International Scholars Journals

African Journal of Infectious Diseases Research ISSN 4729-6836 Vol. 4 (11), pp. 001-009, November, 2017. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Comparison of pathogen enrichment methods for detecting *Acidovorax avenae* subsp*. citrulli"* from watermelon seeds

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Accepted 18 May, 2017

It is well known that bacterial fruit blotch (BFB) of watermelon caused by *Acidovorax avenae* subsp. *citrulli* threatens watermelon production. There is no pathogen enrichment or concentrated steps before detection in conventional PCR-based diagnostic technique, which consequently leads to the lower detection sensitivity. In the present study, two different bacterial suspensions were prepared, and four pathogen enrichment protocols including Biological Preparation (BP), Membrane Filtration (MF), Immune-Magnetic Separation (IMS) and Immune-Capture Preparation (ICP) were employed to prepare the PCR template for the pathogen detection. Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) was also conducted as a parallel comparison. The results showed that IC-PCR was the optimum method through comparing the detection limits, time and expenditure among those methods. The detection limit of IC-PCR for both bacterial suspension and seeds suspension can reach 10^2 CFU/ml, which was 10 times lower than that of IMS-PCR when the seed suspension was used as template. In addition, the results of IC-PCR showed higher degree of precision than those of IMS-PCR. The time and expenditure of IC-PCR were comparable with other methods. The present study demonstrated that the procedures of immune-capture PCR is a sensitive, specific, rapid, reproducible, and economical method to detect *A. avenae* subsp. *citrulli* in watermelon seeds.

Key words: Bacterial Fruit Blotch, watermelon seed, pathogen enrichment, PCR.

INTRODUCTION

Bacterial fruit blotch (BFB) is a seed borne disease, which is caused by gram-negative bacterium *Acidovorax avenae* subsp. *citrulli* (Willems et al., 1992). It has become one of the most serious disease threatening watermelon production since its first outbreak in the late 1988s in United States (Wall and VM, 1988; 1990; Latin and Hopkins, 1995; Schaad et al., 2003). The disease has spread worldwide, and been reported in many other cucurbits such as pumpkin, squash, and cucumber (Langston Jr et al., 1999; Martin and O'Brien, 1999). Contaminated watermelon seed is the important primary inoculum source for BFB (Rane and Latin, 1992; Kucharek et al., 1993; Lessl et al., 2007). As a seed-borne pathogen with highly destructive potential, *A. avenae* subsp. *citrulli* can spread rapidly into different regions via contaminated seeds. The risk of disease outbreak is even higher when seedling transplants are employed to promote the rapid growth and uniform crop establishment. Under the favorable environmental conditions such as high

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temperature, high plant densities, overhead irrigation, and high relative humidity, a few contaminated seeds could result in a huge damage on both the quality and the yield of watermelon (Schaad et al., 2003).

To eliminate contaminated seeds from the watermelon production systems is one of the most feasible disease-management strategies (Kucharek et al., 1993; Walcott and Gitaitis, 2000a). The seed testing procedure includes two steps: 1) to remove the contaminated seeds prior to planting by seed health testing; and 2) to eradicate the bacterium from the seeds by chemical treatments. However, it has been shown that seed testing alone could not guarantee the pathogen-free seeds (Lessl et al., 2007); and the chemical control has also shown its limitation on preventing BFB (Rane and Latin, 1992). Although the seed assays were developed and available for the detection of *A. avenae* subsp. *citrulli* (Walcott et al., 2003), the conventional detection methods such as biochemical identification were relatively less sensitive and accurate. In addition, those methods were also very time-consuming due to the inefficiency on the pathogen isolation, culture and enrichment. The serological detection such as ELISA has low sensitivity and accuracy as well. Therefore, the conventional methods are outdated in practice (Kucharek et al., 1993).

With the rapid development of modern biotechnology, the molecular detection methods such as IM-PCR, and real-time PCR targeting rDNA and ITS region were developed. Those techniques are more sensitive and accurate for detection, however, the DNA/RNA extraction in those methods might increase the cost of the detection. It is thus limited the application of those techniques on detecting the BFB in practice. Another obstacle for the application of the PCR-based methods in practice is the lack of efficient pathogen enrichment methods (Schaad et al., 1995; 2007).

Many pathogen enrichment preparation methods have been reported for the PCR-based detection including biological preparation (Schaad et al., 1995), membrane filtration +-(Schaad et al., 2007; Priou et al., 1999), immune-magnetic preparation (Walcott and Gitaitis, 2000a; Amagliani et al., 2006; Ha et al., 2009) and immune-capture preparation (Peng et al., 2002; Buchhop et al., 2009). The sensitivity, time and expenditure varies among these methods, while those factors should be taken into accounted together to develop a successful PCR strategy on detecting a target pathogen in practice. However, a thoroughly comparison of those pathogen enrichment protocols on detecting *A. avenae* subsp. *citrulli* in watermelon seeds has not yet been reported. Furthermore, establishment of an accurate, rapid and sensitive method to detect BFB contaminated watermelon seed can not only benefit the local watermelon growers, but significantly prevent the invasion and spread of other diseases.

In this study, *A. avenae* subsp. *citrulli* from the watermelon seed was enriched by four pathogen enrichment methods prior to PCR amplification. The detection limits, accuracy, reliability, consumed time and expenditure of those enrichment procedures were thoroughly compared in order to develop an optimum method to detect the BFB contaminated seeds.

MATERIALS AND METHODS

Bacterial culture

A. avenae subsp. *citrulli* strain（NCPPB 3679）were obtained from the International Collection of Phytopathogenic Bacteria (ICPB) in United Kingdom. Strains were stored in 40% sterilized glycerol at –80°C until use. Bacteria were maintained on KB agar plate and incubated at 28°C for 72 h. The single colony was picked with sterilized toothpick and transferred into King's medium B (KB) liquid medium. After 48 h incubation at 28°C in a water-bathing shaker, the concentration of the cell reached 4×10^8 CFU/ml (OD600 = 0.627). The culture of *A. avenae* subsp. *citrulli* was then diluted with PBS into the final concentration at10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/ml, respectively (referred as bacterial suspension).

Seeds of watermelon

All BFB-free watermelon seeds were from Gansu Entry-Exit Inspection and Quarantine Bureau in China. Fifty gram of dry BFB-free watermelon seeds were grinded into fine powder, and mixed with 300 ml general extraction buffer (9.15 mM Na_2SO_4, 0.5 mM $(C_6H_9NO)_{24\text{-}40000}$, 3.08 mM NaN_3, 0.03 mM albumen, 1.77 mM Tween-20, in 1 L sterile distilled water, pH 7.4). The mixture was incubated at 4°C overnight to let the insoluble substance settle down. Eighteen ml supernatant was mixed with 2 ml of each gradient of *A. avenae* subsp. *citrulli* cell suspension. Thus, the cell concentration of each inoculated seed suspension was 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/ml, respectively (referred as seeds suspension later).

PCR template preparation protocols

For direct PCR

The bacterial suspension and seeds suspension were directly used as PCR template without any pathogen enrichment operation. Five microliters of suspension and 31.6 µl sterilized distilled-water added in the tubes, boiled at 99°C for 15 min, and then chilled on ice for 5 min, and then this tube was used for the PCR reaction.

For biological PCR (Bio-PCR): template was prepared according to previous reported method with slight modification (Schaad et al., 2007; Schaad et al., 1995; Schaad et al., 2003). Briefly, 100 µl of sample from bacterial suspensions or seeds suspension were spread on the KB agar plate. After 72 h incubation at 28°C, a white single colony was picked and suspended in 36.6 µl of sterilized distilled-water in PCR reaction tube, The suspension was boiled at 99°C for 15 min, and then chilled on ice for 5 min,and then this tube was used for the PCR reaction .

For single tube immune-capture PCR (IC-PCR)

Template was prepared according to the previous reported method with slight modification (Buchhop et al., 2009; Peng et al., 2002). Briefly, *A. avenae* subsp. *citrulli* specific antibody (American Agdia Company, USA) was diluted to 2.5 µg/ml with coating buffer (15mM Na_2CO_3, 35 mM $NaHCO_3$, in 1 L sterilized distilled water, pH 9.6). Fifty µl diluted antibody were incubated in polypropylene PCR tube at 4°C for 4 h. After removing the non-bound antibodies, the PCR

tube was rinsed three times with 100 µl sterilized phosphate buffer saline (PBS) containing 0.05% Tween-20. Fifty µl of each gradient bacterial suspension or seeds suspension was added into the antibody coated PCR tubes. After 4 h incubation at 28°C, the PCR tubes were rinsed with 100 µl sterilized distilled-water, and after removing the fluid, 36.6 µl sterilized distilled-water added in the tubes boiled at 99°C for 15 min, and then chilled on ice for 5 min, and then this tube was used for the PCR reaction.

For immune-magnetic separation PCR (IMS-PCR)

Template was prepared according to the previous reported method with slight modification (Walcott and Gitaitis, 2000a; Amagliani et al., 2006; Ha et al., 2009). The NHS-biotin (Chinese Treasure Biological Limited Company, Dalian, Liaoning, China) was firstly dissolved in DFM (Dimethylformamide) to the concentration of 50 µg/ml. *A. avenae* subsp. *citrulli* specific antibody was diluted to the concentration of 10 µg/ml with the carbonate buffer saline (pH 9.6). Afterwards, the antibody was mixed with biotin (v/v: 10:1) and shaken at room temperature for 4 h. The streptavidin-magnetic beads (American Promega company, USA) were washed four times with PBS and then held on a magnetic particle concentrator to remove the NaN3. The streptavidin-magnetic beads were incubated with 2 ml of biotin-antibody mixture, and gently agitated at 4°C for 4 h. The antibody-coated beads were obtained due to the strong interaction of streptavidin and biotin. The antibody-coated beads were rinsed three times with the PBS buffer containing 0.05% Tween 20 and 0.1% bovine serum albumin (PBSTB) to remove the unbound antibodies. The coated immune-magnetic beads (approximately 106 beads /ml in 50 µl PBS) were incubated with 1 ml seeds or bacterial suspension and agitated at 20°C room temperature) for 2 h. The suspension was held on a magnetic particle concentrator for 5 min to remove the debris. After washing three times with PBSTB, the beads were suspended in 50 µl sterilized water and boiled at 99°C for 15 min, followed by chilling on ice for 5 min. Finally, it was centrifuged at 5000 rpm for 2 min and 36.6 µl supernatant was used as template for the PCR reaction.

Membrane filtration

The bacterial or seeds suspension was filtered through the 0.22 µm membrane filters (Chinese PenChen Biotechnology Company, Shanghai, China). The membrane was then cut into small pieces and washed with 0.5 ml PBS. After 5 min centrifugation at 2500 rpm, 5 µl supernatant were boiled at 99°C for 15 min, followed by chilling on ice for 5 min and then performed the PCR reaction.

PCR procedure

Each 50-µl PCR reaction mixture contained 5 µl of 10× reaction buffer (25 mM $MgCl_2$), 0.4 µl of *Taq* DNA Polymerase (DR001B, 5 U/µL), 2 µl of each primer (25 µM), 4 µl of 2.5 mM dNTP mixture (D4030RA), ddH_2O was added to make the final reaction volume of 50 µL.PCR The thermal cycles were started with a 2 min denaturation step at 95°C, followed by 35 cycles of 35 s denaturation at 95°C, 35 s annealing at 63°C and 45 s elongation at 72°C. PCR was completed by incubating the tubes at 72°C for 7 min. Specific primers for 16 s ribosomal RNA (rRNA) from *A. avenae* subsp. *citrulli* strain (AAC 94-85) (forward primer: 5'-CAG CCA CAC TGG GAC-3'; reverse primer: 5'-CTG CCG TAC TCC AGC GAT-3') were selected for PCR amplification (Walcott et al., 2000b). The primers were synthesized by Shanghai Sheng-Gong Biological Engineering Company (Shanghai, China).For bacterial suspension, the sterilized PBS buffer and 10^8 CFU/ml *A. avenae* subsp. *citrulli* cell suspension were used as negative and positive control, respectively. For seeds suspension, the seeds extracts and 10^8 CFU/ml *A. avenae* subsp. *citrulli* cell suspension were used as negative and positive control, respectively. PCR amplification products were visualized on 1.5% agarose gel. For individual experiment, it was assessed that the limit reached a special level only if the bands from all experiments can be imaged clearly under the same condition.

Double-antibody sandwich enzyme-linked immunosorbent assay

The experiment was performed according to the instruction of the commercial DAS-ELISA detection kit with *A. avenae* subsp. *citrulli* polyclonal antibodies (SRA14800/0500). Firstly, capture antibody was incubated in 96-well microtitre plate at 37°C for 4 h. The samples were then incubated in the microplate at 4°C for overnight. The alkaline phosphatase labeled antibody was added and incubated at 37°C for 4 h. Microtitre plate was rinsed 5 times after each incubation procedure with PBS containing 0.05% Tween-20. The plate was detected in a micro-plate reader (Thermo Labsystems, multiskan ascent) at a wavelength of 405 nm. The cell suspension (10^8 CFU/ml) were used as positive control, the seeds extracts and the sterilized PBS buffer were used as the negative control, respectively.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. One-way ANOVA was used to compare OD_{405} in different groups in DAS-ELISA test. The probability value of $p < 0.05$ was set as the level for statistical significance.

RESULTS

Direct PCR

Figure 1 shows the PCR product derived from the bacterial suspension or seeds suspension without any enrichment operation. It indicated that the detection limit of the seeds suspension was 10^5 CFU/ml, while the detection limit of the bacterial suspension was 10^4 CFU/ml.

Bio-PCR

The bacterial suspension or seeds suspension (100 µl) was incubated on KB agar plate for 72 h at 28°C. The colonies were then picked and diluted as described in materials and methods section. The diluted suspension was used as template for the PCR reaction. The PCR products were visualized on 1.5% agarose gel (Figure 2). The present results indicated that the detection limits of bacterial and seeds suspensions both reached 10^1 CFU/ml.

IC-PCR

The seeds or bacterial suspension was incubated in the PCR tube that coated with the bacterial specific antibody. After removing the non-specific binding antibody, the PCR reaction tube coated with antibody was used for PCR

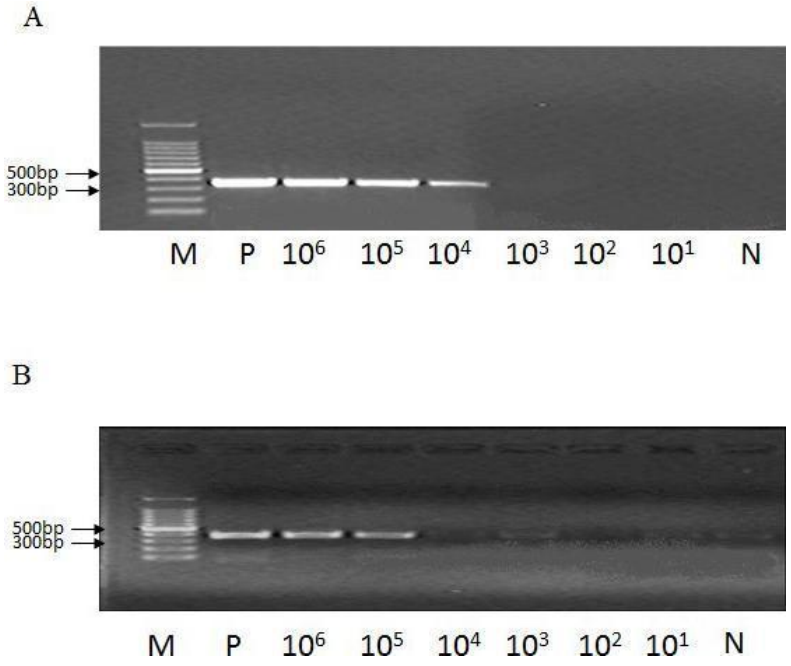

Figure 1. Direct PCR Results. A series dilution of bacterial suspension (A) or seeds suspension (B) were performed by direct-PCR, and visualized on 1.5% agarose gel. Prepresents positive control (10^8 CFU/ml *Aac* cell suspension) N represents negative control (bacterial suspension: PBS buffer; seeds suspension: seeds extract), and M represents 100 bp Ladder.

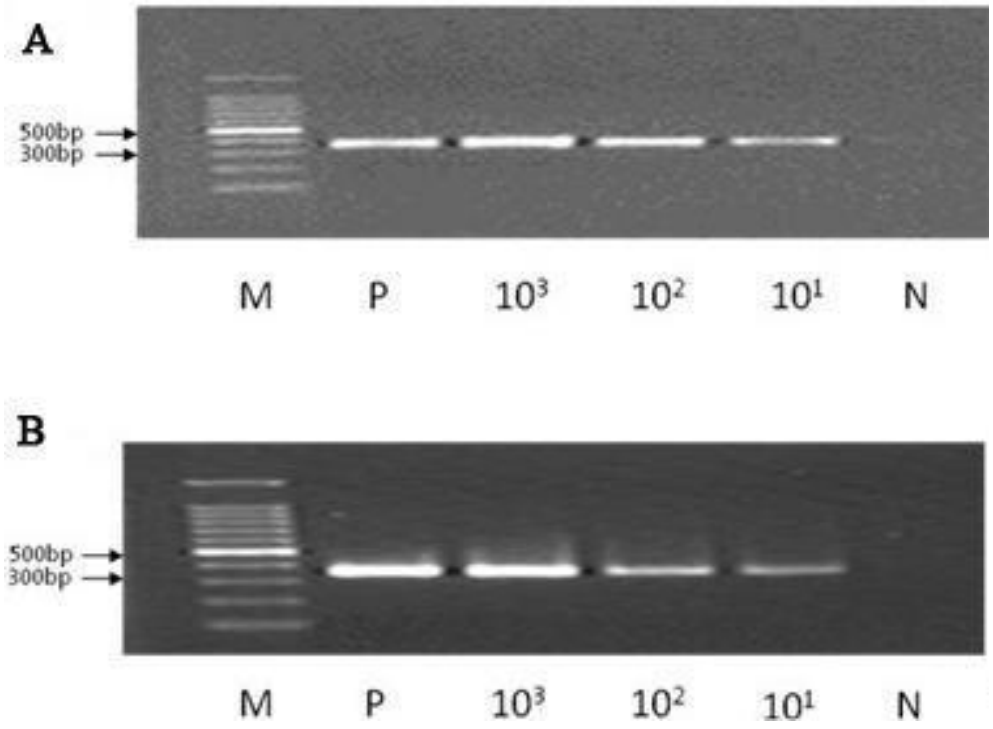

Figure 2. Bio-PCR Results. A series dilution of bacterial suspension (A) or seeds suspension (B) were performed by bio-PCR, and visualized on 1.5% agarose gel. P represents positive control (10^8 CFU/ml *Aac* cell suspension) N represents negative control (bacterial suspension: PBS buffer; seeds suspension: seeds extract), and M represents 100 bp Ladder.

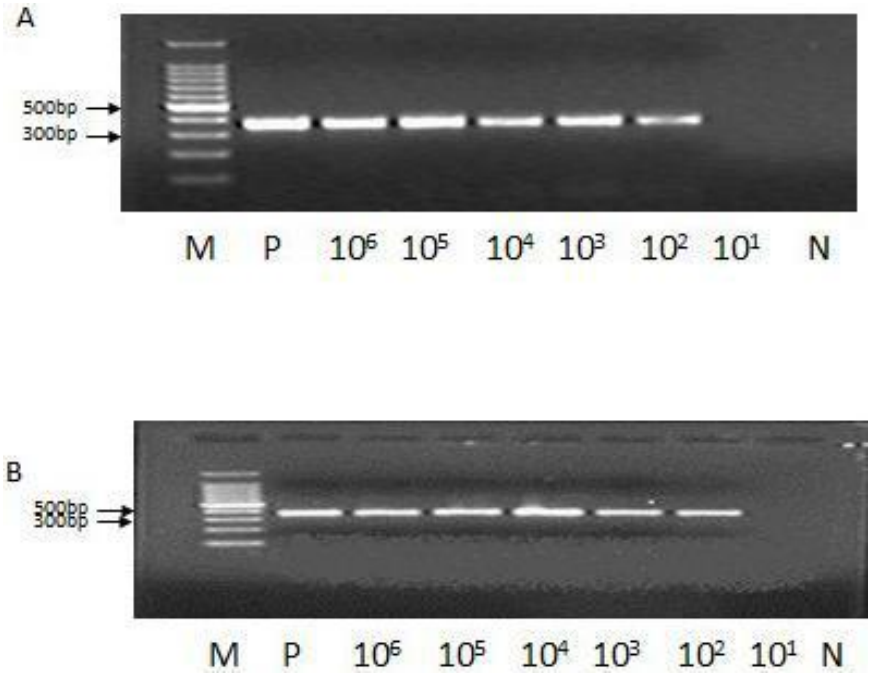

Figure 3. Immune-capture PCR Results. A series dilution of bacterial suspension (A) or seeds suspension (B) were performed by IC-PCR, and visualized on 1.5% agarose gel. P represents positive control (10^8 CFU/ml *Aac* cell suspension). N represents negative control (bacterial suspension: PBS buffer; seeds suspension: seeds extract), and M represents 100 bp Ladder.

reaction. As shown in Figure 3, the detection limits of bacterial and seeds suspensions both reached 10^2 CFU/ml.

IMS-PCR

When bacterial suspension was captured by the antibody-coated magnetic beads, the average detection limit was 10^2 CFU/ml (Figure 4), although two out of six replicates could reach 10^1 CFU/ml. When seeds suspension was used for the PCR, the detection limit was increased to 10^3 CFU/ml.

Membrane filtration PCR

The bacterial suspension was passed through the 0.22 µl membrane filter. Afterwards, the membrane was collected and washed with PBS. The washing solution was used as template for the PCR reaction. As shown in Fig. 5, the detection limit for the bacterial suspension was 10^1 CFU/ml. However, no result was obtained from the seeds suspension due to that it was difficult to be filtrated.

DAS- ELISA

The results of DAS-ELISA for the bacterial suspension were listed in Table 1. The data shows that the $OD_{405} \geqq 0.2$ was considered as positive results. Therefore, both of the sensitivities of DAS-ELISA for the bacterial suspension and the seeds suspension were 10^4 CFU/ml. The results may indicated that particles in the seeds suspension did not affect the sensitivity of DAS-ELISA.

Comparison of different pathogen enrichment methods

A comprehensive comparison was made according to the sensitivity, time, expenditure, and reproducibility (Table 2). It clearly showed that direct-PCR was the least sensitive method but it required less time and lower expense than others. Bio-PCR and Filtration-PCR were the two most sensitive methods. However, Bio-PCR took 72 h to complete the procedures, and the membrane filtration was not applicable for the seeds suspension. Both IC-PCR and IMS-PCR showed considerable higher sensitivity, while the expenditure of single tube IC-PCR is relative lower than that of IMS-PCR (Figure 5). Therefore, the single tube IC-PCR was an ideal pathogen enrichment method for detecting *A. avenae* subsp. *citrulli*.

DISCUSSION

Many conventional methods for detecting the seed-borne

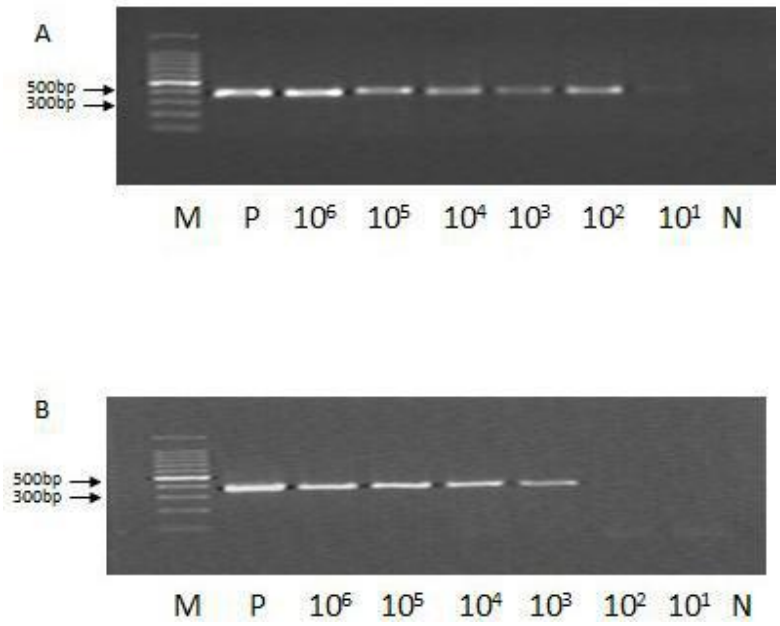

Figure 4. Immune-magnetic PCR Results. A series dilution of bacterial suspension (A) or seeds suspension (B) were performed by IMS-PCR, and visualized on 1.5% agarose gel. P represents positive control (10^8 CFU/ml *Aac* cell suspension). N represents negative control (bacterial suspension: PBS buffer; seeds suspension: seeds extract), and M represents 100 bp Ladder.

Table 1. DSA-ELISA detection results of the bacterial suspension and the seed suspension.

Pathogen Concentration (CFU/ml)	BS*		SS#	
	OD_{405nm}	Difference test	OD_{405nm}	Difference test
10^7	3.957 ± 0.012	a	3.947 ± 0.012	a
10^6	3.941 ± 0.007	a	3.861 ± 0.014	b
10^5	2.344 ± 0.235	b	1.227 ± 0.096	c
10^4	0.334 ± 0.002	c	0.216 ± 0.002	d
10^3	0.126 ± 0.016	d	0.097 ± 0.005	e
10^2	0.100 ± 0.008	d	0.079 ± 0.006	e
10^1	0.085 ± 0.007	d	0.085 ± 0.004	e
Positive CK	3.863 ± 0.123	a	3.884 ± 0.052	ab
Negative CK(PBS)	0.067 ± 0.021	d	0.108 ± 0.025	e
Negative CK(GEB)	0.059 ± 0.026	d	0.112 ± 0.022	e
Blank CK	0.051 ± 0.003	d	0.082 ± 0.012	e

The data indicates mean ± SD. Different letters mean significant difference at P ≤ 0.05. *BS denotes the bacterial suspension #SS denotes the seeds suspension.

pathogens are insensitive, cumbersome and time consuming. It usually takes several days to identify the target bacterial species, which significantly limits the application of those methods. Instead, molecular biological techniques, especially the polymerase chain reaction (PCR) based assays, have been developed and extensively utilized for identifying and characterizing the seed-borne bacteria.

Although PCR is one of sensitive methods to identify a specific bacterium from a small amount of the sample, many factors might affect its application. One obstacle that impact the accuracy of PCR based detection is the presence of inhibitory substances or other bacteria from the seeds (Schaad et al., 2007). Another major holdback of the PCR-based methods is the relatively low population of pathogenic bacteria from the infected sample (Wernars

Table 2. Comparison of different pathogen enrichment methods based on detection limit, time, and labor expense.

Enrichment methods	Detection limits		Reproducibility		Time (hour)	Expense
	BS*	SS#	BS*	SS#		
ELISA	10^4	10^4	6/6	6/6	24	medium
Direct PCR	10^4	10^5	6/6	6/6	3	low
Bio-PCR	10^1	10^1	6/6	3/6	72	low
IC-PCR	10^2	10^2	6/6	6/6	8	medium
IMS-PCR	10^2	10^3	6/6	4/6	9	high
Filtration-PCR	10^1	NA	6/6	0/6	5	medium

*BS denotes the bacterial suspension. #SS denotes the seeds suspension. NA: data is not available.

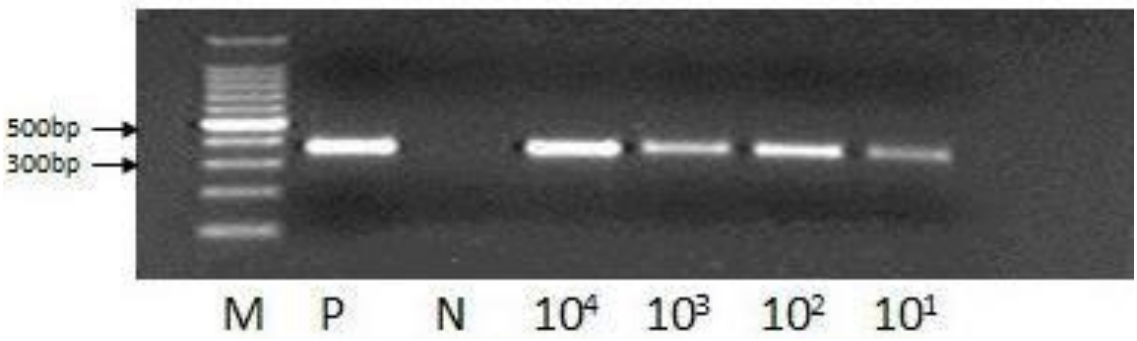

Figure 5. Membrane filtration-PCR Results. A series dilution of bacterial suspension were performed by PCR after membrane filtration, and visualized on 1.5% agarose gel. P represents positive control (10^8 CFU/ml *Aac* cell suspension); N represents negative control (PBS buffer); M represents 100 bp Ladder.

et al., 1991; Schaad et al., 2007; Badosa et al., 2009). Therefore, the preparation of the high quality sample is the key step to develop a successful application of the PCR detection method. In the case of detecting *A.avenae* subsp. *citrulli* from infected watermelon seed, an efficient pathogen enrichment method became crucial for the rapid diagnostication. With the assistance of appropriate pathogen enrichment strategies, the background that might impact the accuracy of the detection methods could be eliminated prior to the PCR reaction.

In the present study, four pathogen enrichment protocols on detecting the BFB from watermelon seeds were compared. Considering the time factor, the Direct-PCR showed advantages, followed by single tube IC-PCR and IMS-PCR (Table 1). After the primary sample preparation, the Direct-PCR needs extra 3 h to complete, while IC-PCR and IMS-PCR need approximately 8 and 9 hr, respectively. As a control, the template of direct-PCR might contain lots of inhibitors that severely affected the PCR due to no pathogen enrichment procedure. It thus significantly increased the detection limit (10^5) and the probability of false positive. These results suggested that the Direct-PCR is not an efficient BFB detection method even though it showed advantage on time and expenditure.

One of the efficient methods to avoid inhibitors from suspension is to enrich the target bacteria on the agar plate or in liquid media prior to PCR. Bio-PCR is a technique based on such principle prior to the PCR reaction (Song et al., 2004; Schaad et al., 2007). After a 15 to 24 h enrichment step, the method could detect the target bacteria as few as 10^1 CFU/ml(Song et al., 2004, Song et al., 2003). The present results showed that Bio-PCR enrichment method had the highest detection sensitivity compared to other methods and considerable low cost. However, it should take at least 3 days to complete the whole procedures for detecting *A.avenae* subsp. *citrulli*.

Immune-magnetic separation (IMS) method is also commonly used to enrich the pathogen (Walcott and Gitaitis, 2000a). IMS is an efficient technique for selectively concentrating and recovering target bacteria from heterogeneous mixtures (Walcott and Gitaitis, 2000a; Fernandes et al., 2008; Lee et al., 2009). However, the antibody concentration, incubation time, and numbers of rinses would affect the sensitivity of IMS on detecting *A. avenae* subsp. *citrulli*. In the present study, the sensitivity of IMS method could reach 10^2 CFU/ml for bacterial suspension and 10^3 CFU/ml for seeds suspension. These results were consistent with previous reports that the

sensitivity of IMS-PCR could reach 10^2 CFU/ml (Walcott and Gitaitis, 2000a)Previous study only detected the *A. avenae* subsp. *citrulli* on the seeds surface, which would lead to the lack of information from the inner parts of seeds. The grinding procedure employed in the present study would help to reveal the full information of the seeds contamination and increase the detection limit. On the other hand, the bacteria as PCR templates avoided the DNA extraction and decreased the cross-contamination.

Similar to IMS-PCR, the single tube IC-PCR enriches the pathogen based on the principle of antibody capture (Peng et al., 2002). The advantage of single tube IC-PCR over the IMS-PCR is that only one test tube is needed for each sample in the whole procedure. It dramatically reduced the time, the cross-contamination, and loss of pathogen DNA. Therefore, IC-PCR not only simplified the procedures, but increased the detection limit. The present study firstly attempted to employ single tube IC-PCR methods to enrich and detect *A. avenae* subsp. *citrulli* without extracting DNA from infected seed samples. The detection limits of single tube IC-PCR for both bacterial and seed suspension were 10^2 CFU/ml. The detection limit of single tube IC-PCR was 10 times lower than that of IMS-PCR when the seed suspension was used as template. In addition, single tube IC-PCR showed better reproducibility than IMS-PCR. Three out of six replicates in single tube IC-PCR could reach 10^1 CFU/ml, and the rest could reach 10^2 CFU/ml. For IMS-PCR, 4 out of 6 replicates reached 10^2 CFU/ml, and none of IMS-PCR could reach 10^1 CFU/ml. Meanwhile, single tube IC-PCR had advantages on the expense.

DAS-ELISA is one of the efficient and accurate methods on detecting *A. avenae* subsp. *citrulli* from the infected seed, of which the detection limits for both bacterial suspension and seeds suspension were 10^4 CFU/ml. However, it took at least 2 days to complete the whole procedures, which suggested that DAS-ELISA might not a suitable BFB detection method in practice.

To physically remove the debris from suspension and concentrate the target bacteria, the sample was passed through a filter prior to the PCR reaction. The present results showed that when the bacterial suspension was used as template, the filtration could significantly increase the detection sensitivity compared with the direct-PCR. However, the filtration method is not applicable for the seeds suspension because it was too sticky to be filtrated. To our knowledge, there seems to be a severe lack of information focusing on pathogen enrichment from plant seeds. The present study firstly succeeded to identify the pathogen from seeds using a combination of immunological method and PCR technique. It avoided the DNA extraction and eliminated the inhibitors in the PCR, which make the methods more applicable. It should be noted that individual bacterium employed in the present study was inconsistent with the field sample which contained a number of bacteria. Whether the existence of other bacteria affect the enrichment procedure and PCR result in practice needs further investigation.

Conclusion

The present study suggested that single tube IC-PCR is a simple, rapid, reproducible and economical method for detecting *A. avenae* subsp. *citrulli* from infected seeds. This method is a good choice for the researchers who need to detect a small amount of *A. avenae* subsp. *citrulli* in a short period of time.

ACKNOWLEDGEMENTS

This work was supported by the key program of science and technology of Gansu Province, China (Grant No. 1102NKDA025)".

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