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Biocontrol potential of bacteriophage ϕsp1 against bacterial wilt-causing *Ralstonia solanacearum* in Solanaceae crops

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Abstract

Background: Bacteriophages are effective biocontrol strategy as well as ecofriendly remedy for the emerging antibiotic and chemical resistance in bacterial phytopathogens such as bacterial wilt-causing *Ralstonia solanacearum*. One of the major challenges in the use of bacteriophage therapy for agricultural phytopathogens is maintaining their viability even during variations in pH, temperature, ultraviolet irradiation, and desiccation during field application for sustainable agriculture.

Results: In this study, the isolation and characterization of phage ϕsp1 for its efficacy against wilt-causing *R. solanacearum* performed on *Solanum lycopersicum* (tomato) seedlings and *Solanum tuberosum* (potato) tuber assay are reported. Bacteriophage was found to be viable and stable at a wide pH range (3.0–9.0) and at temperatures up to 55 °C. Phage ϕsp1 required ~15 min for adsorption and completed its life cycle in 25–30 min by host cell lysis with a burst size of ~250–300. Phage ϕsp1 eradicated 94.73% preformed *R. solanacearum* biofilm and inhibited biofilm formation by 73.68% as determined by the static crystal violet microtiter biofilm assay. Transmission electron microscope revealed the phage ϕsp1 to be approximately 208±15 nm in size, comprising of icosahedral head (100 ±15 nm) and tail, as belonging to Myoviridae family. Plant bioassays showed 81.39 and 87.75% reduction in pathogen count using phages ϕsp1 in potato tuber and tomato seedlings, respectively. Reversal in disease symptoms was 100% in phage-treated tuber and tomato plant (pot assay) compared to only pathogen-treated controls.

Conclusion: Isolated bacteriophage ϕsp1 was found to be highly host specific, effective in biofilm prevention, and capable of inhibiting bacterial wilt at low multiplicity of infection (1.0 MOI) in tomato as well as potato tuber bioassays. Phages ϕsp1 were environmentally stable as they survive at variable pH and temperature. Bacteriophage ϕsp1 shows a promise for development into a biocontrol formulation for the prevention of *R. solanacearum* bacterial wilt disease.

Keywords: Bacterial wilt, Bacteriophage, Biocontrol, Biofilm, Potato tuber, Tomato seedlings

Background

Ralstonia solanacearum has been categorized as the second most devastating pathogen causing bacterial wilt in crops of mainly the *Solanaceae* family worldwide (Ramesh et al. 2014). It is a Gram-negative bacterial phytopathogen with a wide host range of over 200 different crops (Álvarez et al. 2010). *R. solanacearum* has been recently reclassified into three distinct species, namely, *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotypes I and III), and *R. syzygii* (phylotype IV), that have different host ranges and geographic origin/distribution (Safni et al. 2014). Following infection, the pathogen enters through damaged plant systems and colonizes the vascular tissue, secreting copious amounts of exopolysaccharides, blocking water uptake, and

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causing xylem destruction. This results in wilting of leaves and stem and eventual collapse and death of the plant (Yadeta and Thomma 2013). Control of the phytopathogen is difficult due to increasing resistance of the organisms to traditional control mechanisms such as copper, pesticides, and antibiotic treatments. Other treatments that have been studied for its control include the use of antagonistic metabolites producing rhizosphere, endophytic bacteria, fungi and endophytes, organic residues, and various soil amendments (Yuliar and Toyota 2015).

Most control strategies become ineffective in controlling biofilm forms of growth which contribute to the virulence of the phytopathogen. Biofilm formation is the ability of the pathogen to form communities enclosed within an exopolymeric substance (EPS) that protects it from various stresses. This ability to form biofilm can protect the pathogen from plant defenses as well as external control measures (Mina et al. 2019). Mori et al. (2018) have reported the ability of *R. solanacearum* strain OE1-1 to form mushroom type of biofilms within intercellular spaces in tomato plant root and stem. Many biofilm factors such as sugars (ralfuranones) and extracellular nucleases contributed to *R. solanacearum* virulence (Minh Tran et al. 2016; Mori et al. 2018).

Bacteriophages, viruses that are intracellular parasites of bacterial cells, that can penetrate biofilms and cause lysis of phytopathogens are a promising biocontrol strategy (Kaistha et al. 2018). The control of many bacterial phytopathogenic agents such as *Pseudomonas syringae*, *Xanthomonas campestris*, *X. axonopodis*, *Pectobacterium carotovorum*, *Dickeya solani*, and *Erwinia amylovora* has been reported using bacteriophage (Buttimer et al. 2017). For the effective biocontrol of bacterial phytopathogen, lytic phages are particularly useful due to their ability to cause targeted lysis of host cells, their abundance in nature, environmental non-toxicity, and the auto-dosing effect (Kaistha et al. 2018). Many studies have isolated and characterized *R. solanacearum* bacteriophages. These include temperate phages responsible for enhanced virulence, lytic phages that have demonstrated effective biocontrol as well as phages with no demonstrable biocontrol effect in plant assay studies (Álvarez and Biosca 2017). Phage PE226 having the properties of lytic and lysogeny both exhibited virulence towards *R. solanacearum* (Murugaiyan et al. 2011). Another phage Rs551 demonstrated diminished virulence stress of *R. solanacearum* race 3 biovar 2 by decreasing exopolysaccharide production (Ahmad et al. 2017). On the other hand, RSS1 a filamentous phage enhanced virulence of phytopathogen *R. solanacearum* upon infection. This was attributed to induction of virulence factors such as extracellular polysaccharide (EPS) synthesis and twitching motility, leading to early wilting in tomato plants (Addy et al. 2012). Recently, bacteriophage, namely, RsoM1USA, infecting *R. solanacearum* strain RUN302 significantly reduced growth of the infected bacterium in vitro but was unable to reduce virulence in tomato plants, as compared to uninfected strain (Addy et al. 2019). In the Indian context, in vitro phage lytic activity against *R. solanacearum* has been reported (Barua and Nath 2019). There are however, to the best of our knowledge, no studies that report phages capable of inhibiting biofilm formation in *R. solanacearum* and acting as an effective biocontrol agent using plant bioassays.

Herein, isolation of lytic bacteriophage sp1 against *R. solanacearum* strain 1629 that is capable of inhibiting and eradicating *R. solanacearum* biofilms was report. In order to test the phage in field applications, its sensitivity to variations in pH, temperature, and ultraviolet radiation was characterized. The biocontrol efficiency of bacteriophages was demonstrated through plant bioassays performed on *Solanum lycopersicum* (tomato) seedlings and *S. tuberosum* (potato) tuber slice.

Methods

Bacterial strains and culture conditions

R. solanacearum strain F1C1 (obtained from Dr Suvendra Roy, Department of Molecular & Biotechnology, Tezpur University, Assam) and *R. solanacearum* (NAIMCC-F01629) stock cultures were maintained at −20 °C and propagated on the tryptone soy agar (TSA) or casamino acid-peptone glucose (CPG) (Hi Media, India) at 28 to 30°C. 2,3,5-Triphenyl tetrazolium chloride (TTC) medium (Hi Media, India) was used for differentiating virulent colony from non-virulent or mutant type colonies.

Isolation and characterization of bacteriophages

Sampling and isolation of bacteriophage

Bacteriophages were isolated from soil samples collected from rhizosphere of different cultivars of solanaceous crops from Kanpur and Fatehpur, Uttar Pradesh, India. Soil sampling was carried out preferentially in April (summer season) and July (after precipitation). For phage enrichment, soil pool collected from various cultivars was mixed well. Five grams of soil were dissolved in 10 ml 1X phosphate-buffered saline (PBS) in 45-ml Falcon tube by vigorous shaking to release phages from soil particles, centrifuged (10,000 rpm for 10 min at 4°C), supernatant collected, and filtered through PES membrane syringe (0.45-μm pore size) (Hi Media Pvt. Ltd. India). Plaque assay was performed using double-layer agar (DLA) overlay method (Kropinski et al. 2009). Equal aliquots of log phase host bacteria *R. solanacearum* strain 1629 (NAIMCC-F01629) and phage filtrate (100 μl) were mixed in 0.3–0.4% soft agar and poured over a 1.5%

hard agar plate in TSA media. Specificity of isolated phages was also checked against *R. solanacearum* strain F1C1 by employing DLA. Plate was incubated at 30±2°C for 24 to 48 h for observing plaque formation.

Bacteriophage purification and propagation

Isolated bacteriophages were purified through single successive plaque isolation and propagated by picking a well-separated plaque with the help of a sterile inoculation loop and inoculated into 200 ml TSB medium, containing overnight grown log culture of 10^9 host cells and incubated at 30±2°C at 140 rpm for phages stock preparation.

The phage suspension was centrifuged (10,000 rpm, 10 min, 4°C), filter sterilized, and treated with chloroform (1% v/v) to remove bacterial contamination. High titer phage purification was further conducted by using PEG-8000 method with some modification in SM buffer (50 mmol L^{-1} Tris-HCl at pH 7.4, 100 mmol L^{-1} NaCl, 10 mmol L^{-1} $MgSO_4$, and 0.01% gelatin) (Yamamoto et al. 1970). Purified concentrated phages were stored in aliquots at −20°C for long-term storage. Short-term stock preparations were maintained at 4°C for further use.

Spot assay and double-layer agar (DLA) overlay assay

To check phage viability, spot assay was carried out. Briefly, phage suspension was serially diluted, and 10 μl diluted phage was spot inoculated on molten agar (0.4% agar, w/v) containing host cells of 10^7 CFU ml^{-1}. Clear zones of plaques were observed after incubating the plates overnight at 30±2°C. Further, the phage titer was determined by plaque assay by employing DLA technique as described previously (Kropinski et al. 2009).

Adsorption assay, one-step growth curve

Adsorption assay and one-step growth curve were performed with some modification (Delbrück 1940). Adsorption time and burst size for phages ϕsp1 were determined. An equal amount of phages (titer 10^6) and bacterial suspension was taken, incubated for 5 min, diluted ($1:10^2$), and DLA performed at 5 min intervals till 30 min in order to determine the phage titer.

Temperature, pH, and UV irradiation sensitivity

Thermostability of bacteriophage was determined by incubating phage titer 10^6 PFU ml^{-1} for 10 min at 37, 45, and 55°C with intermittent shaking as per protocol of Sagar et al. (2017). For the determination of pH stability, the same titer was incubated at pH 3.0, 5.0, 7.0, and 9.0 for 20 min (Sagar et al. 2017). For UV irradiation sensitivity, phage titer 10^6 PFU was exposed to UV C irradiation (UV254 nm) for 5, 10, and 15 min. The treatments were followed by DLA as described previously.

Host range

Host range of phage ϕsp1 was tested by performing spot assay against *R. solanacearum* isolates RS1, RS2, RS3, and RS4 isolated from stem of potato plants collected from agricultural fields and *R. solanacearum* strain F1C1. Rhizospheric bacterial hosts such as *P. aeruginosa* ATCC 15442, *P. aeruginosa* R32 and GD2 (obtained from Department of Microbiology, CSJMU University, Kanpur) were also used to determine host specificity.

Transmission electron microscopy

Transmission electron microscope (TEM) observation to study bacteriophage morphology was performed with some modifications (Goodridge et al. 2003). High titer purified phage suspension in SM buffer was dropped on copper-coated grids (diameter, 3 mm; 300 meshes) and allowed to adsorb for 5 min. The bacteriophage particles were stained by the addition of 2% (w/v) phosphotungstic acid (PTA) for 10 s. The grid was allowed to dry for 20 min and examined under a TEM (FEI Tecnai S Twin) at 200 kv (SAIF, AIIMS, Delhi, India).

Scanning electron microscopy

Biofilm development on glass cover slip surfaces was visualized by scanning electron microscopy (SEM) with some modification (Sagar et al. 2017). Cover slip was washed gently in sterile 1X phosphate-buffered saline (PBS) to remove planktonic cells, fixed in 5% (v/v) glutaraldehyde in PBS buffer for 2 h, followed by fixing with post-fixative 1% osmium tetroxide. This was followed by dehydration steps through a graded series of 10-min ethanol immersions (30, 50, 70, 90, and 100%). Specimens were mounted on aluminum stubs and observed on SEM (SM 6490, BBAU, Lucknow, UP, India). The entire cover slip surface was examined, and images were chosen that represented the typical field of view.

Biofilm assay

Effect of phage treatment on biofilm inhibition was determined using crystal violet biofilm microtiter assay (Umrao et al. 2020). Briefly, log phase 10^6 CFU ml^{-1} *R. solanacearum* strain 1629 was simultaneously treated by phage ϕsp1 for biofilm formation assay, while 24-h preformed biofilms were phage treated at 1.0 multiplicity of infection (MOI) for biofilm eradication assay. Post 48 h incubation, the biofilm was washed with 1X PBS and stained with (1% w/v) crystal violet for 20 min. Excess stain was removed, plates washed with PBS, and dimethyl sulfoxide (DMSO) was used to solubilize crystal violet-stained biofilm. Results were evaluated by using spectrophotometry (Thermo Scientific Multiscan EX, USA).

Plant bioassay

Plant bioassay study was carried out, using *Solanum lycopersicum* (tomato seedlings and plants) and in *S. tuberosum* (potato tuber slices).

Solanum lycopersicum (tomato) seedling assay

Variety S-22 of tomato seeds were selected and sterilized with 70% ethanol, grown on sterile wet cotton bed on plastic tray (Singh et al. 2018). The tray was covered up to maintain humidity and accessibility of light for seed germination in the month of November. Seedling started appearing from the 5th day onwards. Tomato seedlings of 4–5 cm in height with two cotyledon leaves were used for pathogenicity test. The experiment was designed for 2.0-ml microcentrifuge tubes with phosphate buffer containing pathogen of 10^8 CFU ml^{-1} for pathogenicity by root inoculation method (Singh et al. 2018). Phage ϕsp1 (1.0 MOI) was used for biocontrol, and untreated tomato seedlings in phosphate buffer were used as control. The experiment was performed in triplicates. The seedlings were observed daily, and disease parameters included wilting of stem and leaves. Fresh weight of the infected, treated, and control seedlings was measured at 72 h post-inoculation. Spectrophotometric reading of cell suspensions in microcentrifuge tubes containing the tomato seedling was taken at 620 nm (Thermo Scientific Multiscan EX, USA).

Solanum lycopersicum (tomato) pot assay

Plant bioassay study was undertaken to check bacteriophage biocontrol efficiency and persistence of phage by using soil-drenching method in greenhouse setup in pots containing 250 g per pot (soil, sand, coconut fiber in 20:4:1). Tomato plants (variety S-22) of 5–6-cm length in triplicate were used for the experiment. The base of plant's stem was scratched by a sterilized needle, and plants were inoculated by 20 ml of 10^8 CFU ml^{-1} *R. solanacearum* strain 1629 (8×10^6 CFU g^{-1}) and treated with phage ϕsp1 (1.0 MOI) poured around tomato seedlings in the soil. Disease symptoms of tomato plants were recorded twice in a week by using wilting grade scale according to Kempe and Sequerie (1983). Grade 1, 25% plant leaves wilted; grade 2, 26–50% of plant leaves wilted; grade 3, 51–75% plant leaves wilted; grade 4, 76% or more plant leaves wilted and stem collapses; and grade 5, death of plants (Kempe and Sequeira 1983). All experiments were performed in triplicates.

Solanum tuberosum (potato) tuber slices assay

The experiment was designed for direct inoculation method on potato tuber slices to check phage biocontrol efficiency against *R. solanacearum* strain 1629 as described previously (Champoiseau et al. 2009). Surface-sterilized (70% ethanol) potato tuber was pieced into slices (4.0 × 3.5 × 0.6 cm^3) and inoculated with 10^8 CFU ml^{-1} of host *R. solanacearum* strain 1629 for pathogenicity control. Host + phage ϕsp1 (1.0 MOI), phage ϕsp1 (1.0 MOI), and uninoculated tuber slices were also incubated at 28°C under daily observation. The experiment was performed in triplicate. Diseased symptoms which included vascular browning, bacterial ooze, and tuber necrosis were recorded daily up to day 10 post-incubation. Grading scale used for qualitative measure of tuber disease symptoms was as follows: grade 0, no symptoms; grade 1, yellow discoloration, no ooze, and no necrosis; grade 2, brown discoloration, no ooze, softening of tissue at center of lesion but hard to scoop or pick with inoculating loop; grade 3, brown discoloration with ring formation, whitish ooze from tuber, necrotic tissue softening which is easy to scoop; and grade 4, dark brown-blackish discoloration within the ringed lesion, copious whitish ooze from lesion, total tissue necrosis which is easy to scoop.

Standard plate count was performed at day 10 by plating (100 μl) of (0.1 g ml^{-1}) infected inoculated tuber slices and control tubers. Colony count was recorded after plating for 48 h at 28°C incubation. Cell density of infected tuber tissue solution (0.1 g ml^{-1}) was also quantified by spectrophotometry (A620nm) (Thermo Scientific Multiscan EX, USA). The presence of *R. solanacearum* was confirmed by simple staining with crystal violet using bright field microscopy.

Ethics approval and consent to participate

Not applicable.

Statistical analysis

Statistical analysis was done using Student's *t* test. All experiments were repeated at least twice in triplicates. $p \leq 0.05$ was considered as biologically significant.

Results

Isolation and characterization of bacteriophages

Lytic bacteriophages having ability to infect bacterial wilt-causing *R. solanacearum* (ϕsp1, ϕS1, ϕS2, ϕS3, ϕS4, ϕV3, ϕP1, ϕP2, ϕP3, and ϕP4) were isolated from solanaceous cultivar's rhizosphere soil samples from Kanpur and Fatehpur regions. Phage isolation was performed using DLA with different dilutions of the soil samples. Twelve phages of different plaque morphology were isolated, out of which ϕsp2 was turbid 1–2 mm plaque size, ϕW was pinpoint, and the rest were of clear characteristics (Table 1). Turbid and pinpoint plaque morphology phages were screened to be lysogenic and not considered for further characterization. Phage ϕsp1 was selected for further research based on its clear and consistent lytic activity against a wide host range of *R. solanacearum spp.* One clear large size plaque (4–5 mm) of ϕsp1 was

Table 1 List of isolated bacteriophages for host *Ralstonia solanacearum* strain 1629 used in this study

Phage name	Location (district)	Source	Plaque morphology	Phage type
ϕsp1	Fatehpur	Mixed soil pool	Clear, 4–5mm	Lytic
ϕsp2	Fatehpur	Mixed soil pool	Turbid, 1–2mm	Lysogenic
ϕS1	Fatehpur	Tomato field soil	Clear, 2–3mm	Lytic
ϕS2	Fatehpur	Tomato field soil	Clear, 2–3mm	Lytic
ϕS3	Fatehpur	Tomato field soil	Clear, 2–3mm	Lytic
ϕS4	Fatehpur	Tomato field soil	Clear, 2–3mm	Lytic
ϕV3	Kanpur	Water	Clear, 2–3mm	Lytic
ϕW	Kanpur	Water	Pinpoint	Lysogenic
ϕP1	Kanpur	Potato field soil	Clear, 2–3mm	Lytic
ϕP2	Kanpur	Potato field soil	Clear, 2–3mm	Lytic
ϕP3	Kanpur	Potato field soil	Clear, 2–3mm	Lytic
ϕP4	Kanpur	Potato field soil	turbid, 2–3mm	Lytic

purified and concentrated as described previously and confirmed using the spot test and double layer agar overlay assay (Fig. 1a, b, and c).

Morphological characteristics of phage ϕsp1, using TEM imaging, was found to be of long contractile tailed virus with icosahedral head 85±10 nm and 208±10 nm in size (Fig. 1d). Bacteriophage ϕsp1 morphology resembles phages belonging to Myoviridae family containing ds DNA classified under Caudovirales. Based on these characteristic, phage ϕsp1 was designated as vB_RsoMSP1 (Ackermann 2011; Adriaenssens and Brister 2017). Phage ϕsp1 was further characterized by one-step growth curve and adsorption assay. These phages took ~15 min for adsorption and completed their life cycle in 25–30 min by lysis of the host cell with a burst size of 250–300 (Fig. 2a).

Stability characterization of bacteriophage (pH, temperature, and UV)

Bacteriophage ϕsp1-based biocontrol of *R. solanacearum* is an effective measure of controlling the phytopathogen. However, environmental factors such as pH, temperature, desiccation, and UV irradiation limited the phage survivability and persistence in the agriculture soil (Jones et al. 2018). Phage ϕsp1 isolated from solanaceous cultivar's rhizosphere soil against *R. solanacearum* was found to be stable at temperature up to 55°C and a wide pH range of 3–9 (Fig. 2b and c). Optimal lytic activity of ϕsp1 was found at neutral pH and temperature 37°C, which decreased by increasing temperature and decreasing plaque size (Fig. 2b). The loss of virus viability was more than 36.84% at 45°C in laboratory condition. However, phage ϕsp1 demonstrated viability at variable pH (3–9) in acidic and alkaline condition, but with reduced plaque sizes (Fig. 2c). The burst size of phage decreased in acidic and alkaline pH as well as increasing temperature, but phage infectivity was not affected. Hence, phage ϕsp1 survival will not be affected drastically with changes in rhizospheric soil pH and temperature and help keep the bacterial population under control. Phage ϕsp1 was however drastically affected by UV

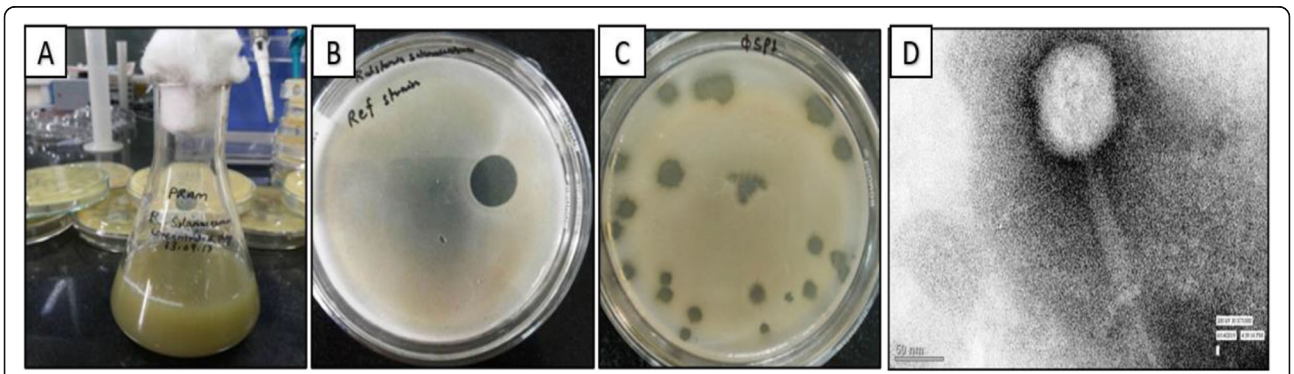

Fig. 1 Isolation and characterization of phage ϕsp1 against host *Ralstonia solanacearum* strain 1629. **a** Propagated phages stock. **b** Spot test. **c** ϕsp1 plaques on double-layer agar plate. **d** TEM image of phage ϕsp1 morphology at 200kv ×71000

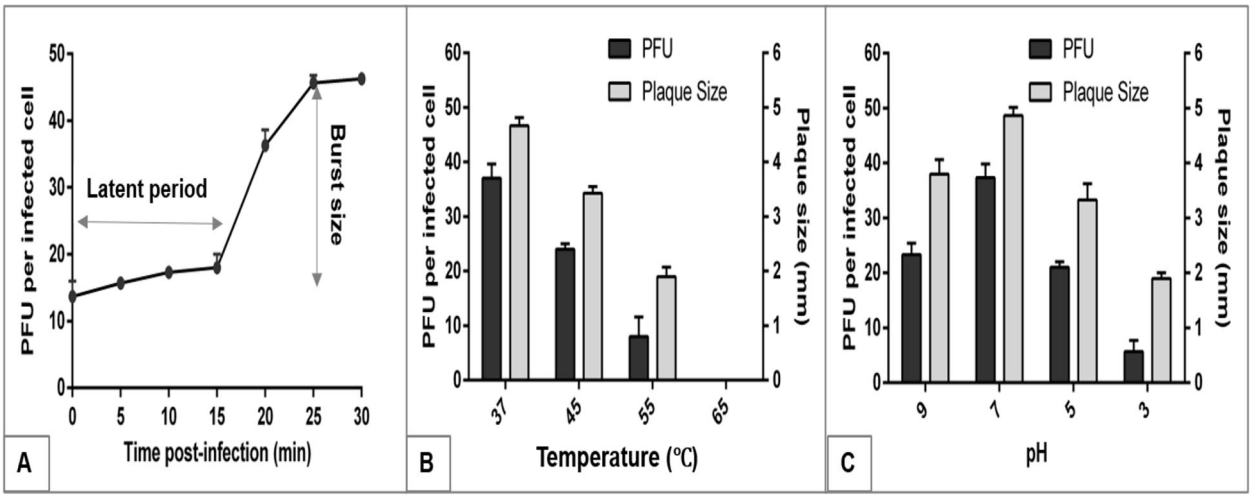

Fig. 2 One-step growth curve and stability characterization of phage ϕsp1 growing on *Ralstonia solanacearum* strain 1629 at varying growth temperatures and pH. **a** Phage ϕsp1 PFUs per infected cell at 5-min interval post-infection up to 30 min with dilution factor 10^2. Phage titer was determined by performing DLA. Latent period 15 min and burst size of virus 250–300. **b** The relative number and size of visible plaques under different temperature conditions were compared for phage variability. **c** Phage ϕsp1 stability at variable pH range 3–9 plaque size deceased in acidic and alkaline conditions

C irradiation (254 nm) as no plaques were observed in UV-treated DLA plates.

Host range determination

Host range of phage ϕsp1 was determined by spot assay on different bacteria such as plant growth promoting Pseudomonads and *R. solanacearum* RS1, RS2, RS3, RS4. *R. solanacearum* strain 1629 was used as positive control (Table 2). Positive spot assay with *R. solanacearum* strain F1C1 indicated biovar similarity between *R. solanacearum* strain 1629 and strain F1C1. Phage ϕsp1 can be a good candidate as biocontrol agent, as it showed no lytic activity against plant growth promoting *Pseudomonas* GD2 and *Pseudomonas* R32 and can be safely used without affecting beneficial soil microflora present in rhizosphere and host plants. The data also indicated that the phages are host specific for *R. solanacearum* and can be useful for diagnosis purpose using phage typing. Due to specificity against targeted host, bacteriophages can be used as diagnostic tools for plant pathogenic bacterial species (Vu and Oh 2020).

Table 2 Host range determination for phage ϕsp1 on different *Ralstonia solanacearum* strains and plant growth-promoting bacteria (PGPB)

Sr No	Host	Spot assay
1.	*R. solanacearum* 1629	+
2.	*R. solanacearum* F1C1	+
3.	*R. solanacearum* RS1	-
4.	*R. solanacearum* RS2	-
5.	*R. solanacearum* RS3	-
6.	*R. solanacearum* RS4	-
7.	*Pseudomonas aeruginosa* ATCC 15442	-
8.	*Pseudomonad* GD2 (PGPB)	-
9.	*Ps. aeruginosa* R32 (PGPB)	-

Bacteriophage ϕsp1 biocontrol efficacy against wilt pathogen *R. solanacearum*

Application of bacteriophages in biofilm inhibition

One of the potent pathogenicity factors of bacterial wilt-causing *R. solanacearum* is its ability to form biofilms (Mori et al. 2016). Efficacy of phage ϕsp1 in inhibiting biofilm formation by *R. solanacearum* strain 1629 by using SEM as well as in static biofilm assay was tested. *R. solanacearum* was found to be a strong biofilm former, and phage ϕsp1 infection inside the biofilm was characterized by scanning electron microscopy (SEM). In the presence of phage ϕsp1, bacterial cells appear to be shrunken, and the surface ruptured unlike untreated control cells (Fig. 3a, b, c, and d).

Using the static crystal violet assay, 73.68 and 94.73% reduction in biofilm formation and biofilm eradication, respectively, was found when bacterial pathogen was treated by phages (1.0 MOI) (Fig. 3e). Phage ϕsp1 (1.0 MOI) was thus capable of inhibiting biofilm formation and biofilm eradication. Reduction in EPS production decreased bacterial wilt incidence significantly in wilt-susceptible plants, and EPS mutants triggered noticeably less production of defense-associated reactive oxygen species in wilt-resistant tomato plants (Milling et al.

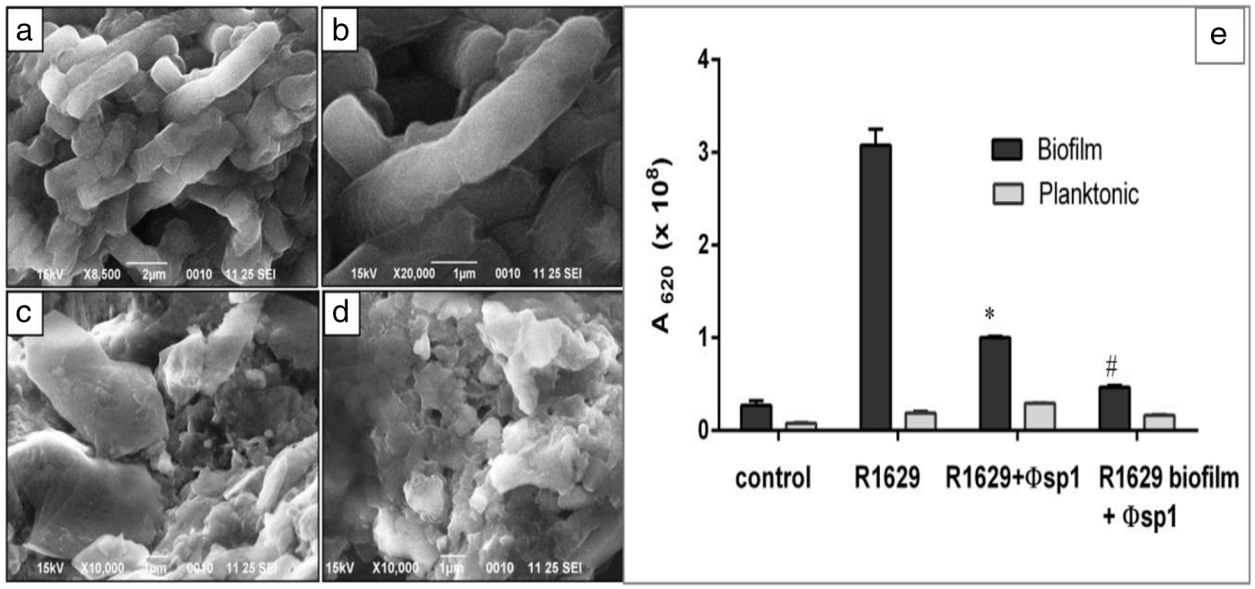

Fig. 3 SEM images of *Ralstonia solanacearum* strain 1629 biofilm. **a** Uninfected biofilm ×8500. **b** Uninfected biofilm at ×20,000. **c** Biofilm treated with phage ϕsp1 (1 MOI) at ×10,000 showing disrupted cells. **d** Biofilm treated with phage ϕsp1 (10 MOI) at ×10,000 showing cell debris. **e** Phage ϕsp1 biocontrol of biofilm formation and eradication of *R. solanacearum* strain 1629 (R1629). $^{*}p \leq 0.0073$ (for biofilm formation), $^{\#}p \leq 0.0017$ (for biofilm eradication) in comparison with phage-untreated R1629 biofilm

2011; Prakasha et al. 2017). Phages are also known to secrete depolymerase enzymes on their capsids, which in addition to host lysis can also degrade biofilm EPS, permitting the phage anti-receptor to gain entry to the receptors on the surface of their host bacterium (Pires et al. 2016).

The present study showed that phage ϕsp1 can be useful for prevention of biofilm formation, a pathogenicity factor of *R. solanacearum*. In addition, phage can also be used as treatment for preformed biofilm that can be eradicated by direct inoculation method.

Phage biocontrol efficacy in plant bioassays

The efficacy of phage ϕsp1 treatment on pathogenicity of *R. solanacearum* strain 1629 was demonstrated in tomato seedlings grown on sterilized wet cotton bed on plastic trays as described previously. After 72 h observation, pathogen-treated tomato

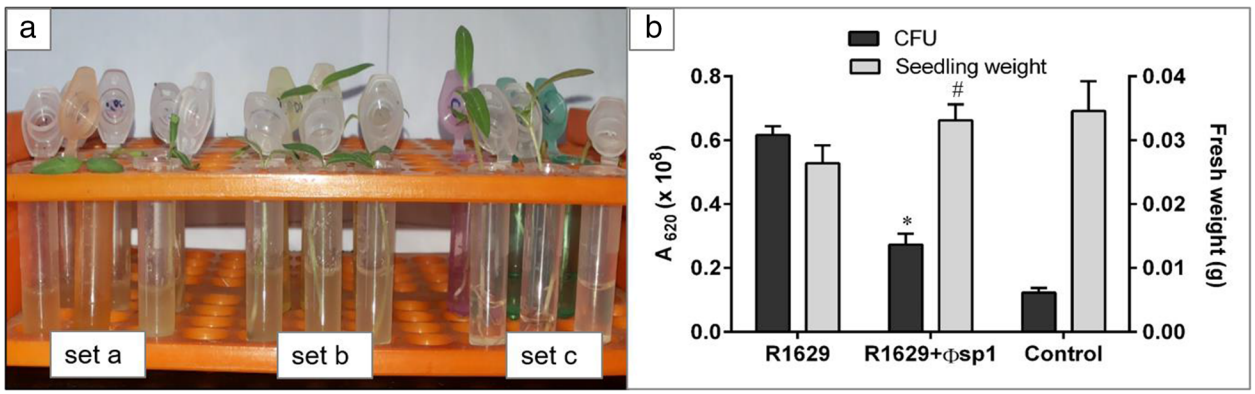

Fig. 4 Phage ϕsp1 biocontrol assay of *Ralstonia solanacearum* strain 1629 (R1629) on tomato seedlings in vitro. **a** Phage (ϕsp1) biocontrol on tomato seedlings in microcentrifuge tubes; set a—*R. solanacearum* strain 1629 inoculated seedling wilted in 72 h, set b—R 1629 + ϕsp1 (1 MOI) treated seedlings remained healthy, set c—uninoculated buffer control seedlings were found completely healthy. **b** Phage ϕsp1 biocontrol efficacy on tomato seedlings after 72 h treatment. Primary axis shows reduced pathogen count (CFU) in phage ϕsp1-treated R1629-infected tomato seedlings (R1629+ ϕsp1) compared to phage untreated, R1629-infected tomato seedlings ($^{*}p \leq 0.0024$). Secondary axis shows fresh weight of tomato seedlings in R1629 + ϕsp1-treated and phage-untreated R1629-infected tomato seedlings ($\# p \leq 0.031$). Data are mean values of experiment performed in triplicates with standard deviation

seedlings containing 10^8 cfu ml^{-1} were found to be completely wilted, while phage-treated seedlings showed partial wilting, and buffer control seedlings remained healthy (Fig. 4a). Furthermore, reduction of bacterial load in phage ϕsp1-treated tomato seedlings was calculated at 87.75% as compared to non-treated pathogen control as evaluated by spectrophotometry. In addition, comparison of fresh weight of tomato seedlings showed biologically statistical difference between phage-treated and non-treated pathogens (Fig. 4b).

In direct inoculation method on tuber slices, diseased symptoms were recorded after 10 days' incubation in pathogen inoculated, while no lesion was found on phage ϕsp1-treated tuber slices. The infected tuber slices showed symptoms of vascular browning and bacterial ooze. The disease symptoms started to appear at 96 h post-inoculation. By day 10, grade 3.0 disease symptoms with brown vascular discoloration, oozing, and soft tissue collapse were observed in the infected tuber. A slight yellow discoloration with no ooze or tissue necrosis observed for phage-treated infected tuber was scored as grade 1.0 disease symptoms. No such features were observed in control uninoculated tuber. Further, to check the presence of phage, samples were obtained from inoculated infected tuber slice (0.1 g ml^{-1}) and quantified using the DLA assay (Fig. 5). Up to 81.39% reduction in bacterial population was found in phage-treated tuber sample confirming the biocontrol potential of phage ϕsp1 against wilt-causing *R. solanacearum* strain 1629 (Fig. 5). In order to determine phage persistence and pathogen survivability in the tubers, tuber samples were processed for DLA and standard plate count on TTC media respectively. 9.68×10^{10} CFU g^{-1} bacteria were isolated from pathogen-infected tuber, whereas only 2.5×10^4 CFU g^{-1} pathogen was recovered from phage-treated tubers. Phage 2×10^4 PFU g^{-1} was reisolated from *R. solanacearum* strain 1629 + ϕsp1 samples by DLA assay. Further, *R. solanacearum* or plaques were not isolated from uninfected control tuber (Table 3). Similarly, phage application (0.01 MOI) to control *Pectobacterium carotovorum* ssp. *carotovorum* and *P. wasabie* destruction prevented damage of up to 80% on tuber slices and up to 95% on entire tubers against tissue maceration from combined bacterial infection (Czajkowski et al. 2015).

To confirm phage persistence in soil microcosm, tomato plant bioassay was performed in pots within the greenhouse setup as described in methods. Plants were inoculated with 10^8 CFU ml^{-1} *R. solanacearum* strain 1629 on scratched stem cells at day 0 and by soil drenching method on day 7. For biocontrol experiments, plants were inoculated with phage ϕsp1 suspension concentration of 1.0 MOI. At day 10 of second inoculation, pathogen-inoculated tomato plants were found to be stunted, yellowing to browning of the leaves was

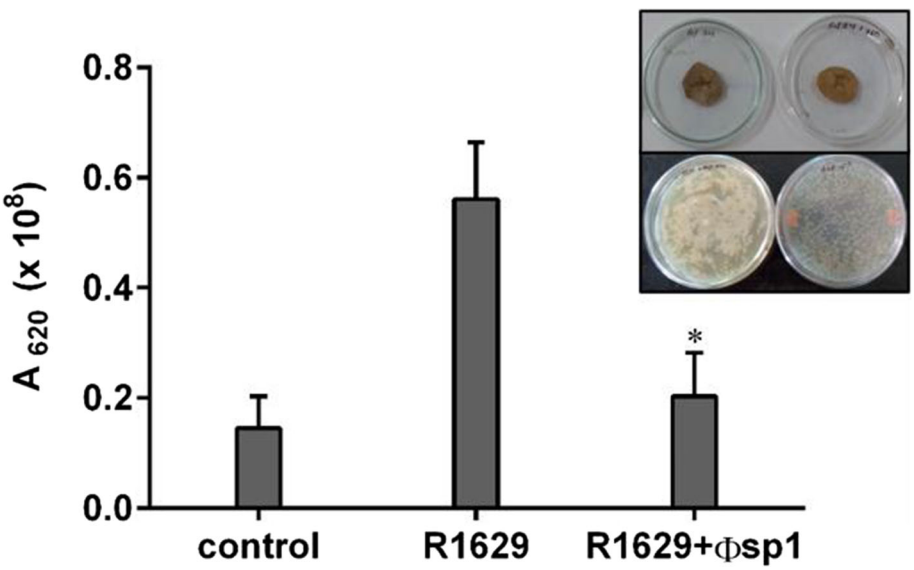

Fig. 5 Pathogenicity of *Ralstonia solanacearum* strain 1629 (R1629) and phage ϕsp1 biocontrol efficacy on potato tuber slice. In vitro phage ϕsp1 bioassay on potato tuber slices against R1629 and phage-untreated tuber. Plating of reisolated R1629 from phage ϕsp1-treated R1629-infected potato tubers showing plaques on bacterial lawn. Standard plate count for R1629 from same potato tuber slices on TTC medium (figure inset). Reduction in *R. solanacearum* strain 1629 (R1629) following phage ϕsp1 treatment in inoculated tuber sample suspension (0.1g/ml) post 10 days of inoculation. Phage ϕsp1 + R1629 inoculated tuber slices show significantly reduced bacterial population compared to R1629 inoculated (*$p \leq 0.05$). Growth measured with optical density (OD) by spectrophotometry absorbance at 620 nm (1.0 OD = 10^8 cells)

Table 3 Phage ϕsp1 biocontrol efficacy in potato tuber slice bioassay using *Ralstonia solanacearum* strain 1629 (R 1629)

Experimental set-up	Disease symptoms	Grade	Microbial count CFU g^{-1}	Phage re-isolated PFU g^{-1}
Control	No vascular discoloration, ooze absent with no change in tissue architecture	0	No *Ralstonia*	No plaques
R1629	Vascular discoloration with brown ring-like appearance, slimy ooze, tissue necrosis	3	9.68×10^{10}	No plaques
R1629 + phage ϕsp1	Yellowing, ooze absent, tissue intact	1	2.5×10^{4}	2×10^{4}

recorded, and two leaves out of six were found to be wilted by *R. solanacearum* strain 1629. The disease progression was recorded to be at grade 2.0 (Fig. 6). The uninoculated plants (control plants) were found to be healthy plants, and height of the plants at the same day was recorded to be 15–16 cm with 6 leaves. Phage ϕsp1 + *R. solanacearum*-treated plants were found intact, standing with 6 leaves at 15–16 cm with no effects of disease symptoms. At 15-day post-infection, the experimental plant sets were reinoculated with 10^8 CFU ml^{-1} *R. solanacearum* strain 1629 with no further phage treatment of plants. Within a week, the pathogen-infected tomato whole plants collapsed resulting in death showing wilting of grade 5.0. However, phage-treated plants were found to remain completely healthy and growing in the same manner as control plants. Hence, phage ϕsp1 showed 100% control of bacterial wilt symptoms in *R. solanacearum*-infected tomato plants. Furthermore, phage treatment was effective even after subsequent re-exposure to pathogen.

Discussion

The role of bacteriophage-based biocontrol of wilt-causing *R. solanacearum* in India has not been explored widely although it has been studied in other geographical areas with promising results. Myoviridae jumbo phage ϕRSL1 showed a limited growth, penetration, and movement of inoculated *R. solanacearum* by maintaining a sustainable host phage population (Fujiwara et al. 2011). Post-treatment with phage PE204 of Podoviridae family showed delayed development of wilt, whereas simultaneous application was found to completely inhibit disease in tomato plants (Bae et al. 2012). The utilization of 6 lytic phage cocktail applied to the rhizosphere soil of the tomato plants as soil drench resulted in reduced bacterial wilt incidence by around 10–20%

Fig. 6 Plant bioassay on tomato seedlings infected with *Ralstonia solanacearum* 1629 (R1629). Set a—R 1629 inoculated plants showing wilting symptoms of grade 2. Set b—plants with ϕsp1+ R 1629. Set c—uninoculated control plants

(Kalpage and De Costa 2014). The mixture of two podoviridae bacteriophages (J2 and ϕRSB2) efficiently lysed *R. solanacearum* cells in contaminated soil, while only J2 phage treatment resulted in disease prevention in tomato plants (Bhunchoth et al. 2015). Podovirus RsPod1-EGY, stable at pH range 5–9 and temperatures up to 60°C, was shown to suppress *R. solanacearum* in 4-week-old tomato seedlings under greenhouse conditions (Elhalag et al. 2018). Phage cocktail (P1) showed diverse inhibition patterns up to 98% against development of the potato plant wilt-causing *R. solanacearum* (Wei et al. 2017). Recently, the isolation of lytic bacteriophages belonging to T7-like virus genus of Podoviridae was found to be effective in reducing *R. solanacearum* in environmental waters. Watering with one or combination of phages was shown to prevent wilt in more than 300 plants (Álvarez et al. 2019). Different phage combination-treated tomato plants reduced *Ralstonia* wilt disease incidence by 80% in greenhouse and field experiments (Wang et al. 2019). These effective biocontrol studies made use of single-phage treatment that was highly stable with strong lytic activity or phage cocktails for effective management of phage resistance and persistence issues. In this study, environmentally stable phage ϕsp1 demonstrated *R. solanacearum* wilt control in potato tuber, tomato seedlings in buffer media, as well as in tomato plants in soil environment in greenhouse conditions. Further characterization of phage biocontrol efficacy in open field conditions using different formulations for maintaining phage persistence and activity need to be explored.

Bacteriophage-based biocontrol of bacterial wilt-causing *R. solanacearum* in tomato plants depends on phage multiplicity of infection (MOI), plant age as well as variety including environmental factors such as soil type, pH, temperature, moisture content, and presence of organic matter in the soil (Buttimer et al. 2017). Phage population also drastically reduces following UV exposure (Iriarte et al. 2007). In order for phage persistence in the soil, protective formulations can be used which significantly reduce the deleterious effects of environmental stress on phage viability. Non-formulated *Ralstonia* phage ϕXacm was found to survive on tomato leaves at 28 °C at 6 and 8 days but were significantly reduced at 15 and 32°C. Phage ϕRSL1 was found to be stable in temperature range of 37–55°C and effectively limit penetration and movement of *R. solanacearum* strain M4S in tomato plantlings (Fujiwara et al. 2011). Lytic phage PE205 was reported to be stable at wide temperature and pH range in artificial soil microcosm (Bae et al. 2012). Phages isolated against *Ralstonia* strain IVIA-1602.1 were found to be stable between 14 and 31 °C at pH 7.0 in a modified Wilbrink broth (MWB) (Álvarez et al. 2019). Obtained results showed that phage ϕsp1 is environmentally stable, but its survival is significantly reduced in increasing temperatures, pH extremes, and exposure time of sunlight (exposure up to UV 254 nm). Hence, in order to enhance in-field phage persistence, phage application by soil-drenching method would be most effective and not affected by exposure to UV irradiation (Iriarte et al. 2012). In addition, use of carrier formulations as well as non-pathogenic or avirulent *Ralstonia* host can be used as strategies for enhancing phage persistence in the soil environment. The ability of phage to survive variations in temperature and pH makes it a suitable candidate for field applications.

The occurrence of phage-resistant pathogen remains an important issue to address while developing biocontrol phage agents. It is important to measure the dynamics of naturally occurring *R. solanacearum* populations in order to determine the ratio of phage to host for adequate control. It is also recommended to re-isolate it seasonally to obtain newly evolved virulent ones as phage populations tend not to be effective from 1 year to the next. In addition, mutant phages as well as phage cocktails can be used to minimize the occurrence of phage-resistant *R. solanacearum* strains (Buttimer et al. 2017; Ye et al. 2019).

Bacteriophage-based control of pathogens in the form of agricultural biopesticides is garnering support.

Conclusion

In obtained findings, Myoviridae lytic phage ϕsp1 infecting bacterial population at low multiplicity of infection (1.0 MOI) can be used as an efficient biological control agent against *R. solanacearum.* The phage was found to be highly host specific and, hence, can be efficiently useful in race/biovar determination of *R. solanacearum*. Phages specific to infecting particular races of *R. solanacearum* can also be useful for the development of highly specific phytopathogen diagnostic tools termed as phage typing in contaminated soil or infected plant and vegetables. The potential to reduce the pathogenic wilting activity of *R. solanacearum* in tomato seedlings as well as potato tuber will be helpful to prevent infections in cold storage.

Abbreviations

TSA: Tryptone soy agar; TSB: Tryptone soya broth; TTC: Triphenyl tetrazolium chloride; CFU: Colony-forming unit; PFU: Plaque-forming unit; MOI: Multiplicity of infection; DLA: Double-layer agar; SEM: Scanning electron microscope; TEM: Transmission electron microscope; DW: Distilled water; EPS: Extracellular polysaccharide

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Authors' contributions
PDU performed the experiments and wrote the manuscript. VK helped in the analysis and interpretation of the data regarding graphical representation of the data using software graph pad prism. All the experiments were performed under the supervision and guidance of SDK, who revised and gave final touch in manuscript preparation. All authors read and approved the final manuscript.

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Availability of data and materials
Data and materials given in this study can be used as a reference by other researchers.

Declarations

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Consent for publication
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