A Rapid, Sensitive Assay for *Ralstonia solanacearum* Race 3 Biovar 2 in Plant and Soil Samples Using Magnetic Beads and Real-Time PCR

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Abstract

Ha, Y., Kim, J.-S., Denny, T. P., and Schell, M. A. 2012. A rapid, sensitive assay for *Ralstonia solanacearum* race 3 biovar 2 in plant and soil samples using magnetic beads and real-time PCR. Plant Dis. 96:258-264.

The *Ralstonia solanacearum* species complex causes economically significant diseases in many plant families worldwide. Although generally limited to the tropics and subtropics, strains designated race 3 biovar 2 (R3Bv2) cause disease in cooler tropical highlands and temperate regions. R3Bv2 has not become established in North America but, due to concerns that it could devastate the U.S. potato industry, it has been designated a Select Agent, and is subject to strict quarantine regulations. Quarantine screening for R3Bv2 requires rapid and robust assays applicable to small populations present in plant tissues or soil, and must distinguish R3Bv2 from the multiple other *R. solanacearum* subgroups. We developed a 100%-accurate real-time polymerase chain

reaction (RT-PCR) assay that can detect R3Bv2 populations >1,000 cells ml⁻¹. However, detection by RT-PCR was inhibited by compounds present in some plant and soil samples. Therefore, we developed simple immunomagnetic separation (IMS) and magnetic capture hybridization (MCH) methods to purify *R. solanacearum* cells or DNA from PCR inhibitors. When coupled with RT-PCR, these tools permitted detection of R3Bv2 at levels >500 cells ml⁻¹ in stem, tuber, and soil samples when direct RT-PCR failed, and reduced detection time from days to hours. IMS-RT-PCR was usually more sensitive than MCH-RT-PCR, especially at lower population levels.

Ralstonia solanacearum causes bacterial wilt or brown rot disease of many plants in over 50 different families (9,22). This pathogen is endemic in most tropical and subtropical regions, and its diseases often result in severe losses in a variety of commercial and subsistence crops. There is extensive genetic diversity among strains and, based on multilocus DNA sequence typing, *R. solanacearum* is now considered to be a species complex divided into four groups called phylotypes, which are subdivided into sequevars based on the DNA sequence of the *egl* endoglucanase gene (5,19,56). Historically, however, *R. solanacearum* strains have been classified into five races based loosely on host range or into six biovars based on their differential ability to produce acid from several carbohydrate substrates (9). There is no general correlation between races and biovars, except that race 3 and race 5 strains are usually biovars 2 and 5, respectively.

Race 3 biovar 2 (R3Bv2) strains have an unusual ability to cause wilt disease at cooler temperatures than other *R. solanacearum* strains (9,13,22,37,53). They can also survive in latent infections of potato tubers and other plants (9,22). These traits and seed potato commerce have contributed to the dissemination of R3Bv2 strains to most potato-growing regions of the world, including tropical highlands and northern Europe (14). Outbreaks in Europe have significantly damaged seed potato production and resulted in quarantine-related crop losses (27,28). Latent infections of geraniums with R3Bv2 in some parts of Africa and Central America have been a problem for commercial production of this exported crop

(28,61). Once R3Bv2 is introduced into a cropping system, it is difficult to manage because (i) host resistance is limited, (ii) it can survive long-term in weeds and soil, and (iii) it can be further disseminated via latently infected plant tissues or tubers, irrigation water, and soil-contaminated equipment (6,9,24).

R3Bv2 is not established in North America but, due to concerns that it threatens potato in the United States and Canada, it is designated as a Select Agent and subject to strict quarantine and biosecurity regulations (6,32). These regulations identify the pathogen as R3Bv2 (rather than describing it as phylotype II, sequevar 1); therefore, we will do likewise in this article. Preventing introduction of R3Bv2 into North America requires rapid, robust, and reliable detection of this pathogen when it is present in relatively low numbers in asymptomatic tissues or in associated soil or debris. However, as with many pathogens, the methods presently available for specifically detecting R3Bv2 strains are inadequate. For example, conventional identification techniques, which often require isolation in pure culture, are laborious and time consuming. Several semiselective media are available for *R. solanacearum* but growth of competing or antagonistic organisms from soil can prevent its recovery, especially when it is present in low numbers (45). Even when *R. solanacearum* has been recovered from plant tissue or soil, additional cultural, biochemical, or genetic tests are required to determine if a strain is R3Bv2 (10,37,39,63). Although relatively simple, the entire process can take several weeks.

Testing plant tissue or soil extracts for a pathogen directly using immunological or DNA-based techniques is considerably more rapid, usually requiring less than 1 day. A number of immunodiagnostic assays, including enzyme-linked immunosorbent assay (ELISA), immunofluorescence-antibody staining, immunofluorescence-colony staining, and commercial serological kits, have been developed to detect and identify *R. solanacearum* (9). Although these techniques can display detection thresholds as low as 10⁴ cells g⁻¹ or ml⁻¹ of sample, the presently available *R. solanacearum*-specific antibodies cannot differentiate between R3Bv2 and other *R. solanacearum* strains (9). Diagnostic polymerase chain reaction (PCR) assays, which are rapid and sensitive, have been successfully employed in the medical, veterinary, agricultural, and food-testing arenas (16). Species-, phylotype- and biovar-specific primers for *R. solanacearum* have been developed for conventional

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Accepted for publication 24 September 2011.

<http://dx.doi.org/10.1094/PDIS-05-11-0426>
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and real-time (RT)-PCR (3,4,8,18,19,25,31,39,41,51,54,59). Unfortunately, conventional and RT-PCR reactions are often inhibited by excessive nontarget DNA, as well as by inhibitory compounds present in tissue or soil extracts (40,43,47,62). Current attempts to reduce or eliminate these contaminants by dilution or neutralization of PCR inhibitors, isolation of target cells, or DNA purification can increase assay time and costs while reducing sensitivity (23,43,44,59).

An alternative strategy is to adapt magnetic bead separation techniques to quickly, specifically, and easily purify cells or high-quality DNA from contaminated soil and infected plant material. Immunomagnetic separation (IMS) uses magnetic beads coated with microbe-specific antibodies to isolate target bacteria free from PCR inhibitors before DNA is released for analysis. Alternatively, in magnetic capture hybridization (MCH), beads coated with a microbe-specific single-stranded oligonucleotide are hybridized to total DNA released from a sample, and PCR inhibitors are washed away before purified target DNA is released. IMS and MCH followed by conventional or RT-PCR have been used to detect microorganisms in soil, food, plant tissue, and seed (1,7,21,26,43,57). Here, we report development of IMS and MCH methods for R3Bv2 strains which, when coupled with RT-PCR, provide rapid and specific assays to detect low concentrations of this select agent and quarantine pathogen in complex plant tissue samples and soil.

Materials and Methods

Bacterial strains and culture conditions. *R. solanacearum* strains (Table 1) were cultured in BG broth or on BG agar (34) at 30°C. Other bacteria—*R. pickettii* American Type Culture Collection (ATCC) 27511, *R. pickettii* PH2, *Burkholderia thailandensis* E264, *Sinorhizobium meliloti* 54, *Pseudomonas putida* ATCC 12633, *R. eutropha* JH16, *B. cepacia* J2315, *Acinetobacter (calcoaceticus) baylii* ADP1, *P. aeruginosa* PAO1, and *Klebsiella (aerogenes) pneumoniae* ATCC 9621—were cultured on Luria-Bertani (LB) agar or in LB broth at 30°C. *R. solanacearum* extracellular polysaccharide-deficient (EPS−) strain IPO1609 EPS− was generated by transformation (34) using genomic DNA purified from strain UW551 EPS−.

Preparation of antisera. The R3Bv2 *R. solanacearum* strains UW24, UW551, UW349, IPO1609, and UW551 EPS− were grown for 36 h with agitation. Cells were harvested by centrifugation and resuspended in water to an optical density at 600 nm (OD₆₀₀) of 10. Equal volumes of each suspension were combined, heated to 55°C for 40 min, and then diluted with water to 2.5 mg ml^{−1} of cell protein. A 0.5-ml aliquot was emulsified with an equal volume of Freund's complete adjuvant and the mixture was injected intradermally at multiple sites into an adult NZW-SPF rabbit by personnel at The University of Georgia University Research Animal Resources lab (<http://www.ovpr.uga.edu/urar/lsl/>). Four weeks later, the rabbit was injected with the same volume of cell mixture that had been emulsified with incomplete Freund's adjuvant. Blood was harvested 10 days later, and the serum prepared from it was frozen.

Dot-blot assays of antisera. Strains were grown to an OD₆₀₀ of approximately 1 and 2 µl of each culture, or dilutions thereof, were spotted onto nitrocellulose membranes. In some cases, 1 ml of a culture was centrifuged at 7,000 × g for 1 min and the cell pellets resuspended in 0.05 volume of water before spotting. After air

drying, the membranes were blocked by shaking overnight at 25°C in 50 mM Tris-HCl (pH 7.6) + 0.15 M NaCl + 0.1% Tween 20 + 2% Difco skim milk (TBSTS) and then shaken at 25°C with a 1:10,000 dilution of polyclonal antiserum in TBSTS for 1 h. After washing with TBSTS for 10 min, membranes were shaken with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, CA) in TBSTS for 1 h and then washed with TBSTS as described above. The membranes were rinsed with water; then, ready-to-use, stabilized 3,3',5,5'-tetramethylbenzidine substrate (TMB; Promega Corp., Madison, WI) was added. After 10 min of shaking, the membrane was rinsed with water to stop the color development, air dried, and photographed.

Preparation of anti-*R. solanacearum* magnetic beads. Dynabeads sheep anti-rabbit immunoglobulin G (IgG)-coated magnetic beads (1 ml, approximately 10⁹ beads; Invitrogen, Carlsbad, CA) were transferred to a 2-ml cylindrical microfuge tube (Axygen Inc., Union City, CA; catalog number MCT-200-C). The tube was placed in a magnetic particle collector (MPC; Promega Corp.; catalog number Z5333) for 2 min while the beads became immobilized on the rear wall of the tube. Supernatant was carefully removed while the tube remained in the MPC. The beads were washed by adding 1 ml of phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4) containing 0.5% bovine serum albumin (PBSB) along the wall where the beads were bound and, after 1 min, carefully removing the PBSB. The washed beads were then mixed with 300 mg of IgG that had been purified from anti-*R. solanacearum* rabbit serum using a Protein A Antibody Purification Kit (Sigma-Aldrich, St. Louis). After shaking for 18 h at 7°C, the beads were washed twice with 1 ml of PBS using an MPC as described above, and stored at 4°C in PBS + 0.02% NaN₃.

Preparation of MCH beads. Dynabeads M-280 Streptavidin magnetic beads (1 ml, approximately 10⁹ beads; Invitrogen) were washed three times with 1 ml of 2× bead wash buffer (BW; 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 2.0 M NaCl) using an MPC as described above. The beads were mixed with 1 ml of BW containing 200 pmol of 2403CAPYH capture oligonucleotide (5' biotin-ATCCTTTGTGCTGAAGGCCGTTATCGGCCTTGACAGGC CCGCAAGGTGGGATGGATGTGTT-3'; targets between 65 and 5 bp upstream of the R3Bv2 diagnostic amplicon). After shaking for 1 h at 25°C, tubes were placed in the MPC and beads were washed three times with 1 ml of BW. The beads were suspended in 0.4 ml of 0.125 M NaOH plus 0.5 M NaCl, incubated at 25°C for 15 min, rinsed three times with 1 ml of BW, and stored at 4°C in 0.15 ml of BW + 0.02% NaN₃.

IMS procedure. Fivefold concentrated PBSB (0.2 ml) was added to 0.8-ml test samples in 2-ml cylindrical microfuge tubes and 7 µl (approximately 5 × 10⁹ beads) of anti-*R. solanacearum* magnetic beads was added. The tubes were manually inverted two or three times every 15 min at 25°C for 60 min and then placed in the MPC. After 2 min, the supernatant was carefully removed, leaving the beads immobilized on the rear wall of the tubes undisturbed, and then 2 ml of PBSB was added and removed after approximately 30 s. Beads were then similarly washed with 1 ml of PBS followed by 1 ml of sterile water. Finally, after the tubes were removed from the MPC, 1 ml of sterile water was added, beads

Table 1. *Ralstonia solanacearum* strains used

Strain	Relevant characteristics	Reference or source
AW1	Race 1 biovar 1 (phylotype II sequevar 7)	11
GMI1000	Race 1 biovar 3 (phylotype I sequevar 18)	49
GMI1000 EPS−	GMI1000 <i>epsB</i> ::pCR2.1, EPS−	M. A. Schell and J. Wang, <i>unpublished</i>
UW551	Race 3 biovar 2 (phylotype II sequevar 1)	20
UW551 EPS−	UW551 <i>epsB</i> ::pVIK112, EPS−	36
IPO1609	Race 3 biovar 2 (phylotype II sequevar 1)	55
IPO1609 EPS−	IPO1609 <i>epsB</i> ::pVIK112, EPS−	This study
UW24	Race 3 biovar 2 (phylotype II)	C. Allen, University of Wisconsin
UW349	Race 3 biovar 2 (phylotype II)	C. Allen, University of Wisconsin

were resuspended by pipetting, and the suspension was transferred to a 1.5-ml conical centrifuge tube. After placing this tube in the MPC for 2 min, the supernatant was carefully removed and 30 μ l of water was added. The tube was removed from the MPC and centrifuged for 5 s to force all beads and liquid to the bottom. After resuspension by pipetting, appropriate aliquots of the bead suspension, or dilutions thereof, were spread plated on BG agar and colonies were counted after 2 days. The remaining portion of the bead suspension was heated at 97°C for 7 min and centrifuged for 30 s, and the bead-free supernatant was saved for RT-PCR. IMS assays involving geranium or soil extracts required more extensive washing to remove PCR inhibitors: once with 2 ml of PBSB, three times with 1 ml of PBS, and twice with 1 ml of sterile water before transfer to a new tube for heat elution of DNA.

MCH procedure. Tris-EDTA buffer (0.1 ml; 10 mM Tris-HCl [pH 8.0] plus 1 mM EDTA) was added to 0.4-ml test samples in 2-ml cylindrical tubes. After heating at 95°C for 5 min, 0.4 ml of DIG Easy Hyb solution (Hoffmann-La Roche, Inc., Nutley, NJ) was added. After incubation at 95°C for 10 min, samples were chilled on ice for 5 min, and 20 μ l (approximately 10^8 beads) of 2403CAPYH magnetic beads was added. After shaking at 25°C for 2 h, tubes were placed in an MPC for 2 min, the supernatant was removed, and the beads were washed once with 2 ml of water, followed by two more washes with 1 ml of water as described for IMS above. Finally, beads were suspended in 20 μ l of water, incubated at 95°C for 10 min, and centrifuged for 5 s, and the supernatant was removed and saved for RT-PCR. MCH assays involving geranium or soil extracts also required more extensive washing: once with 2 ml of water followed by five washes with 1 ml of water.

RT-PCR. Design and characterization of R3Bv2-specific PCR primers targeting the RRSL_2403 gene were previously described (31). RT-PCR was performed using either iQ SYBR Green Supermix (Bio-Rad) or SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies Inc., Carlsbad, CA) with the Smart Cycler II (Cepheid, Sunnyvale, CA) or StepOne (Applied Biosystems) instruments, respectively. The 25- μ l reactions containing 12.5 μ l of master mix, 10 pmol each primer (RRSL2403F/R), and 5 μ l of test sample were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s. The detection threshold of the StepOne system was set automatically whereas, for the Smart Cycler II, it was set to 15 fluorescence units. The cycle threshold (Ct) value was determined as the RT-PCR cycle at which fluorescence exceeded the detection threshold.

Fig. 1. Specificity and detection sensitivity of rabbit antiserum against *Ralstonia solanacearum*. Serial dilutions of cells were spotted on to a nitrocellulose membrane. After drying and blocking and incubation with anti-*R. solanacearum* antibodies, membranes were developed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and TMB substrate. *R. solanacearum* strains: GM, GMI1000; 551, UW551; EPS-, deficient in exopolysaccharide production. Other bacteria: Rp1, *R. pickettii* ATCC 27511; Rp2, *R. pickettii* PH2; Bt, *Burkholderia thailandensis*; Sm, *Sinorhizobium meliloti*; Pp, *Pseudomonas putida*; Re, *R. eutropha*; Bc, *B. cepacia*; Ab, *Acinetobacter (calcoaceticus) baylii*; Pa, *P. aeruginosa*; Kp, *Klebsiella (aerogenes) pneumoniae*.

Preparation of plant tissue and soil extracts. For potato tubers, a number 5 cork borer was used to obtain a 0.8-by-2-cm cylindrical tissue sample. After removing the skin, the cylinder was cut into approximately 10 slices and 1 g was added to 10 ml of water in a 15-ml Falcon tube (BD Biosciences, Bedford, MA). After vortexing for 15 s, tubes were inverted by hand five times every 5 min for 15 min. Insoluble debris was allowed to settle for 15 min and the supernatant was removed. For stems, an approximately 2-cm stem segment weighing approximately 1 g was cut from the center of the plant, placed in a 15-ml Falcon tube, and crushed with the tip of a 5-ml plastic pipette, and 10 ml of water was added. The tube was vortexed vigorously and further processed as described for potato tubers. For soil, 1 g of soil (sandy loam with 5.9% organic matter, pH 6.6) was suspended in 10 ml of water and processed as described for stem samples. The amount of dissolved solids in each extract was determined gravimetrically and found to be 1.0 mg ml⁻¹ for tubers, 3.5 mg ml⁻¹ for plant stems, and 0.3 mg ml⁻¹ for soil.

Results

Characterization of titer and specificity of anti-*R. solanacearum* serum. Efficient IMS requires a high-titer and high-affinity antibody. Because several attempts to generate an R3Bv2-specific monoclonal antibody were unsuccessful (G. D. Peckham and A. M. Alvarez, unpublished), we prepared polyclonal antiserum against a mixture of five R3Bv2 strains, one of which is deficient in EPS production. Ability of the antisera to strongly react with EPS- strains was considered critical because EPS is produced at very low levels, if at all, when *R. solanacearum* is growing at low cell density (29,50). Of three available *R. solanacearum*-specific monoclonal antibodies we tested, none reacted with EPS- strains (unpublished data). At a 1:10,000 dilution, our antiserum reacted strongly with cells of both *R. solanacearum* GMI1000 (race 1, biovar 3), and UW551 (R3Bv2), as well as their EPS- mutants (GMI1000 EPS- and UW551 EPS-, respectively) (Fig. 1). This antiserum also reacted strongly with *R. pickettii* cells. The other species of *Burkholderiaceae* that were tested reacted weakly (*R. eutropha* and *B. cepacia*) or not at all (*B. thailandensis*), while six other α - and β -proteobacteria did not react. In the dot-blot assay, this antiserum detected as few as 10^4 cells of *R. solanacearum* (Fig. 1) and, at a 1:200 dilution, it detected 10^3 cells (results not shown).

Prominent antibody-reactive haloes surrounded the spots with 10^6 cells of wild-type *R. solanacearum* strains GMI1000 and UW551 but the haloes were dramatically reduced in size and color intensity for their EPS- mutants (Fig. 1). Haloes also were essentially absent when wild-type cells were washed three times with water before spotting on the membrane (not shown). Haloes were present when the 0.45- μ m filtered cell-free culture supernatant from EPS-producing cells but not EPS- cells was applied to the membrane (not shown). These results suggested that the anti-*R. solanacearum* polyclonal antibodies bound not only to cells but also to cell-free EPS.

Specificity and sensitivity of IMS for *R. solanacearum*. Anti-*R. solanacearum* magnetic beads were prepared by conjugation with IgG purified from the polyclonal serum. When employed in an IMS procedure, the beads were more specific and sensitive in detecting a variety of *R. solanacearum* strains than the dot blot method (Table 2). On average, the IMS beads recovered 22% of the approximately 10^5 *R. solanacearum* cells in the 1-ml reaction but, in contrast to the dot blot assay, *R. pickettii* or *B. cepacia* cells were not detected. This was not surprising, because the spots on the dot blot contained dried cells that likely had released cytoplasmic antigens for reaction with the antiserum, whereas the anti-*R. solanacearum* magnetic beads only reacted with antigens on the surface of intact cells. *R. eutropha*, one of the strains that was weakly positive in dot blot analysis, was also recovered by IMS but at levels nearly 100-fold less than the *R. solanacearum* strains. Nonspecific binding of *R. solanacearum* cells to unconjugated beads was negligible. The recovery of EPS- cells was consistently

three- to fivefold less than for EPS-producing wild-type cells, again indicating that EPS is a major recognition determinant and that its presence enhances detection of *R. solanacearum* cells. Tests to determine the recovery efficiency of *R. solanacearum* from IMS reactions with decreasing concentrations of cells showed that this technique worked with as few as 10 cells ml⁻¹ (Table 3). These results show that the IMS beads provide a consistent, robust, and accurate detection method for *R. solanacearum* cells.

Coupling IMS to RT-PCR increased speed and sensitivity of detecting *R. solanacearum*. Although the IMS procedure is fairly rapid (approximately 2 h), obtaining final results from plate counts takes about 2 days. More importantly, IMS does not differentiate between races or biovars of *R. solanacearum*. An R3Bv2-specific PCR primer set targeting the RRSL_2403 gene of strain UW551 was previously been reported (31). An RT-PCR assay using these primers detected as few as five R3Bv2 cells (using 5 µl of a 10³ CFU ml⁻¹ suspension) in 2 h (Table 4), which was nearly as sensitive as plating and approximately 25 times faster. Therefore, we evaluated coupling IMS to R3Bv2-specific RT-PCR to enhance both sensitivity (e.g., by concentrating cells from 1 ml to 30 µl) and speed. When we subjected a 200 CFU ml⁻¹ R3Bv2 cell suspension to IMS and assayed one-half of the eluted sample by RT-PCR, we obtained a Ct value of 31, whereas the other half of the sample contained approximately 12 cells, as determined by plate counts. Not surprisingly, direct RT-PCR assay of the 200 CFU ml⁻¹ R3Bv2 cell suspension did not give a Ct value, because the 5-µl aliquot used in RT-PCR would have contained less than one cell. Thus, by concentrating *R. solanacearum* cells, IMS increased sensitivity for R3Bv2 detection by RT-PCR 8- to 64-fold, depending on the target population size. The process also reduced detection time approximately 10-fold.

MCH coupled to RT-PCR also increased speed and sensitivity of detecting *R. solanacearum*. Antiserum for use in IMS is not always readily available and can be difficult or costly to prepare. As an alternative, MCH using a bacterium- or strain-specific oligonucleotide (the capture probe) coupled to magnetic beads can be used to capture, via hybridization, a diagnostic DNA sequence that can be detected by RT-PCR. We coupled a 60-bp oligonucleotide (complementary to an RRSL_2403 sequence adjacent to the R3Bv2-diagnostic amplicon) to magnetic beads. When used in an MCH procedure, these beads consistently allowed RT-PCR detection of *R. solanacearum* R3Bv2 at concentrations as low as 10³ CFU ml⁻¹, whereas direct RT-PCR detection at this concentration occurred only about half the time. Although MCH functioned nearly as well or better than IMS at cell concentrations >10⁵ CFU

ml⁻¹ (Table 4), IMS showed better sensitivity at cell concentrations <10⁵ CFU ml⁻¹.

IMS and MCH allow rapid detection of cryptic populations of *R. solanacearum* in field sample mimics. Detection of R3Bv2 in quarantine screening situations requires rapid and accurate analysis of tissues from plants such as potato, tomato, and geranium, and also soil and water samples. Such samples are extremely complex in molecular and cellular content, and inhibition of PCR by soil or plant materials is well documented (2,64). Therefore, we set up a model system to evaluate the use of IMS and MCH to purify and concentrate R3Bv2 cells or DNA away from PCR inhibitors likely to be encountered in real-world test samples. Extracts of noninfected plant tissues or soil were prepared and R3Bv2 cells added to simulate complex positive samples. In preliminary experiments, we found that detection of R3Bv2 by RT-PCR was blocked if simulation mixtures contained >2% of extracts of potato stem, potato tuber, geranium stem, or soil unless the concentration of R3Bv2 cells exceeded 10⁶ CFU ml⁻¹. At 5%, all extracts except those from tomato stems completely blocked RT-PCR detection, even at 10⁷ CFU ml⁻¹. In general, only when the ratio of cells to extract exceeded 10⁷ cells g⁻¹ for potato tuber or stem extract or 10⁸ cells g⁻¹ for soil or geranium stem extracts could detection by direct RT-PCR be assured. In contrast, when positive field sample mimics with extract concentrations of 3 to 7% and cell concentrations of 10⁶ to 10³ CFU of R3Bv2 ml⁻¹ were subjected to IMS or MCH and then analyzed by RT-PCR, R3Bv2 cells were detected in most of the samples whereas direct detection by RT-PCR failed (Table 5). The Ct values obtained from IMS-coupled RT-PCR for samples with 10³ CFU ml⁻¹ suggested that R3Bv2 could be reliably and unambiguously detected by our method well below this concentration in extracts of both potato stem and tuber and, likewise, well below the 10⁴ CFU ml⁻¹ level in soil and geranium ex-

Table 2. Efficiency and specificity of immunomagnetic separation (IMS) for detection of *Ralstonia solanacearum* strains as determined by plate counts

Strain	Cells bound ^a
<i>R. solanacearum</i> UW551	23,500
<i>R. solanacearum</i> IPO1609	42,000
<i>R. solanacearum</i> GM11000	32,000
<i>R. solanacearum</i> AW1	11,500
<i>R. solanacearum</i> IPO1609 EPS-	8,400
<i>R. solanacearum</i> UW551 EPS-	12,000
<i>R. solanacearum</i> GM11000 ^b	50
<i>R. eutropha</i> JH16	1,500
<i>R. pickettii</i> ATCC 27511	ND
<i>Sinorhizobium meliloti</i> 54	ND
<i>Burkholderia cepacia</i> J2315	ND

^a Cells were diluted in phosphate-buffered saline containing 0.5% bovine serum albumin (to 10⁵ CFU ml⁻¹) and 1-ml aliquots were subject to IMS with anti-*R. solanacearum* immunoglobulin G (IgG)-conjugated magnetic beads. Aliquots of thrice-washed beads were plated on BG agar to determine the number of bound cells. Values are average of two independent experiments with less than twofold variation and representative of more than three other determinations. ND = none detected; no colonies were observed after 5-µl aliquots of beads were plated.

^b Unconjugated goat anti-rabbit IgG beads were used instead of anti-*R. solanacearum* beads.

Table 3. Sensitivity of immunomagnetic separation (IMS) for detection of *Ralstonia solanacearum*

Initial cell number ^a	Cells bound (recovery [%])
10 ⁵	35,000 (35)
10 ⁴	3,900 (39)
10 ³	170 (17)
10 ²	18 (18)
10 ¹	3 (30)

^a IMS was performed on 1-ml reactions containing the indicated number of GM11000 cells as described for Table 2. Number of cells bound to beads was determined by plate counts and are the average of more than four independent tests with less than twofold variation. IMS reactions using unconjugated goat anti-rabbit immunoglobulin G beads instead of anti-*R. solanacearum* beads resulted in a recovery of <0.01%.

Table 4. Comparison of immunomagnetic separation (IMS) and magnetic capture hybridization (MCH) coupled with real-time polymerase chain reaction (RT-PCR) for detection of *Ralstonia solanacearum* R3Bv2

Conc. (CFU ml ⁻¹) ^a	Cycle threshold (Ct) value		
	Direct PCR	IMS-RT-PCR	MCH-RT-PCR
10 ⁶	21.2	22	20.3
10 ⁵	26.1	22.9	24.4
10 ⁴	30.5	24.6	27.7
10 ³	32.3	26.7	31.2
200	>33	31	33

^a Cell concentration. *R. solanacearum* UW551 cells were diluted to the indicated concentrations in phosphate-buffered saline containing 0.5% bovine serum albumin for IMS and in water for MCH; magnetic bead separations were carried out as described in Materials and Methods. For MCH, samples were adjusted to 0.2× Tris-EDTA and heated at 97°C for 7 min before beads were added. Ct values were determined using 5 µl of DNA eluted from beads and are the average of more than three independent experiments; Ct values varied by no more than 5% between experiments. Ct values >33 cycles were not considered reliable indicators of the presence of R3Bv2 cells or DNA because Ct values of 34 to 38 were sometimes obtained with samples lacking cells or DNA.

tracts. Therefore, IMS and, to a lesser degree, MCH are simple and highly effective methods to dramatically increase the sensitivity of RT-PCR detection of R3Bv2 in field-sample mimics. In some cases, they were indispensable, because direct PCR was not possible.

Discussion

We developed two magnetic-bead-based assays to facilitate specific and sensitive detection of R3Bv2 in less than 4 h from samples that mimic the conditions expected to be present in field samples. Beads conjugated to *R. solanacearum*-specific antibodies or an R3Bv2-specific DNA capture oligonucleotide purified target cells or DNA away from PCR inhibitors so that the subsequent RT-PCR routinely detected R3Bv2 at population levels as low as 500 CFU ml⁻¹. A number of methods have been previously used as pretreatments to reduce the level of PCR inhibitors and excess nontarget DNA in field samples (30,33,35,38,42,60,62). Diluting samples 10- to 100-fold can increase success of subsequent detection by PCR (44) but this also dilutes the target cells, thereby reducing sensitivity and, thus, is unsuitable for samples with low target cell populations, such as latently infected tissues or soil. Swanson et al. (52) used 2 µm- and 0.4 µm-filtration to concentrate target cells, as well as to remove inhibitors from the irrigation effluent of pots containing infected geranium plants; however, subsequent RT-PCR did not reliably detect R3Bv2 when populations were less than 10⁵ cells ml⁻¹. Alternatively, DNA can be extracted directly from soil or plants and partially purified with a variety of methods to enhance detection by PCR (4,8,25,43, 45,47,51). However, these methods do not guarantee removal of inhibitors and, because they are time consuming and can use hazardous reagents or expensive kits, they are not suitable for routine diagnostic or detection procedures.

The most successful strategy to remove PCR inhibitors or enhance detection sensitivity has been to incubate the sample in a semiselective medium prior to PCR analysis (40,60). Because such enrichment dramatically increases the target cells present in complex samples, and also greatly reduces the relative concentration of PCR inhibitors, this simple approach can be used with currently available immunological methods or PCR (15,46,60). Unfortunately, this approach adds 24 to 96 h of incubation time and, especially in the case of soil samples, involves a risk that other bacteria will also multiply enough to interfere with PCR and increase the chance of contamination (4,45,60).

The use of paramagnetic beads is a relatively simple and rapid way to partially purify targets and eliminate contaminants (26). In addition, concentration of the captured targets into a smaller volume enhances detection without time-consuming enrichment culture. Both our IMS and MCH effectively removed PCR inhibitors and permitted detection of R3Bv2 down to 1,000 cells ml⁻¹ in extracts of various plant tissues and soil that otherwise inhibited PCR. However, the inhibitory potency of each extract varied and,

in part, depended on a sample's origin and the concentration of dissolved solids. As expected (47,58,62), we found that extracts of soil were >25 times more potent in blocking PCR than those prepared from a potato stem or tuber, while similarly prepared tomato stem extracts were not inhibitory. Surprisingly, extracts of geranium stem were the most potent at inhibiting PCR. When testing for R3Bv2 in extracts with potent inhibitors, we found that it was critical to increase the number of times that the magnetic beads and tube surfaces were washed to attain positive test results. Hence, the extent of washing to remove PCR inhibitors must be empirically determined for different types of samples.

Magnetic bead technologies are also amenable to field use, because the equipment needs are minimal. Field applicability and efficacy of our IMS and MCH methods were demonstrated by analysis of stem and rhizosphere soil samples from wilted tomato plants prepared on site in Arenal, Guatemala. Stem samples tested positive for R3Bv2 using either direct, IMS-, or MCH-coupled RT-PCR, whereas rhizosphere soil samples tested R3Bv2-positive only after MCH (*results not shown*).

There are two earlier reports where IMS was used to enhance detection of *R. solanacearum*. Expert et al. (17) recovered up to approximately 15% of the *R. solanacearum* cells when present at >10⁶ cells ml⁻¹ in water or potato tuber macerates but they did not test samples with lower cell concentrations. Our IMS procedure, however, captured approximately 25% of the *R. solanacearum* cells in water suspensions at concentrations of 10¹ to 10⁵ cells ml⁻¹. Although IMS is excellent for capturing *R. solanacearum* cells, it currently cannot be used alone to detect R3Bv2, because R3Bv2-specific polyclonal or monoclonal antibodies are not available. Dittapongpitch and Surat (12) reported that IMS followed by standard PCR had a detection threshold of approximately 10⁴ *R. solanacearum* cells ml⁻¹ in water but they were unable to detect cells in soil extracts containing <10⁵ cells g⁻¹. In contrast, our IMS removed PCR inhibitors and nontarget cells in various plant and soil extracts and permitted specific detection of *R. solanacearum* R3Bv2 by RT-PCR down to the level of 200 cells ml⁻¹. Thus, in addition to being R3Bv2 specific, our IMS-coupled RT-PCR is at least 10-fold more sensitive than previously used IMS procedures and other immunodiagnostic assays that do not involve enrichment culture or plating (such as indirect ELISA, double-antibody sandwich ELISA, immunofluorescence-colony staining, and dip sticks), which have detection thresholds of 10⁴ to 10⁵ cells g⁻¹ or ml⁻¹ (9,15,48).

There are also two reports describing MCH coupled to PCR for detecting *R. solanacearum*. Expert et al. (17) used beads coated with a nonspecific DNA-binding compound followed by standard PCR with *R. solanacearum*-specific primers to detect *R. solanacearum* at as few as 10² cells ml⁻¹ in tomato stem and potato tuber extracts, whereas detection by direct PCR required >10⁴ cells ml⁻¹. Poussier et al. (43) compared the use of MCH beads coated

Table 5. Evaluation of immunomagnetic separation (IMS) and magnetic capture hybridization (MCH) for detection of *Ralstonia solanacearum* R3Bv2 cells in complex environmental samples

Conc. (CFU ml ⁻¹) ^b	Cycle threshold (Ct) values ^a											
	Potato tuber extract			Potato stem extract			Geranium stem extract			Soil extract		
	IMS	MCH	PCR	IMS	MCH	PCR	IMS	MCH	PCR	IMS	MCH	PCR
10 ⁶	19.3	23	>33	20.6	24.5	>33	23.8	23.3	>33	23.6	24.4	>33
10 ⁵	19.3	24.5	>33	21	26.3	>33	26.4	27.2	>33	26.9	27.3	>33
10 ⁴	24	26.5	>33	23.2	29	>33	27.8	30	>33	28.2	30	>33
10 ³	28	>33	>33	27.7	33	>33	30.3	33	>33	32.4	>33	>33

^a PCR = direct real-time polymerase chain reaction.

^b Cell concentration. For IMS, appropriate amounts of *R. solanacearum* UW551 cells were added to 0.9 ml of indicated extract solution followed by addition of 0.1 ml of 10× phosphate-buffered saline containing 0.5% bovine serum albumin (PBSB) and IMS beads. Samples for MCH were prepared in the same manner, except 20 µl of 10× Tris-EDTA was added instead of 10× PBSB and then heated for 7 min at 97°C before beads were added. Potato extract samples were 7% while geranium and soil samples were 3%. All samples with cells and soil extract were filtered through a 5-µm Versapore filter (Pall Life Sciences number 4489T) before beads were added. Processing on the magnetic particle collector was as before, except beads from all reactions with geranium or soil extracts were washed six instead of three times. Ct values are average of more than three independent experiments; Ct values varied by <7% between experiments. Ct values > 33 are not considered positive for detection because Ct values of 34 to 38 were sometimes obtained with samples lacking cells or DNA.

with *R. solanacearum*-specific oligonucleotides to several other standard methods to reduce the effect of PCR inhibitors in total DNA prepared from complex samples prior to detection by nested PCR with *R. solanacearum*-specific primers. In general, their MCH did not perform as well as the other methods when applied to soil or to tissue samples from five different plants, including tomato and geranium. Nonetheless, both MCH and the QIAamp kit allowed detection of the pathogen in various soil suspensions down to approximately 10^3 cells g⁻¹ (the lowest concentration tested). Our MCH procedure displayed as good as or better sensitivity thresholds and performance when applied to plant and soil extract samples. Additionally, our MCH-coupled RT-PCR is highly specific and 100% accurate for R3Bv2 strains.

Our MCH method gave an approximately 10-fold increase in detection sensitivity with pure cells in water at $>10^4$ cells ml⁻¹ but not at lower concentrations. This is similar to detection enhancements reported for other MCH-based detections of bacteria (1,21,26). The magnitude of the improvement largely depends on the hybridization efficiency of the capture oligonucleotide. In preliminary screening, we tested seven capture oligonucleotides (30 to 65 bases long) targeting four R3Bv2-specific genes (RRSL_1249, RRSL_2402, RRSL_2403, and RRSL_2208) (31). Two capture oligonucleotides gave approximately 10-fold increases in sensitivity when compared with Ct values for direct PCR (including the 2403CAPYH oligonucleotide described here), three others gave two- to fourfold increases, and two were no better than direct PCR. Neither the length of the capture oligonucleotides nor their proximity to the R3Bv2-specific target (up to 120 bases distant) correlated with MCH efficiency (*results not shown*). We also found that adding 10-fold more MCH beads into the reactions or extending hybridization times increased the signal by an additional approximately threefold (*results not shown*) but the extra cost and time detracted from this approach. Although not tested, use of multiple-capture oligonucleotides targeting both DNA strands in one MCH reaction might improve detection sensitivity.

In conclusion, we demonstrated the applicability and potential of IMS and MCH followed by RT-PCR for reliable detection of R3Bv2 in a variety of samples that mimic those expected to be encountered in field situations. These methods are amenable to adaptation to many venues from remote field locations to high-throughput diagnostic laboratories and now can be evaluated by additional field trials. Overall, IMS-coupled RT-PCR displayed lower Ct values than MCH-coupled RT-PCR. However, the sensitivity of MCH should be adequate if IMS reagents are unavailable. In addition, when quarantine regulations preclude shipping or handling samples potentially containing live R3Bv2 cells, they can be heat-treated to kill the pathogen before shipment. Obviously, neither MCH nor IMS when followed by RT-PCR will be applicable in cases where isolation of R3Bv2 in pure culture is required for legal reasons prior to enacting official quarantine-mandated countermeasures. Additional research in locations where R3Bv2 is endemic will be required to determine whether these methods are sufficiently reliable to identify samples that deserve more rigorous examination using methods that recover viable cells.

Acknowledgments

This research was funded by the United States Department of Agriculture Cooperative State Research, Education and Extension Service Integrated Grant number 2007-55605-17843 under Animal and Plant Health Inspection Permit for Select Agents. We thank R. Walcott for advice and assistance in developing MCH and L. Lipscomb for reading and providing comments on the manuscript.

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