Isolation and biochemical characterization of *Pseudomonas syringae* causing citrus blast disease and its sensitivity test against some antibiotics

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Abstract— Citrus blast disease caused by *Pseudomonas syringae* is a very demolishing illness in different parts of the world, but with a very limited information about the structure and nature of the pathogen involved. Considering from the above fact, this study reports characterization of *P. syringae* isolates from infected plant portion using biochemical approaches and determined its antibiotic sensitivity assay. Several biochemical tests including gram staining, motility, MacConkey agar, catalase, potassium hydroxide, Kligler iron agar, triple sugar iron agar, methyl red, and carbohydrate utilization tests showed positive results against the isolated bacteria, while negative to Simmons citrate agar, urease, tween 80, sulfur indole motility, King's B and mannitol fermentation medium. All biochemical tests ensuring the isolated bacteria is gram negative. Antibiotic sensitivity assay showed that, Carbenicillin (5 mcg/disk) revealed the highest sensitivity pattern with 30±0.5 mm diameter of inhibition zone against the isolated bacteria, where others disk showed moderate antibiotic spectrum. This study help to identify the causal agent of the citrus blast disease and antibiotic sensitivity assays confirm its efficacy.

Index Terms— Citrus blast, *Pseudomonas syringae*, Isolation, Biochemical characterization, Carbohydrate test, OD measurement, Antibiotic sensitivity assay

1 Introduction

Citrus genus play a vital induction as a dietary supplement and therapeutic agent [1]. Citrus belongs to a family of Rutaceae of tribe Citrae and unique among all other fruits [2]. Citrus fruits have a great nutritional significance and also good source of vitamin C, folate, carotenoids, flavonoids and other essential nutrients required for the health [3][4]. Flavonoids of citrus have radical scavenging and antilipoperoxidant activity [5]. Many pharmacological studies have been suggested that, citrus have active components which act as cancer prevention [6]. Citrus used as toothpowder for maintaining our dental health and leaves are used in numerous health problems such as asthma, constipation, dysentery, diarrhea, fever, jaundice, piles, pulmonary, skin disease, vomiting etc [7][8]. Essential oil of citrus fruit shown better antioxidant action and exerts an antinociceptive effect by the central inhibitory mechanisms [9]. Basically, citrus fruit juice used in numerous periodic application for healing blow, scabies and other skin problems [10]. Besides these, fruit juice of citrus have good antibacterial action against some pathogenic bacteria such as *S. aureus, E. coli, K. aerogenes, K. pneumonia* and also have reliable antifungal activity against some *Colletotrichum* species [11][12]. The poor quality of citrus plants is scare to attack for many bacterial diseases such as citrus canker, bacterial spot, bacterial blast, black pit, citrus greening etc [13]. Among these diseases citrus blast has its own consideration and it is caused by *Pseudomonas syringae*. It is one of the top most plant pathogenic disease species in the world [14]. The symptoms of the disease is initially occur lesions on the leaves and twigs and turn brown to black necrotic areas [15]. *P. syringae* is a gram negative, necrogenic pathogen that attacks a variety of symptoms on plants. Generally, this causal pathogen have been identified from other species according to their colony morphology, biochemical and nutritional tests and expression of symptoms in host plant [16]. Biochemical tests play an important role to identify the responsible pathogen for causing the disease. The main aim of this study are isolate the causal organism and identify the pathogen from the infected plant portion. So, all the findings will be very much effective and helpful for management of post-harvested disease of citrus.

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2 MATERIALS AND METHODS

2.1 Plant Collection

The present investigation was done during the timescale of 2016-2017 at Professor Joarder DNA and Chromosome Research Laboratory in Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh. Infected leaves samples were collected from the University of Rajshahi botanical garden and was confirmed by a scientific officer of Bangladesh Fruits Research Institute, Binodpur, Rajshahi, Bangladesh. The infected leaves sample was cut using sterilized surgical blade, then washed with distilled water, and finally kept into plastic bag and placed in a refrigerator or in a cooler box.

2.2 Sterilization of infected leaves sample

Generally the leaves sample was cut into 1 cm^2 and sterilized by 70% ethanol for 1-2 minute and lastly the leaves sample washed with distilled water [17].

2.3 Isolation and purification of isolated bacteria

Isoplation of *P. syringae* was done in LB media [18]. Sterile plant leaves then crudhed with sterile mortar pestle and put into LB liquid media. Then media was incubated overnight at 37^0C. After overnight culture, a sterile loop was taken to streak the bacteria on LB agar media plate and again incubated overnight at 37^0C. In order to identify the isolated bacteria, it is very crucial to obtain a pure culture. That's why streak-plate method was done.

2.4 Morphological test

Colony morphology: The *P. syringae* colonies on LB agar plate were white, convex, mucoid, and different in colony size (1-2 mm).

Gram staining: Gram staining was done to determined size, shape, and staining reaction of the isolated bacteria. Basically four reagents such as crystal violet, gram iodine, decolorizers (alcohol), and safranin was used to perform this test [19].

Motility test: Soft agar media were used for determine to isolated bacteria was motile or not. One colony from the plate was pick and inoculated the motility agar medium by stabbing the center to a depth of 2 inch [20].

2.5 Biochemical test

MacConkey agar test: This test is a selective and differential medium designed to isolate and differentiate enteric based on their ability to ferment lactose. One colony were picked from the media and streak the plate and incubated overnight at 37^0C [21].

Catalase test: Take a clean sterile glass slide and place a drop of hrdrogen peroxide and then transferred a single bacterial colony on the slide [22].

Potassium hydroxide (KOH) test: For identification of gram negative bacteria it is much quicker and easier to perform than the traditional Gram stain [23].

Kligler iron agar (KIA) test: This test is basically done to see the bacteria can ferment carbohydrate sucrose as a carbon source. If the bacteria were able to ferment sucrose then the media changes its color to indicate acid production [24].

Simmons citrate agar test: This test determines the capability of a bacterium to utilize citrate as side carbon source [25].

Triple sugar iron agar (TSI) test: TSI is a differential medium that contains lactose, sucrose, dextrose, ferrous sulfate, and the pH indicator phenol red. Basically this test differentiate the isolated bacteria was able to reduce sulfur and ferment carbohydrates [26].

Urease test: This test was done to determine the isolated bacterium able to hydrolyze urea with the help of urease enzyme or not. If the bacteria produce urease, the color of the slant changes from yellow to pink.

Tween 80 hydrolysis test: In this test the milky white precipitation confirmed the isolated bacteria was able to positive tween 80 reagents [25].

Sulfur-indole-motility (SIM) test: SIM medium is recommended for the differentiation of gram negative enteric bacteria on the basis of sulfide production, indole formation and motility

Methyl red (MR) test: The tube containing MR medium were inoculated the isolated pure colony and incubated overnight at 37^0C. The results were recorded after the addition of one or two drop of MR reagent [25].

Fluorescence under UV test: Colonies of *P. syringae* on King's B non-fluorescent under UV light at 366 nm after 24 hour of bacterial growth [27].

Mannitol fermentation test: This is a selective and differential medium in gram positive bacteria. But some gram negative bacteria can also live in such medium and change the medium color light yellow [27].

Carbohydrate utilization test

The main purpose of this test is measuring the concentration of bacteria on different carbohydrate medium and also determine the isolated bacteria was able to grown in different carbohydrate such as Sucrose, fructose, glucose, lactose, and maltose [28].

2.6 Antibiotic sensitivity test

Sensitivity of isolates was obtained *in-vitro* by disk diffusion method of Kirby-Bauer [29]. The procedure involved to measuring the zone of inhibition in diameter into the medium surrounding the disc. Antibiotic discs Amoxicillin (10 μg), Azithromycin (15 μg), Carbenicillin (100 μg), Cefixime (5 μg), Clarithromycin (15 μg), Doxycycline (30 μg), Erythromycin (15 μg), Gentamycin (30 μg), Kanamycin (30 μg), Nalidixic acid (30 μg), Neomycin (30 μg), Oxytetracycline (30 μg), Penicillin (10 μg), Rifampicin (5 μg), Streptomycin (10 μg), Tetracycline (30 μg), and Vancomycin (30 μg) were used for this analysis. This test was performed by using LB agar medium. The prepared medium sterilized by autoclaving at 121^0C for 20 minutes. The medium was placed on sterile petri plates and allowed to solidify. For each sensitivity test, 100 μl of microbial inoculums from overnight culture medium was spread on the surface of LB agar plate. Then the antibiotic discs were placed on the center of the plates with the help of sterile forceps and incubated overnight at 37^0C in an inverted position. The results and their concentrations are given at table 2. The results were recorded as resistant, intermediate, and susceptible to specific antibiotic disc based on the areas of inhibition zone diameter [30].

Statistical analysis: Above tests were conducted in repeated triplicate for significant results. All the inhibition zone were revealed as mean and standard error (M±SE). P<0.5 was considered statistically significant.

3 Results

3.1 Isolation and identification of isolated bacteria

The affected leaves placed on LB broth medium, then incubated at 37^{0}C for overnight. The turbid condition in the LB media indicates the bacteria were grown and colonies size and shape were small, white, convex and mucoid.

3.2 Morphological test

Gram staining: Isolated bacteria were gram negative and rod shaped.

Motility test: The growth area extending away from the inoculation line, which indicates isolated bacteria were motile. Some morphological test result are shown in figure 1.

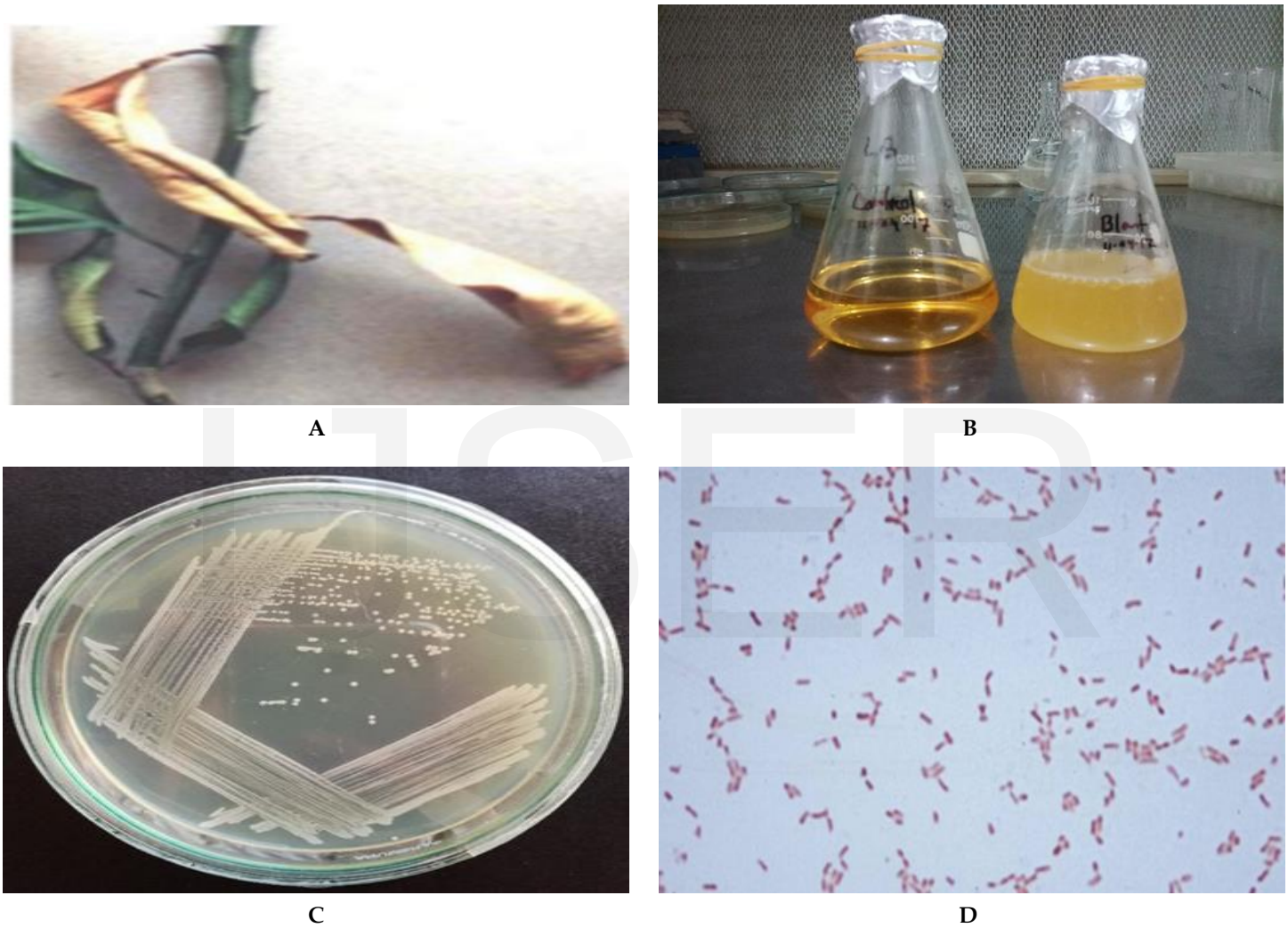

Fig 1: Citrus blast infected leaves (A). Overnight culture of isolated bacterial strain in LB liquid medium (B). Obtain single colony by streaking method (C). Microscopic view of *Pseudomonas syringae* (D).

3.3 Biochemical test

A series of biochemical tests were performed for characterized with the suspected gram negative bacteria. All biochemical tests results are given in table 1. After analyzing the all biochemical tests results, it was confirmed that isolated bacteria are *P. syringae*. Identification was done by morphological and biochemical tests according to the EPPO/CABI standard diagnostic protocol [31].

Table 1 Summary of morphological and biochemical test results of isolated bacteria

Tests	Results	Findings
Gram staining	+	Small, rod shaped, pink color
Motility	+	Growth area extending away from the line of inoculation
MacConkey agar	-	No color
Catalase	+	Oxygen bubbles
KOH	+	Viscous and sticky slime

KIA	+	Lactose fermenting, but no gas form
Simmons citrate	-	No color
TSI	+	Lactose fermenting, but no gas and H_2S form
Urease	-	No color
Tween 80 hydrolysis	-	No color
SIM	-	No color, but motile
MR	+	Utilize glucose

Fluorescence under UV	-	No color
Mannitol fermentation	-	Isolates were not able to grow high salt condition area
Carbohydrate utilization test	+	**Carbohydrate OD** Sucrose 0.49 Fructose 0.24 Glucose 0.25 Lactose 0.23 Maltose 0.19

3.4 Antibiotic sensitivity test

For antibiotic sensitivity test. Seventeen different antibiotics discs were used against *P. syringae.* Figure 2 showed all inhibition zone pattern against the *P. syringae* and table 2 provides the sensitivity pattern result against the used antibiotics. Among these antibiotics, Carbenicillin revealed the highest inhibition zone of 30±0.5 mm in diameter, while Azithromycin showed lowest 5±0.5 mm in diameter inhibition zone against *P. syringae*. Most of the antibiotic showed better antibiotic spectrum against *P. syringae*.

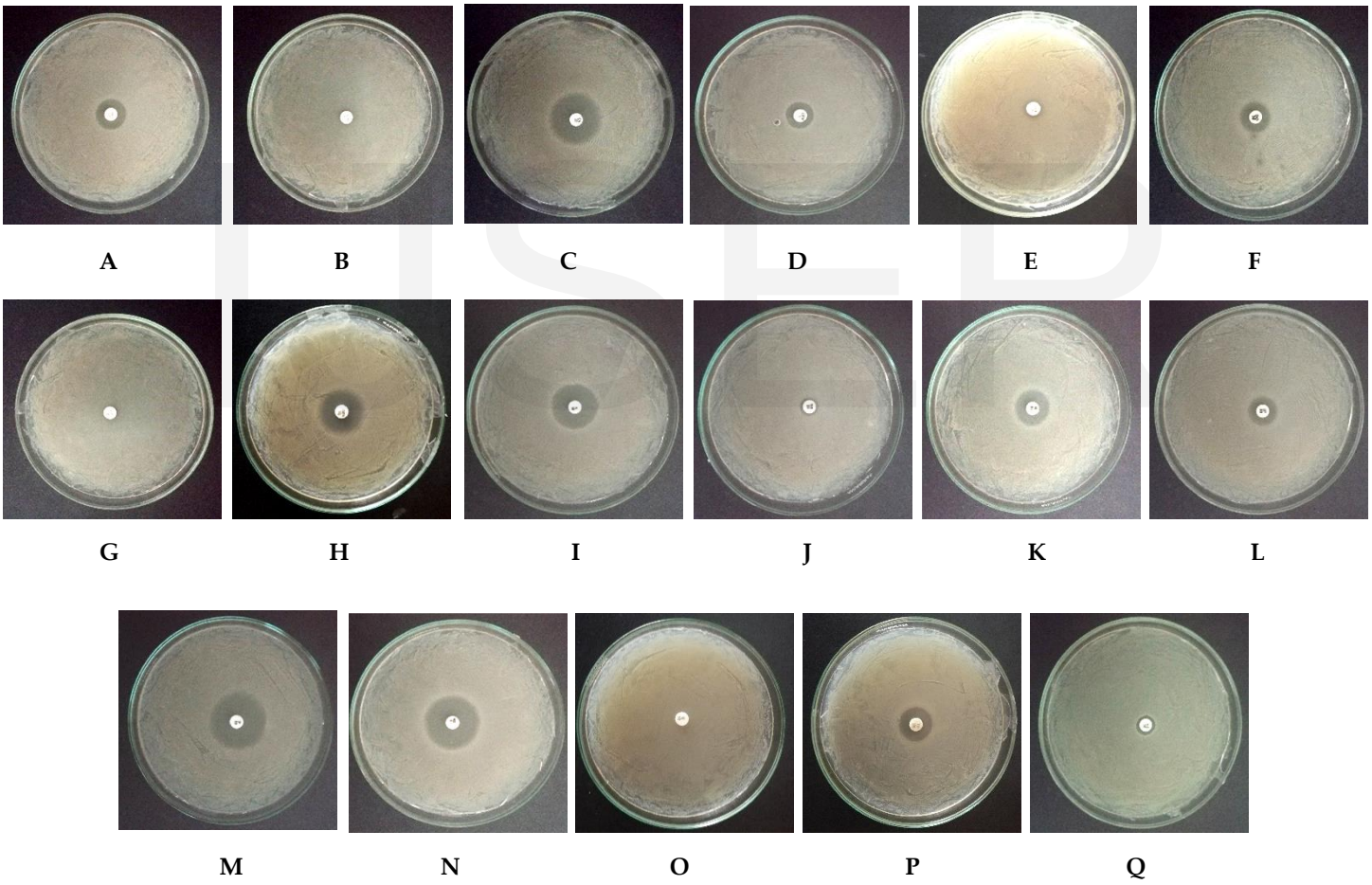

Fig 2: Result showing zone of inhibition against *P. syringae*. Amoxicillin (A), Azithromycin (B), Carbenicillin (C), Cefixime (D), Clarithromycin (E), Doxycycline (F), Erythromycin (G), Gentamycin (H), Kanamycin (I), Nalidixic acid (J), Neomycin (K), Oxytetracycline (L), Penicillin (M), Rifampicin (N), Streptomycin (O), Tetracycline (P), and Vancomycin (Q)

Table 2 Effects of antibiotics against the isolated bacteria

Antibiotic Name	Disc Potency (μg/disc)	Zone of inhibition (M±SE)	Response
Amoxicillin	10	16±0.5	S
Azithromycin	15	5±0.5	R
Carbenicillin	100	30±0.5	S
Cefixime	5	14±0.5	I
Clarithromycin	15	7±0.5	R
Doxycycline	30	14±0.5	I
Erythromycin	15	8±0.5	R
Gentamycin	30	25±0.5	S
Kanamycin	30	21±0.5	S
Nalidixic acid	30	7±0.5	R
Neomycin	30	17±0.5	S
Oxytetracycline	30	13±0.5	I
Penicillin	10	27±0.5	S
Rifampicin	5	26±0.5	S
Streptomycin	10	6±0.5	R
Tetracycline	30	16±0.5	S
Vancomycine	30	8±0.5	R
R= Resistant (5-10 mm) I= Intermediate (11-15 mm) S= Susceptible (16 mm ≥) [30]			

4 Discussion

Citrus blast disease is a very devastating threat to citrus fruits and its production. Generally the symptoms of this disease starts as black lesions in the leaves petiole and progress into the leaves exile. Proper illustration of the pathogenic characterization is very useful for preventing this disease. The study was conducted to determine the presence and characterize *P. syringae* in infected leaves samples with the symptoms of citrus blast and also find out its sensitivity to various standard antibiotic discs. The responsible bacteria were isolated from infected leave samples and identified using different morphological and biochemical tests. *P. syringae* was a gram negative bacterium with small size, rod shaped and polar flagellum [19]. The optimum growth temperature range for 30^0C to 37^0C. The motility test indicates, the isolated bacterium were motile, because growth area extending away from the inoculation line. *P. syringae* did not showed any color in the media plate which indicates our bacterium were not able to ferment lactose. Similar findings were observed by Brodsky and Nixon (1973) in *P. aeruginosa* bacterium. The oxygen bubbles clearly indicates isolated bacterium were positive to catalase test. In addition, Suslow *et al.*, (1982) performed KOH test in gram negative bacteria of wheat, where our isolated bacterium clearly showed positive results [32]. In case of KIA and TSI test, our isolated bacterium were able to ferment lactose but no gas was formed. Arshad *et al.*, (2015), were performed Simmons citrate, tween 80 hydrolysis, and MR test in *Xanthomonas oryzae* pv. *oryzae*, where negative results was found in citrate and tween 80 hydrolysis test and positive to MR test. Our isolated bacterium showed similar results against citrate utilization, tween 80 hydrolysis, and MR test. *P. syringae* showed negative observation against urease test, which confirmed that, our bacterium were not able to hydrolyze urea. According to Lelliott and Stead (1987), the book of Methods in Plant Pathology, SIM test was carried out. Our isolated bacteria showed negative to sulfur and indole production, but motile [33]. In case of King's B medium and mannitol fermentation test, *P. syringae* bacteria were non fluorescent under UV and unable to ferment mannitol salt medium [27]. Liu (1952) was done carbohydrate test in *P. aeruginosa* species, where he found bacteria were able to grow in different carbohydrate media tubes with moderate bacterial concentration. Our isolated bacteria was also showed similar results against different carbohydrates such as sucrose, fructose, glucose, maltose, and lactose [28]. Most antibiotics with great effectiveness *in vitro*. Carbenicillin showed the highest inhibition zone of 30±0.5 mm in diameter, while Gentamycin, Kanamycin, Penicillin, and Rifampicin showed average spectrum against the isolated bacterium. This test possess better emphasize of effective bactericides and gives us proper knowledge of the appropriate dosage.

5 Conclusion

Since 1990s, scientific authority and regulatory personnel world-wide have made necessary steps to eradicate bacterial blast disease, based on beneficial ratios for the planned actions and its cost. So, this research provides an overview of the bacterial blast disease in citrus spp. The above tests provided detail information about the isolation, characterization, and antibiotic sensitivity assay against *P. syringae*.

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7 Competing interests

The authors have declared that no competing interests exist regarding this publication.

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