virulence factor flagellin and its evaluation in screening *Ralstonia pseudosolanacearum*

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Abstract

The bacterial wilt disease caused by *Ralstonia pseudosolanacearum* presents a notable economic risk to a variety of crucial crops worldwide. During preliminary isolation of this phytopathogen, several colonies of other saprophytic bacteria may be mistaken with it. So, the present study aims to address this issue by proposing the application of immunogenic proteins, particularly flagellin (FliC), to enable a rapid and early identification of bacterial wilt. In this study, a novel approach is unveiled for the early detection of *R. pseudosolanacearum*. The study exploits the immunogenic attributes of flagellin (FliC), by generating polyclonal antibodies against recombinant FliC within model organisms—rabbits and mice. The efficacy of these antibodies is meticulously assessed through discerning techniques, including DAS-ELISA and Western blot analyses, which elucidate their remarkable specificity in identifying various *R. pseudosolanacearum* strains. Furthermore, the introduction of antibody-coated latex agglutinating reagents offers an additional layer of confirmation, substantiating the feasibility of establishing a laboratory-based toolkit for swift screening and unambiguous identification of the bacterial wilt pathogen. This study presents a significant stride toward enhancing early diagnostic capabilities, potentially revolutionizing agricultural practices by safeguarding crop yield and quality through proactive pathogen detection and mitigation strategies.

Keywords Agglutination · DAS-ELISA · Flagellin · Polyclonal antibodies · *Ralstonia pseudosolanacearum*

Abbreviations

BSA	Bovine serum albumin
CFU	Colony-forming unit
CPG	Casamino acid-peptone-glucose
DAB	3,3′-Diaminobenzidine
DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay
DVC	Direct viable count
EDAC	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide
ELISA	Enzyme-linked immunosorbent assay
FliC	Flagellin
FP	Forward primer
His	Histidine
HRP	Horseradish peroxide
H202	Hydrogen peroxide
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAT	Latex agglutination test
MES	2-morpholino ethane sulfonic acid
mSMSA	Modified semi-selective medium South Africa
Ni-NTA	Nickel-nitrilotriacetic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PVDF	Ppolyvinylidene difluoride
RP	Reverse primer
RPS	*Ralstonia pseudosolanacearum*
RS	*Ralstonia solanacearum*
RSSC	Ralstonia solanacearum species complex
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WC	Whole cell

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Introduction

Ralstonia pseudosolanacearum (*RPS*) is a gram-negative soil-borne phytopathogen that causes bacterial wilt in many plant species including the economically important Solanaceae family crops [1, 2]. Previously, the pathogen was a member of four phylotype groups, the *Ralstonia solanacearum* species complex (RSSC). Recently, RSSC was rearranged into three different species [3, 4]: *R. solanacearum* (*RS*) (phylotype II), *RPS* (phylotype I and III), and *R. syzygii* (phylotype IV and blood disease bacterium) [5–7]. *RPS* shares most of the virulence genes and other characteristics with *RS* [8, 9].

The pathogen invades the susceptible host through open wounds thereby infecting the cortex and spreading to the xylem blocking its flow, leading to the occurrence of marked symptoms of wilting [10, 11]. The infection can be preliminarily diagnosed by an onsite ooze test. For confirmation, the etiological agent firstly needs to be isolated in Kelman's tetrazolium medium or modified semi-selective medium South Africa (mSMSA), followed by subsequent confirmation using molecular or immune-detection tools. The challenge lies in precisely identifying colonies of the etiological agent among the colonies of saprophytic bacterial cells and other secondary pathogens [12]. In such circumstances, immune-detection tools can provide a rapid and handy method to screen colonies for later confirmatory laboratory assays.

To our knowledge, no rapid immune-based detection platforms for the bacterial wilt pathogen have been described in scientific literature. Most of the studies were based on ELISA using either polyclonal or monoclonal antibodies raised against the whole cell or LPS of the pathogen [13–15]. The polyclonal antibodies raised against whole cells and lipopolysaccharides (LPS) have specificity limitations [13, 16–18]. Conversely, several immune-based detection systems targeting specific virulent proteins were described for other gram-negative bacteria [19, 20].

In the genus *Ralstonia*, several virulence factors including type III secretion system and effectors [21, 22], extracellular polysaccharide I [23, 24], flagella [25, 26], type II secretion system [27, 28], and cell wall degrading enzymes such as exo-and endo-polygalacturonase (PGs) [29, 30] contribute to the pathogenicity [31]. Among these, flagella-mediated swimming motility plays a crucial role in the ecological fitness and virulence of the pathogen [25, 26]. Flagella is a composite organelle comprising of flagellar filament, basal body, etc. [32]. The flagellar filament is a polymer of flagellin protein (FliC), encoded by the *flagellin* gene (*fliC*). In many gram-negative pathogens including human pathogens, the flagellar protein, FliC, has been successfully used in immune-based detection procedures [33–35]. Intended to explore the possibility of using flagellar protein FliC as an immune target for rapid and specific identification of *RP* colonies and cells in general, polyclonal antibodies were generated against the recombinant form of flagellin. The antibodies were then evaluated using ELISA and Western Blot to confirm the presence of flagellin protein in *RPS* cells. Thereafter, a latex agglutination reagent (LAT) was prepared and evaluated for screening colonies of *RPS*. Figure 1 embodies the schematic representation of the proposed detection pipeline involved in the present study.

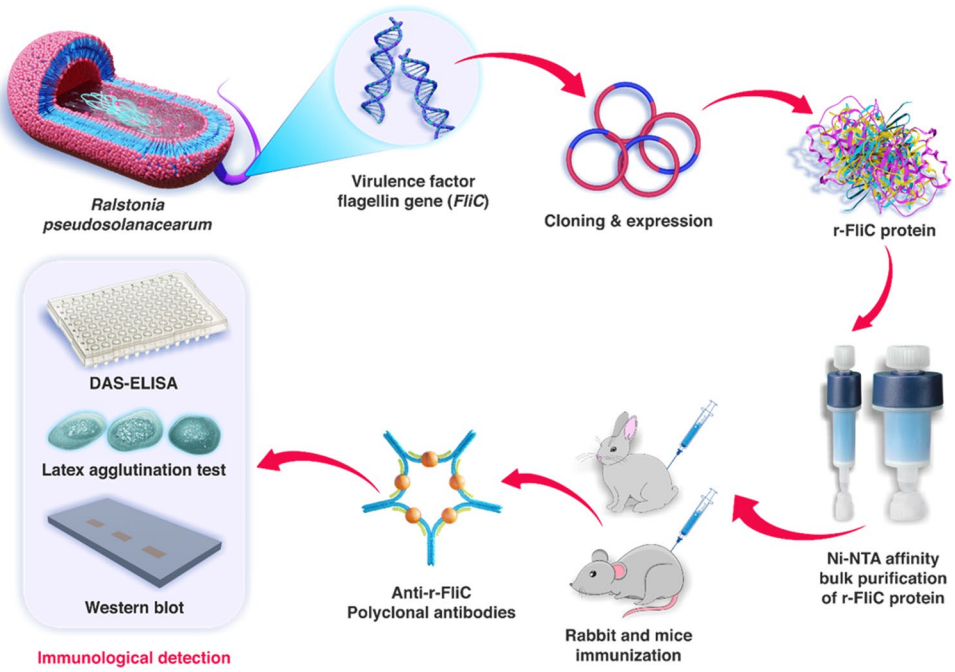

Fig. 1 Schematic representation of the immune-based detection scheme for the *Ralstonia pseudosolanacearum* induced bacterial wilt

Materials and methods

Bacterial strains, vector, and host strains used in the study

R. pseudosolanaceaerum and *non-R. pseudosolanaceaerum strains*

Six bacterial wilt strains including five *RPS* strains, i.e., DIBER115 (MG266193), DIBER116 (MG266202), DIBER117 (MG266199), DIBER118 (MG266203), and DIBER119 (MG266201), and one *RS* strain NAIMCC-B-00418 (National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau Nath Bhanjan, India) were used in this study. The non-bacterial wilt strains used were *Ralstonia pickettii* (*R. pickettii*) (MTCC 648), *Ralstonia insidiosa* (*R. insidiosa*) (ATCC 49129), *Pseudomonas* spp., *Ochroboctrum* spp., *Cupriavidus paculus* (*C. paculus*) (All DIBER culture collection), and Escherichia *coli* (*E. coli*) (ATCC 10536).

Vector and host strains used

Commercially available Qiagen's pQE-30 vector, containing an ampicillin resistance gene, was utilized for both gene cloning and expression purposes. This vector is specifically designed for the expression of the cloned gene, accompanied by an N-terminal 6x histidine (His)-tag. For host strains, *E. coli* strain M15 [pREP4] was chosen, serving as both a cloning and expression host. This strain is known for its capability of high-level expression and harbors a native plasmid pREP4 rendering resistance to antibiotic kanamycin. Therefore, the growth of M15 cells was conducted in Luria-Bertani broth medium supplemented with 25 µg/mL kanamycin, maintained at 37°C for 12 h.

Molecular procedure for screening bacterial wilt isolates

Six bacterial wilt strains including five *RPS* strains, i.e., DIBER115 (MG266193), DIBER116 (MG266202), DIBER117 (MG266199), DIBER118 (MG266203), and DIBER119 (MG266201), and one *RS* strain NAIMCC-B-00418 (National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau Nath Bhanjan, India) were used in this study. Isolation of the pathogen, *RPS*, was carried out from the infected plants of tomato, pepper, and eggplants from diverse regions of the state Uttarakhand, India. Plants manifesting bacterial wilt symptoms were collected, as well as onsite screening by the stem streaming test (Ooze test) was performed [12]. Tissues from suspected plants were transported to the laboratory and crushed in 1× phosphate-buffered saline (PBS) (pH 7.4) to isolate the etiological agent in modified semi-selective medium South Africa (mSMSA) agar plates [36]. Colonies that resemble *RPS* and *RS* were selected and sub-cultured in casamino acid-peptone-glucose (CPG) broth [37–39]. Thereafter, the isolates were screened by commercially available DAS-ELISA (Adgia Co.) and PCR (Q-cycler 96 Hain Life sciences, UK, Ltd.). In-house designed primer pairs Pbh2F (5′-AAT GCC AGC AAG TGG AGC ACC-3′) and Pbh1/2R (5′-TAC GAC AAC GTG AGG ATG AAC G-3′) (ThermoFisher Scientific) targeting the 683bp region spanning nucleotides 1075 and 1757 of the exo-polygalacturonase (*pehB*) gene in *RS* strain GMI1000 (accession number NC_003295 available at National library of medicine, NCBI). Qiagen's HotStarTaq DNA polymerase kit with Cat No. 203205 was used for conducting PCR. The thermal cycling encompasses initial denaturation at 95°C for 3 min, and 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 20 s, and extension at 72°C for 45 s. Consequently, the isolates were confirmed by 16S rDNA sequencing outsourced to professional sequencing services (AgriGenome Labs Pvt. Ltd., Kochi). The strains were preserved (30% glycerol stock) in ultra-low-temperature freezer at −80°C to prevent spontaneous phenotypic conversion from pathogenic fluidal to non-pathogenic non-fluidal colony morphology [40, 41].

Cloning of flagellin gene in *E. coli* and its expression

Nucleotides encoding the amino acids 4 to 266 of the *fliC* gene were amplified from the genomic DNA of NAIMCC-B-00418 strain. A standardized PCR protocol was employed with the temperature profile as shown in Table 1, involving an initial denaturation step at 95°C for 3 min, followed by 35 cycles of amplification at 95°C for 30 s, 60°C for 20 s, and 72°C for 1 min (Qiagen's HotStarTaq DNA polymerase Kit; Cat No. 203205). This PCR amplified the 10th to 798th bp

Table 1 Primer pair sequence with nucleotide targeted and encoded amino acids

Target gene	Primer pair	Primer sequence	Nucleotide targeted	Amino acids encoded
FliC	FliC F	5´AAGAT**GGATCC** AGCCTCAATACC AACATCTCG-3´	10th to 798th	4th to 266th
	FliC R	5'CAGCA**AAGCTT** AGGTTCAGGATG CTGTTGGG-3´		

Inserted restriction enzymes sites are marked with bold and underlined

region of the *fliC* gene using specific cloning primers namely FliC forward and reverse primer, FliC-FP and FliC-RP. Table 2 shows sequence of the primer sets with incorporated restriction enzyme sites underlined and bold. The resulting amplicon was purified and subsequently cloned into the pQE-30 expression vector (Qiagen, Germany), known for its ability to co-express a poly-histidine tag. After transformation into chemically competent *E. coli* M15 cells using standard techniques, antibiotic selection employing ampicillin and kanamycin was used to isolate transformed cells [42]. Confirmation of successful clones was achieved through PCR screening using FliC-FP and FliC-FP. Thereafter, selected clones were induced to produce recombinant FliC protein (r-FliC) using 1.5 mM IPTG. This involved inoculating 5 mL of prewarmed Luria-Bertani broth with 100 µL of overnight cultures of the selected clones and incubating them at 300 rpm until reaching an OD600 of 0.6, typically taking around 3 h under our experimental conditions. Subsequently, induction with IPTG at a final concentration of 1 mM was performed. Verification of induced cultures expressing r-FliC was conducted via Western blot using anti-His-HRP conjugated antibodies [42]. These antibodies specifically detect the poly-histidine tag co-expressed at the N-terminal of the r-FliC protein. Clones exhibiting relatively higher expression levels of r-FliC were chosen for further experimentation.

Purification of r-flagellin protein

Determining protein solubility

Recombinant proteins expressed in *E. coli* are in vivo stored either in soluble form or as insoluble inclusion bodies [43]. Therefore, prior to bulk purification, the solubility of recombinant proteins was determined. One milliliter of IPTG-induced culture of the selected clone was harvested by centrifugation at 6500 rpm for 10 min and sonicated at 6× 10s with a 10-s pause at 40 amplitudes in a sonicator (Vibra-Cell™ Sonics, USA). The cell lysate was kept on ice throughout the procedure. Sonicated cell lysate was then centrifuged at 10,000 rpm for 10 min at 4 °C; supernatant and pelleted cells were then individually analyzed by SDS–PAGE and Western blot [42].

Table 2 Temperature profile used in PCR for the amplification of *fliC* gene with primer pair FliC-FP and RP

Step	Temperature (°C)	Time (minutes)	No. of cycles
Initial denaturation	95	3	1
Denaturation	95	0.5	35
Annealing	60	0.5	
Extension	72	0.7	
Final extension	72	3	1

Recombinant proteins expressed in *E. coli*, *M15* were purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity purification column (Qiagen, Germany).

Preparation of cleared *E. coli* lysates under denaturing conditions

One liter culture of each of the selected clones was induced with IPTG. Induced cells were harvested by centrifugation at 5000 rpm for 20 min in 25-mL batches. Then the cell pellet was resuspended in lysis buffer pH 8.0 (100mM NaH_2PO_4, 10mM Tris-Cl, 8M Urea) at 5 mL per gram wet weight of the harvested pellet. Resuspended cells were stirred for 60 min (till the solution becomes translucent) at room temperature in a tube rotor (Tarsons Products Pvt. Ltd.). Then the lysate was centrifuged at 10,000 rpm for 30 min at room temperature to pellet the cellular debris. Supernatant or cleared lysate was further processed for purification of recombinant protein.

Purification of 6xHis-tagged proteins from *E. coli* under denaturing conditions

The denatured lysate was used for the purification of 6xHis-tagged proteins as per the manufacturer's instruction (Qiagen Ni-NTA Spin Kit). Collected fractions of eluates were analyzed by SDS-PAGE [42].

Recombinant protein concentration and quantification

Eluates giving a single band corresponding to the molecular weight of the protein were pooled in respective 15-mL eppendorf tubes. Thereafter, the protein was concentrated using Amicon® Ultra centrifugal (EMD Millipore, Germany) of appropriate size according to the molecular weight of the recombinant protein. Pooled r-FliC protein was concentrated using an Amicon® Ultra 15K device with a molecular weight cut-off (MWCO) of 15,000 Da. Thereafter, the concentration of each of the proteins was determined spectrophotometrically using Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, USA).

Production of polyclonal antibodies

Polyclonal antibodies against the recombinant proteins and whole cell of strain NAIMCC-B-00418 were produced in model animals, rabbits, and mice, through outsourcing to Abgenex Private Limited, Bhubaneswar, India. For the preparation of inactivated whole cells, 20 mL of 48 h CPG broth culture of Rs0418 was pelleted. Then the harvested cells were washed twice with 10 mL of sterile 1× PBS. Finally, the washed cells were resuspended in 5mL of sterile 1× PBS. The cell count of the washed cell suspension was done using the serial dilution technique by the direct

viable counting (DVC) method. The plates were incubated at 28°C for 48 h and the resultant colonies were counted and the respective cell suspensions were standardized to 1×10^6 CFU/mL using 1× PBS. Thereafter, formalin inactivation was done by adding formalin to the cell suspension at a final concentration of 0.5% (v/v).

Evaluation of antibody titer

Evaluation of antibody titer for anti-rFliC was done by indirect ELISA. Briefly, 100 µL of purified protein at a concentration of 20 ng/100 µL in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) were coated onto the 96-well ELISA plates (Nunc™ PolySorp™), followed by incubation at 37°C for 1 h. Then, the plates were rinsed three times with 1× PBS-Tween 20 (PBS-T) washing solution for 3 min, followed by blocking the unbound sites in the wells by adding 200 µL of 1% BSA solution (in 1× PBS) and incubation overnight at 4°C. Doubling dilutions of polyclonal antisera (each of rabbit and mice) ranging from 1:1500 to 1:286,000 dilutions in 1× PBS were made, and 100 µL from each dilution was added in respective wells and the plates were incubated at 37°C for 1 h and washed. Then, either Goat anti-rabbit IgG or Goat anti-mice IgG conjugated to horse radish peroxidase (HRP) diluted to 1:5000 in 1× PBS was prepared, and 100 µL of the diluted conjugate was added onto the respective wells and incubated at 37°C for 1 h and washed. 100 µL of the development buffer [5 mg o-phenylenediamine (OPD) and 20 µL H_2O_2 in 20 mL of sodium phosphate buffer] was added to each well and incubated in dark at room temperature for 10 min. Then 50 µL of 5M H_2SO_4 was added to each well to stop enzyme activity. Thereafter, absorbance was measured at 405 nm using a microplate reader (Synergy HT Multi-Detection Microplate Reader, Bio Tek Instruments, Inc., USA). Further, cut-off value was calculated as described by Classen et al. [44] using the following formula:

$$\text{Cut} - \text{off value} = \text{mean of negatives} + 5\text{time the standard deviation}$$

ELISA optimization

Before performing further experiments for specificity evaluation, the optimal concentrations of capture antibody and detection antibody for each of the raised antibodies were determined by checkerboard titration. For this, Indirect ELISA in checkerboard titration format was performed in 96-well ELISA plates (Nunc™ PolySorp™) as described previously. The highest dilution which gives OD values greater than the cut-off values and can detect minimum antigen is the optimum antigen and antibody concentration.

Briefly, wells were coated with an array of doubly diluted rFliC protein. Diluted protein in the range 5–320 ng/well in coating buffer was coated while anti-FliC antibodies were titrated in doubling dilutions in 1× PBS from 1:2500 to 1:80000. For anti-WC antibodies optimization, the coating was done with tenfold dilutions of *RS* strain NAIMCC-B-00418 cells in the range 1×10^6 to 1 CFU/100 µL in coating buffer and antisera in the range of 1:2500 to 1:80000 in 1× PBS were used.

Evaluation of raised polyclonal antibodies

Evaluation of anti-rFliC Ab, as well as anti-WC Ab, was done by Indirect Double Antibody Sandwich (Indirect DAS) ELISA and Western blot.

Preparation of washed cell suspensions

For using *RPS* and non-*RPS* cells in Western blot and DAS-ELISA, washed cell suspensions of each of the strains were prepared. For this, respective *RPS* and non-*RPS* strains were grown for 48 h and 24 h respectively in 10 mL of CPG broth medium. Cells were then harvested by centrifugation at 10,000 rpm for 5 min, and harvested cells pellet were washed twice with 1× PBS. Finally, the washed cells were resuspended in 1 mL of 1× PBS. The cell counts were performed using the serial dilution technique by the DVC method. The plates were incubated at 28°C for 48 h and the resultant colonies were counted. And finally, the respective cell suspensions were standardized to 1×10^6 CFU/ 100 µL using 1× PBS.

Specificity evaluation by Western blot

Western blot was performed to reveal the presence of target proteins in test microcosms. 1 mL of each of the prepared washed cells were pelleted and used for sample preparation for SDS-PAGE (10% polyacrylamide, 0.1% SDS). Proteins expressed in the respective pellets were resolved in the gel and electro-transferred onto the PVDF (polyvinylidene difluoride) membrane following standard protocol with slight modifications [45]. Briefly, the membrane was washed three times by soaking it in 1× PBS-T for 5 min each. Thereafter, unbound sites in the membrane were blocked by soaking the membrane in 1×-BSA in 1×-PBS and incubating overnight at 4°C. After consecutive incubation with the Rabbit anti-FliC polyclonal serum (1:10000) and anti-rabbit secondary antibody (1:5000) (Sigma-Aldrich, USA), water-soluble chromogenic substrate 3,3′-Diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2) were used to visualize the immuno-labeled bands. Washing was performed after every incubation as explained above.

Specificity evaluation by Indirect DAS-ELISA

Washed cell suspension of *RPS* and non-*RPS* cells (1×10^6 CFU/100 µL) were coated onto the 96-well ELISA (Nunc™ PolySorp™) plates. In DAS-ELISA, as performed previously, mice anti-WC polyclonal serum (1:10000) was used as capture antibodies, and Rabbit anti-FliC polyclonal serum (1:10000) as revealing antibodies to detect flagellin protein in the cells. Goat anti-rab IgG HRP conjugate (1:5000) was used as a secondary antibody-enzyme conjugate.

Latex agglutination tests

Preparation of latex agglutinating reagent

The preparation of polystyrene latex beads (Sigma-Aldrich, USA) for agglutination was done according to the manufacturer's protocol with slight modifications. Briefly, the latex beads were preliminary washed in 1 mL of MES (2-morpholino ethane sulfonic acid) buffer, and 500 µg of anti-FliC antibodies was coupled to latex beads using 0.2 mM EDAC [1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide]. After 15 min incubation in a rocker and subsequent washing with wash buffer (50 mM MES, 0.1% Triton x-100; pH 6.1), the antibody coupled beads pellet was resuspended in 1 mL of storage buffer (50 mM glycine in MES buffer, 0.03% Triton x-100 and 0.1% sodium azide, pH 8.1).

Test procedure

Latex agglutination tests were performed in two sets of bacterial cells. In the first set, washed cell suspensions of bacterial wilt and non-bacterial wilt strains were tested for agglutination. Briefly, 1 mL of 24 h grown culture was pelleted and resuspended in 100 µL of PBS. 10 µL of each of this prepared cell suspension was mixed with an equal volume of coated particles on a clean grease-free slide and hand tilted gently for 2 min. Results were evaluated macroscopically and recorded qualitatively as positive (+) and negative (−). In the second set of experiments, a single colony from CPG-grown microcosms was washed twice, resuspended in 100 µL of saline, and tested for agglutination. The purified recombinant protein was used as the positive control. All the latex agglutination tests were performed in triplicate with cells from different batches of microcosms.

Results

Bacterial strains, vector, and host strains

Colonies namely DIBER115-DIBER119 showed the characteristic feature of *RPS* colonies such as white colonies with pink centers generating reddish-brown pigmentation (Fig. 2). These colonies were further confirmed by PCR using the primer pairs Pbh2F/Pbh1/2R targeting the gene *pehB* region respectively of *RS/RPS* [38]. Figure 3 shows the agarose gel electrophoresis of the PCR products for the primer sets Pbh2F/Pbh1/2R and 760F/759R. NAIMCC-B-00418 strain was used as the positive control, and PCR reaction with no template was used as the negative control. PCR product of all the stains showed a 683bp band in electrophoresis which corresponds to the exo-polygalacturonase (*pehB*) gene targeted by Pbh2F/Pbh1/2R. DIBER120 strain gave weak amplification with primer set Pbh2F/Pbh1/2R.

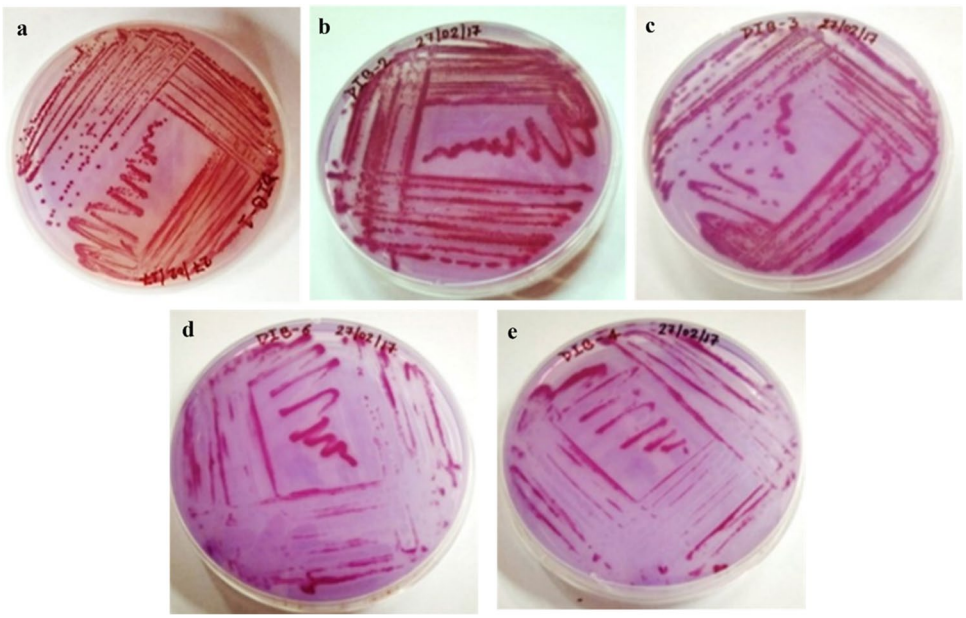

Fig. 2 Colony morphology of *Ralstonia pseudosolanacearum* strains. **a** DIBER115; **b** DIBER116; **c** DIBER117; **d** DIBER118; **e** DIBER119

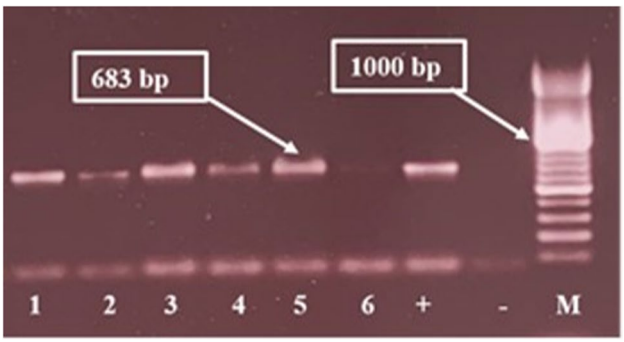

Fig. 3 PCR for confirmation of the selected colonies. Lane 1–lane 5: DIBER115-DIBER119; +: positive control (NAIMCC-B-00418); – ve: non-template control. PCR results reveal that all the isolates were *Ralstonia pseudosolanacearum*

Further 16SrDNA sequencing confirms that all the positives were identified as Phylotype-I of RSSC which is taxonomically reclassified as RPS [46]. Hence, the strains were rechristened *RPS* DIBER115–DIBER120. The background colonies were identified as strains belonging to *Pseudomonas*, *Ochroboctrum*, *Cupriavidus*, and *Burkholderia* genera by 16S rDNA sequencing. Isolates were submitted to National Center for Biotechnology Information (NCBI) and received the NCBI gene bank accession numbers MG266193 (DIBER115), MG266202 (DIBER116), MG266199 (DIBER117), MG266203 (DIBER118), MG266201 (DIBER119), and MG266200 (DIBER120) respectively. Refer to supplementary files S1 for16S rRNA sequences of each strain and supplementary material for BLAST results. Information regarding *RPS* and non-*RPS* as well as host strains used in the study along with the culture media and growth conditions required to cultivate them is provided in supplementary file S1 (Table S1).

Cloning of flagellin gene in *E. coli* and its expression

Growth of recombinant clones of the *fliC* genes is observed in the form of colonies (>300) on the Luria-Bertani agar plates supplemented with ampicillin. Upon PCR of the extracted plasmid from the broth cultures of the selected clones, 7 clones showed a band corresponding to the 748 bp of the *fliC* gene. These PCR-positive clones were further sub-cultured for IPTG induction to express the recombinant protein and confirmed by Western blot [42]. In Western blot, it was observed that the 2 clones gave a band corresponding to the molecular weight of the protein, i.e., 28.7 kDa. Figure 4 shows transformation and screening results for recombinant FliC protein. The cloned sequence of the *fliC* gene was submitted to NCBI and received the accession number MW759461. Partial coding sequence of cloned *fliC* gene is provided in supplementary file S1 and refer to supplementary material for BLAST analysis.

Purification of r-flagellin protein

Western blot analysis of sonicated M15 cell lysate transformed with the *fliC* gene showed that the r-FliC protein

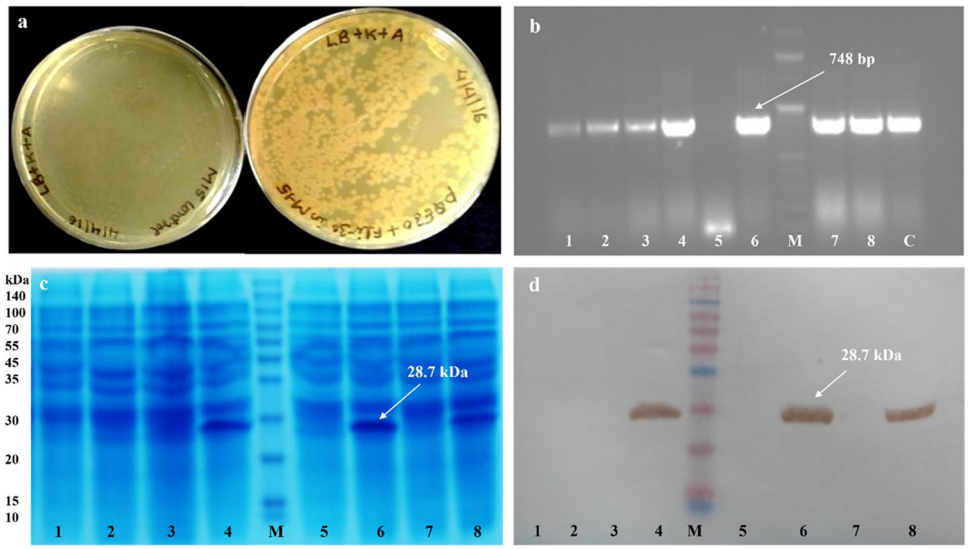

Fig. 4 Confirmation of observed clones. **a** Growth of clones in Luria-Bertani agar + Amp + Kan Plates. **b** Confirmation of positive clones by agarose gel electrophoresis of the PCR product showing band corresponding to *fliC* gene. Lanes 1–8: PCR product of the selected clones. **c** SDS PAGE and **d** Western Blot, analysis of induced and non-induced clones of M15 cells giving r-FliC protein expression. Lanes 1, 2, 3: non-induced cells of selected clones; lanes 4, 5, 6, 7: 4 h induced cells of selected clones, showing band corresponding to the molecular weight of FliC protein, i.e., 28.7 kDa; lane 8: positive control (purified r-FliC Protein); M—clearly stained Protein Ladder (Takara Bio USA, Inc. Code No. 3454A, 10–210 kDa)

was present in the sonicated pellet, indicating that the protein was expressed in an insoluble form inside the inclusion bodies (Fig. 5). So, further purification of the proteins was done under denaturing conditions. Figure 4 shows the result of SDS-PAGE for solubility determination.

During affinity purification, recombinant protein was successfully purified as observed by band corresponding to the molecular weight of the protein. Eluates 12–17 gave single band of 28.7 kDa, so these were pooled (Fig. 5). Purification was repeated five times, and all the eluates giving a single band corresponding to the molecular weight of r-FliC protein were pooled and concentrated using an amicon filter (Sigma-Aldrich, USA) of 15 kDa. The purified and concentrated recombinant protein was quantified using Total Protein Kit, Micro Lowry, Peterson's Modification by Sigma-Aldrich, USA, and the concentration of protein was determined to be 3.4 mg/ mL.

Production of polyclonal antibodies

Outsourced polyclonal antibodies against concentrated r-FliC and formalin-inactivated whole cells of Rs0418 raised in rabbits and mice were received and used in further experiments.

Antibody titer

The optimum antibody titer for the produced anti-WC and anti-r-FliC antibodies was determined by indirect ELISA. The highest dilution of the antisera giving an O.D. value greater than the cut-off values of the respective normal rabbit and mice antisera was considered the optimum antibody titer for the respective antibodies produced against the whole cell as well as the recombinant protein. Cut-off value and optimum antibody titer for the respective antibody are summarized in Table 3.

ELISA optimization

Absorbance greater than the *cut-off* value was selected and antiserum dilution corresponding to that absorbance was further used in the experiments. The mean absorbance of the checkerboard titration involving serially diluted WC and rFliC protein with their corresponding rabbit/mice antiserum suspension is provided in the supplementary file S1 (Tables S2 and Table S3).

For rabbit/mice anti-r-WC antisera:

Cut-off value (for rabbit antisera) = 0.317 + 5(0.061) = 0.622

Cut-off (for mice antisera) = 0.3205 + 5(0.038) = 0.510

Antiserum dilution of 1:5000 and 1:10000 respectively for rabbit and mice antisera were further used for evaluating the anti-r-WC antibodies using DAS-ELISA.

For rabbit/mice anti-r-FliC antisera:

Table 3 Antibody titer for the synthesized anti-whole cell and anti-FliC antibodies

Protein/whole cell	Antibody	Cut-off value	Ab titer
Whole cell	Anti-WC^{rabbit}	0.230	1:62000
	Anti-WC^{mice}	0.215	1:32000
r-FliC	Anti- r-FliC ^{rabbit}	0.230	1:128000
	Anti- r-FliC^{mice}	0.208	1:128000

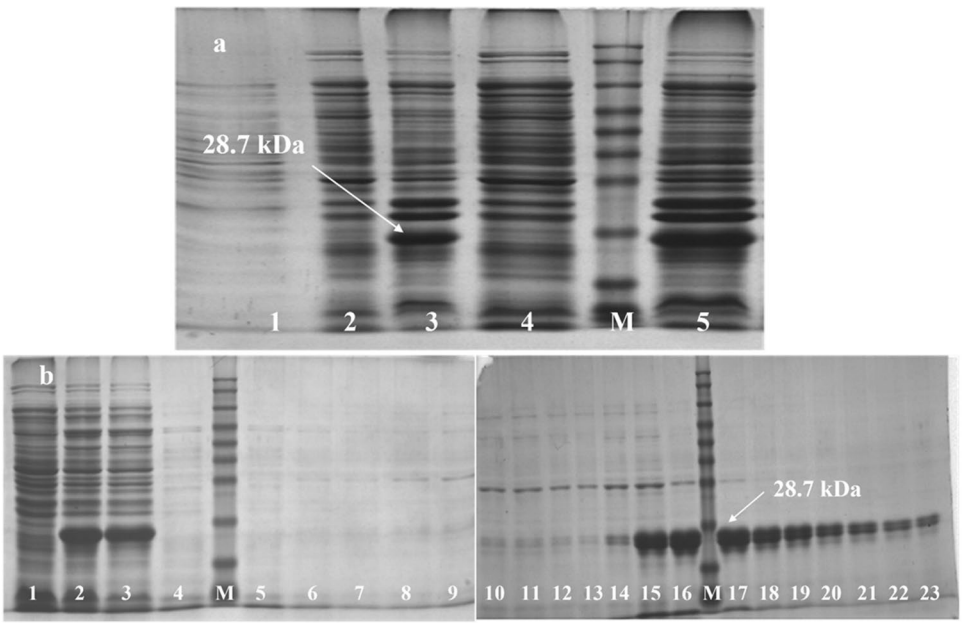

Fig. 5 **a** SDS-PAGE for solubility determination of the expressed r-FliC protein. Lane 1: pelleted suspension of clone; lane 2: non-induced clone; lane 3: induced clone; lane 4: sonicated supernatant; lane 5: sonicated pellet; M: stained protein ladder (Takara Bio USA, Inc. Code No. 3454A, 10-210 kDa). **b** SDS-PAGE of collected fractions of r-FliC purification. Lane 1: non-induced clone; lane 2: induced clone; lane 3: resuspended pellet; lane 4: flow through; lanes 5 and 6: wash fraction 1 and 2; lanes 7 to 23: eluates 1 to 17; M: stained protein ladder (Takara Bio USA, Inc. Code No. 3454A, 10–210 kDa)

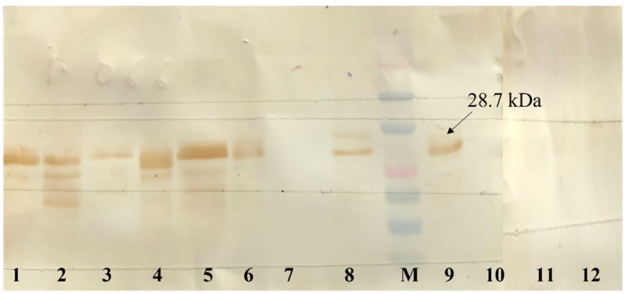

Fig. 6 Western Blot results of washed cell suspensions (10^6 CFU/mL) of *RPS* and non RSSC strains. Lane 1: NAIMCC-B-00418; lane 2: DIBER115; lane 3: DIBER116; lane 4: DIBER117; lane 5: DIBER118; lane 6: DIBER119; lane 7: negative control-PBS; lane 8: positive control (recombinant protein rFliC); lane 9: *R. pickettii*; lane 10: *Pseudomonas sp*.; lane 11: *E. coli*; lane 12: *Ochrobactrum* sp.; lane M: low-range protein ladder

Cut-off (for rabbit antisera) = 0.248 + 5(0.032) = 0.408

Cut-off (for mice antisera) = 0.26 + 5(0.025) = 0.385

Antiserum dilution of 1:10000 for rabbit anti-r-FliC and 1:5000 for mice anti-r-FliC antibodies were used for evaluating the anti-r-FliC antibodies in further ELISA.

Evaluation of antibodies

Anti-r-WC Cut-off value of the absorbance of non-immunized sera was calculated and strains giving absorbance greater than the cut-off value were marked as positive for the reactivity with the anti-WC antibodies. Cut-off value of the negatives is:

Cut-off value = 0.18 + 5(0.010) = 0.230

High degree of nonspecific reactions with non-bacterial wilt strains was observed in DAS-ELISA using anti-WC antibodies. Therefore, anti-WC antibodies were not chosen for agglutination studies.

Anti-r-FliC In Western blot, all the *RS* strains reacted with the antibodies as bands corresponding to the molecular weight of flagellin (FliC) were observed, while no reactivity was seen for non-*RS* strains except for *R. pickettii* strain (Fig. 6 and Table 4). For indirect DAS-ELISA, cut-off value of the absorbance of non-immunized sera was calculated and strains giving absorbance greater than the cut-off value were marked as positive for the reactivity with the anti-FliC antibodies. Cut-off value of the negatives is:

Cut-off value = 0.19 + 5(0.009) = 0.235

Similar results were observed in indirect DAS-ELISA.

Latex agglutination test

Latex beads coated with anti-FliC antibodies agglutinated *RPS*, *RS*, and *R. pickettii* colony suspensions. No visible agglutination was observed with *Pseudomonas* spp., *Ochrabactrum* spp., and *E. coli*, which were routinely identified as background colonies (Fig. 7), whereas agglutination reactions with washed cell suspensions derived from liquid broth showed variable results similar to DAS-ELISA. Table 4 summarizes the evaluation results for anti-WC and anti-FliC antibodies.

Table 4 Evaluation results of synthesized anti-WC and anti-FliC antibodies

Culture	Source of the strain	DAS-ELISA using anti WC antibodies	Immunological tests with anti-flagellin antibodies			
			Western Blotting	DAS-ELISA	Latex agglutination	
					Washed cell suspension	Colony
DIBER 115	Tomato	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
DIBER 116	Tomato	+/+/+	+/+/+	+/−/−	+/−/+	+/+/+
DIBER 117	Tomato	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
DIBER 118	Tomato	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
DIBER 119	Tomato	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
NAIMCC-B-00418	NBAIMCC	+/+/+	+/+/+	+/−/+	+/−/−	+/+/+
R. picketti	ATCC 648	+/+/+	+/+/+	+/+/−	+/+/+	+/+/+
Pseudomonas sp.	DIBER114	+/+/+	−/−/−	−/−/−	−/−/−	−/−/−
E. coli	ATCC10536	+/+/+	−/−/−	−/−/−	−/−/−	−/−/−
Ochrabactrum sp.	DIBER102	+/+/+	−/−/−	−/−/−	−/−/−	−/−/−

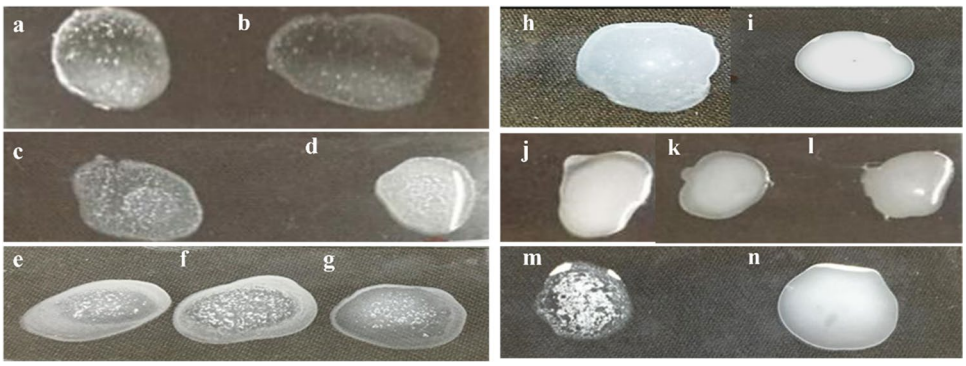

Fig. 7 Representative picture of agglutination using anti-r-FliC polyclonal antibody-coated latex particles. **a** NAIMCC-B-00418; **b** DIBER115; **c** DIBER116; **d** DIBER117; **e** DIBER118; **f** DIBER119; **g** *R. pickettii*; **h** *Pseudomonas* sp.; **i** *E. coli*; **j** *Ochroboctrum* sp.; **k**, **l** *Cupriavidus paculus*; **m** positive control (purified recombinant protein); **n** negative control (PBS)

Discussion

Various media such as 2,3,5-triphenyl tetrazolium chloride medium (TZC) and mSMSA are being employed for isolation of the bacterial wilt pathogens, *RPS* and *RS* [37, 47–49]. Along with pathogens, several saprophytes and secondary pathogens grow in the media. These nonspecific colonies account for more than 95% of colonies [50]. Most of them belong to the genus *Pseudomonas*, *Ochroboctrum*, *Cupriavidus*, and *Burkholderia*. In this work, the suspected colonies were identified by PCR targeting the exo-polygalacturonase gene (*PehB*) and the results were compared with commercially available DAS-ELISA. The PCR primer pairs Pbh2F (5′-AAT GCC AGC AAG TGG AGC ACC-3′) and Pbh1/2R (5′-TAC GAC AAC GTG AGG ATG AAC G-3′) were found theoretically specific and practically successful in identifying colonies of bacterial wilt pathogen.

During the identification of the bacterial wilt pathogen from the suspected plant samples, screening every colony by PCR or DAS-ELISA constitutes a complex tedious process. It involves colonies being subcultured in liquid media, a process requiring at least 24–48 h. This long procedure can be overcome by the introduction of a rapid immuno-based screening system. To our knowledge, such a rapid system has not been described for members of bacterial wilt pathogens in scholarly publications. In most of the immunological systems described, polyclonal antibodies (anti-WC) were directed toward the whole cell of the pathogen. This often results in non-specific antigen-antibody interactions with background organisms, which were also evident in this study [18, 51]. Henceforth, an effort was undertaken to determine the potential of the FliC protein encoded by the *fliC* gene as an immuno-target.

Flagella plays an important role in the virulence of bacterial wilt strains, *RS* and *RPS*. They mediate swimming motility required for host infection and plant colonization. Cells lacking flagella or deformed flagella either fail to infect the host or have reduced virulence [25, 52]. Recent experiments have proved that both twitching and swimming motility, respectively, mediated by type IV pili (TFP) and flagella are required for full virulence [26, 53]. The component proteins of these motility mediators can be excellent targets for antibodies as their in situ position is exocellular. Type IV pili component protein is conserved across many bacterial genera, while the amino acid sequences of flagellin are found to be specific for phylotypes I, II, and III of RSSC with 98–100% sequence similarity. Hence, considering the possibility of using the flagellin protein as an immuno-target, the sequence of the nucleotides encoding the 4th amino acid to 266th amino acid was cloned and expressed in recombinant form (r-FliC) and antibodies (anti-FliC) were raised against the purified recombinant protein.

The antibody evaluation was performed in two stages. In the first stage, the presence/absence of flagellin was evaluated in lysed cells by SDS-Western blotting. In the second stage, the whole cells of the strains were probed with anti-FliC antibodies by DAS-ELISA, to evaluate the presence of in-situ flagellin, and the results were compared with anti-WC mediated DAS-ELISA. In the third stage, latex beads coated with anti-FliC antibodies were evaluated for rapid identification of bacterial colonies and cells grown in broth culture.

Motility in RSSC is predominant when the concentration of cells in liquid broth reaches 10^6–10^8 CFU/mL [54]. Hence, all microcosms were harvested once the concentration reached 10^7 and their counts were normalized to 5×10^6 CFU/ mL for uniform analysis. In Western blot, flagellin protein was detected in all *RPS* strains and *RS* strain NAIMCC-B-00418. Among the non-bacterial wilt strains, the protein was detected in *R. pickettii*. In the genus *Ralstonia*, the flagellin protein was shared across *RPS*, *RS*, *R. pickettii*, *R. mannitolilytica*, and *R. insidiosa* as revealed by NCBI-BLAST analysis. This explains the positive reaction of antibodies with *R. pickettii*.

Results of DAS-ELISA using anti-fliC polyclonal antibodies were inconclusive about flagellin's in situ presence on some batches of strains DIBER116 and NAIMCC-B-00418 (Table 4). In this species, motility is a quantitative trait; only 60% of cells exhibit flagella during the maximum motility phase [54]. Further, the minimum

detection sensitivity of anti-fliC-mediated DAS-ELISA was 10^5 CFU/mL. Colorimetric signals from cell counts below this were masked by background signals emerging from antibodies reacting with non-flagellin epitopes. Hence, the inconsistency in results may be due to the synergistic effect of the low sensitivity of DAS-ELISA and fewer cells with flagellin in some batches of broth culture (Table 4). This assertion can be true for inconclusive results observed in agglutination reactions of cells from broth cultures. However, cells derived from colonies were consistently agglutinated with the latex reagent (Table 4). Colony-derived cells are considered non-motile until exposed to the liquid broth for a few hours [52, 55]. However, this nonmotility is not due to the absence of flagella, but owing to deformed straight flagella [52]. Our tests with agglutination reagent endorse that flagellin is indeed present in cells affiliated with colonies of genus *Ralstonia*. The repeatable and reproducible agglutination results observed are due to the high cell numbers in colonies ($>10^8$ CFU). Our results affirm the reliability of anti-FliC coated latex agglutinating reagent in identifying bacterial wilt strain.

The non-specific agglutination observed with *R. pickettii* and *R. insidiosa* will not influence the outcome of the final results, as this agglutination test will be used only for preliminary screening of colonies for subsequent confirmation by other molecular and immune tools. Further to note, *R. pickettii*, *Ralstonia mannitolilytica* (*R. mannitolilytica*) and *Ralstonia insidiosa* (*R. insidiosa*) are emerging nosocomial pathogens, which cause life-threatening bacteremia/septicemia in immunocompromised individuals and children [56–58]. Hence, the flagellin can be evaluated as a suitable immune target for detecting these pathogens.

The main aim of this study is to develop a proof of concept for immunodetection using flagellin as a target and to devise a prototype latex agglutinating reagent based on anti-flagellin antibodies for screening colonies. In a nutshell, two conclusions can be drawn from our results: (1) The flagellin protein can be used as a potential immune target for the identification of bacterial wilt pathogen; (2) anti-FliC polyclonal-coated latex beads can be used for preliminary screening of suspected *RPS* and *RS* colonies.

In conclusion, the present study illustrates the possible use of flagellin protein as an immune target for detecting *RPS* and *RS* cells. Antibodies raised against the flagellin protein coated over latex particles can be utilized for preliminary screening of the colonies obtained during the isolation of the bacterial wilt strains from the infected plant samples. Further, this proof of concept and the prototype need to be evaluated with bacterial wilt strains isolated from different hosts across various geographical regions.

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Author contribution Study conceptualization: Shalini Bhatt, S. Merwyn P. Raj; methodology: Shalini Bhatt, S. Merwyn P. Raj, Neha Faridi; formal analysis and investigation: Shalini Bhatt, S. Merwyn P. Raj, Shraddha Mishra; writing—original draft preparation: Shalini Bhatt; writing—review and editing: Shalini Bhatt; supervision: Ankur Agarwal, S. Merwyn.

Declarations

Polyclonal antibodies used in the study were outsourced by Abgenex Private Limited, Bhubaneswar, India.

Competing interests The authors declare no competing interests.

References

1. Prameela TP, Suseela Bhai R (2020) Bacterial wilt of ginger (Zingiber officinale Rosc.) incited by Ralstonia pseudosolanacearum - a review based on pathogen diversity, diagnostics and management. J Plant Pathol 102:709–719
2. Peeters N, Guidot A, Vailleau F, Valls M (2013) *Ralstonia solanacearum* , a widespread bacterial plant pathogen in the post-genomic era. Mol Plant Pathol 14:651–662
3. Gonçalves OS, Campos KF, de Assis JCS, Fernandes AS et al (2020) Transposable elements contribute to the genome plasticity of Ralstonia solanacearum species complex. Microb Genom 6:1–12
4. Sharma P, Johnson MA, Mazloom R, Allen C, Heath LS, Lowe-Power TM, Vinatzer BA (2022) Meta-analysis of the Ralstonia solanacearum species complex (RSSC) based on comparative evolutionary genomics and reverse ecology. Microb Genom 8:000791
5. Greenrod STE, Stoycheva M, Elphinstone J, Friman VP (2022) Global diversity and distribution of prophages are lineage-specific within the Ralstonia solanacearum species complex. BMC Genomics 23:1–19
6. Safni I, Cleenwerck I, de Vos P, Fegan M et al (2014) Polyphasic taxonomic revision of the Ralstonia solanacearum species complex: proposal to emend the descriptions of Ralstonia solanacearum and Ralstonia syzygii and reclassify current R. syzygii strains as Ralstonia syzygii subsp. syzygii subsp. nov., R. s. Int J Syst Evol Microbiol 64:3087–3103
7. Lee I, Kim YS, Kim JW, Park DH (2020) Genetic and pathogenic characterization of bacterial wilt pathogen, Ralstonia pseudosolanacearum (Ralstonia solanacearum Phylotype I), on Roses in Korea. Plant Pathol J 36:440
8. Goncalves OS, de Queiroz MV, Santana FM (2020) Potential evolutionary impact of integrative and conjugative elements (ices) and genomic islands in the Ralstonia solanacearum species complex. 10:12498
9. Salanoubat M, Genin S, Artiguenave F, Gouzy Mangenot S, Arlat M, Billault A, Brottier P, Camus JC, Cattolico L, Chandler M, Choisne N, Claudel-Renard C, Cunnac S, Demange N, Gaspin C, Lavie M, Moisan A, Robert C, Saurin W, Schiex T, Siguier P, Thébault P, Whalen M, Wincker P, Levy M, Weissenbach J,

Boucher CA (2002) Genome sequence of the plant pathogen Ralstonia solanacearum. Nature 415:497–502
10. Catara V, Bella P (2020) Bacterial diseases. In: Integrated Pest and Disease Management in Greenhouse Crops. Plant Pathology in the 21st Century. 9:33–54. Switzerland, AG: Springer Nature
11. Genin S, Denny TP (2012) Pathogenomics of the *Ralstonia solanacearum* Species Complex. Annu Rev Phytopathol 50:67–89
12. EPPO standards (2018) PM 7/21 (2) Ralstonia solanacearum, R. pseudosolanacearum and R. syzygii (Ralstonia solanacearum species complex). EPPO Bulletin 48:32–63
13. Robinson-Smith A, Jones P, Elphinstone JG, Forde SMD (1995) Production of antibodies to *pseudomonas solanacearum* the causative agent of bacterial wilt. Food Agric Immunol 7:67–79
14. Behiry SI, Mohamed AA, Younes HA, Salem MZM, Salem AZM (2018) Antigenic and pathogenicity activities of Ralstonia solanacearum race 3 biovar 2 molecularly identified and detected by indirect ELISA using polyclonal antibodies generated in rabbits. Microb Pathog 115:216–221
15. Bellstedt DU (2009) Enzyme-linked immunosorbent assay detection of Ralstonia solanacearum in potatoes: the South African experience. Methods Mol Biol 508:51–62
16. Griep RA, van Twisk C, van Beckhoven JRCM, van der Wolf JM, Schots A (1998) Development of specific recombinant monoclonal antibodies against the lipopolysaccharide of *Ralstonia solanacearum* Race 3. Phytopathology 88:795–803
17. Caruso P, Llop P, Palomo JL, Garcia P, Morente C, López MM. Evaluation of methods for detection of potato seed contamination by Ralstonia solanacearum. InBacterial Wilt Disease: Molecular and Ecological Aspects. 128-132. Berlin, Heidelberg: Springer Berlin Heidelberg.
18. Rajeshwari N, Shylaja M, Krishnappa M, Shetty HS et al (1998) World J Microbiol Biotechnol 14:697–704
19. Han Y, Zhang XH, Chen Y, Li Y, Chen H, Fang L (2011) High-level expression, purification, polyclonal antibody preparation against recombinant OprD from Pseudomonas aeruginosa. Afr J Biotechnol 10:1246–1251
20. Hotinger JA, May AE (2020) Antibodies inhibiting the type III secretion system of Gram-negative pathogenic bacteria. Antibodies 9:35
21. Landry D, González-Fuente M, Deslandes L, Peeters N (2020) The large, diverse, and robust arsenal of *Ralstonia solanacearum* type III effectors and their in planta functions. Mol Plant Pathol 21:1377–1388
22. Suraby EJ, Sruthi KB, Antony G (2022) Genome-wide identification of type III effectors and other virulence factors in Ralstonia pseudosolanacearum causing bacterial wilt in ginger (Zingiber officinale). Mol Genet Genomics 297:1371–1388
23. Denny TP (1991) Genetic evidence that extracellular polysaccharide is a virulence factor of *Pseudomonas solanacearum*. Mol Plant Microbe Interact 3:293–300
24. Hayashi K, Senuma W, Kai K, Kiba A, Ohnishi K, Hikichi Y (2019) Major exopolysaccharide, EPS I, is associated with the feedback loop in the quorum sensing of Ralstonia solanacearum strain OE1-1. Mol Plant Pathol 20:1740–1747
25. Tans-Kersten J, Huang H, Allen C (2001) *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J Bacteriol 183:3597–3605
26. Corral J, Sebastià P, Coll NS, Barbé J, Aranda J, Valls M (2020) Twitching and swimming motility play a role in Ralstonia solanacearum pathogenicity. MSphere 5:e00740-19
27. Liu H, Zhang S, Schell MA, Denny TP (2005) Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol Plant Microbe Interact 18:1296–1305
28. Xavier AD, de Melo AG, Hendrich CG, Tremblay DM, Rousseau GM, Plante PL, Forest KT, Alfenas-Zerbini P, Allen C, Moineau S (2022) In through the out door: a functional virulence factor secretion system is necessary for phage infection in Ralstonia solanacearum. MBio 13:e01475-22
29. Huang Q, Allen C (1997) An exo-poly-alpha-D-galacturonosidase, PehB, is required for wild-type virulence of Ralstonia solanacearum. J Bacteriol 179:7369–7378
30. Lowe-Power TM, Khokhani D, Allen C (2018) How Ralstonia solanacearum exploits and thrives in the flowing plant xylem environment. Trends Microbiol 26:929–942
31. Umrao PD, Kumar V, Kaistha SD (2021) Study of pathogenic traits of bacterial wilt-causing phytopathogens around Kanpur and Fatehpur regions, Uttar Pradesh, India. J Appl Biol Biotechnol 9:45–55
32. Armitage JP, Berry RM (2020) Assembly and dynamics of the bacterial flagellum. Annu Rev Microbiol 74:181–200
33. Jindal G, Tewari R, Gautam A, Pandey SK, Rishi P (2012) Immunological characterization of recombinant Salmonella enterica serovar Typhi FliC protein expressed in Escherichia coli. AMB Express 2:55–63
34. Okamura M, Matsumoto W, Seike F, Tanaka Y et al (2012) Efficacy of soluble recombinant FliC protein from Salmonella enterica Serovar Enteritidis as a potential vaccine candidate against homologous challenge in chickens. Avian Dis 56:354–358
35. Mirhosseini SA, Fooladi AAI, Amani J, Sedighian H (2017) Production of recombinant flagellin to develop ELISA-based detection of Salmonella Enteritidis. Braz J Microbiol 48:774–781
36. Elphinstone JG, Hennessy J, Wilson JK, Stead DE (1996) Sensitivity of different methods for the detection of Ralstonia solanacearum in potato tuber extracts. EPPO Bulletin 26:663–678
37. Kelman A (1954) The relationship of pathogenicity of Pseudomonas solanacearum to colony appearance in a tetrazolium medium. Phytopathology 44:693–695
38. Opina N, Tavner F, Hollway G, Wang JF, Li TH, Maghirang R, Fegan M, Hayward A, Krishnapillai V, Hong W, Holloway B (1997) A novel method for development of species and strain-specific DNA probes and PCR primers for identifying Burkholderia solanacearum (formerly Pseudomonas solanacearum). Asia Pac J Mol Biol Biotechnol 5:19–30
39. Umesha S, Avinash P (2015) Multiplex PCR for simultaneous identification of Ralstonia solanacearum and Xanthomonas perforans. 3 Biotech 5:245–252
40. Thomas P, Upreti R (2014) Significant effects due to peptone in Kelman Medium on colony characteristics and virulence of Ralstonia solanacearum in tomato. Open Microbiol J 8:95–113
41. Nakahara H, Mori T, Matsuzoe N (2021) Screening of phenotypic conversion mutant strains of Ralstonia solanacearum for effective biological control of Verticillium wilt in eggplant. Crop Prot 142:105530
42. Sambrook J, Russell D, Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, vol 1, 3rd edn. Cold Spring Harbor Laboratory Press, New York
43. Wingfield PT (2015) Overview of the purification of recombinant proteins. Curr Protoc Protein Sci 80:6–1
44. Classen DC, Morningstar JM, Shanley JD (1987) Detection of antibody to murine cytomegalovirus by enzyme-linked immunosorbent and indirect immunofluorescence assays. J Clin Microbiol 25:600–604
45. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
46. Safni I, Cleenwerck I, De Vos P, Fegan M, Sly L, Kappler U (2014) Polyphasic taxonomic revision of the .....sp.nov. Int J Syst Evol Microbiol 64:3087–3103
47. Zheng XF, Zhu YJ, Liu B, Zhou Y, Che JM, Lin NQ (2014) Relationship between *Ralstonia solanacearum* diversity and severity of bacterial wilt disease in tomato fields in China. J Phytopathol 162:607–616

48. Rostand RC, Joel M, Viviene M, Steven R (2018) Isolation and characterization of Ralstonia solanacearum strains causing bacterial wilt of potato in Nakuru County of Kenya. Afr J Biotechnol 17:1455–1465
49. Seleim MAA, Abo-Elyousr KAM, Abd-El-Moneem KM, Saead FA (2014) First report of bacterial wilt caused by Ralstonia solanacearum biovar 2 race 1 on tomato in Egypt. Plant Pathol J 30:299–303
50. Kumar R, Barman A, Jha G, Kumar R (2013) Identification and establishment of genomic identity of Ralstonia solanacearum isolated from a wilted chilli plant at Tezpur, North East India. Curr Sci 106:1571
51. Pradhanang PM, Elphinstone JG, Fox RTV (2000) Sensitive detection of Ralstonia solanacearum in soil: a comparison of different detection techniques. Plant Pathol 49:414–422
52. Kelman A, Hruschka J (1973) The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of Pseudomonas solanacearum. J Gen Microbiol 76:177–188
53. Kang Y, Liu H, Genin S, Schell MA, Denny TP (2002) *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol Microbiol 46:427–437
54. Clough SJ, Flavier AB, Schell MA, Denny TP (1997) Differential expression of virulence genes and motility in Ralstonia (Pseudomonas) solanacearum during exponential growth. Appl Environ Microbiol 63:844–850
55. Brumbley SM, Denny TP (1990) Cloning of wild-type Pseudomonas solanacearum phcA, a gene that when mutated alters expression of multiple traits that contribute to virulence. J Bacteriol 172:5677–5685
56. Stelzmueller I, Biebl M, Wiesmayr S, Eller M, Hoeller E, Fille M, Weiss G, Lass-Floerl C, Bonatti H (2006) Ralstonia pickettii—innocent bystander or a potential threat? Clin Microbiol Infect 12:99–101
57. Ryan MP, Adley CC (2014) Ralstonia spp.: emerging global opportunistic pathogens. Eur J Clin Microbiol Infect Dis 33:291–304
58. Fang Q, Feng Y, Feng P, Wang X, Zong Z (2019) Nosocomial bloodstream infection and the emerging carbapenem-resistant pathogen Ralstonia insidiosa. BMC Infect Dis 19:334–342