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An enrichment microsphere immunoassay for the detection of *Pectobacterium atrosepticum* and *Dickeya dianthicola* in potato tuber extracts

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Abstract

An enrichment microsphere immunoassay (MIA) was developed, based on the Luminex xMAP® technology, for the simultaneous (duplex) detection of *Pectobacterium atrosepticum* (former name *Erwinia carotovora* subsp. *atroseptica*) (Pca) and *Dickeya dianthicola* (former name *Erwinia chrysanthemi*) (Dcd) in potato plant extracts. Target bacteria in the extracts were enriched for 48 h in a semi-selective broth containing polypectate under low oxygen conditions. Samples were subsequently incubated with antibody-coated colour-coded microspheres (beads) and with secondary antibodies conjugated with Alexa Fluor® 532, a reporter dye. Samples were analyzed with the Luminex analyzer, in which one laser identified each microsphere and another laser the reporter dye conjugated to the secondary antibodies. The assay required minimal sample preparation, could be completed in 1 h, was performed in 96 wells microtitreplates and required no wash steps.The limit of detection for the duplex enrichment MIA was 100–1000 cfu ml^{-1}, which was a hundred times lower than of an enrichment-ELISA. Without enrichment, the sensitivity of MIA and ELISA was largely similar and ranged between 10^6 and 10^7 cells ml^{-1}. No difference in sensitivity was found between a MIA in a single or duplex format. In a comparative test with non-infected potato plant extracts and extracts from plants infected with Pca or Dcd, results of the enrichment MIA correlated well with those of the enrichment ELISA and enrichment PCR. These results indicate that MIA can be reliably used for multiplex detection of soft rot *Enterbacteriaceae* in crude potato plant extracts. The technology is an attractive and cost-effective alternative to other detection methods, including ELISA.

Introduction

Pectobacterium atrosepticum (Dye, 1969; Gardan et al., 2003) (Pca) and *Dickeya dianthicola* (Burkholder, 1953; Samson et al., 2005) (Dcd) are the causative agents of blackleg and aerial stem rot in potato, and cause considerable yield loss and economic damage in potato production world-wide (Pérombelon and Kelman, 1980). Based on DNA–DNA hybridization studies, serology and numerical taxonomy, Pca (formerly named *Erwinia carotovora* subsp. *atroseptica*) was recently classified as *P. atrosepticum* (Gardan et al., 2003), whereas Dcd (formerly named *E. chrysanthemi*) was distributed over six genomic species within the genus *Dickeya* (Samson et al., 2005). Control of these soft rot bacteria is mainly based on exclusion and reduction of inoculum, as

bactericides are generally ineffective. Therefore, the use of seed-testing methods is indispensable.

For seed testing, serological methods are still favoured above nucleic acid-based techniques, because of their robustness, cost effectiveness and simplicity. Serological methods for detection of Pca and Dcd include the use of immunofluorescence cell-staining (Vruggink and De Boer, 1978), immunofluorescence colony-staining (Van Vuurde and Roozen, 1990), immunomagnetic separation followed by dilution plating (Van der Wolf et al., 1996) and ELISA, which can be used either directly on plant extracts or after enrichment of bacteria in a semi-selective broth (Gorris et al., 1994). Enrichment, conducted under anaerobic conditions in a semi-selective broth with polypectate, increased the specificity for ELISA from ca. 10^5 to as low as 2×10^2 cells ml^{-1}. Enrichment under anaerobic conditions can also increase the specificity of the assay, as saprophytic bacteria shown to cross-react with the polyclonal antibodies against Pca in ELISA, were not detected under anaerobic conditions that favour enrichment of target bacteria (Lopez, Valencia, personal communication). In the Netherlands, the enrichment ELISA is applied in routine testing programmes.

Here we describe an enrichment immunoassay for the simultaneous detection of Pca and Dcd based on the use of the Luminex 100 system. The Luminex 100 is a small flow cytometer designed with the sole purpose of detecting microspheres (Earley et al., 2002). The microspheres have a diameter of 5.3 μm and are made from polystyrene. They are internally stained with two fluorochromes. An assortment of 100 beads are available, each with a unique composition of red and infrared fluorochromes. The Luminex technology has proven its value already for multiplex detection of target compounds in the field of human diagnostics (Kellar, 2003), food microbiology (Dunbar et al., 2003), environmental microbiology (Spiro and Lowe, 2002) and biological warfare (McBride et al., 2003; Biagini et al., 2004) by using protein and nucleic acid-based techniques. Beads can be covalently coupled with proteins, peptides, polysaccharides, lipids and oligonucleotides (Kellar and Ianonne, 2002; Dunbar et al., 2003).

In the microsphere immunoassay (MIA), samples are subsequently incubated with the antibody-coated beads and with secondary antibodies conjugated with a reporter fluorochrome. Within the Luminex analyzer, one laser excites the internal dyes of the microspheres and another laser the reporter fluorochrome, captured by the antigen (Figure 1).

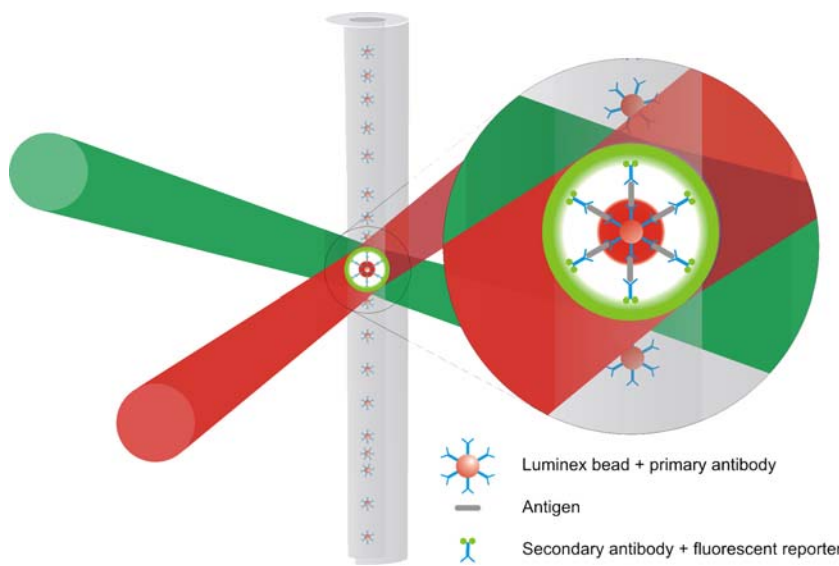

Figure 1. Diagram of the microsphere immunoassay using the Luminex Technology. Samples are subsequently incubated with colour-coded antibody-coated microspheres and with secondary antibodies conjugated with a fluorescent reporter. Samples are analyzed in the Luminex analyzer, in which a red laser identifies the microsphere and a green laser the fluorescent reporter.

In comparative testings, MIA shows a comparable sensitivity to ELISA but an improved dynamic range (Vignali, 2000; Biagini et al., 2003; DuPont et al., 2005). Moreover, MIA is less labour-intensive and different analytes can be measured at the same time (Dasso et al., 2002; Biagini et al., 2003). Also when compared to antibody microarrays, the Luminex technology has better features, such as a lower detection limit and a better dynamic range, although microarrays are more suitable for miniaturization (Rao et al., 2004). Once coupled, the beads can be easily stored without any loss of activity for at least 1 year. A multiplex MIA can be easily adapted to the user's demand by making the desired combinations of single assays. The goal of this study is to develop and evaluate an enrichment MIA for duplex detection of Pca and Dcd in potato peel extracts.

Materials and methods

Plant material

Two potato seed lots of cv. Agria and cv. Remarka, essentially free of Pca and Dcd, respectively, were used in this study. Tubers were stored in the dark at 4 °C until required. The stolon ends of 10 tubers were placed in a plastic bag with 5 ml of quarter strength Ringer solution (Sigma) and crushed with a hammer. These extracts were directly used in the experiments.

Samples from two potato fields were used to validate the method. From a naturally infected field with potato plants showing wilting of foliage, individual stem segments (approx. 5 cm) were taken and individually crushed in a plastic bag with 5 ml of the Ringer solution. From a test field of plants grown from tubers vacuum-infiltrated with Pca, Dcd or water, a composite sample of stems, stolons and stolon ends of tubers of indivual plants were crushed in a plastic bag with 5 ml of the Ringer solution. Prior to use, the extracts were stored at 4 °C (for a maximum of 1 day).

Bacterial strains

Two strains were used: Pca IPO 1987, a spontaneous streptomycin resistant mutant of Pca wild-type strain IPO 161 and Dcd IPO 1991, a spontaneous nalidixic acid-resistant strain of Dcd wild-type strain 502. Both strains were isolated from Dutch potatoes. Bacteria were maintained on plates with TSA medium with streptomycin (100 μml^{-1}) for Pca and nalidixic acid (100 $\mu g\ ml^{-1}$) for Dcd. Ten-fold serial dilutions of bacterial suspensions in Ringer buffer and potato extract were used.

Enrichment assays

To enrich the target bacteria, 100 µl of the potato peel extract supplemented with Pca or Dcd was added to 1 ml Eppendorf tubes containing 900 µl medium D-PEM (Meneley and Stanghellini, 1976). The tubes were tightly closed to create low oxygen conditions and were incubated for 72 h at 25 °C.

Double antibody sandwich (DAS) ELISA

DAS-ELISA was performed essentially as described by Van der Wolf and Gussenhoven (1992). Polystyrene 96-well plates were coated with 200 µl of IgG purified polyclonal antibodies against Pca (9022-01) or Dcd (8276-01) (Prime Diagnostics, Wageningen) at a concentration of 0.5 $\mu g\ ml^{-1}$ in sodium carbonate buffer. Plates were sealed with plastic foil and incubated overnight at 4 °C. After incubation the plates were washed three times with running tap water. To each well, 200 µl of sample was added and plates were incubated for 4 h at 37 °C. Plates were washed again three times with running tap water. To each well, 200 µl of IgG purified polyclonal antibodies conjugated with alkaline phosphatase against Pca or Dcd were added at a concentration of 0.3 $\mu g\ ml^{-1}$ in PBS (8 g NaCl, 1 g KH_2PO_4, 14.5 g $Na_2HPO_4 \cdot 12\ H_2O$ (pH 7.4) in 1000 ml distilled demineralized water) with 0.1% Skimmed Milk Powder (Oxoid). Plates were incubated for 2 h at 37 °C and washed three times under running tap water. To each well 200 µl of *p*-nitrophenyl phosphate (1 $mg\ ml^{-1}$ substrate in diethanolamine buffer) was added. The absorbance was measured with an automatic ELISA reader at 405 nm after incubation for 60 min at room temperature. Results were considered positive if absorbance values of tested samples exceeded twice the negative control.

Conjugation of beads with antibodies

Carboxylated beads from Luminex (Austin, USA) were coated with the identical Pca- or Dcd-specific polyclonal antibodies used in the ELISA. The bead suspension (100 µl of 1×10^6 beads ml^{-1}) was vortexed vigorously for 5 min at room temperature prior to use. The bead suspension was centrifuged for 2 min at room temperature at 10,000 *g* in an Eppendorf centrifuge. Beads were resuspended and activated in 100 µl of 10 mM NaH_2PO_4 (pH 6.3) containing 500 µg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (Fluka) and 500 µg of *N*-hydroxysulfosuccinimide (Fluka) for 20 min at room temperature on a vortex with gentle agitation. After incubation, beads were centrifuged for 2 min at 10,000 *g*, and the supernatant was discarded. Both Pca and Dcd specific antibodies were dialysed against 50 mM HEPES (pH 7.4) and concentrations were adjusted to 125 µg ml^{-1}. After, the activated beads were resuspended in 0.5 ml of antibody solution and incubated for 1 h at room temperature in the dark. The beads were centrifuged for 2 min at 10,000 *g*, the supernatant was discarded and the beads were resuspended in PBS buffer and centrifuged for 2 min at 10,000 *g*. The pelleted beads were resuspended once again in 200 µl of PBS supplemented with 1% (w/v) BSA (PBSB) and stored at 4 °C. The bead concentration was determined using the Luminex system and the concentration was adjusted to 10^5 beads μl^{-1} in PBSB. To prevent microbial growth, sodium azide 0.02% (w/v) was added.

Conjugation of secondary antibodies with Alexa Fluor 532

Antibodies were purified from serum using a Protein G sepharose column (Amersham) according to the manufacturer's instructions. A bed volume of 1 ml resin was used for 1 ml of serum. The buffer was exchanged to 0.1 M sodium bicarbonate using a PD-10 column (Amersham). Alexa Fluor 532 (Ex. 532 nm, Em. 554 nm, Molecular Probes) was dissolved in dimethyl formamide (DMF) to a final concentration of 10 mg ml^{-1}. For each labelling, 50 µl of this solution was added to 200 µl of 6 mg ml^{-1} antibody solution. The mixture was incubated at room temperature for 1 h with gentle agitation. Conjugated antibodies were separated from the unbound dye by passing it over a Sephadex G50 (DNA grade, Amersham) column (300 mm × 10 mm). The protein concentration was measured and adjusted to a final concentration of 2 mg ml^{-1} in PBS with 0.02% (w/v) sodium azide.

Microsphere immunoassay (MIA)

In a 96-well plate, 50 µl of sample extract was mixed with 50 µl of 2× concentrated PBS supplemented with 0.1% (w/v) Tween 20, 1% (w/v) skimmed milk powder and 1000 beads of each bead set. Plates were incubated at room temperature, in the dark, for 20 min while shaking at 600 rpm using a vortex with a plate support. After incubation 1 µl (2 mg ml^{-1}) of each secondary Alexa Fluor 532 conjugated antibody was added to each well and plates were incubated for another 30 min at the same speed and temperature, in the dark. After this final incubation, the samples were ready for analysis on the Luminex system. Analysis was complete after 100 beads of each bead set were measured in each well. This resulted in average run times of 10 s per well and 20 min total run time for each plate.

Enrichment PCR

A PCR amplification procedure was developed with primers adapted for equal annealing temperatures. For Pca, primers were designed based on those described by De Boer and Ward (1995) and for Dcd based on those described by Nassar et al. (1996). From the naturally infected samples, 200 µl of enriched culture was used to extract genomic DNA using the QIAamp DNA mini kit, according to the manufacturer's instructions (Qiagen). Enrichment cultures were centrifuged at 7000 *g* for 5 min at room temperature. DNA was extracted from the pellet with the tissue protocol. For unknown infections, 90 µl of enriched culture was mixed with 10 µl of 50 mM NaOH and incubated at 96 °C for 3 min. The suspension was cleared by centrifugation for 5 min at 10,000 *g*. From each sample, 2 µl of extract was used for a PCR. The PCR mix consisted of 10 pmol of each primer (Pca Forw GATCGGCATCATAAAAACACG, Pca Rev CGCACACTTCATCCAGCGAG, Dcd Forw GAAAGCCCGCAGCCAGATC and Dcd Rev TCAGGATGGTTTTGTCATGC), 200 µM of each dNTP, 5 µl

of SuperTaq buffer (SphaeroQ) 2 U of SuperTaq (SphaeroQ), 1 µl of 25 mM $MgSO_4$ made up to 50 µl total with sterile purified water. After denaturing at 96 °C for 1 min, amplification was done in 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. The ramp for the annealing step was set to 1 °C per second. The programme ended with one 5 min cycle at 72 °C. Fragment lengths were 667 and 392 basepairs respectively for Pca and Dcd. Reactions were either stored at 4 °C or directly loaded and run on a 1.5% agarose gel in 1 × TBE at 100 volts.

Results

Detection levels of ELISA and MIA for enriched and non-enriched samples

The limit of detection (LOD) of MIA in a duplex assay format was compared with ELISA using potato tuber peel extracts seeded with a serial dilution of Pca and Dcd. All samples in MIA and ELISA were tested in duplicate. Without prior enrichment, for Pca a LOD of 10^6 cfu ml^{-1} and for Dcd a LOD of 10^7 cfu ml^{-1} was found both in MIA and ELISA (Figure 2). After incubation of seeded peel extracts in the semi-selective DPEM-broth for enrichment of target bacteria, a greater sensitivity resulting in a decreased LOD was found for both ELISA and MIA. For ELISA the sensitivity increased 100-fold compared to non-enriched samples resulting in a LOD of 10^4 cfu ml^{-1} for Pca and 10^5 cfu ml^{-1} for Dcd. For MIA, the sensitivity increased 10,000-fold resulting in a LOD of 10^2 cfu ml^{-1} for Pca and 10^3 cfu ml^{-1} for Dcd. Seeding of samples with densities of bacteria exceeding 10^4 cfu ml^{-1} for Pca and 10^5 cfu ml^{-1} for Dcd, resulted in a decrease of the mean fluorescence in the enrichment Luminex assay.

Comparison of duplex and single assays

Each sample was analysed in a duplex assay as well as a single assay format, in which beads and secondary antibodies for both Pca and Dcd were added. Results of non-enriched and enriched samples in the single assay were largely similar to those in the duplex assay (Figure 3.). For Pca the same LODs in both assays were obtained. For Dcd, the LOD of the enriched samples was higher in the single assay (10^4 versus 10^3 cfu ml^{-1}), but the LOD of the non-enriched samples in the single assay was lower (10^6 versus 10^7 cfu ml^{-1})

Naturally infected samples

The duplex MIA was evaluated using potato tubers from field experiments infected with Pca and Dcd. Enrichments were made with 10 tubers likely to be infected with Pca and 10 tubers likely to be infected with Dcd. These tubers were harvested from a potato crop (cv. Kondor) grown from potato seed vacuum-infiltrated with Pca strain IPO 1987 or Dcd strain IPO 1991. As a negative control, enriched samples of 10 tubers from an soft rot-free crop (cv. Kondor) were used.

Tubers from the soft rot-free crop were negative in MIA and ELISA as well as in PCR, which was used as a reference method (Figure 4). Samples were considered positive if values exceeded twice the mean value of the extracts of the soft rot-free tubers. All 10 tubers from the Pca-infected potato crop showed significant positive signals in MIA, whereas only eight samples were positive in ELISA (Figure 5). The MIA for Dcd showed nine significant positive samples. The only negative Dcd sample (Dcd*1*) appeared to be Pca-positive (Figure 4). Sample Dcd*3* showed a double infection with Pca and Dcd. In ELISA only six samples were Dcd-positive, sample Dcd*1* was Dcd-negative as in MIA but did not appear to be Pca-positive (Figure 5). The enrichment PCR confirmed the MIA results, except for sample Dcd*1*, which was negative with both primer sets.

In addition, five tubers were analysed from a naturally infected crop (cv. Bintje) that showed typical blackleg symptoms. Before and after enrichment, samples were negative for Pca. All five samples showed a low, but significant Dcd signal before enrichment (results not shown). After enrichment the Dcd signals strongly increased.

Discussion

In this study a duplex immunoassay has been developed for the sensitive detection of Pca and Dcd in potato peel extracts, based on enrichment in a semi-selective broth and a microsphere immunoassay using a Luminex platform.

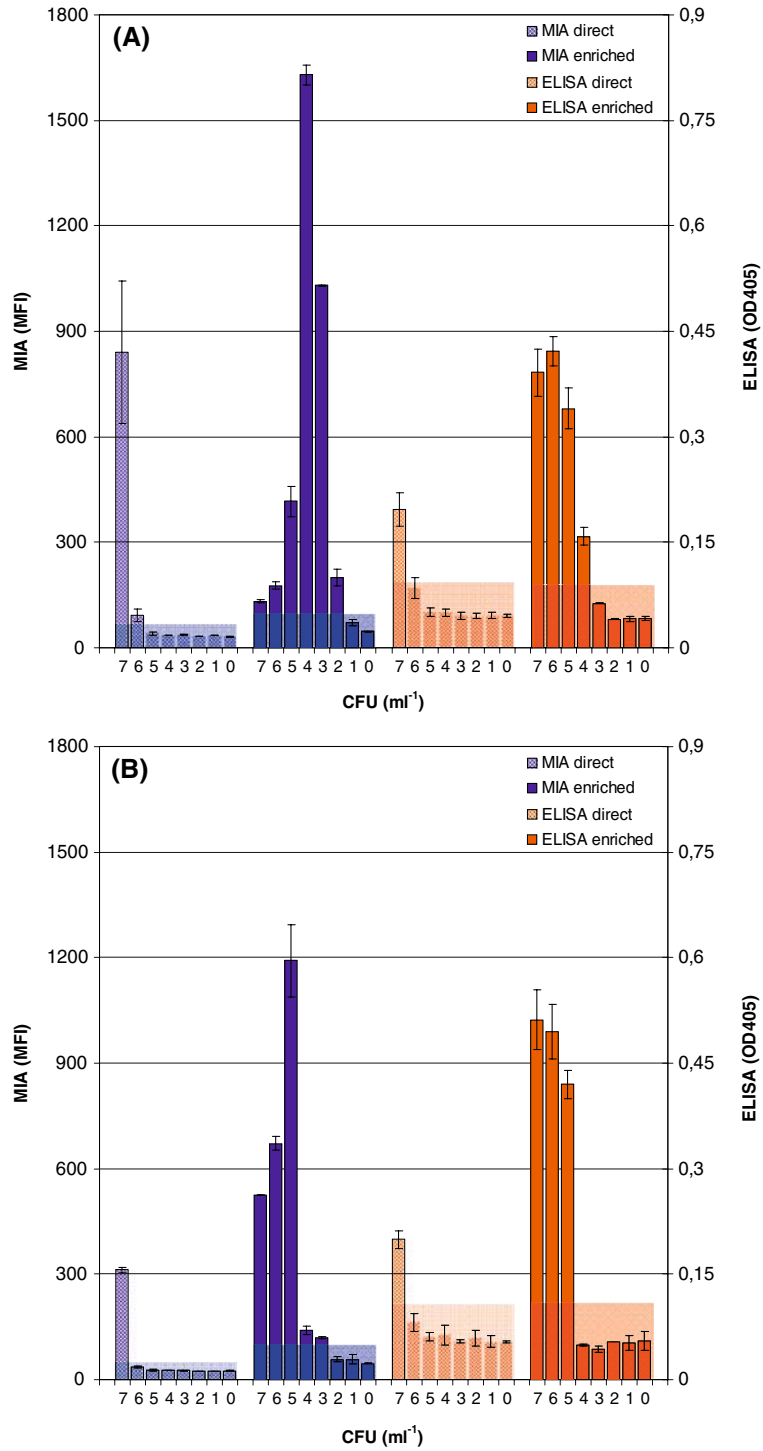

Figure 2. Dose–response bar diagram for Pca (A) and Dcd (B) from seeded potato peel extract in a microsphere immuno-assay (MIA) and in ELISA, before and after enrichment in a semi-selective broth for 72 h at 25 °C. Threshold for positive samples indicated with shaded boxes (2× background).

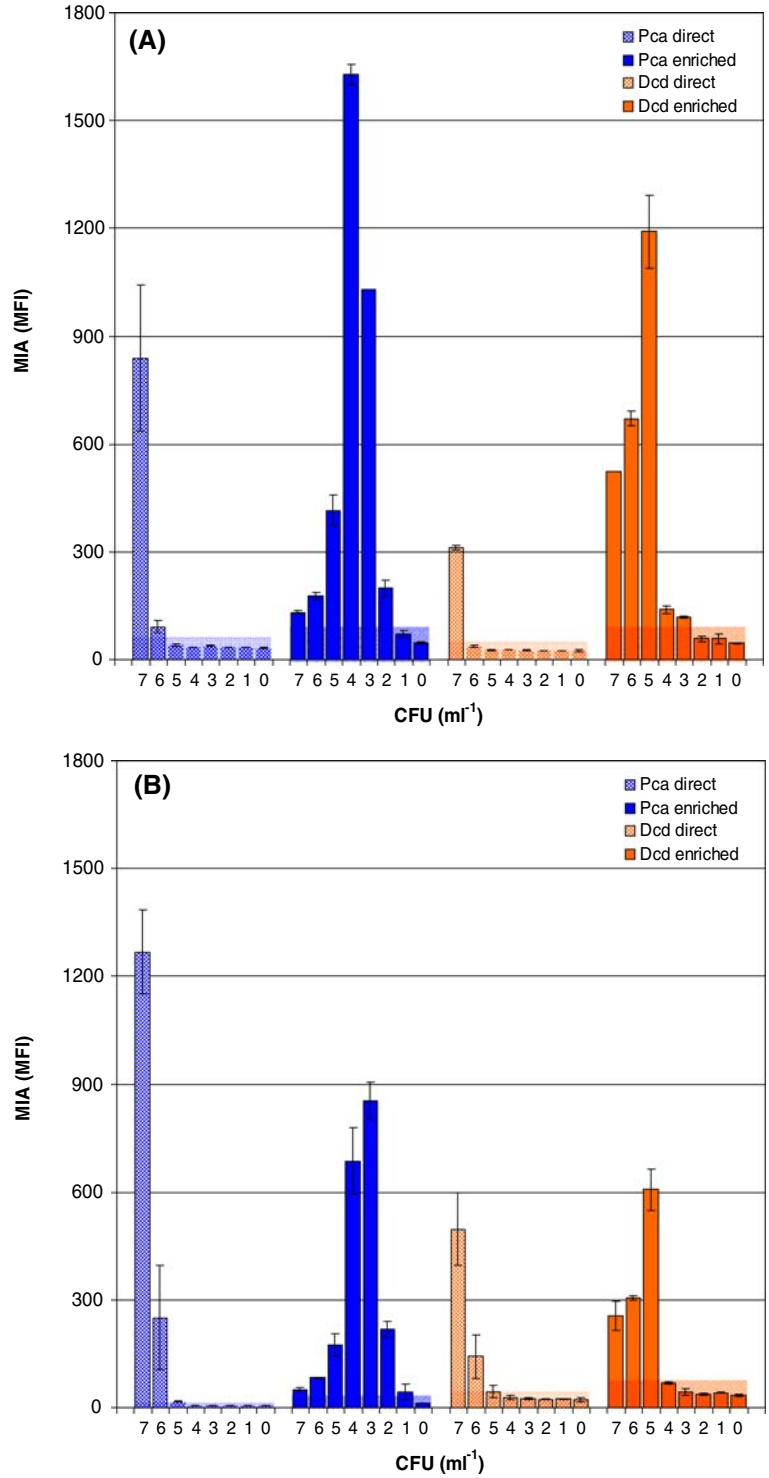

Figure 3. Dose–response bar diagram for Pca and Dcd from seeded potato peel extract before and after enrichment in a semi-selective broth for 72 h at 25 °C using a microsphere immuno-assay in a single (B) and duplex (A) format. Threshold for positive samples indicated with shaded boxes (2× background).

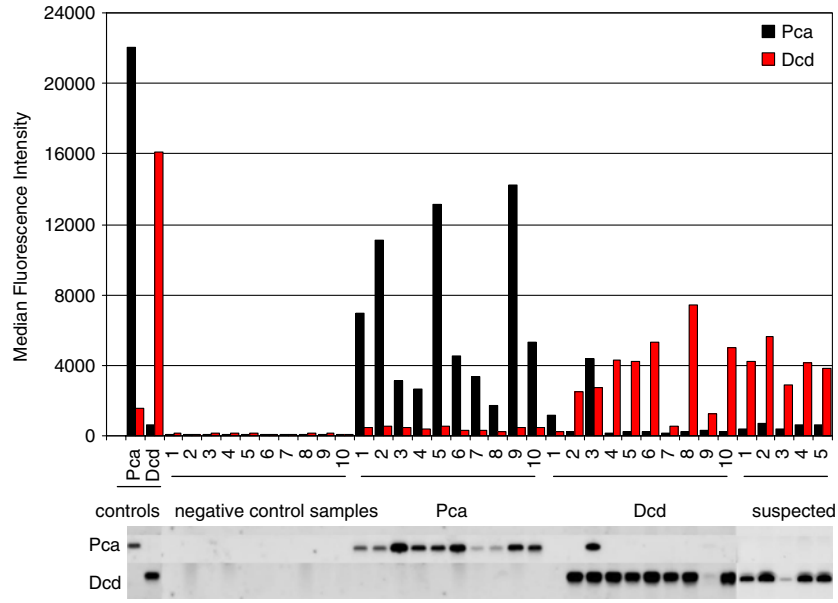

Figure 4. MIA multiplex detection and PCR confirmation of potato tuber extracts, naturally infected with Pca and Dcd. The first two samples are positive controls (peel extracts seeded with Pca and Dcd at a density of 10^7 cfu ml^{-1}). Negative control samples were prepared from tubers virtually free from infection. Pca and Dcd samples were tuber extracts prepared from plants grown from Pca and Dcd inoculated tubers. Suspected samples were tuber extracts made from plants naturally infected with blackleg.

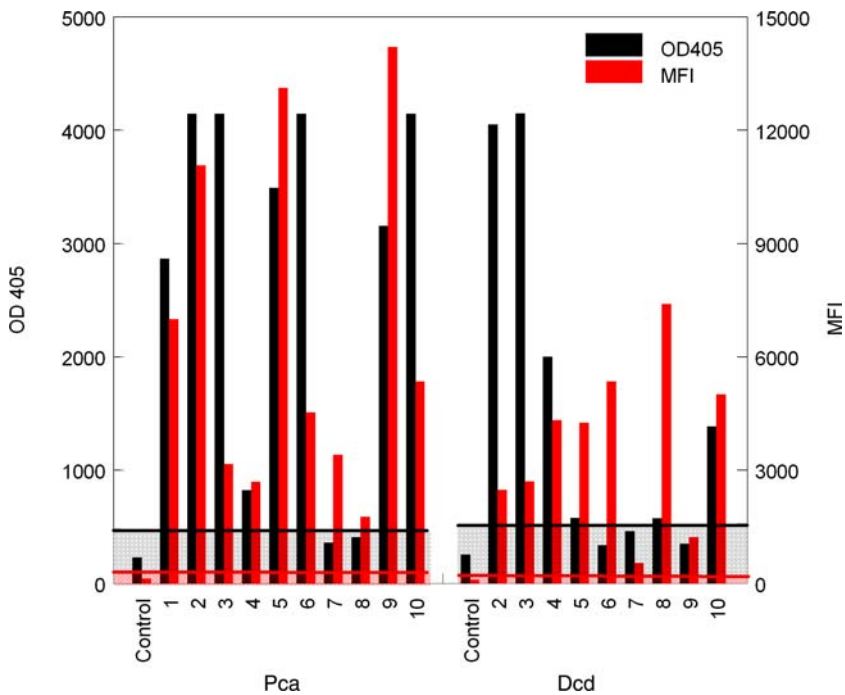

Figure 5. Comparison of MIA and ELISA for samples naturally infected with Pca and Dcd. Control: the mean value of ten infection-free samples. Threshold for positive samples indicated with shaded boxes (2× background). Red boxes indicate Luminex, black boxes indicate ELISA.

The LOD of MIA was similar to ELISA for extracts in which bacteria were detected without previous enrichment. Also in previous comparative studies, the sensitivity of MIA was often similar to ELISA (Dasso et al., 2002; McBride et al., 2003). The LODs of MIA for detection of Pca and Dcd in peel extracts were rather high compared to those published for other bacteria (Dunbar et al., 2003) in which the authors reported a sensitivity of ca. 500 cfu ml^{-1} for four human pathogens; *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes* and *Campylobacteri jejuni*. The differences may be explained by the type of antigen, the antibodies and in particular by the assay format. Dunbar et al. (2003) used a biotin-labelled secondary antibody and a streptavidin-phycoerythrin for detection of captured antigens. This format required several washing steps and cannot be used for crude plant extracts.

The LOD of the MIA for Pca and Dca in seeded peel extracts was 100-fold lower than an enrichment ELISA for the individual pathogens and was respectively 100 and 1000 cfu ml^{-1}. Obviously, during enrichment, antigens are produced that are detected more readily by MIA than by ELISA. Possibly antibodies conjugated with alkaline phosphatase, which has a relative high molecular weight of 140 kD, encounter more problems with binding to antigens with repetitive motifs such as lipopolysaccharides, due to steric hindrance, than antibodies labelled with the Alexa Fluor 532, which is a relatively small molecule.

Apart from the increased sensitivity, the MIA offers several other advantages compared to ELISA. First, in the MIA multiple beads per sample (100–200) are measured in comparison to the few replicate wells normally used in ELISA per sample. The higher number of replicates result in a higher precision for the MIA (Dasso et al., 2002). Second, the entire MIA can be completed in 1 h, including measurement by the Luminex analyzer which typically takes 20 min per 96-well plate, compared to a minimum of 6 h for ELISA. Third, Dcd and Pca can be detected in a duplex assay. Only little interference was found between assays for Pca and Dcd in the duplex MIA compared to the MIA conducted for the single pathogens, resulting in slightly higher MFI values for both pathogens.

The specificity of the assay was determined by the enrichment procedure and the specificity of the antibodies. Antibodies against Dcd can cross-react with *Pseudomonas fluorescens* strains (Van der Wolf et al., 1993) and Pca antibodies against *Comamonas* spp. (Hyman et al., 1995). This potential problem seems to be overcome by the use of anaerobic conditions during selective growth of Pca and Dcd, which represses the growth of the strictly aerobic *P. fluorescens* and *Comamonas* strains. The specificity of the assay can be further increased by the use of monoclonal antibodies which excludes some of these cross-reactions (De Boer and Mcnaughton, 1987; Vernon-Shirley and Burns, 1992; Gorris et al., 1994; Hyman et al., 1995).

The use of immunoassays for detection of Pca and Dcd requires a thorough knowledge of the existing serogroups. For Pca, eight different serogroups have been described (De Boer et al., 1979; De Boer and McNaughton, 1987; Perminow, 1997). In several important seed potato growing countries in Europe such as the Netherlands and Scotland serogroup I strains of Pca dominate in potato (Van Beckhoven et al., 2001). However, in other European countries, up to 40% of the Pca strains isolated from blackleg diseased plants belonged to another serogroup (De Boer and McNaughton, 1987; Sledz et al., 2000). In this case it would be necessary to include antibodies against other dominant serogroups in the assay. For Dcd nine serogroups have been described, although so far all Dcd strains isolated from potato in Europe belonged to serogroup O_1 (Samson et al., 1987, 2005; Janse and Ruissen, 1988).

The enrichment MIA in its current format cannot be used for quantification of the pathogens. Multiplication of the target bacteria during the enrichment will be strongly dependent on the plant extract and the microbial population associated with the extract and the MIA shows a hook effect (Selby, 1999). At high antigen concentrations the signal decreases due to the presence of unbound antigens in solution which compete with bound antigens for the limited amount of secondary antibodies. To avoid severe infections being overlooked, it is recommended to test different sample dilutions. In the near future, paramagnetic Luminex microspheres will be released, which will allow unbound antigens to be removed by washing, and in this way avoid the hook effect. Using a magnetic support for a 96-well plate, the total processing time will remain at 1 h. During

washing, the potato extract components will also be removed. This may enhance signals and further decrease the background.

For the detection of these target bacteria in naturally infected potato samples, the enrichment MIA showed better properties than the enrichment ELISA. The results of the enrichment MIA correlated almost entirely with those of the enrichment PCR; whereas in the enrichment ELISA more samples were found to be false-negative. For most samples, the signal to noise ratios were higher in the enrichment MIA than in the enrichment ELISA. Only at high antigen concentrations, the ELISA resulted in higher signal to noise ratios due to the hook effect on the MIA signal.

It is concluded that enrichment MIA is a sensitive, easy to perform and cost-effective method for the detection of Pca and Dcd in potato plant extracts. Similar to ELISA, MIA can be automated for high throughput analysis using pipetting robots and the HTS version of the Luminex analyzer.

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