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Development of a sensitive serological method for detection of *Burkholderia gladioli* pv. *gladioli*, the causative agent of saffron bacterial corm rot

Mohammad Ramezani Kaporchali [a,1], Mohammadreza Safarnejad [b,*], Hashem Kazemzadeh Beneh [c], Abolghasem Ghasemi [b], Pär K. Ingvarsson [d,**], Naser Farrokhi [a,***]

[a] Department of Cell and Molecular Biology, Faculty of Life Sciences & Biotechnology, Shahid Beheshti University, Tehran, Iran
[b] Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization, Tehran, Iran
[c] Department of Horticulture Science, Biotechnology & Plant Molecular Genetic Section, University of Hormozgan, Bandar Abbas, Iran
[d] Department of Plant Biology, Swedish University of Agricultural Sciences, Uppsala, Sweden

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ABSTRACT

Pathogenic microorganisms cause infectious plant diseases, which limit crop productivity and cause economic losses. Early detection enables improved pathogen recognition and more effective control through integrated pest management strategies. *Burkholderia gladioli* pv. *gladioli* (*Bgg*) cause saffron bacterial corm rot (BCR), a hard-to-detect disease, which has a devastating effect with up to 80 % field destruction rates and corresponding heavy yield losses. In this study, (two isolates of *Bgg* (A and B)) were identified via TEM and SEM image analysis followed by molecular identification by 23S rRNA PCR. A *Bgg*-specific polyclonal antibody was developed and was conjugated to alkaline phosphatase for direct assays, and no conjugation was used for indirect assays employing a secondary antibody. ELISA, Western blot, dot blot and immunofluorescence imaging were used to evaluate the efficacy and specificity of the developed antibody. The raised antibody bound specifically to *Bgg* with very low cross-reactivities to structurally related bacteria. Dot blot was shown as a suitable, cheap, and rapid field test. This work represents an important step forward to diagnose and prevent the spread of BCR.

1. Introduction

Saffron (*Crocus sativus* L.) is an herbaceous perennial and medicinal plant from the family Iridaceae that has valuable health benefits due to its bioactive substances. Saffron has been, cultivated primarily in Asia for over 3000 years and Iran currently has the largest production (440 tons) and cultivated area (120,000 ha) of saffron in the world [1,2]. Saffron production can be affected by varieties of phytopathogens including *Burkholderia gladioli* pv. *gladioli* (*Bgg*) [3,4].

Burkholderia is a genus of bacteria resistant to known antibiotics, which are found in diverse environments [5]. It contains more than 60 species active in animals, plants, and humans [6,7]. *Bgg* has been reported to cause cystic fibrosis in humans [8]. *Bgg* infiltrates diverse arrays of plants, including *Crocus* spp., where it is the causative agent of saffron corm rot disease, which is a serious threat to saffron yield [9]. The composition of the soil rhizospheric bacterial community, containing both beneficial non-pathogenic and pathogenic bacteria, is correlated with saffron quality and is known to influence its secondary metabolite contents [10]. *Bgg* attacks all aerial and underground parts of saffron and produces burn-like symptoms, which cause up to 80 % yield 400, especially during wet seasons [11,12]. Field diagnosis of *Bgg* is difficult due to the similarity of its symptoms to nutrient deficiencies.

Saffron corm rot has spread rapidly in recent years. The development of direct, accurate, low-cost, and high-fidelity diagnostic techniques would help distinguish the causative effects as *Bgg* from other potential threats to saffron production. Developing such methodologies will impact the sustainable production of saffron and border control for quarantine purposes of corm transfer. To date, PCR-based assays have been used for detection *Bgg*, but its use requires skills, reagents, and equipment [12]. In contrast, serological tests are fast, cost-effective, and

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: mrsafarnejad@yahoo.com (M. Safarnejad), par.ingvarsson@slu.se (P.K. Ingvarsson), n_farrokhi@sbu.ac.ir (N. Farrokhi).
[1] Current address: Department of Plant Bioproducts, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

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relatively easy to adopt [13].

Antibodies are bio-recognition elements used in direct diagnostic methods and for biosensor development [3]. Antibodies have proven to be a convenient and cost-effective alternative method for reliable diagnosis in field-collected samples [14]. Bacterial LPS, cyclic and linear peptides, bacterial flagella or killed bacterial cells, and heterologously expressed recombinant membrane-bound full- or partial length proteins can stimulate the immune system of animals to produce specific monoclonal and polyclonal antibodies [15–20].

In this study, a specific polyclonal antibody was developed as an effective method for *Bgg* identification. Varieties of serological techniques were tested and dot blot proved to be the best cost-effective, rapid and accurate method for *Bgg* diagnosis. The method does not require bacterial isolation and therefore rapid detection is amenable. Also, in this research, surface antigens of *Bgg* were detected using polyclonal antibodies and by Western blot techniques.

2. Materials and methods

2.1. Bacterial isolation and identification

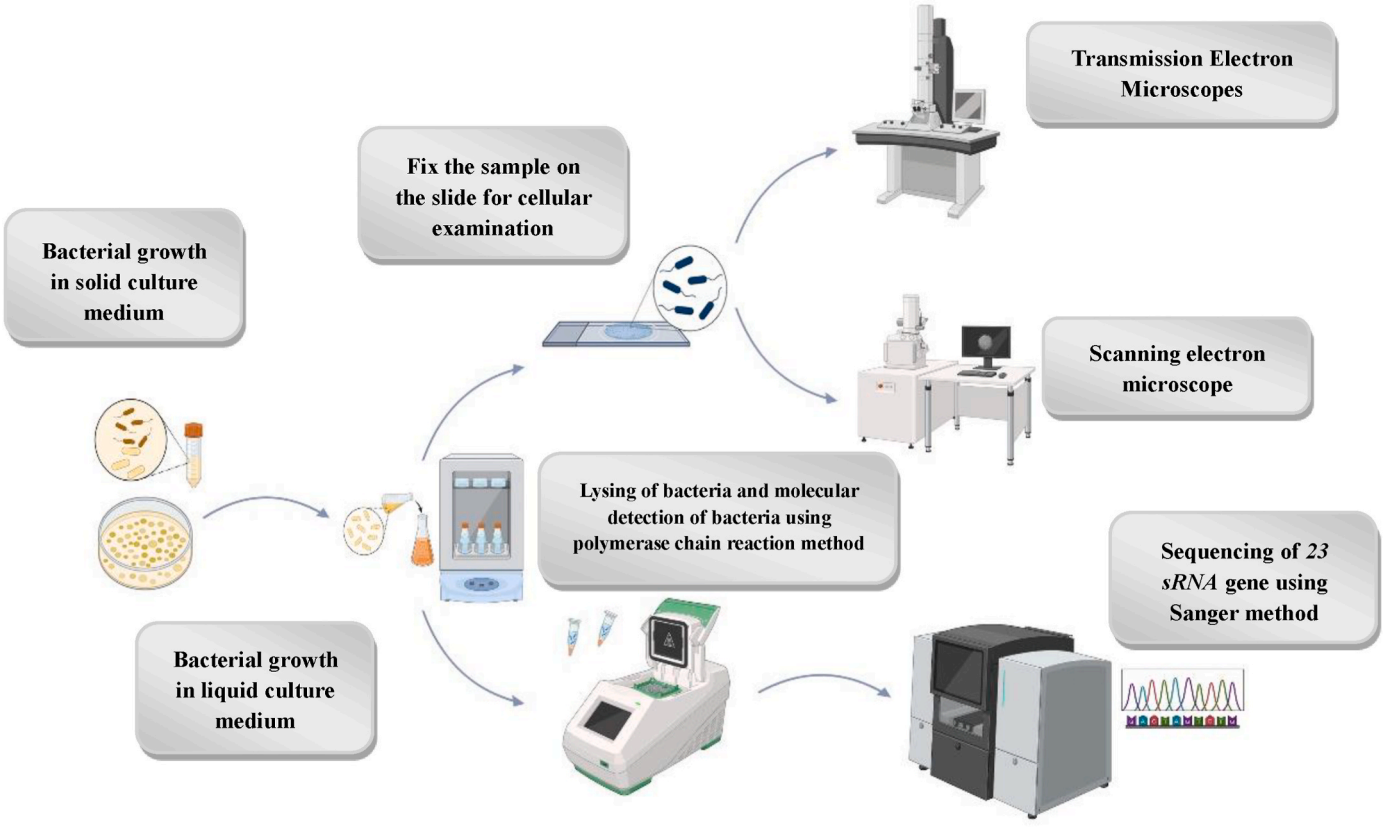
Contaminated corms were sampled from saffron farms in Torbat Heydarieh, Iran in 2019. Bacteria isolation (two isolates named A and B) from infected saffron was performed according to Ref. [21]. Initially, electron microscopy and polymerase chain reaction (PCR) were used to identify the bacterial genus (Scheme 1). Bacterial identification was based on *23S rRNA* gene sequence analysis. DNA of each isolate was extracted according to Ref. [22]. Polymerase chain reaction (PCR) was performed in a PCR express Thermal cycler (Eppendorf) using primers: LP1 (5′-GGGGGGTCCATTGCG-3′) and LP4 (5′-AGAAGCTCGCGCCACG-3′) [23]. Each 24 μL reaction contained 10 μL of master mix (Kalazist, Tehran, Iran), 2 μL of genomic DNA, and 1 μL of each primer (10 μg/μL). PCR was initiated at 94 °C for 3 min, followed by 30-cycle of [94 °C: 10 s, 60 °C: 10 s, and 72 °C: 60 s]. Following amplification, 10 μL of each reaction mixture was subjected to electrophoresis in a 1 % agarose gel in TAE buffer (pH 8.0). The amplicons were separated on 1 % agarose gel electrophoresis, visualized by 1 μg/μL ethidium bromide (Sigma Aldrich, St. Louis, Missouri, USA) and 700 bp band was excised from gel for Sanger DNA sequencing. The obtained sequences have been deposited in GenBank [GenBank accessions number# OP903427 (isolate A) and # OP903428 (isolate B)]. The *23S rDNA* sequences were compared against GenBank using BLASTn. The FDAARGOS_389 strain was used as a model for *Bgg*. A multiple sequence alignment was constructed using approximately 700 bp of *23S rDNA* gene fragments with ClustalW (http://www.ebi.ac.uk/clustalw) and a phylogenetic tree was constructed using neighbor-joining algorithm in the MEGA v.11 software (www.megasoftware.net), with confidence tested by bootstrap analysis (10000 repeats).

2.2. Electron microscopy

For both SEM (Hitachi SU3500, performed at the Shahid Beheshti University) and TEM (JEM-1400Flash, 100 kV; Conducted by Arya Rastak Laboratory), 2 % glutaraldehyde was used to fix the bacteria. Liquid culture medium (100 μL) containing bacteria, incubated at 28 °C for 16 h, was dried on sterile aluminum foil. The foil was placed in a 2 % glutaraldehyde solution for 16 h. Bacterial samples were sequentially dehydrated in 50, 60, 70, 80, 90, 100 percent alcohols and were photographed on the same day [24].

2.3. Bacterial isolates and growth conditions

The bacterial strains used in the study were obtained from the Iranian Research Institute of Plant Protection (IRIPP, Laboratory of Plant Diseases, Tehran, IRAN). These bacterial strains were *Staphylococcus aureus, Agrobacterium tumefaciens, E. coli, Pseudomonas savastanoi, P.*



Scheme 1. Two different cellular and molecular methods were used in bacterial detection and identification.

viridiflava, P. aeruginosa, *Burkholderia cepacia*, *Xanthomonas* spp., *B. gladioli* pv. *gladioli (A, B)*. All strains were cultured at 28 ℃ using Luria-Bertani (LB) agar. Three-to five-day-old cultures of bacterial cells derived from culture medium after double subculturing.

2.4. Preparation of Bgg antigen and production of polyclonal antibodies in rabbits

To produce rabbit-derived antibodies against *Bgg*, we used the A and B isolates by two different methods. For the first method, we used heat-inactivated bacteria. A 10^8 CFU/ml ($OD_{600\ nm}$ = 0.4) suspension was prepared for each strain, and the two strains were combined in a 1:1 ratio. Using a centrifuge at 5000 rpm for 15 min, the precipitated bacterial cells were collected and washed three times with 0.85 % saline. The tubes were incubated at 90 °C for 20 min. For the second method, overnight-grown cultures of *Bgg* in LB were treated with 1 % (v/v) formaldehyde for 1 h at 22 °C. The formaldehyde-fixed cells were precipitated by centrifugation at 10,000 rpm for 5 min. The cell pellet was washed three times in phosphate-buffered saline (PBS) (20 mM sodium phosphate, pH = 7.0; 300 mM NaCl) and resuspended in 0.5 ml PBS [8]. Antigens prepared by both methods were mixed with Freund's incomplete adjuvant (Sigma, USA) in a ratio of 1:1 and suspension (2 ml) were administered four times at 30-day intervals for two New Zealand rabbits [14]. Acquisition of blood from the rabbit and isolation of antibodies were performed according to Refs. [25,26]. Blood was collected from the marginal vein of the ear directly into the centrifuge tubes. After collection, the samples were incubated for 30 min at 37 °C to form a clot and stored for 16 h at 4 °C. The clot was gently separated from the walls of the tube and centrifuged at 1000×*g* at 4 °C for 15 min. The serums of both rabbits were gently decanted from the precipitate and pooled.

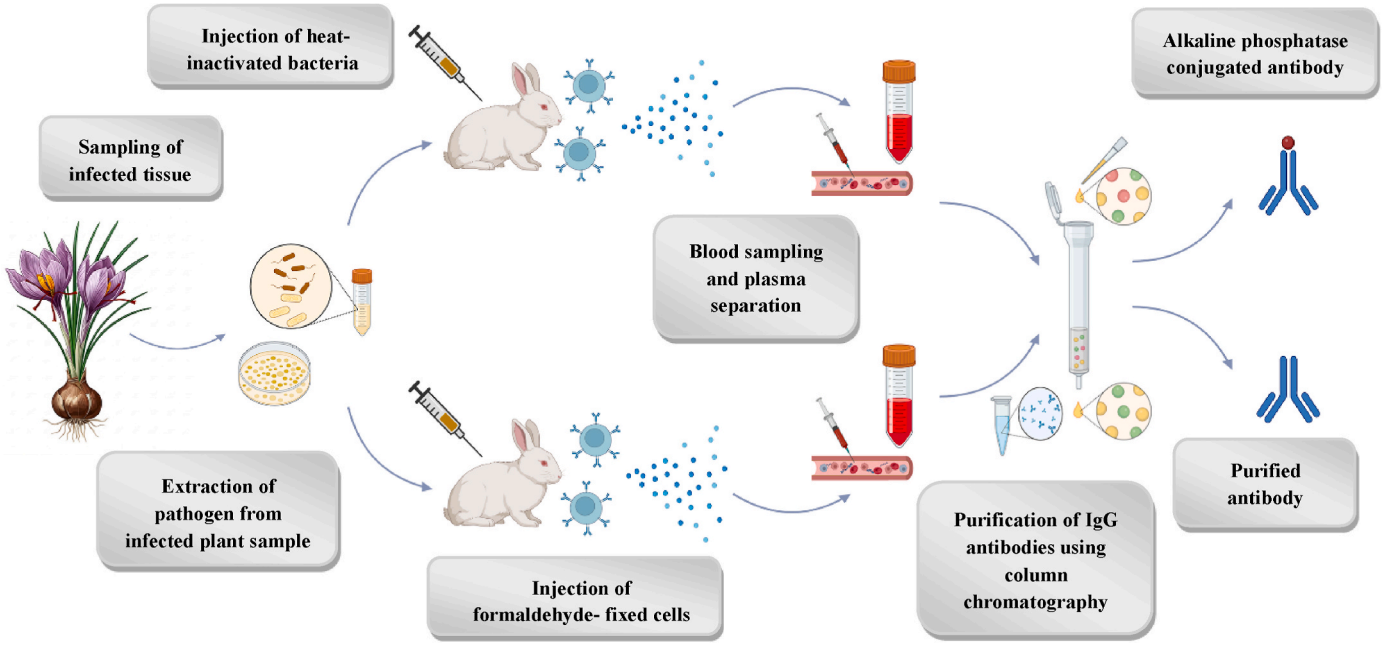
An agar gel immunodiffusion assay was used according to Jenson TA to confirm the presence of *Bgg* specific antibodies in the serum [27]. For purification of immunoglobulins, high-affinity protein G column (0.46 × 11 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) for the Fc region of polyclonal antibodies was used [28,29] (Scheme 2). Rabbit serum (40 ml) was loaded and allowed to flow slowly through the column, which had previously been equilibrated with 20 mM PBS (pH = 7.0). The non-IgG components were washed out with 20 mM PBS, and the bound IgG was eluted with 100 mM citrate buffer (pH = 2.7) at a flow rate of 1 ml min^{-1}. The eluted IgG was neutralized with 1 M Tris-HCl buffer (pH = 9.0) and half of the purified antibody dialyzed (dialysis-bag with 0.45 μm pore size, Sigma-Aldrich, Burlington, Massachusetts, USA) with 20 mM PBS. Half of the purified and dialyzed antibody was conjugated with alkaline phosphatase enzyme according to Winston et al., for direct tests [30]. Both antibodies were used for various tests as shown in Scheme (3) (see Scheme 3).

2.5. ELISA

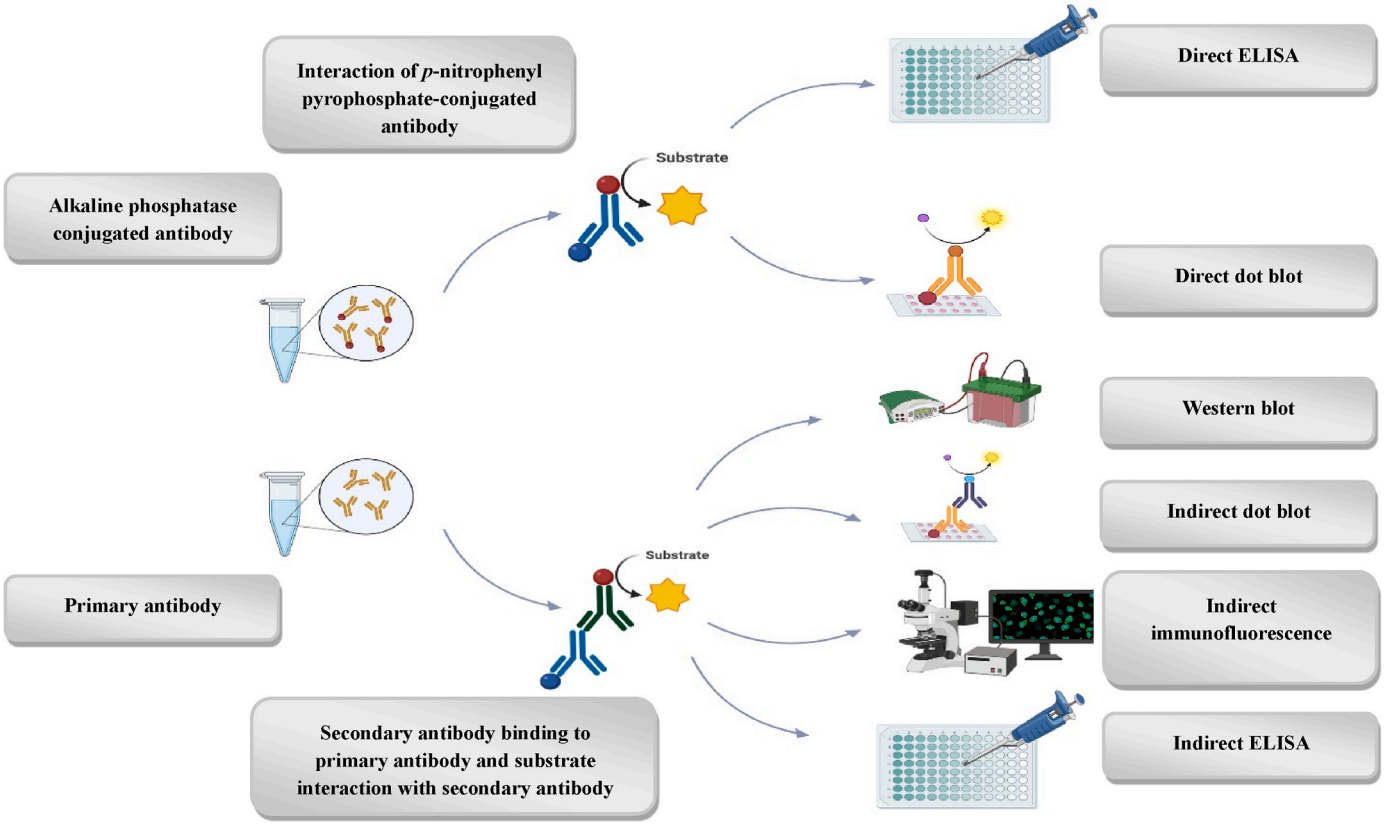
The direct and indirect ELISA were performed in 96-well Jet Bio Filtration Immuno Plates (Guangzhou, China) with at least three replicates. Bacterial suspensions were boiled at 85 °C for 15 min and used as antigens. Infected and healthy plant samples were weighed equally and their suspension (plant tissue was lysed with 1 × PBS and centrifuged at 1000 rpm for 1 min) was used. Paired rows of wells were coated with 100 μL/well of positive and negative lysates or 1: 20 dilutions of the test leaf sap in carbonate-coated buffer (pH = 9.6), for 16 h incubation at 4 °C. The plates were washed three times with 300 μL/well of 1 × PBST (1 × PBS with 0.05 % (v/v) Tween 20, pH = 7.2) wash buffer. The plates were blocked with 200 μL/well of 1 % (w/v) casein/PBS, pH = 7.2 (CPBS), blocking buffer for 2 h at 22 °C. The plates were subjected to 3 × wash at 1 min intervals [14].

For the indirect method, 100 μL of purified primary antibody diluted (1: 1000) was loaded into each well and incubated at 37 °C for 1 h. The plate was washed three times with washing solution. A volume of 100 μL/well of commercial AP-conjugated anti-rabbit immunoglobulins (1:1000 dilution in PBS) was added for 1 h incubation at 37 °C followed by another 3 × wash as previously mentioned. For the direct method, 100 μL of primary antibody conjugated with AP (1: 1000 dilution in PBS) was loaded into each well.

Then, 100 μL of *pNPP* (prepared at a ratio of 1 mg/ml), conjugated by alkaline phosphatase enzyme and produces its own color at a wavelength of 405 nm, was added to each well. Optical densities (OD) were measured in an ELISA plate reader (Conquer Scientific, CA, USA) fitted with a 405 nm filter at 15-, 40- and 60-min. Bacterial strains of *E. coli*, *Pseudomonas* spp., *Xanthomonas*, *B. cepacia* and plant samples of tobacco leaves, pods, corms and healthy leaves of saffron were used as negative controls. The experiments were repeated three times.



Scheme 2. Two different methods in the production of specific antibodies for saffron rot disease and its purification using protein G column.



Scheme 3. An illustration of various plant disease detection techniques (Indirect and Direct).

2.6. Western analysis

All the antigens and extracts of infected and control plants were prepared in the same way as for the ELISA method. Proteins were separated on SDS-PAGE. The transfer from the gel to the nitrocellulose membrane (Bio-Rad, CA, USA) was carried out for 16 h at 25 V and 60 mA. The nitrocellulose membrane was incubated with 5 % blocking buffer (defatted dry milk dissolved in phosphate-buffered saline (PBS) with a small amount of Tween-20) at 37 °C for 1.5 h [31]. The purified antibody was diluted using 1 % bovine serum albumin in phosphate buffer at a ratio of 1: 500 and the membrane was incubated for 1 h in an incubator shaker at 70 rpm and 37 °C. The secondary antibody (GAR) (Abcam, Cambridge, UK) was diluted using 1 % bovine serum albumin in phosphate buffer at a ratio of 1: 1000 and incubated with the nitrocellulose membrane for 1 h at 22 °C on a shaker incubator at 70 rpm. The NBT/BCIP substrate was dissolved in 10 ml of dH_2O and the membrane was treated with the substrate for 5–10 min until the bands appeared [32]. The membrane was washed with dH_2O to stop the reaction.

2.7. Dot blot

Dot blot was done directly and indirectly to determine the specificity of the produced polyclonal antibody according to Garnsey et al. and Cardosa et al. with some modifications [33,34]. All the samples were prepared in the same way as the ELISA, with three replicates. Briefly, for the indirect method, 5 μL of test samples were spotted at 1: 2 dilutions in PBS, pH = 7.2. The nitrocellulose membranes (NCMs) were air-dried and blocked in 5 % defatted skimmed milk/PBS, pH = 7.2 (NFSM-PBS), for 30 min. The membranes were washed 3 times for 10 min intervals with PBST (PBS-0.05 % Tween20, pH = 7.2). The membrane was incubated for 5 h with the primary antibody with a dilution of 1: 1000 at 22 °C. The membranes were washed 3 times with PBST for 30 min each at 10 min intervals before 2 h incubation with AP-conjugated Goat anti-rabbit (GAR) immunoglobulins (diluted in 1 × PBS at a ratio of 1: 1000). NBT/BCIP (nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate) precursor was added to the membrane and covered the container with aluminum foil for 15 min until black spots appeared. For the direct method, the antibody was conjugated with AP alone (with a dilution of 1: 1000) and added onto the membrane and incubated at 4 °C for 16 h.

2.8. Indirect immunofluorescence imaging

Indirect immunofluorescence imaging was used to confirm the efficiency of the antibody produced by the cellular method. To perform this test, bacterial isolate A was used as a treatment and Pseudomonas was used as a negative control. Using an optical microscope, the density of bacteria on the slide was measured as diluted with PBS buffer, 2 M EDTA (pH = 7.0). Goat serum (25 μl) was used to reduce the adhesion of bacteria and their concentration on the slide [35,36]. The bacterial suspension (100 μL) was fixed on the slide at 600 rpm for 5 min with a cytospin. Four different test conditions were performed on both bacteria. A) antigen treatment alone (autofluorescence examination), B) antigen treatment with Goat anti-rabbit antibody (examination of natural antibodies in rabbit not treated with antigen), C) antigen treatment with secondary antibody (non-binding of secondary antibody to antigen), D) the main treatment with the presence of antigen, primary and secondary antibodies. The concentration of the specific antibody was 5 μg/ml, the secondary antibody 10 μg/ml and the concentration of Goat anti-rabbit antibody was 5 μg/ml. Bacteria were fixed with 100 μL of 95 % (v/v) ethanol for 1 min. The samples were washed with washing solution (PBS Tween 0.01 % + BSA 0.03 %) for 3 min. They were treated with 100 μl of blocking solution (PBS Tween + BSA + Goat anti-rabbit antibody 5 %) for 30 min. The primary antibody (100 μL) with the desired concentration was loaded on the D-mode slide and incubated at 22 °C for 1 h. The washing solution was loaded on the A-mode slide and incubated at

22 °C for 1 h. Goat anti-rabbit antibody (100 μL) with the desired concentration was loaded on the B-mode slide and incubated at 22 °C for 1 h. The secondary antibody (100 μL) was loaded on the C-mode slide and incubated at dark, at 22 °C for 1 h. State D and C slides were washed three times (each time for 5 min) using glass jars. The secondary antibody (100 μL) was added to the D-mode slide and placed in the dark for 45 min. We washed the slide three times in glass jars. The combination of glycerol and 1 × PBS was used to mount all the slides for imaging. Antibodies conjugated with the colored fluorescent substance fluorescein isothiocyanate (FITC) with an absorption wavelength of 494 nm and an emission wavelength of 517 nm with a yellow-green color were used [35]. Imaging was performed with a DP71 immunofluorescence microscope (Optoedu, Mantao, Italy).

3. Results

3.1. Phylogenetic inference of 23S rRNA gene sequences and cell detection

Two *Bgg* isolates, A and B, were PCR-identified using LP1 and LP4 primers [12]. Gel electrophoresis failed to differentiate. clarified that size similarities in amplicons limit resolution; actual distinction requires sequencing (Fig. 1a). Sequence and phylogenetic analyses showed that isolates A, B, and strain FDAARGOS are closely related (Fig. 1b). The phylogeny tree also shows that Burkholderia is closely related to *Pseudomonas aeruginosa* (Fig. 1b). A modified method was used to fix the bacteria to visualize the surface appendages and we photographed the *Bgg* flagellum for the first time (Fig. 2). Based on the SEM and TEM images, the flagellum length in the mature state is 3 times the length of the bacterial body (1–3 μm depending on species). All photographed bacteria had a flagellum, but due to the high sensitivity of flagella to fixing solutions, most lost their flagellum during preparations.

3.2. ELISA

ELISA reads at OD_{460} were collected after 120 min of incubation. In direct ELISA, isolates A and B, and infected saffron samples showed a strong reaction from the produced pAb (Fig. 3). According to the direct ELISA results, infected corm samples have shown the highest level of sensitivity compared to infected saffron pod samples and pure bacterial

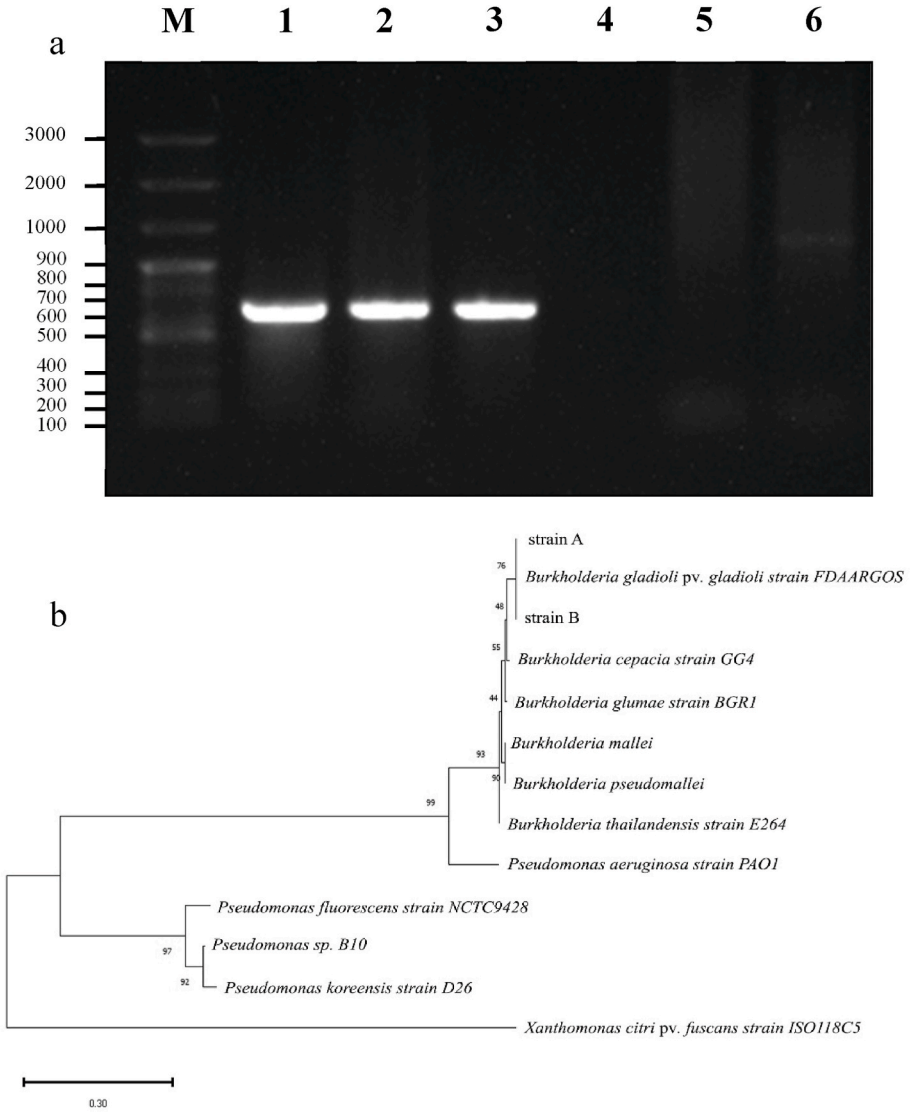


Fig. 1. Sequence amplification of 23s rRNA genes followed by phylogeny analysis of bacterial species using the 23s rRNA sequence. a) Amplification of the 23s rRNA gene from isolates A and B of *Bgg* (lanes 1 and 2) and *P. fluorescens* (lane 3), respectively (600 nucleotides long). Lane 4 represents a negative control, while lane 5 contains Xanthomonas and lane 6 contains *B. cenocepacia*. Lane M is a DNA biotech marker in the size range 100–3000 nucleotides. b) A phylogeny tree from 13 different strains based on the common part of the 23s rRNA gene created using neighbor-joining (NJ) in MEGA v.11.

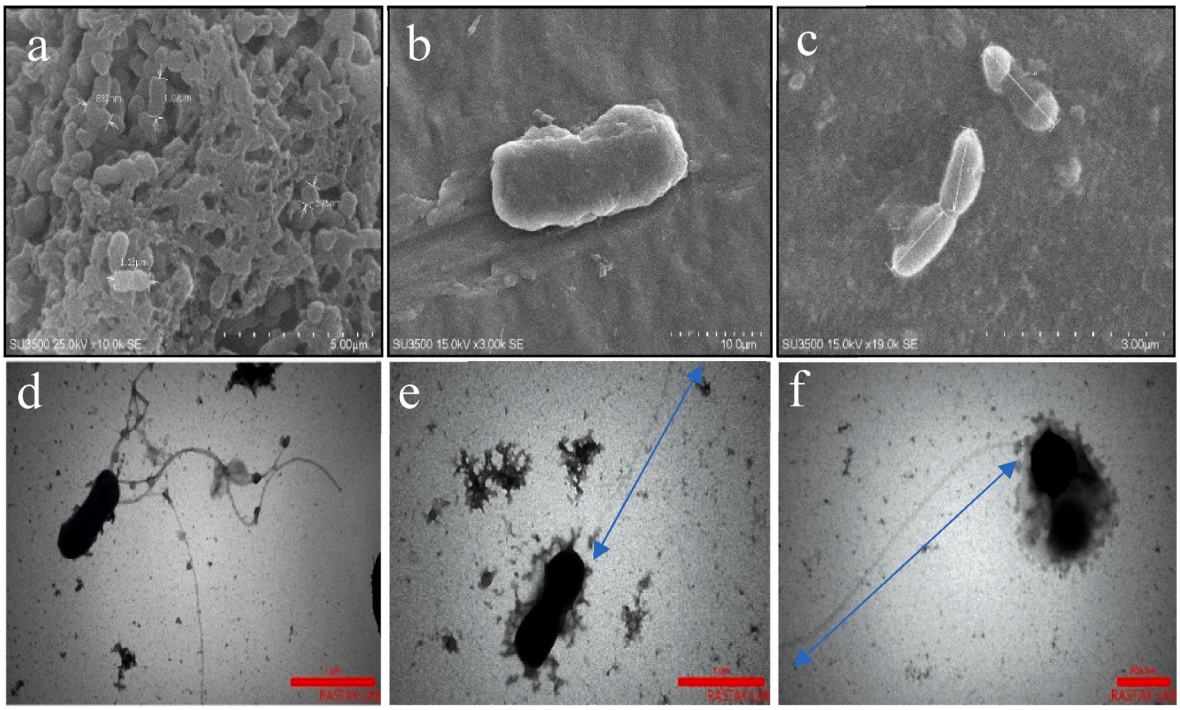


Fig. 2. Electron micrographs of flagellum arrangements (Monotrichous and Lophotrichous) of two *Bgg* pathogenic isolates. a-c corresponds to SEM, showing the general shape and size of the isolate A with 1–1.5 μm bodily length. d-f to correspond to TEM images. d, Lophotrichous flagella recorded from isolate A. e and f show the general and complete view of the bacterial flagellum of isolate B with a length of ~6 μm and Monotrichous arrangement.

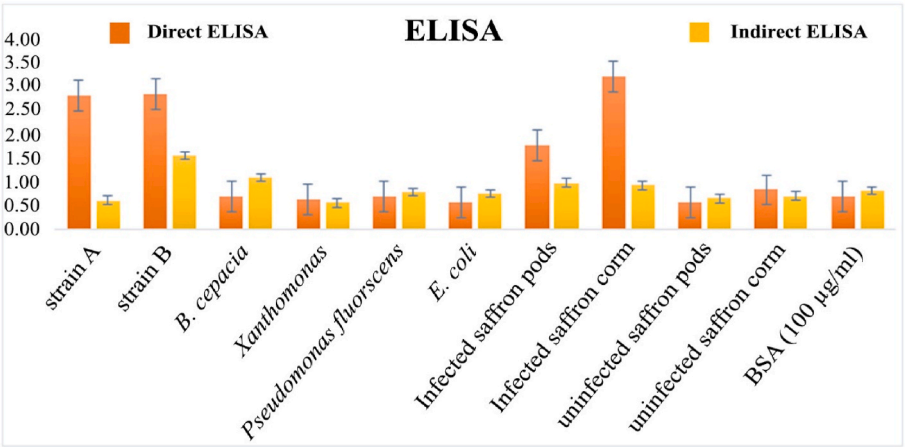


Fig. 3. Enzyme-linked immunosorbent assay (ELISA) of developed antibodies against a range of samples. Orange: direct ELISA, yellow: indirect ELISA. *Bgg* A and B antigens, and infected corm and pod samples had the highest reaction with the produced pAb in direct ELISA.

antigens. The numbers in the graph are the average of three replicates. Other bacterial species and uninfected saffron plant samples showed weak reactions. In indirect ELISA, due to the cross-reactions between the primary and secondary antibodies, there is no proper reaction between the produced pAb and the infected plant samples (Fig. 3).

3.3. Western analysis

The banding pattern (Fig. 4) shows the reaction between the produced pAb with isolates A and B of *Bgg* and the infected samples of saffron corms. In other healthy and bacterial plant samples, no specific banding was seen. The produced pAb detected eight antigens in infected plant samples, while only five bands are visible in pure antigen samples of bacteria (Fig. 4). This band difference can have various reasons with one of the strongest possible arguments being that there were cross-reactions between the *Bgg* pAb and other bacteria on the plant.

3.4. Dot - blot

Dot blots were performed in two ways, direct and indirect, using a polyclonal anti-*Bgg* antibody (antibody with 1:1000 dilutions were used in both methods). For indirect dot blots, various antigens, including infected and healthy saffron plant samples, were covered on nitrocellulose membrane (NCM) dot blot strip, and detection was done by anti-*Bgg* polyclonal antibody and secondary antibody. In the direct dot blots, antigens were covered by pAb conjugated with alkaline phosphatase and detected. The reactions performed using different antigens and the results from the produced pAb demonstrated the high specificity of the antibody. The results obtained from the dot blot strips demonstrate that the direct method has greater sensitivity than the indirect method

Fig. 4. Western blot analysis. Lanes 1 and 2, healthy corms samples. Lane 3, ladder (pre-stained protein ladder) with a size ranging from 3.5 to 240 kDa. Lanes 4 and 5 contain infected corms samples with antigens of different molecular weights. Lanes 6 and 7 contains heated *Bgg* samples (A and B isolates, respectively) with antigens of different molecular weights. Lanes 8 and 10 contains heated *B. cepacia* samples. Lane 9 contains heated *Pseudomonas fluorescens* samples.

(Fig. 5). Fewer cross-reactions are created in the direct method than in the indirect method. Therefore, the direct method is more sensitive. The results show that the direct dot-blot assay can be used as a rapid and low-cost primary screening test for *Bgg* in saffron.

3.5. Indirect immunofluorescence assay (IFA)

IFA is another serological test that we used to confirm the effectiveness of the produced pAb. Using DP71 immunofluorescence microscope, to confirm the specificity of the pAb, we used bacteria from the genus Pseudomonas which are closely related to Burkholderia as a negative control. The produced pAb with high specificity was able to detect *Bgg* (Fig. 6). All negative control images had no fluorescent signal.

4. Discussion

One of the major limitations in saffron production worldwide is the prevalence of diseases caused by bacteria and fungi. Among these, saffron bacterial rot disease caused by *Burkholderia gladioli* pv. *gladioli* (*Bgg*) pose a serious threat to saffron production by attacking all aerial and underground parts of the plant, especially during humid seasons. It causes symptoms resembling scorching on the corms, leaves, and stems of saffron plants. Field diagnosis of this disease is highly challenging due to the similarity of its symptoms to those caused by nutrient deficiencies [11,12]. Currently, no effective solutions other than quarantine measures are known for managing the disease. The situation becomes more critical when the disease enters an area previously free of the bacterium and therefore, rapid identification and removal of infected plants and subsequent quarantine measures are essential in limiting the spread of the pathogen. Considering the rapid spread of the disease, a direct, accurate, low-cost, and highly sensitive diagnostic method is necessary to rapidly identify infected plant samples to prevent the transmission of the pathogen. Molecular methods such as PCR, DNA hybridization, serological diagnostic methods and electron microscopy have been used to identify bacterial diseases. Due to this bacterium's similarity to other symbiotic bacteria in saffron cultivation areas, only serological tests can determine the population of this bacterium, as disease onset is mainly dependent on passing a certain threshold in the bacterial population [37]. Increasing demand for the development of tools to diagnose and control plant infections has led to the development of less-laborious or time-consuming techniques, such as serological methodologies that can be implemented within biosensors. According to the research conducted by Patel et al., antibodies can be used as bio-recognition elements in direct diagnostic methods and biosensors [3]. Lipopolysaccharides (LPS) have been used in immune system stimulation and production of specific antibodies for other bacteria [15–17].

A major limitation of pAbs is their potential for cross-reactivity with non-target bacteria present in infected plant tissues, leading to inaccurate pathogen detection and false positive results. However, using a novel approach, we have generated a specific antibody that exhibits high sensitivity for *Bgg*, effectively eliminating cross-reactivity and enabling reliable detection of this pathogen. Problems with the use of antibodies for bacteria detection occur because bacterial cells are not the only detectable antigens, for example, soluble EPS produced by these cells, the amounts of which may differ depending on the number of cells, also function as antigens [22].

We compared different serological and molecular methods. The results indicate that serological methods, as a sensitive test, can be used as a complement to morphological and other molecular methods for *Bgg* detection in saffron.

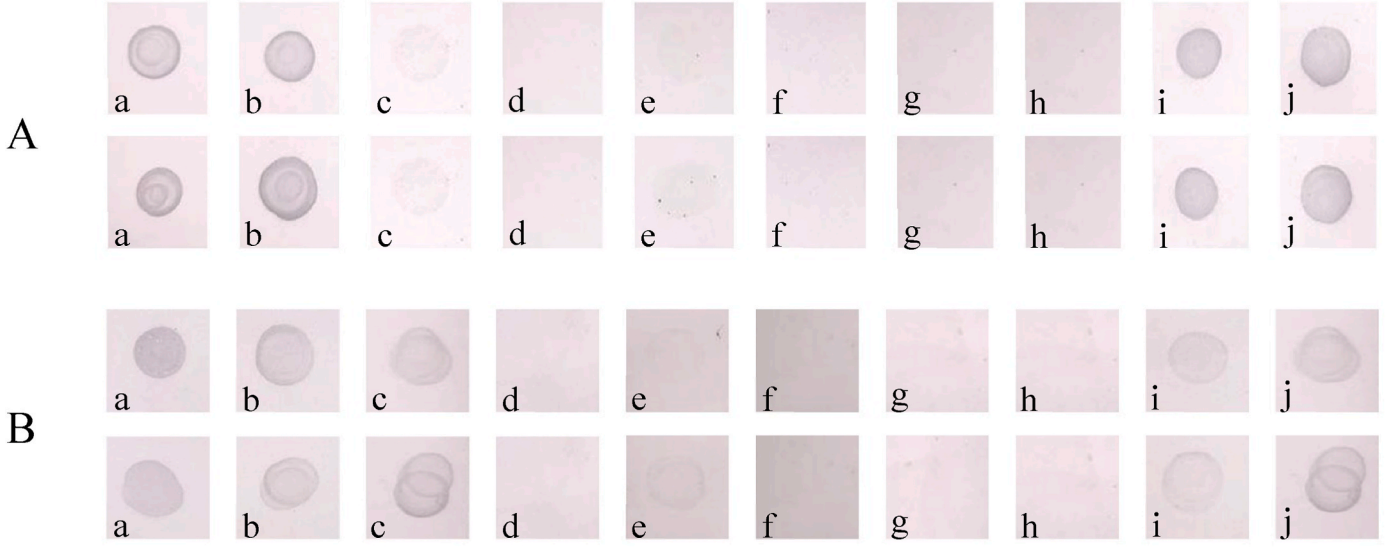


Fig. 5. Direct and indirect dot-blot method. A: Direct dot blot. B: Indirect dot blot. a, *Bgg* isolate A. b, *Bgg* isolate B. c, *B. cepacia*. d, *E. coli*. e, *Pseudomonas fluorscens*. f, Xanthomonas. g, healthy saffron pods. h, healthy saffron corms. i, infected saffron pods. j, infected saffron corms.

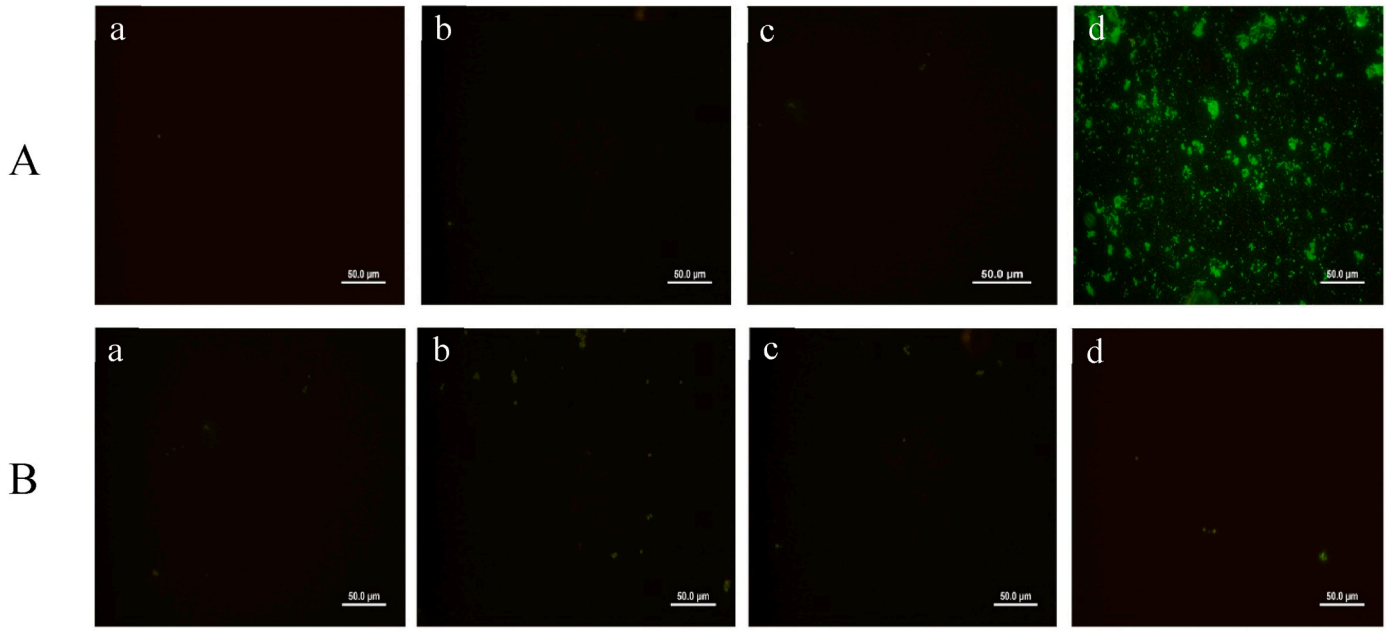


Fig. 6. DP71 immunofluorescence microscopy images. (A) *Bgg*. (B) Pseudomonas. (a) fixed antigen to confirm the absence of bacterial autofluorescence. (b) antigen and normal Goat Anti-rabbit antibody of New Zealand rabbit. (c) antigen and secondary antibody to confirm non-reaction between secondary antibody and antigen. (d) antigen and primary and secondary antibodies to confirm antigen detection with produced pAb.

5. Conclusion

The antibodies developed in the current study are sensitive and specific and they, therefore, provide the opportunity to be immunosorbents, allowing selective binding of *Bgg* and reducing interference from non-target microorganisms in diagnostic assays. Based on the results obtained from this research, the polyclonal antibody produced exhibits high sensitivity. The data from this study also indicate that the dot blot test has higher sensitivity and accuracy compared to other serological tests. Moreover, the dot blot test has the potential for commercial use with many samples. Further observations suggest that direct serological tests (dot blots and ELISA) exhibit lower cross-reactivity compared to indirect tests. Moreover, indirect immunofluorescence imaging was able to successfully identify *Bgg* without any cross-reactivity. This antibody enables the development and commercialization of the first serological *Bgg* detection kit. It would be preferable to continue this work by conducting broader research on field samples from different locations to find the functional gaps of this antibody.

CRediT authorship contribution statement

Mohammad Ramezani Kaporchali: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Mohammadreza Safarnejad:** Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Hashem Kazemzadeh Beneh:** Investigation. **Abolghasem Ghasemi:** Validation. **Pär K. Ingvarsson:** Writing – review & editing, Resources. **Naser Farrokhi:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

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Declaration of competing interest

All authors confirm that the article is the author's original work. The authors declare that they do not have any conflict of interest. This manuscript has not been submitted for publication elsewhere. All authors listed have contributed notably, read the manuscript, and agreed to its submission to Journal of Agriculture and Food Research. On behalf of co-authors, the corresponding authors takes full responsibility for the submission.

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Data availability

Data will be made available on request.

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