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Highly specific and super-sensitive Dot-ELISA and colloidal gold immunochromatographic strip for detecting *Xanthomonas oryzae* pv. *oryzicola* of rice bacterial leaf streak

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Abstract

Rice bacterial leaf streak (BLS), caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), is a worldwide destructive rice bacterial disease, and seriously affects the global rice industry. Effective integrated management measures for BLS depend on the timely and proper detection of *Xoc*. Here, two highly specific and super-sensitive monoclonal antibodies (MAbs) against *Xoc* were first prepared using the cultured *Xoc* RS105 as an immunogen. Then, two serological assays, Dot enzyme-linked immunosorbent assay (Dot-ELISA) and Colloidal gold immunochromatographic strip (CGICS), were developed for the super-sensitive and broad-spectrum detection of *Xoc* in homogenates prepared from field-collected rice plants. The newly created Dot-ELISA and CGICS assays can detect ten different *Xoc* strains from various provinces in China and other countries without cross-reactivity with the other ten tested plant bacteria. Furthermore, Dot-ELISA and CGICS can detect *Xoc* in bacterial suspensions diluted up to 9.78×10^3 CFU/mL and 4.88×10^3 CFU/mL, respectively, or in rice plant tissue homogenates diluted up to 1:51,200 (w/v, g/mL). Surprisingly, both Dot-ELISA and CGICS serological assays were more sensitive than the conventional PCR. Additionally, analysis results using field-collected rice samples showed that the newly created Dot-ELISA and CGICS were reliable in detecting *Xoc* in rice tissues. Thus, the two serological assays were highly valuable and effective for the diagnosis of rice bacterial leaf streak in rice-grown areas and for inspection and quarantine of *Xoc*.

Keywords Rice bacterial leaf streak, *Xanthomonas oryzae* pv. *oryzicola*, Monoclonal antibody, Dot-ELISA, Colloidal gold immunochromatographic strip

Background

Rice is the most important cereal crop, serving as a staple food for more than half of the world's population (Ainsworth 2008). However, bacterial diseases pose significant challenges to the rice industry and cause significant rice yield losses worldwide (Dai et al. 2007). Among them, rice bacterial leaf streak (BLS) is considered one of the most important rice bacterial diseases that seriously threatens rice production in rice-growing areas worldwide. The first reported case of BLS dates back to 1918 in the Philippines. Subsequently, the disease spread widely to other rice-growing regions of Asia (Ou et al. 1985),

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Northern Australia (Aldrick et al. 1973), Africa (Awoderv et al. 1991), and Latin America (Lozano 1997). In China, the first outbreak of BLS was recorded in Guangdong Province in 1953 (Fang et al. 1957). BLS quickly spread to all southern rice-growing regions of China because of its seed transmission and has now become one of the most important rice bacterial diseases (Xie et al. 2014). Contaminated rice seeds play a significant role as a primary source of infection and transmission carrier of this disease (Xie and Mew 1998).

BLS primarily occurs during the early stage of rice growth, especially from the tillering stage to the heading stage. It causes leaf wilting and diminishes the rice plant's ability to undergo photosynthesis, ultimately impacting rice yield (Mew 1993). It reported that BLS can cause a reduction in rice yield ranging from 8 to 32% (Liu et al. 2014). The pathogen responsible for BLS is *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), which is distinct from *X. oryzae* pv. *oryzae* (*Xoo*), the causal agent of rice bacterial blight. They represent different pathogenic variants of the *X. oryzae* species (Schaad 1982). Since the two pathogens belong to the same *X. oryzae* species and share many similar characteristics, such as morphological structure, biological characteristics, and disease epidemic factors, distinguishing between them can be a challenge (Vera Cruz et al. 1984).

Currently, there are no effective and readily available means for preventing, controlling, and curing BLS in rice paddies. The integrated management measures are widely applied to control BLS, but their effectiveness relies on timely and accurate detection of the pathogen causing BLS. Therefore, it is crucial to establish rapid and practical detection techniques that can identify *Xoc* in rice plants and seeds to control this disease. Traditional identification techniques of *Xoc* on rice plants, such as symptom observation of diseased rice plants, and morphological and physiological biochemical identification of isolated pathogens, are unreliable, labor-intensive and time-consuming. Molecular identification techniques, such as polymerase chain reaction (PCR), real-time PCR, and biological and enzymatic amplification (BIO-PCR), can specifically and sensitively detect *Xoc* (Kang et al. 2008; Zhang et al. 2008), but they require specialized instruments, trained technicians, and extended test time, making them unsuitable for on-site testing. By contrast, serological assays, such as Enzyme-linked immunosorbent assay (ELISA) and Colloidal gold immunochromatographic strip (CGICS), are known to be quick, simple, sensitive, specific, and suitable for on-site detection. However, the effectiveness of serological methods depends primarily on the quality of available antibodies (He et al. 2020). Therefore, serological assays have been widely used to detect a variety of plant pathogens (Guo et al. 2020; He et al. 2020, 2021; Li et al. 2021). CGICS is now the fastest technique for detecting various pathogens, including plant pathogens, and is widely applied worldwide (Bin et al. 2018; Guo et al. 2023; Zhang et al. 2023). However, the serological detection methods of *Xoc* were rarely reported because of cross-reaction problems with other bacteria and low sensitivity (Wu et al. 2015).

To develop visual, simple, fast serological test techniques of *Xoc*, in this work, we prepared a pair of *Xoc*-specific monoclonal antibodies (MAbs) and further developed Dot-ELISA and CGICS serological techniques for the supper-sensitive, highly specific, and broad-spectrum monitoring of *Xoc* in rice leaf tissues. Because these two techniques are simple, fast, visual, super-sensitive, and highly specific for the *Xoc* test, we consider that these two serological techniques are particularly useful for the inspection and quarantine of *Xoc*, and the prevention and control of BLS.

Results

Characteristics of MAbs against *Xoc*

Using Xoc strain RS105 as the immunogen, two murine hybridoma lines (11D2 and 11B6) secreting MAbs against the pathogenic bacterial *Xoc* were created through cell fusion, hybridoma screening with a cell medium containing the HAT supplement (Sigma-aldrich), antibody test by an indirect-ELISA, and successive three rounds of cell cloning. Subsequently, the created hybridomas were intraperitoneally injected into pristine-primed BALB/c mice to yield ascites containing anti-*Xoc* MAbs. The immunoglobulin class and subclass of both MAbs were determined to be IgG1, κ light chain. IgG concentrations of MAbs 11D2 and 11B6 were determined to be 6.98 and 9.17 mg/mL, respectively. The analysis results of indirect ELISA indicated that the titers of both MAbs were all up to 10^{-7}.

The indirect-ELISA results showed that MAbs 11D2 and 11B6 reacted strongly and specifically with ten different *Xoc* strains: *Xoc* RS105, *Xoc* BLS256, *Xoc* oxy01, *Xoc* oxy02, *Xoc* oxy04, *Xoc* oxy05, *Xoc* JS, *Xoc* GX, *Xoc* AH, and *Xoc* YN, but not with other ten negative control bacterial strains: *X. oryzae* pv. *oryzae* PXO86, *X. oryza* pv. *oryzae* Y2, *X. citri* pv. *citri* 29–1, *X.campestris* CGMCC 1.3408, *X. sacchari* ACCC 10416, *X.axonopodis* CCTCC AB 2018263, *X. albilineans* FJ1, *Burkholderia glumae* Os48, *Pantoea ananatis* F163, and *Acidovorax oryzae* CGMCC 1.1728 (Fig. 1a), indicating that both prepared MAbs were highly specific and broad-spectrum for *Xoc*. The indirect-ELISA results using serially two-fold diluted *Xoc* strain RS105 and *Xoo* strain PXO86 suspensions as detection samples revealed that the detection sensitivities of MAbs 11D2 and 11B6 were all up to 2.44×10^3 CFU/

mL (Fig. 1b), indicating that both prepared MAbs were super-sensitive for *Xoc* detection.

Dot-ELISA for *Xoc* detection

Two Dot-ELISAs were developed to detect *Xoc* using either MAb 11D2 or 11B6 as the detection antibody. The results from three independent assays indicated that the working dilutions of the detection antibodies (MAb 11D2 or 11B6) and the AP-conjugated goat anti-mouse IgG second antibody were separately determined to be 1:6000 (v/v) and 1:8000 (v/v) in Dot-ELISA. Specificity and broad-spectrum analysis results displayed that both established Dot-ELISAs could successfully detect all ten tested *Xoc* strains but had negative reactions with all ten tested negative control bacterial strains (Fig. 2a). To determine detection endpoints of the Dot-ELISAs, suspensions of *Xoc* strain RS105 and *Xoo* strain PXO86 were serially two-fold diluted in phosphate-buffered saline (PBS) and then detected by the two created Dot-ELISAs. The results showed that detection endpoints of the two developed Dot-ELISAs for *Xoc* were all 9.78×10^3 CFU/mL (Fig. 2b). In addition, to determine the detection sensitivities of the created Dot-ELISAs for *Xoc*-infected rice plants, a homogenate form a *Xoc*-infected or an uninfected rice plant tissue was serially two-fold diluted, and then each dilution was tested for the presence of Xoc. The test results displayed that for the infected rice tissue homogenates, the low detection limits of both Dot-ELISAs were all 1:51,200 (w/v, g/mL) dilution (Fig. 2c).

To compare the sensitivity of the dot-ELISAs developed in this study with PCR, bacterial suspensions of *Xoc* strain RS105 were diluted two-fold and tested for the presence of *Xoc* by PCR. Similarly, homogenates from *Xoc*-infected rice leaf tissues (same as shown in Fig. 2c) were also diluted two-fold and tested for the presence of *Xoc* by PCR. A specific DNA band of 694 bp could be amplified from *Xoc* suspensions diluted from 1×10^7 to 7.81×10^4 CFU/mL (Fig. 2d) and from the *Xoc*-infected rice leaf tissue homogenate diluted from 1:50 to 1:25,600 (w/v, g/mL) (Fig. 2e). This indicated that the low detection limit of PCR for the *Xoc* suspension and the infected rice leaf tissue was separately 7.81×10^4 CFU/mL and 1:25,600 (w/v, g/mL). These above results indicate that the developed dot-ELISAs were about seven times more sensitive than PCR.

Establishment of CGICS for *Xoc* detection

The schematic diagram of the CGICS for *Xoc* detection is shown in Fig. 3. The preliminary assays demonstrated that the CGICS had a good detection effect using MAb 11D2 as the CGNP-labeled antibody and MAb 11B6 as the capture antibody at the T line. The preliminary assays also uncovered that the intensity of the red color on the T line was positively correlated with the concentration of MAb 11B6 used as the capture antibody. However, the non-infected rice plant generated a very weak red band on the T line when the IgG concentration of MAb 11B6 was above 0.90 mg/mL. Thus, 0.90 mg/mL was considered to be the optimal concentration for the capture MAb 11B6 in this CGICS. To ascertain the optimal concentration of the CGNP-labeled MAb solution on the conjugate pad, it was serially diluted in 0.02 mol/L PBS (pH 7.4) containing 0.02% NaN3, 2% BSA, and 3% sucrose. CGICS test results displayed that the 1:25 (v/v) diluted CGNP-labeled MAb solution generated a feeble false-positive band at the T line, whereas the 1:50 diluted CGNP-labeled MAb solution lowered the detection sensitivity of CGICS. Therefore, the 1:40 diluted CGNP-labeled MAb solution was considered the best for CGICS.

Specificity, broad-spectrum, and sensitivity of the CGICS for *Xoc* monitoring

Specificity, broad-spectrum and sensitivity are crucial characteristics of a CGICS. To assess the specificity and broad-spectrum of the newly created CGICS in this study, ten different *Xoc* strains (i.e., *Xoc* RS105, *Xoc* BLS256, *Xoc* oxy01, *Xoc* oxy02, *Xoc* oxy04, *Xoc* oxy05, *Xoc* JS, *Xoc* GX, *Xoc* AH, and *Xoc* YN) and ten different negative control bacterial strains (i.e., *X. oryzae* pv. *oryzae* PXO86, *X. oryza* pv. *oryzae* Y2, *X. citri* pv. *citri* 29-1, *X.campestris* CGMCC 1.3408, *X. sacchari* ACCC 10416, *X.axonopodis* CCTCC AB 2018263, *X. albilineans* FJ1, *Burkholderia glumae* Os48, *Pantoea ananatis* F163, and

(See figure on next page.)

Fig. 1 Specificity, broad-spectrum, and sensitivity analyses of the prepared two MAbs by indirect-ELISA.**a** Specificity and broad-spectrum analyses of the prepared two MAbs by indirect-ELISA using ten *Xoc* strains (*Xoc* RS105, *Xoc* BLS256, *Xoc* oxy01, *Xoc* oxy02, *Xoc* oxy04, *Xoc* oxy05, *Xoc* JS, *Xoc* GX, *Xoc* AH, and *Xoc* YN) and ten negative control bacterial strains (*X. oryzae* pv. *oryzae* PXO86, *X. oryza* pv. *oryzae* Y2, *X. citri* pv. *citri* 29–1, *X.campestris* CGMCC 1.3408, *X. sacchari* ACCC 10416, *X.axonopodis* CCTCC AB 2018263, *X. albilineans* FJ1, *Burkholderia glumae* Os48, *Pantoea ananatis* F163 and *Acidovorax oryzae* CGMCC 1.1728) as the test samples. The optical density at 405 nm (OD_{405}) value of each treatment was measured at 30 min after the addition of the substrate solution and is presented as the mean values ± standard deviation (SD) from three independent assays. Two asterisks (**) indicate a significant difference ($P<0.01$; Student's t-test). **b** Sensitivity analyses of the prepared two MAbs by indirect-ELISA using serially two-fold diluted *Xoc* strain RS105 and *Xoo* strain PXO86 suspensions (from 1×10^7 to 76.3 CFU/mL) as the test samples. The OD_{405} value of each treatment is presented as the mean values ± SD from three independent assays

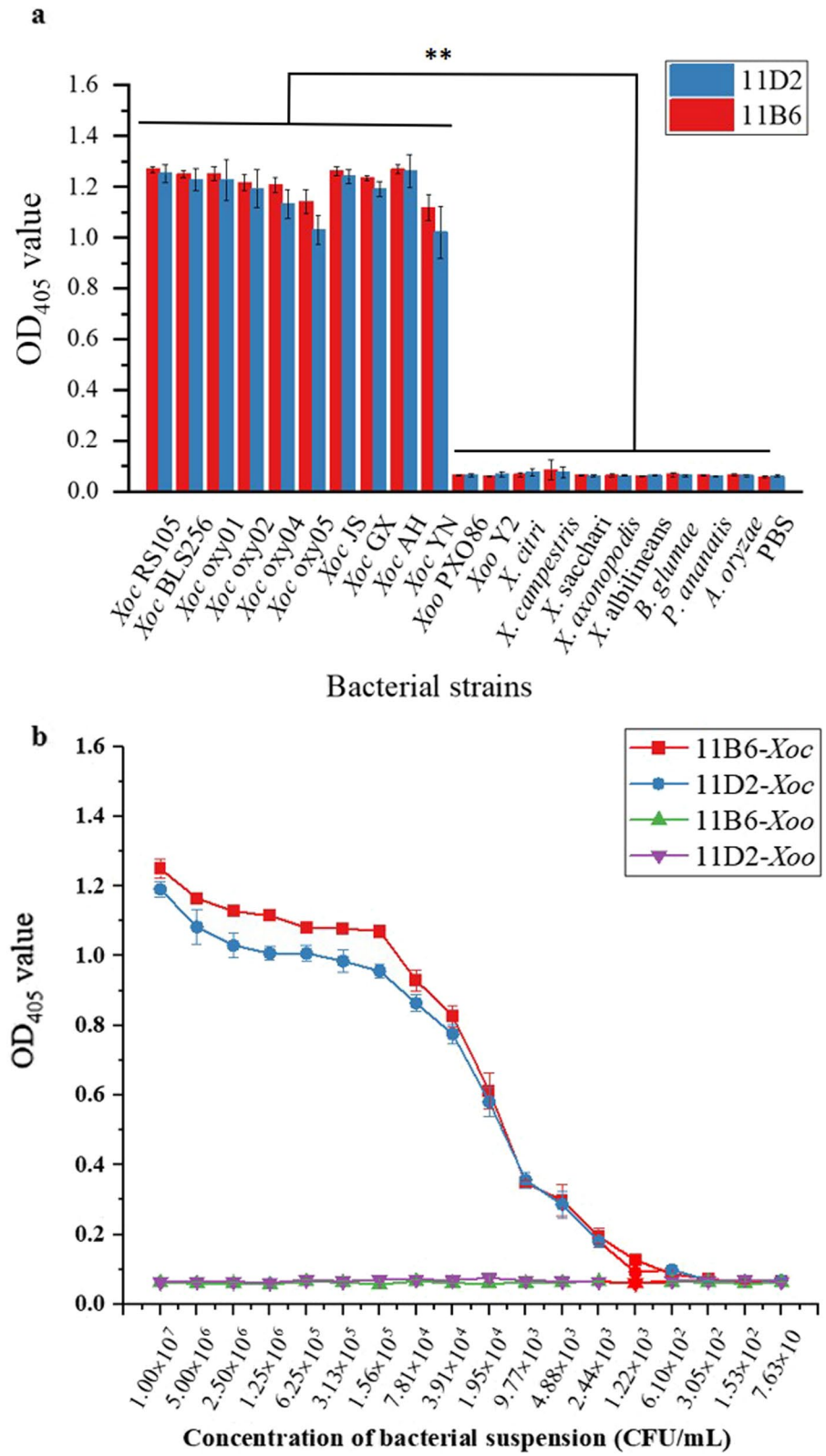


Fig. 1 (See legend on previous page.)

Acidovorax oryzae CGMCC 1.1728) were separately tested by the CGICS. The test results showed that the CGICS could detect all ten tested *Xoc* strains but had negative reactions with all ten tested negative control bacterial strains (Fig. 4a), demonstrating that the developed CGICS is highly specific and broad-spectrum for *Xoc* detection.

To determine the sensitivity of the developed CGICS, the cultured bacterial suspension of *Xoc* strain RS105 was serially two-fold diluted in PBS, and then 50–100 µl of each dilution was detected through the CGICS. The results showed that the CGICS could detect *Xoc* in bacterial suspensions diluted from 1×10^7 to 4.88×10^3 CFU/mL (Fig. 4b), making its low detection limit of 4.88×10^3 CFU/mL. In addition, a homogenate from an *Xoc*-infected rice leaf tissue, as shown in Fig. 2c, was serially two-fold diluted, and then each dilution was tested by the CGICS. The test results showed that the low detection limit of CGICS for this infected rice leaf tissue was 1:51,200 (w/v, g/mL) dilution (Fig. 4c). The above results demonstrate that the developed CGICS is super-sensitive for detecting *Xoc*.

Xoc detection in paddy-collected rice plants

To further assess the accuracy and effectiveness of the dot-ELISAs and CGICS for *Xoc* detection developed in the study, 12 paddy-collected rice plant specimens from Hainan, Guangdong, and Yunnan provinces of China in 2023 were subjected to the Dot-ELISA and CGICS for detecting *Xoc* infection. The results of the dot-ELISA and CGICS tests showed that seven out of the 12 rice leaf samples were infected with *Xoc* (Fig. 5a, b). To verify this serological test result, these paddy specimens were simultaneously tested for *Xoc* infection through conventional PCR. The PCR test confirmed that the seven rice specimens that tested positive for *Xoc* using the dot-ELISA and CGICS tests were indeed infected by *Xoc* (Fig. 5c). The remaining paddy specimens and the uninfected rice control did not give positive products (Fig. 5c). These findings indicate that the newly developed dot-ELISAs and CGICS tests are highly effective and accurate in detecting *Xoc* infection in rice plant specimens.

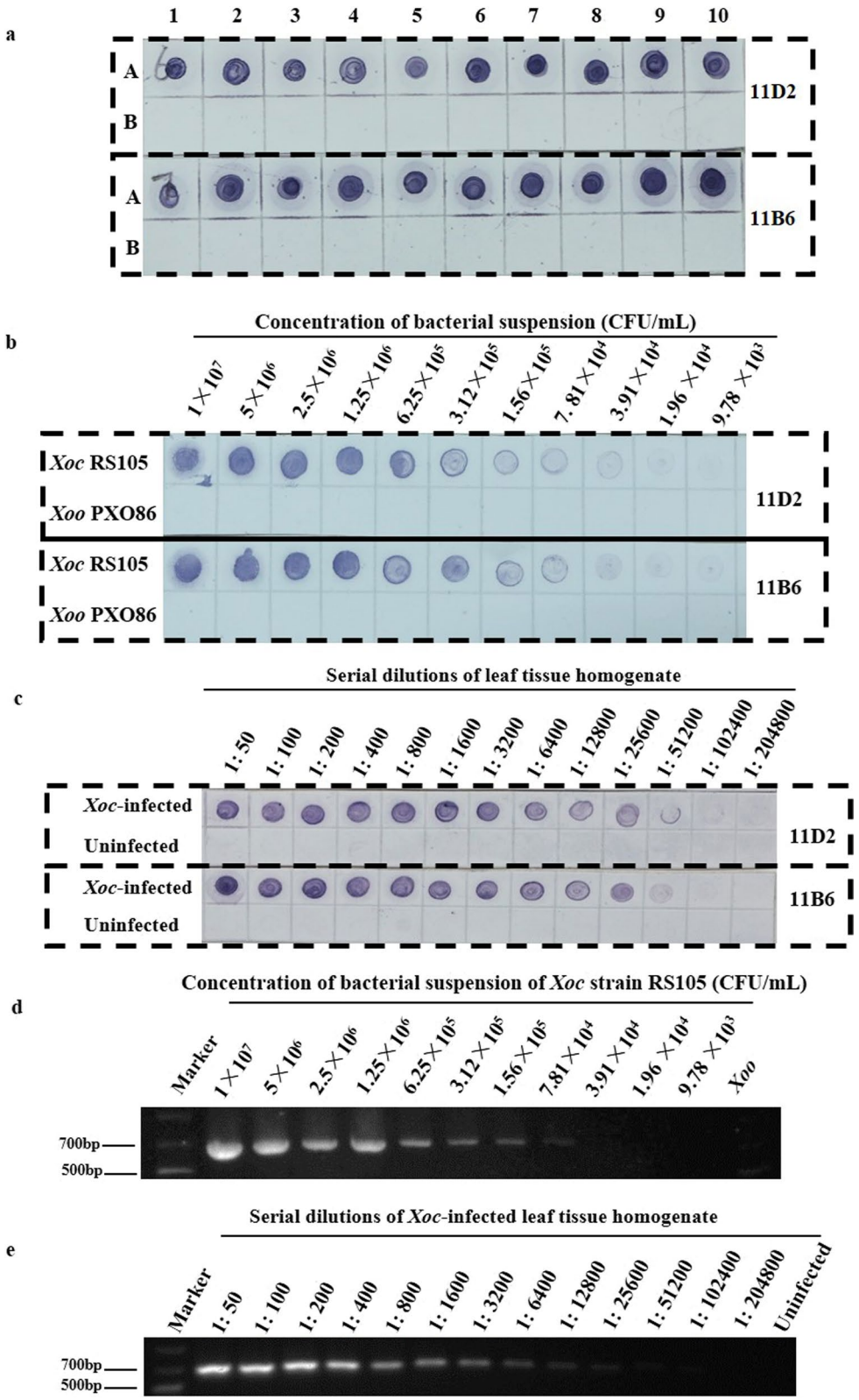
Discussion

BLS is a significant rice quarantine disease that poses an enormous threat to the rice industry worldwide (Thianthavon et al. 2021). In recent years, the severity and spread of BLS have increased, resulting in stricter quarantine (Cao et al. 2020). Therefore, there is an urgent need for rapid and reliable quarantine tools to prevent the large-scale spread of this rice disease and ensure smooth export and import of rice seeds between different countries.

With the advancement of molecular detection technology, PCR-based techniques have become popular for quarantine and detection of plant pathogenies (Sakthivel et al. 2001). Several PCR-based detection techniques have been developed and applied for the detection and identification of *Xoc* (Kang et al. 2008; Zhang et al. 2008). For example, Kang and colleagues designed a pair of primers specific for *Xoc* and established a PCR detection method with a low detection limit of 1.3×10^4 CFU/mL (Kang et al. 2008). However, such PCR-based techniques require expensive equipment and complex operation steps, which makes them unsuitable for quick, large-scale, and on-site detection. On the other hand, antibody-based serological technology is simple, quick, sensitive, and practical for plant pathogeny detection. Serological technology was first applied in 1918 for the detection of plant pathogenic bacteria and subsequently became one of the most effective tools for the identification, detection, and classification of pathogenic bacteria (He et al. 2021). Among serological techniques, enzyme-linked immunosorbent assay (ELISA) is widely applied due to its high sensitivity and specificity, rapidity, and suitability for the detection of large-scale specimens. In 1993, Wang et al. established

(See figure on next page.)

Fig. 2 Specificity, broad-spectrum, and sensitivity analyses of the developed two Dot-ELISAs using MAbs 11D2 and 11B6 as the detection antibodies. **a** Specificity and broad-spectrum analyses of the two developed Dot-ELISAs for the detection of *Xoc*. A1–10 represent *Xoc* RS105, *Xoc* BLS256, *Xoc* oxy01, *Xoc* oxy02, *Xoc* oxy04 *Xoc* oxy05, *Xoc* JS, *Xoc* GX, *Xoc* AH, and *Xoc* YN, used as the positive bacterial strains. B1–10 represent *X. oryzae* pv. *oryzae* PXO86, *X. oryza* pv. *oryzae* Y2, *X. citri* pv. *citri* 29–1, *X.campestris* CGMCC 1.3408, *X. sacchari* ACCC 10416, *X.axonopodis* CCTCC AB 2018263, *X. albilineans* FJ1, *Burkholderia glumae* Os48, *Pantoea ananatis* F163, and *Acidovorax oryzae* CGMCC 1.1728, used as the negative control bacterial strains. Each diluted suspension (2 µL) was separately spotted on the nitrocellulose membranes and then tested by the two developed Dot-ELISAs. Purple dots indicate positive reactions, while no color dots indicate negative reactions. **b** Sensitivity analyses of the two developed dot-ELISAs for the detection of *Xoc*. *Xoc* strain RS105 and *Xoo* strain PXO86 (as negative control) suspensions were serially two-fold diluted, and each resulting diluted suspension (2µL) was loaded onto a nitrocellulose membrane for the Dot-ELISA test. **c** Sensitivity analyses of the two developed dot-ELISAs for the detection of *Xoc* in rice plants. A homogenate from a *Xoc*-infected or an uninfected rice leaf tissue was serially two-fold diluted, and each dilution (2µL) was loaded onto a nitrocellulose membrane for Dot-ELISA test. **d** Sensitivity analysis of conventional PCR for *Xoc* detection. Serially diluted *Xoc* strain RS105 suspensions were tested by PCR using *Xoc*-forward primer/*Xoc*-reverse primer to amplify a specific 694 bp gene fragment. **e** Sensitivity analysis of PCR for a *Xoc*-infected rice leaf tissue, same as in Fig. 2c



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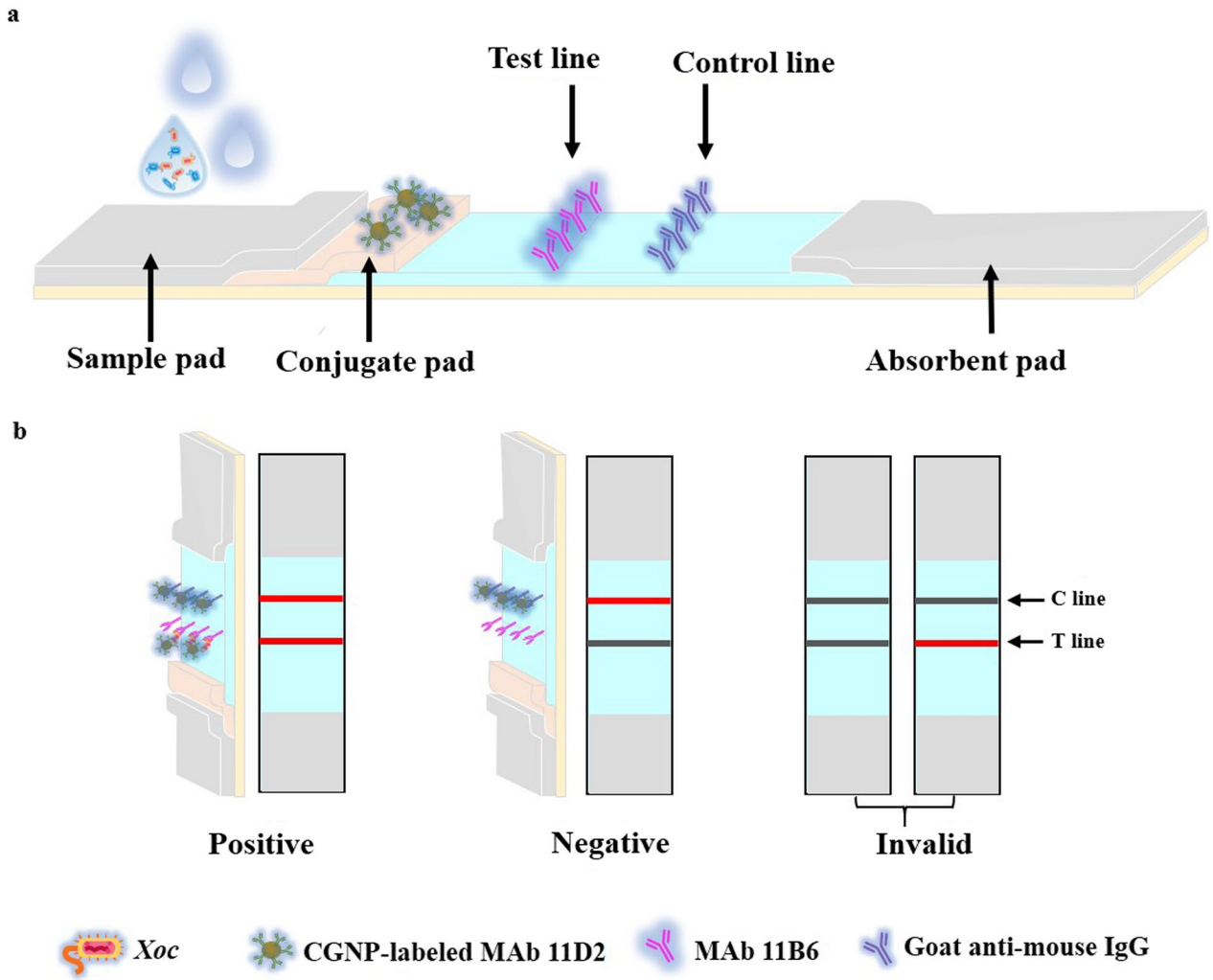

Fig. 3 Schematic diagram of CGICS for the detection of *Xoc*

an ELISA for the *Xoc* test. Unfortunately, this ELISA, based on polyclonal antibodies (PAbs), exhibited cross-reactivity with other bacteria (Wang et al. 1993) and could not distinguish between *Xoo* and *Xoc*. Compared to the aforementioned reported serological methods, the MAb-based Dot-ELISAs developed in this study not only can specifically detect ten analyzed different *Xoc* strains but also have no cross-reactivity with tested ten control bacterial strains such as *Xoo*. Furthermore, the sensitivities of the two Dot-ELISAs developed in this study were all up to 9.78×10^3 CFU/mL, which is about seven times more sensitive than that of PCR (Fig. 2).

The CGICS is the simplest and fastest test technique utilizing the colloidal gold nanoparticles as the tracer. It has been widely used to test all kinds of human, animal, and plant pathogens (Jiang et al. 2011; Jin et al. 2012), toxicants (Gao et al. 2012), drug residues (Byzova et al. 2010; Zhang et al. 2011), and heavy metal ions (Liu et al. 2012). Wu et al. (2015) developed a CGICS capable of detecting both *Xoc* and *Xoo* with a detection limit of 10^6 CFU/mL in rice specimens. However, to date, there are no reports on the establishment and application of CGICS for specifically detecting *Xoc*. In this study, we developed an excellent CGICS assay for *Xoc* detection. Our findings showed this CGICS could broad-spectrum test different *Xoc* strains with a sensitivity of 4.88×10^3 CFU/mL and has no cross-reactivity with other ten control plant pathogens such as *Xoo*. Moreover, the whole detecting process of this CGICS can be completed in 10 min.

Given the high antigen homology between *Xoc* and *Xoo* from *Xanthomonas*, the antibodies against *Xoc* often cross-react with *Xoo*. Surprisingly, the two MAbs prepared in this study can specifically target *Xoc* and do not cross-react with *Xoo*. Additionally, *Xoc* distributes globally and displays considerable genetic variability (Vera Cruz et al. 1984). Intriguingly, the two prepared MAbs

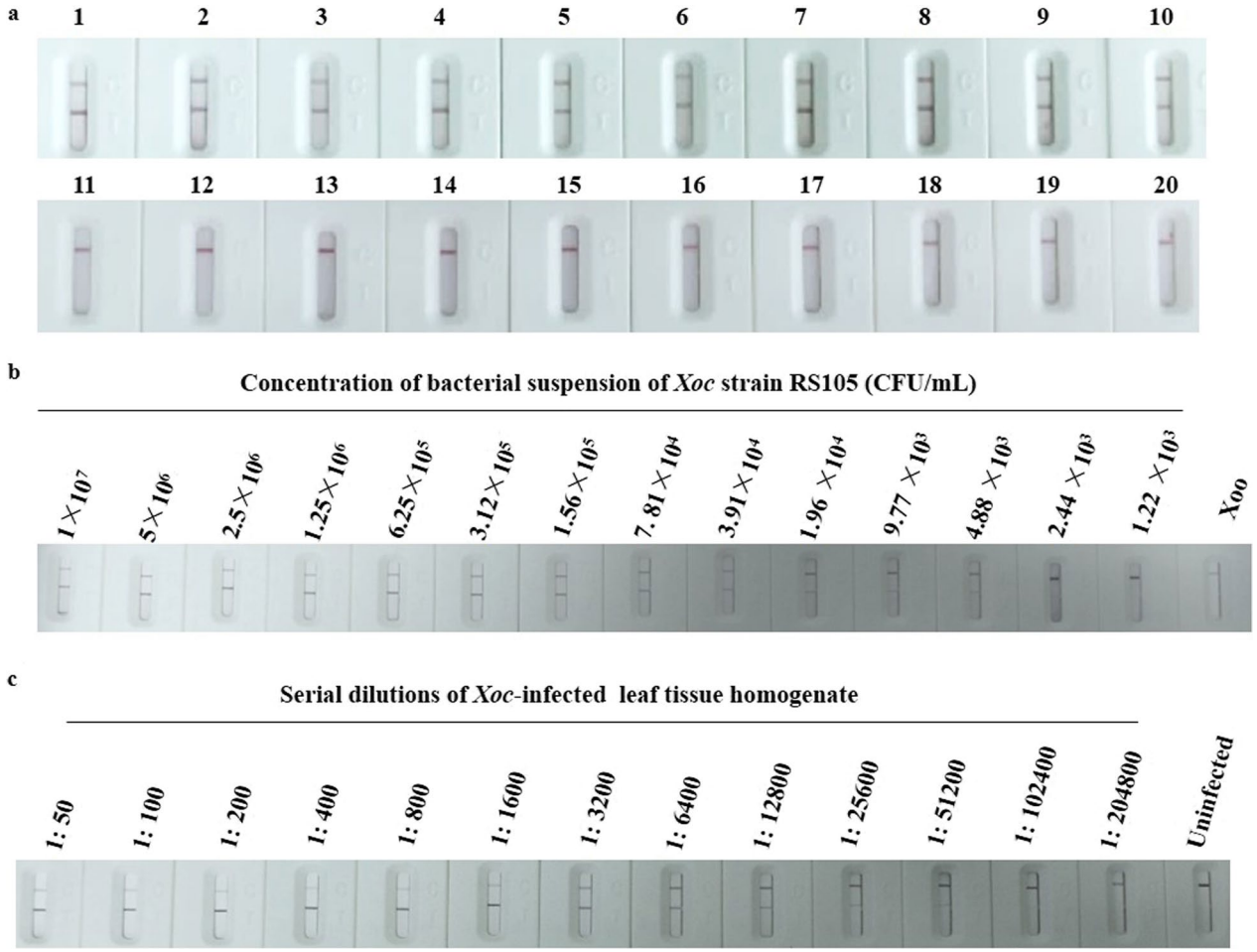

Fig. 4 Specificity, broad-spectrum, and sensitivity analyses of the developed CGICS for Xoc detection. **a** Specificity and broad-spectrum of the developed CGICS were determined using ten different Xoc strains and ten different negative control bacterial strains. 1–10 represent *Xoc* RS105, *Xoc* BLS256, *Xoc* oxy01, *Xoc* oxy02, *Xoc* oxy04 *Xoc* oxy05, *Xoc* JS, *Xoc* GX, *Xoc* AH, and *Xoc* YN, respective. 11–20 represent *X. oryzae* pv. *oryzae* PXO86, *X. oryza* pv. *oryzae* Y2, *X. citri* pv. *citri* 29–1, *X.campestris* CGMCC 1.3408, *X. sacchari* ACCC 10416, *X.axonopodis* CCTCC AB 2018263, *X. albilineans* FJ1, *Burkholderia glumae* Os48, *Pantoea ananatis* F163, and *Acidovorax oryzae* CGMCC 1.1728 respectively. Each bacterial suspension (100 µL) was loaded into the sample pads of the CGICSs, and then the color of the C and T lines was observed with the naked eye in 5–10 min. **b** The sensitivity of the developed CGICS was determined using serially two-fold diluted bacterial suspensions of *Xoc* strain RS105. **c** Sensitivity analysis of the developed CGICS for the detection of *Xoc* in a rice leaf tissue. A homogenate from a *Xoc*-infected was serially two-fold diluted, and each dilution (100 µL) was loaded into the sample pads for the CGICS test. A homogenate from an uninfected rice leaf tissue was used as the negative control

and their two serological technologies developed in this work can detect ten different *Xoc* strains from various provinces in China and other countries (Fig. 2), which indicates that both MAbs prepared in this work and the newly created two serological approaches are broad-spectrum for *Xoc* detection.

Conclusions

In this study, two highly specific, broad-spectrum, and super-sensitive anti-*Xoc* MAbs were successfully prepared, and using the prepared MAbs as the detection antibodies, two efficient and accurate serological techniques, Dot-ELISAs and CGICS, were established for specifically and super-sensitively detecting *Xoc*, the pathogen of rice BLS. These two prepared MAbs and two serological tools in this work will provide reliable technical support for inspection, quarantine, and prevention and control of BLS, and have a promising prospect in *Xoc* detection application.

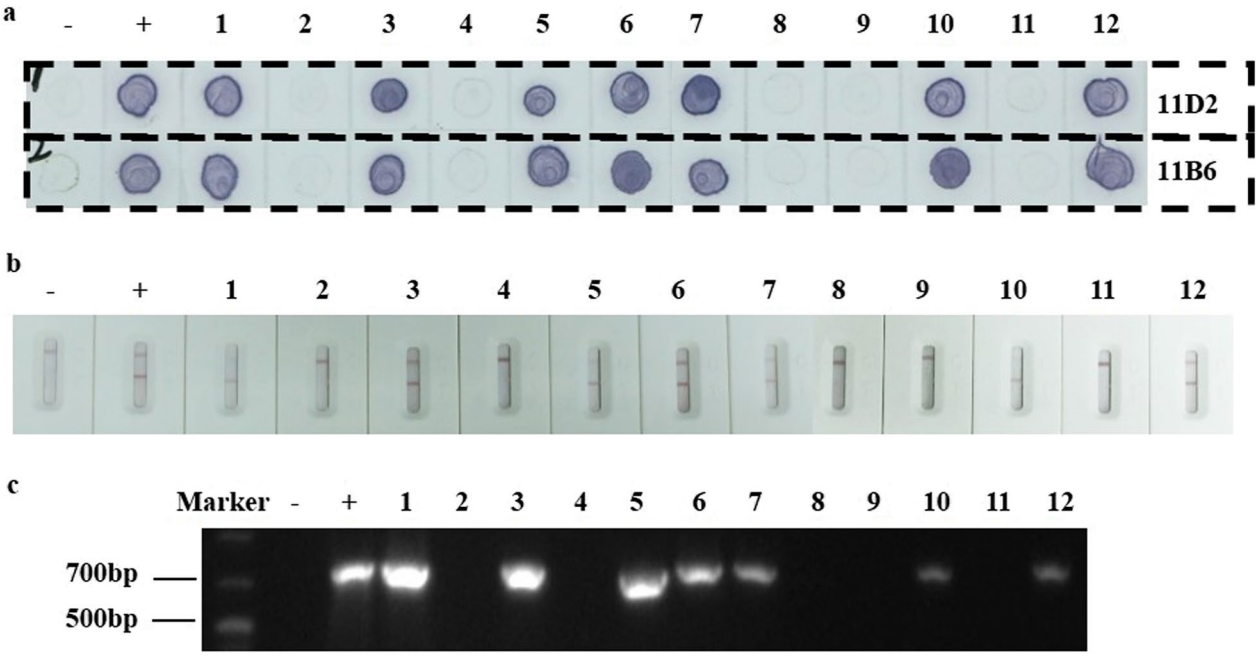

Fig. 5 Test results of *Xoc* infection in paddy-collected rice plants through the newly created Dot-ELISAs,CGICS, and conventional PCR. **a** Test results of *Xoc* infection in 12 paddy-collected rice plants through the newly created Dot-ELISAs. 1–12 represent 12 paddy-collected rice plant specimens. "–" is an uninfected rice leaf tissue, used as the negative control. "+" is a *Xoc*-infected rice leaf tissue, used as the positive control. **b** Test results of *Xoc* infection in 12 paddy-collected rice plant specimens through the newly created CGICS. **c** Test results of *Xoc* infection in 12 paddy-collected rice plant specimens through PCR. Marker representatives a 1 kb DNA marker

Methods

Bacterial strains, bacterium culture and preparation, and field sample collection

Sources of bacterial strains used in this study are listed in Table 1. The bacterial strains were initially streaked on nutrient agar medium and cultured for 24 h at 30°C. After PCR verification, a single bacterial colony was transferred to fresh beef extract peptone liquid culture medium and cultured in a shaking incubator at 200 rpm, 30°C for 10 h. The resulting bacterial cultures (1 mL per sample) were pelleted through 5 min of centrifugation at 13,500×*g*. The pellets were resuspended individually in 1 mL of sterile water, and the bacterial concentration was determined using a standard plate-counting procedure (He et al. 2021) and recorded as colony-forming units (CFU)/mL. Rice plants infected or un-infected *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) were collected in 2023 from rice paddies in Sanya City of Hainan Province, Mangshi of Yunnan Province, and Enping City of Guangdong Province, China.

Production of murine monoclonal antibodies (MAbs) against *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*)

Xoc strain RS105 was cultured in liquid nutrient agar medium for 24 h and then pelleted via 5 min centrifugation at 13,500×*g*. The resulting pellet was resuspended in a physiological saline solution containing 0.5% formaldehyde and incubated at 4°C for 24 h to inactivate bacteria as described previously (He et al. 2021). The inactivated bacteria were used as the immunogen to immunize five seven-week-old female BALB/c mice for MAb preparation (Guo et al. 2023; Zhang et al. 2023). In brief, the mice were immunized twice with the inactivated *Xoc* bacteria (10^6 CFU/each mouse) emulsified in an equal volume of Freund's complete and Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) at a three-week interval. For the third immunization, three weeks after the second immunization, the mice were immunized with the inactivated bacteria (2×10^6 CFU/each mouse) in a physiological saline solution via intraperitoneal injection. At 3 d after the third immunization, spleen lymphocytes from the immunized mouse with the highest serum titer were isolated and fused with Sp2/0 myeloma cells in 50% polyethylene glycol (Sigma-Aldrich, wt/vol, MW = 3500). The hybridomas secreting antibodies against *Xoc* were obtained through an indirect-ELISA screen and then cell-cloned separately by the limited dilution method as described previously (Li et al. 2021). The resulting cloned hybridomas were intraperitoneally injected into BALB/c mice to produce ascites containing MAbs (He et al. 2020). The titers of ascites containing MAbs were determined by an indirect ELISA using *Xoc* as the coating

Table 1 Bacterial strains used in this study

Bacterial strain	Hosts	Origins	Provided organizations
X. oryzae pv. *oryzicola* RS105	Rice	China	Shanghai Entry-Exit Inspection and Quarantine Bureau
X. oryzae pv. *oryzicola* BLS256	Rice	Philippines	Shanghai Entry-Exit Inspection and Quarantine Bureau
X. oryzae pv. *oryzicola* oxy01	Rice	China	Institute of Plant Protection, Chinese Academy of Agricultural Sciences
X. oryzae pv. *oryzicola* oxy02	Rice	China	Institute of Plant Protection, Chinese Academy of Agricultural Sciences
X. oryzae pv. *oryzicola* oxy04	Rice	China	Institute of Plant Protection, Chinese Academy of Agricultural Sciences
X. oryzae pv. *oryzicola* oxy05	Rice	China	Institute of Plant Protection, Chinese Academy of Agricultural Sciences
X. oryzae pv. *oryzicola* JS	Rice	Rice	Zhejiang Academy of Agricultural Sciences
X. oryzae pv. *oryzicola* GX	Rice	Rice	Zhejiang Academy of Agricultural Sciences
X. oryzae pv. *oryzicola* AH	Rice	China	Zhejiang Academy of Agricultural Sciences
X. oryzae pv. *oryzicola* YN	Rice	China	Zhejiang Academy of Agricultural Sciences
X. oryzae pv. *oryzae* PXO86	Rice	Philippines	Shanghai Entry-Exit Inspection and Quarantine Bureau
X. oryzae pv. *oryzae* Y2	Rice	China	Nanjing Agricultural University
X. citri pv. citri 29–1	Citrus	China	Shanghai Entry-Exit Inspection and Quarantine Bureau
X. campestris CGMCC 1.3408	Sarson	United Kingdom	China General Microbiological Culture Collection Center
X. sacchari ACCC 10416	Sarson	China	Agricultural Culture Collection of China
X. axonopodis CCTCC AB 2018263	Carpet Grass	China	China Center for Type Culture Collection
X. albilineans FJ1	Sweet Potato	China	Shanghai Entry-Exit Inspection and Quarantine Bureau
Burkholderia glumae Os48	Rice	China	Institute of Biotechnology, Zhejiang University
Pantoea ananatis F163	Rice	China	Institute of Biotechnology, Zhejiang University
Acidovorax oryzae CGMCC 1.1728	Rice	Japan	China General Microbiological Culture Collection Center

antigen (Zhang et al. 2020). The IgG of MAb was purified from ascites by the saturated ammonium sulfate precipitation method (Guo et al. 2020).

Indirect-ELISA and Dot-ELISA

Using *Xoc* strain RS105 (10^5 CFU/mL) in 50 mmol/L sodium bicarbonate buffer (pH 9.6) as the coating antigen, indirect-ELISA was performed as described by He et al. (2021) for antibody screening and antibody titer determination. Briefly, the *Xoc* suspension (100 µL/well) was added into wells of a 96-well ELISA microplate and incubated overnight at 4°C. The microplate was washed three times with 0.01 mol/L PBS containing 0.05% Tween-20 (PBST, pH 7.4) and blocked with 3% skimmed milk powder in PBS (200 µL/well) for 30 min at room temperature (RT). After three times washes with PBST, hybridoma culture supernatants or diluted MAbs (100 µL/well) were separately added to the microplate wells and incubated for 1 h at RT. After three times washes with PBST, an alkaline phosphatase (AP)-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich) solution (100 µL/well) was added into each microplate well and incubated for another one hourat RT. After washed four times with PBST, the microplate wells were loaded with p-nitrophenyl phosphate (Sigma-Aldrich) substrate solution (100 µL/well) and incubated for 30 min at RT. The absorbance at 405 nm was measured using a multifunctional microplate reader. Each sample was assayed in triplicate, and the mean value was reported. A value at least three times greater than that of the negative control was considered positive.

Dot-ELISA for the detection of *Xoc* was carried out as described by He et al.(2021). In brief, different *Xoc* strain and negative control bacterial suspensions and homogenates from rice leaf tissues were prepared, and then the diluted bacterial suspensions and rice leaf homogenates (2µL per sample) were dotted separately onto a nitrocellulose membrane (GE Healthcare, Bucks, UK). After air-drying at RT for 10 min, the dotted membrane was blocked in PBS containing 5% skimmed milk powder for 30 min, and then incubated in anti-*Xoc* specific MAb dilution for 1 h. After rinsing three times with PBST, the membrane was incubated in suitable diluted AP-labeled goat anti-mouse IgG secondary antibody solution for another hour. After four times rinses with PBST, the color reaction on the membrane was visualized after the addition of a nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) substrate solution for 20 min at RT.

Production of colloidal gold nanoparticle (CGNP)-labeled MAb

Colloidal gold nanoparticles (CGNPs) with 40 nm in diameter were synthesized using the citrate reduction method described by Huang et al. (2019). To label MAb with CGNPs, 1.0 mg of purified MAb was dropwise

added into 100 mL of CGNP solution under stirring, and the mixture was gently stirred for 30 min at RT, followed by the addition of 1 mL of 0.01 mol/L borate solution containing 10% bovine serum albumin (BSA). The above mixture was stirred gently for another 30 min at RT and then centrifugated at 20,000 × g at 4°C for 20 min. The resulting CGNP-labeled MAb pellet was dissolved in 10 mL of 0.02 mol/L PBS (pH 7.4) containing 0.02% NaN3, 2% BSA, and 3% sucrose, and stored at 4°C until use.

Establishment of Colloidal gold immunochromatographic strip (CGICS)

The CGICS for the detection of *Xoc* was established as our previously reported literatures (Guo et al. 2023; Zhang et al. 2023) and consisted successively of the sample pad, conjugate pad, nitrocellulose membrane (Sartorius AG. Millipore, Billerica, MA, USA), and absorbent pad (Zhang et al. 2023). Briefly, the sample pad of glass fibers was soaked in 0.01 M PBS containing 1% BSA and 0.05% Tween-20 for 30 min to minimize nonspecific binding and then dried at 37°C. The CGNP-labeled MAb was dispensed onto the conjugate pad and incubated at 37°C for two hours. The capture MAb and the goat anti-mouse antibody were respectively dispensed to the test (T) line and control (C) line on the nitrocellulose membrane using Bio-Dot XYZ-3000 dispensing platform (Bio-Dot, CA, USA). The absorbent pad consisted of cellulose fiber membrances was soaked in PBST, and then dried at 37°C for two hours. After that, all above components were assembled successively onto the polyvinyl chloride backing plate with a 2 mm overlap at each joint. Finally, the resulting assembled plate was longitudinally cut with a guillotine cutter to produce strips with 3 mm wide.

Characteristic analysis of CGICS

For the specificity and broad-spectrum analyses, ten different *Xoc* strains, ten other phytopathogenic bacteria, a homogenate from a non-infected rice plant (used as a negative control), and 0.01 M PBS (used as a blank control) were respectively tested by the newly developed CGICS. For the sensitivity analysis, *Xoc* strain RS105 and X. oryzae pv. oryzae (*Xoo*) strain YN (used as a negative control) were serially two-fold diluted in 0.01 M PBS from 1×10^7 CFU/mL to 9.78×10^3 CFU/mL, and the resulting each dilution was separately monitored by the newly developed CGICS. The above sample solutions were individually dropped into the sample pads of CGICSs, and samples showing two red lines in 5–10 min were considered *Xoc*-positive, while samples showing only one red control line were considered *Xoc*-negative. If the strip lacked a red control line, the test result was considered as invalid.

PCR detection of *Xoc*

To verify serological test results of 12 field-collected rice plant specimens, *Xoc* infection was simultaneously monitored through PCR using a pair of primers (*Xoc*-forward primer: 5′-TATCTCCCAGCATGT TGATCG -3′ and *Xoc*-reverse primer: 5′-ACGCGT TCAATCTCCTCCATGT -3′) specific for *Xoc* LPS O-antigen biosynthesis protein gene (GenBank Accession no. AY572534) to amplify a 694 bp gene fragment. Rice total DNA was extracted with FastPure Plant DNA Isolation MiniKit-BOX2 (Vazyme, Nanjing, China) according to the manufacture's instructions. To determine PCR sensitivity, a cultured *Xoc* suspension (1×10^7 CFU/mL) was serially two-fold diluted, and 1 µL of each bacterial dilution was used as a PCR template. PCR reaction system (25 µL each) consisted of 12.5 µL 2 × Green Taq mix (Vazyme), 1 µL for each primer (10 µmol/L each), 1 µL diluted bacterial suspension/diluted leaf tissue homogenate, and 9.5 µL sterile deionized water. The thermal cycles program comprised an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 10 s, and a final extension at 72°C for 10 min.

Detection of *Xoc* infection in field-collected rice plant specimens

Approximately 50 mg of 12 field-collected rice leaf samples were ground into powder in liquid nitrogen, and a homogenate was further prepared in 0.01 M PBS at a ratio of 1:20 (w/v, g/mL). Subsequently, the specimen homogenates were simultaneously detected *Xoc* infection by the established Dot-ELISA, CGICS, and conventional PCR.

Abbreviations

AP	Alkaline phosphatas
BIO-PCR	Biological and enzymatic amplification
BLS	Rice bacterial leaf streak
CGNP	Colloidal gold nanoparticle
CGICS	Colloidal gold immunochromatographic strip
Dot-ELISA	Dot enzyme-linked immunosorbent assay
MAb	Monoclonal antibodie
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RT	Room temperature

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Author contributions
JW conceived and designed the experiments. SD and JW wrote the manuscript. SD and JW designed the figures. NL, XZ, CZ, BL, QA, and XZ participated in this research. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
The animal study protocol was approved by the Animal Experimentation Ethics Committee of Zhejiang University, Hangzhou, China (Protocol code ZJU20210215, 30 August 2021).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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