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Development of a magnetic bead fluorescence microscopy immunoassay to detect and quantify *Leptospira* in environmental water samples

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ABSTRACT

Climate change, world population growth, and poverty have led to an increase in the incidence of leptospirosis. Leptospirosis is caused by pathogenic spirochaete bacteria that belong to the genus *Leptospira*. The bacteria are maintained in the renal tubules of the reservoir hosts (typically a rodent), then shed into the environment via the urine. Water is key for environmental survival and transmission, as leptospires can survive for several weeks in a moist environment. Therefore, environmental epidemiological studies are needed to study the contamination of environmental water sources. However, few such studies have been performed using cultivation of the isolates and PCR assays. But, leptospira cultivation can be easily contaminated by other organisms and takes usually several weeks. Moreover, PCR is a complex and costly analysis for the underdeveloped countries that have the highest incidence of leptospirosis. In this study, we describe two modifications of a fluorescence microscopy assay based on immuno-magnetic separation (IMS) to detect leptospires in environmental water samples that mainly differ in fluorescent dye staining. The first type uses acridine orange fluorescent dye staining combined with multiplexed IMS for sample screening. The detection limit ranged from 10^2 to 10^3 organisms per mL and largely depended on the capture efficiency (CE) of the immuno-magnetic particles. The second type uses serogroup-specific immuno-particles and direct fluorescence antibody staining (DFA) to detect leptospires; the detection limit of this second assay was approximately 10^1 cells per mL. Both assay types were applied to natural and experimentally infected water samples, which were also analysed with DFM and real-time PCR. Our data show that the fluorescent microscopy immunoassay successfully identified experimental leptospire contamination and was as sensitive as PCR. This modified immune-fluorescence assay may therefore enable epidemiological studies of leptospirosis.

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1. Introduction

Pathogenic spirochaetes that belong to the genus *Leptospira* cause leptospirosis, which is a zoonotic disease that is prevalent all over the world (Faine et al., 1994; WHO, 2010; Hartskeerl et al., 2011). Leptospires infect and are carried by maintenance hosts, which are typically rodents. The parasites infect the host renal tubules and are shed into the environment via urination, where they can survive in moist soil and surface water for up to several months (Trueba et al., 2004; Smith and Self, 1955). Leptospirosis outbreaks often occur during seasonal rainfall and floods, especially in warm and humid climates that facilitate leptospire survival (Hartskeerl et al., 2011; Lau et al., 2010; Coelho and Massad, 2011; Hui-ming et al., 2003). Therefore,

Abbreviations: IMS, immuno-magnetic separation; mIMS, multiplexed IMS; DFA, direct fluorescence antibody assay; CE, capture efficiency; AO, acridine orange; DAOS, direct acridine orange staining; EPA, environmental protection agency; EMJH, Ellinghausen and McCullough liquid medium as modified by Johnson and Harris; BSA, bovine serum albumin; PBS, phosphate buffered saline; MAT, microscopic agglutination test; RT, room temperature; FITC, fluorescein isothiocyanate; Ct, cycle-to-threshold.

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occupation- and recreation-related human leptospirosis is considered to be predominantly waterborne (Ricaldi and Vinetz, 2006; WHO, 2010; Diesch and McCulloch, 1966). Leptospirosis epidemiological investigations have been largely limited to human and animal studies (WHO, 2003). However, environmental reservoirs should be studied to survey for contamination, assess the risk of transmission, and prevent outbreaks. Because specific leptospire serovars are typically associated with chronic carriers, information regarding serovars is important for the design of effective control and prevention measures.

Detecting leptospiral contamination in environmental water samples can be challenging, and consequentially few methods have been applied to detect leptospires in environmental samples (Wilson and Fujioka, 1995; Ridzlan et al., 2010; Henry et al., 1971). One applied method is cultivation of isolates in specialised media; although this assay is relatively simple to perform, it is laborious, time-consuming, and can be easily contaminated (Henry et al., 1971; Alexander et al., 1975). Previously, PCR-assays alone or in combination with cultivation techniques have been used to detect leptospiral genetic material in water (Ridzlan et al., 2010; Ganoza et al., 2006; Murgia et al., 1997; Aviat et al., 2009). However, PCR analysis cannot specify serovars (Cerqueira and Picardeau, 2009). Finally, complicated PCR assays, such as nested or duplex PCR assay, are required for assay specificity, which makes this technique complex and expensive (Tansuphasiri et al., 2006).

The direct fluorescent antibody assay (DFA) is a common antigen detection method that has been applied to environmental analysis as well (Henry et al., 1971). Unlike PCR, which amplifies genetic material regardless of whether the antigen is fragmented or intact, DFA may yield more information regarding the quantity of intact leptospiral organisms. However, fluorescence background and high detection limits are inherent limitations during environmental sample analysis.

In this study, we combine immuno-magnetic separation (IMS) and fluorescent microscopy in an alternative method to detect leptospire contamination of environmental water samples. This modified assay does not require complex and time consuming pre-enrichment techniques, has low background fluorescence, and has a low limit of detection (LOD). We developed two types of the assay that mainly differ in fluorescence staining. The first type is a serogroup-specific assay, where polyclonal anti-*Leptospira* antibody based IMS is used in combination with direct fluorescent antibody staining (IMS-DFA). The second type is multiple serovar reactive that combines multiplexed IMS (mIMS) and direct acridine orange (AO) fluorescent staining (mIMS-DAOS). Both assay types were used to analyse experimentally inoculated and untreated water samples and were compared to *LipL32* real-time PCR analysis (Stoddard et al., 2009). The main goal of this study was to determine if a fluorescence microscopy assay in combination with immuno-magnetic separation can be used to study the environmental epidemiology of leptospirosis.

2. Materials and methods

2.1. Bacteria and antibodies

Pathogenic *Leptospira interrogans* serovars Rachmati, Sejroe, Bratislava, Shermani, Canicola and Ranarum, as well as non-pathogenic *L. biflexa* serovar Patoc (Table 1), were obtained from the National Leptospirosis Reference Centre, the National Institute of Health, Thailand. The stock cultures were subcultured weekly in Ellinghausen and McCullough liquid medium, as modified by Johnson and Harris (EMJH), at 27 °C. The log phase leptospiral culture was washed by centrifugation at 12,000 × *g* for 15 min and resuspension in wash buffer (10 mM phosphate buffered saline (PBS), pH 7.4). The cell count was determined by manual counting with a dark-field microscope (DFM) (Olympus BX51) and a Neubauer counting chamber. Antibody individually reactive against the Canicola, Sejroe, Shermani, Bratislava, Ranarum, and Rachmati serovars was prepared as previously described and used to conjugate to fluorescein isothiocyanate (FITC) (Doungchawee et al., 2005) as well as to magnetic particles (Schreier et al., 2011). The antibody concentration was determined with a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies). The conjugate antibody solution was diluted to a concentration of approximately 2 mg/mL and stored at 4 °C prior to use.

Table 1
Leptospira reference strains used for seeding experiments.

Serogroup	Serovar	Strain
L. interrogans		
1. Sejroe	Sejroe	M84
2. Autamnalis	Rachmati	Rachmat
3. Australis	Bratislava	Jez Bratislava
4. Shermani	Shermani	LT821
5. Ranarum	Ranaram	Ranarum
6. Canicola	Canicola	Hond Utrecht IV
L. biflexa		
7. Semaranga	Patoc	Patoc 1

2.2. Water samples

PCR, IMS-DFA, and mIMS-DAOS were used to analyse sterile PBS, tap water, and 6 representative experimentally inoculated water samples that differed in their turbidity, debris content, and microbial concentration according to dark-field microscopy. The untreated water samples were analysed within 5 days after collection and were subsequently stored at 4 °C until experimental inoculation. The following 6 environmental water samples were used in the study:

CS: Water from Samsen Creek, Phayathai district Bangkok that was malodorous and contained a high concentration of dark debris and microbes. The resting water appeared slightly dark in colour.
CA: Water from a creek in the Ayuthaya province that contained a high content of debris and microbes. The resting water was brown in colour.
RS: Water from the Tamyae Village, Sisaket Province that contained a high content of debris and low amount of microbes. The resting water was slightly brown in colour.
LN: Water from a park lake in Nonthaburi Province, Bangkok area that contained moderate content of debris and microbes. The resting water had no colour and was relatively clear.
RC: Water from the ChaoPraya River sampled in Bangkok that contained a moderate content of debris and microbes. The resting water had no colour and was relatively clear.
KN: Water from a creek in the Nan province, Isan region that contained a large content of debris and a low amount of microbes. The resting water had no colour and was relatively clear.

2.3. Immuno-magnetic bead preparation

The immuno-magnetic beads were prepared as previously reported with modifications (Schreier et al., 2011). Briefly, the purified precipitate was immobilised onto activated carboxylated magnetic particles (FluidMAG-ARA, 0.2 μm/2 × 10^{11} beads per mg, chemicell GmbH, Berlin) by the zero-crosslinker method (Grabarek and Gergely, 1990). The magnetic particles were activated according to the manufacturer's instructions. The conjugation was performed in 0.5 mL of phosphate buffered de-ionised water

(10 mM, pH 4.5) at RT for 1.5 h with 120 μg of antibody per 1 mg of particles. The conjugated beads were stored in storage buffer (10 mM PBS pH 7.4, 5% bovine serum albumin (BSA), 5% glycine, and 0.05% NaN_3) at 4 °C at a final concentration of 2–4 mg/mL. A capture efficiency (CE) quality control test was performed with freshly prepared immuno-magnetic beads as previously described (Schreier et al., 2011) with minor modifications. The CE of the IMS system is expressed as the fraction of bead-bound target leptospires divided by the original quantity of free organisms. Thus, 100% CE refers to complete capture of the target. Briefly, 2 μg of single serovar reactive beads was incubated with 2×10^5 cells of the corresponding serovars in 0.1 mL of pure culture for 30 min under constant rotation at RT. The particles were considered to be highly reactive if the CE was at least 95%, which implies that fewer than 1×10^4 cells were not bound to the beads after magnetic separation.

2.4. Immunoassay detection experiments

Before testing, approximately 50 mL of the water sample was left untouched for 5–10 min to allow for large-particle and debris sedimentation. The supernatant was pipetted into another vial and inoculated with an initial concentration of 10^6 washed leptospires per mL. This initial dilution was further serially diluted 10-fold to final concentrations ranging from 10^4 to 10^1 cells per mL. Two variants of the same fluorescence microscopy assay were tested: (i) an IMS-DFA assay that uses IMS for serogroup specific detection of leptospires and (ii) mIMS-DAOS for multiplexed serovar detection of the Canicola, Rachmati, Ranarum, Sejroe, Bratislava, and Shermani serovars, which are prevalent in Thailand. The assay procedures are outlined in Fig. 1. The inoculated water samples were placed in a 1.5 mL Eppendorf tube, BSA (0.4 mL) was added to a final concentration of 2% (w/v), and 20 μg (IMS-DFA) or 36 μg (mIMS-DAOS) of the immuno-magnetic beads was added. The suspension was incubated at RT for 25 min with constant rotation (~50 rpm). The magnetic beads were subsequently precipitated by placing a strong permanent magnet (~1.3 T) next to the tube wall. The supernatant was removed, and the beads were resuspended in 1.5 mL of wash buffer (10 mM PBS, pH 7.4). The optimal time for magnetic separation and concentration was approximately 4 min. The magnetic beads were thoroughly washed by resuspension, vortexing, and magnetic separation. For IMS-DFA, the bead bound leptospires were resuspended in 3.5 μL of FITC conjugate solution (2 ± 1 mg/mL) and incubated for 15 min in the dark with occasional manual agitation. For mIMS-DAOS, the bead-bound leptospires were stained with 3,6-acridinediamine (acridine orange, AO) (1 mg/mL stock solution in ddH_2O) at a final concentration of 5 μg/mL in 1.5 mL of wash buffer (see Fig. 1) for at least 10 min. The beads were magnetically separated and resuspended in 1.5 mL of wash buffer (10 mM PBS pH 7.4) to remove the unbound fluorescent dye and reduce nonspecific binding. A total of four washes were required for sufficient background reduction. The final volume of the bead suspension was adjusted to 6 μL. The beads were then loaded onto a Neubauer counting chamber slide and observed with an Olympus BX50 fluorescence microscope equipped with FITC filter set (Olympus Optical, Tokyo, Japan).

2.5. Read-out system

The leptospires were manually counted with a fluorescence microscope at 200× and 400× magnification. The counting area (field = 9 mm^2) was adjusted, depending on the initial concentration of leptospiral cells. For concentrations between 10^3 and 10^4 cells per mL, the counting area was 1 mm^2. For 10^2 cells per mL, the counting area was 2 mm^2, and for 10^1 cells per mL, the counting area was 4–9 mm^2. All of the figure data have been normalised to one counting field. The pictures were taken at 200× magnification

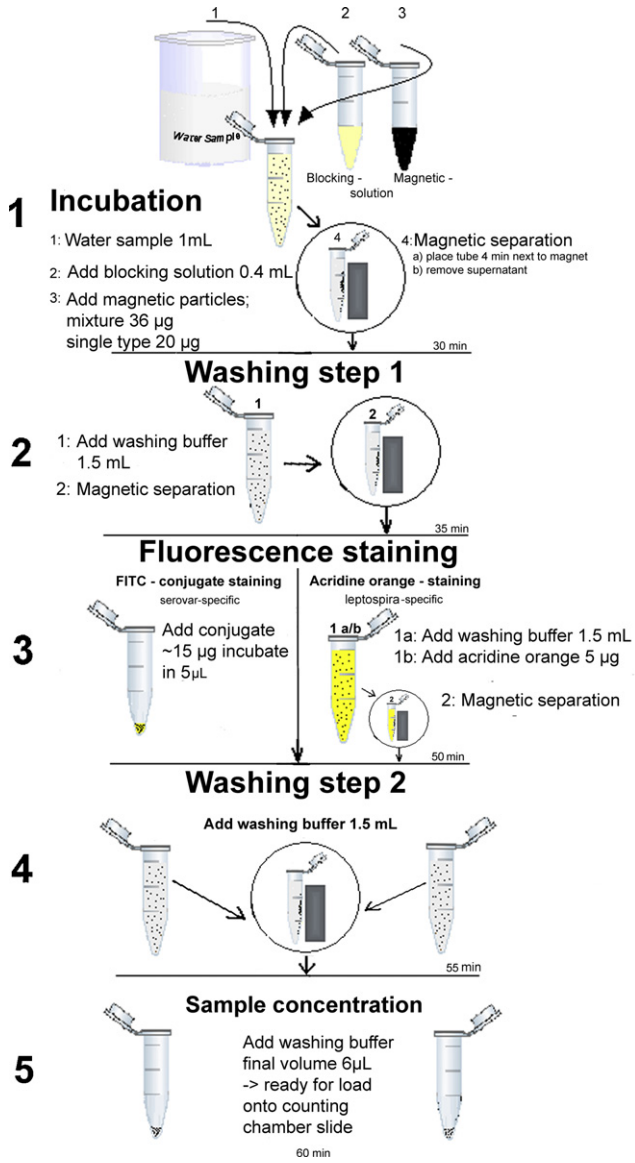

Fig. 1. A diagram of the detection method. Step 1: The sample water is mixed with blocking solution and immuno-magnetic particles. The reaction is incubated for 25 min, and the beads are magnetically separated. Step 2: The beads are resuspended in wash buffer and magnetically separated. Step 3 (IMS-DFA, left and mIMS-DAOS, right): For IMS-DFA, the particles are resuspended in 3.5 μL of the antibody fluorescent conjugate. The reaction is incubated for 15 min, subsequently washed by 1.5 mL with PBS buffer, and magnetically separated: For mIMS-DAOS, the particles are resuspended in 1.5 mL of wash buffer and stained with acridine orange fluorescence dye for 10 min, and the beads are magnetically separated. Step 4: As step 2. Step 5: The particles are concentrated to a final volume of 6 μL.

with a DP50 CCD camera mounted on a BX50 fluorescence microscope; the picture represents an area of approximately 0.075 mm^2, taken at a 1375 × 1200 resolution within 1–3 s of exposure time.

2.6. PCR

2.6.1. Primers

The real-time PCR primers and cycle conditions were previously described by Stoddard et al. (2009). PCR amplification was performed with a BioRad C1000 Thermo Cycler (Bio-Rad Laboratories).

2.6.2. Template DNA preparation

L. interrogans from the Canicola serovar culture was harvested at the log phase of growth and used to inoculate the samples.

Table 2
Particle quality control.

Bead type	Bead CE: target/non-target[a] in %
Anti-Sejroe	80/6
Anti-Bratislava	94/2
Anti-Rachmati	99/1
Anti-Canicola	81/7
Anti-Shermani	80/12
Anti-Ranarum	92/9

[a] Non-target leptospira: serovar Patoc, the bead-bound fraction was determined indirectly by counting the remaining leptospires after incubation with beads and expressed as a percentage in relation to the original amount applied.

The bacterial concentration was determined in duplicate with a Neubauer counting chamber. The water samples were prepared as described by Aviat et al. (2009) with minor modifications. Briefly, 1 mL of PBS, tap water, or fresh water was inoculated with leptospires. Serial tenfold dilutions were made to final concentrations of 2×10^4 to 2×10^1 leptospires per mL. The sample was centrifuged at $1200 \times g$ for 20 s to remove the debris, and 0.95 mL of the supernatant was collected and recentrifuged at $12{,}000 \times g$ for 30 min. The DNA was extracted from the pellet with a QIAamp DNA Minikit (QIAGEN, Germany), eluted in 100 μL of ddH_2O, and finally 5 μL of the dilution was used as the PCR template.

3. Results and discussion

3.1. Assay optimisation

The immuno-magnetic bead capture efficiency (CE) of the fluorescence microscopy assay largely determines the assay accuracy. Therefore, the assay can be optimised by generating highly reactive immuno-magnetic beads. A standardised bead testing procedure was applied to ensure assay quality and consistency. Table 2 shows the CE-values of the Canicola, Rachmati, Ranarum, Sejroe, Bratislava, and Shermani serovar reactive immuno-magnetic beads. The beads and leptospiral concentrations used in the test protocols correlate with the parameters that were used for the IMS-DFA assay. The CE of all of the immuno-beads was between 80% and 99%. Low-quality particles (CE and specificity) may affect the limit of detection and lead to false positives, and the particle quality mainly depends on the reactivity of the antibody that was used to conjugate the particles, as well as the fluorescent dye. Therefore, only highly specific and reactive antibody should be used for these assays.

Initial experiments with untreated environmental water showed substantial fluorescent background for both types of staining, despite rigorous washing. To reduce the background, BSA was added as blocking agent to the beads during the incubation step; BSA blocking significantly reduced the noise level. Thus, we hypothesise that initial signal was due to nonspecific binding to the immuno-magnetic beads, as well as to FITC-labelled antibodies. The addition of BSA as a blocking agent significantly reduced the background signal, negating the need for additional time-consuming pre-treatment steps, such as filtration or centrifugation. The positive correlation between the amount of beads and the degree of nonspecific binding, thus fluorescent background in environmental samples, necessitate a low bead concentration. However, effective target capture also requires an optimal concentration of immuno-magnetic beads in the sample solution. Thus, the maximum particle amount per sample was limited to 40 μg per mL.

3.2. Leptospira detection with IMS-DFA and IMS-DAOS

The RS water samples that were inoculated with the Sejroe or Bratislava serovars at a concentration of 3×10^4 organisms per mL were analysed with IMS-DFA and IMS-DAOS. The fluorescent microscopic read-out of both assays showed that there were numerous crescent-shaped microorganisms with hook-like ends and bright fluorescence intensity (Fig. 2A and B). While FITC staining is by dye conjugation to the leptospire surface components, the AO fluorochrome selectively labels nucleic acids. Both FITC and

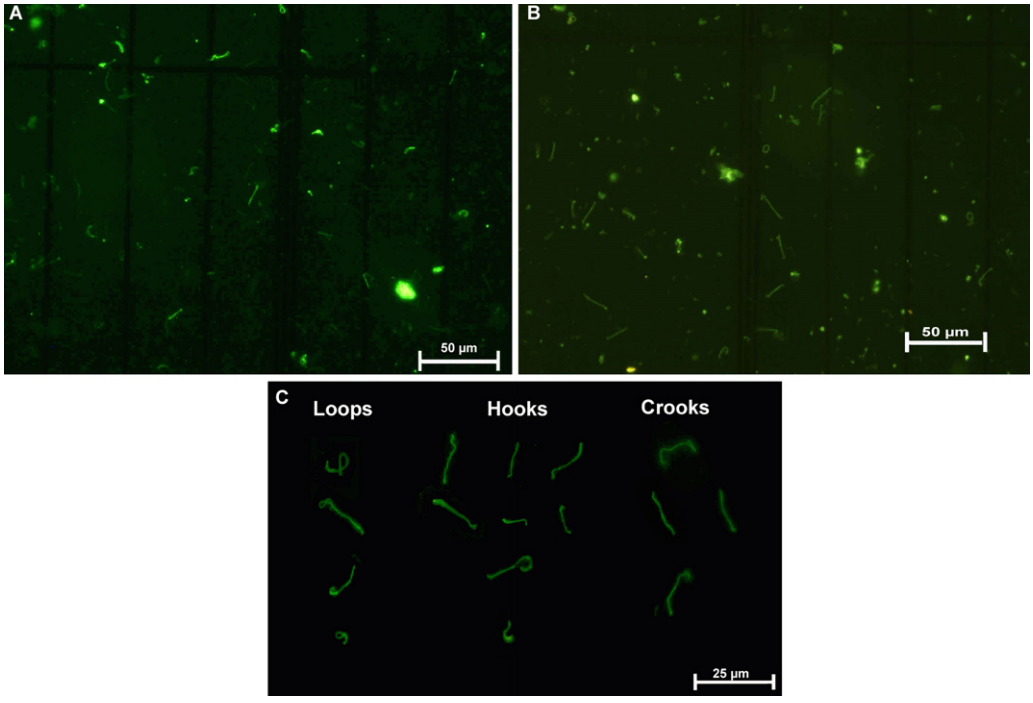

Fig. 2. *Leptospira* cells under a fluorescence microscope detected by IMS-DFA/DAOS. (A) IMS-DFA of a sample inoculated with 3×10^4 serovar Sejroe leptospires per mL, and (B) IMS-DAOS of a sample inoculated with the same concentration of serovar Bratislava (200× magnification). (C) Morphological classification of leptospires based on three identifying characteristics. The picture shows a collection of typical leptospiral morphologies.

AO have a similar excitation and emission spectrum at neutral pHs (Darzynkiewicz et al., 1992), which enables the visualisation of morphologically intact green fluorescent leptospires by both approaches using the same microscopic settings. Previous reports have shown that AO staining can detect other pathogenic bacteria in clinical samples (Lauer et al., 1981), and leptospires stained with AO are visible in blood smears (Kingscote, 1986). However, because there is a relatively high level of nonspecific binding, AO had a higher background than FITC staining. Moreover, AO staining may create artefacts that resemble *Leptospira* and may result in false positives. Therefore, the correct interpretation of the fluorescent signal depends on two criteria. The first characteristic is a distinctive morphological pattern, as is shown in Fig. 2C. In addition to a thin rod-like appearance, leptospires are characterised by a curved or/and looped bodies and hook-like ends that are visible at 400× magnification. The second criteria for positive fluorescence identification is an even fluorescence staining intensity. Leptospires are brightly stained compared to the background (DFA and AO), and the staining intensity is equally distributed along the bacterial cell without interruption.

3.3. Effects of multiplexing

IMS-DAOS was further modified to capture a mixture of different leptospiral serovars and to facilitate sample screening. For this procedure, a mixture of serovar-specific immuno-magnetic beads (mIMS) was used. Recent reports have shown that mIMS can be used to simultaneously detect different human pathogens (Kim et al., 2010; Tu et al., 2010). The mIMS-DAOS assay, which is capable of simultaneously detecting three or six serovars, was compared to the IMS-DAOS assay, which can only detect one serovar. The efficiency of detecting the Rachmati, Bratislava, and Sejroe serovars in CS water samples with mIMS decreased with an increasing number of mixed serovar-specific IMS-types (Fig. 3), which is expected because mixing the beads together dilutes each individual type of bead. However, the decrease in capture was not proportional to the number of different beads types in the test. The decrease in capture from the mono-bead system to the 3-type multiplexed system was markedly larger than the decrease from 3-type to 6-type multiplex systems. This result suggests that there is a nonlinear correlation between the quantity of different beads and the CE. Moreover, expansion of the multiplex system with additional serovars may not be successful. The CE of beads against serovar Rachmati was higher compared to other serovars (Table 2), and consequently there was a significantly increased overall cell count for Rachmati compared to the Sejroe and Bratislava serovars (Fig. 4). Although the CE was 94% for the anti-Bratislava beads, the count dropped dramatically when the beads were applied to a multiplex system.

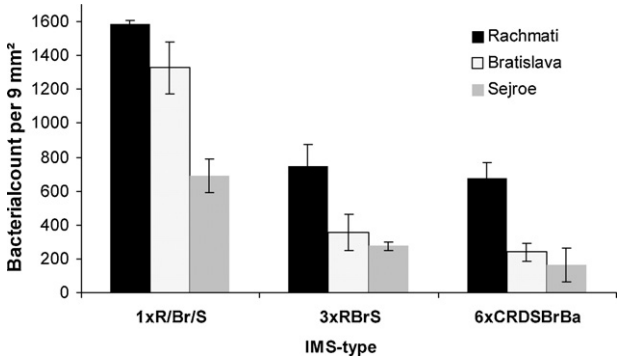

Fig. 3. A Comparison of the mIMS-DAOS capture efficiency using 3 different IMS-types. CS water samples that were inoculated with 3 different *Leptospira* serovars at a concentration of approximately 3×10^4 leptospires per mL: Rachmati (black), Bratislava (speckled), and Sejroe (grey). The leptospires were incubated with a single serovar type, and 2 types of multiplexed beads were added (36 μg of each bead type). The single bead types were reactive against serovars Rachmati (1xR), Bratislava (1xBr), and Sejroe (1xS). The 3-fold multiplexed beads were simultaneously reactive against serovars Rachmati, Bratislava, and Sejroe (3xRBrS), and the 6-fold multiplexed beads (6xCRDSBrBa) were reactive against the previously mentioned serovars, as well as the Canicola, Ranarum, and Shermani serovars. The number of leptospiral cells within a 4-mm² area of the counting chamber area was manually counted, and the data were normalised to a 9-mm² area. The data shown are the mean of 3 independent experiments.

Thus, we suggest that only highly reactive beads (CE >97%) be used for a multiplexed IMS system.

3.4. Detection limit

The lower limit of detection (LLOD) was defined as the appearance of morphologically intact leptospires at the lowest concentration. For both types of assays, non-inoculated CS and RS water samples were tested prior to LLOD analysis to ensure that no leptospire-like structures were present. Because it is unlikely that concentrations greater than 10^5 leptospires per mL are present in the environment (Ganoza et al., 2006), our fluorescence immunoassay dynamic range was from 10^1 to 10^5 leptospires per mL. Although theoretically, one leptospire cell per mL of sample can be detected by studying the entire slide area (~60 mm²), we chose to analyse a 9-mm² area on the counting grid. The recovery rate did not exceed 35% (value obtained from experiments with IMS-DFA assay), which results in the LLOD of approximately 20 cells per mL. The number of leptospires visible under the fluorescence microscope at each concentration appeared to depend on the sample type. The rural water samples generally had higher counts of leptospires compared to the urban samples (Fig. 4A). It is possible that antibody binding was nonspecifically blocked by various

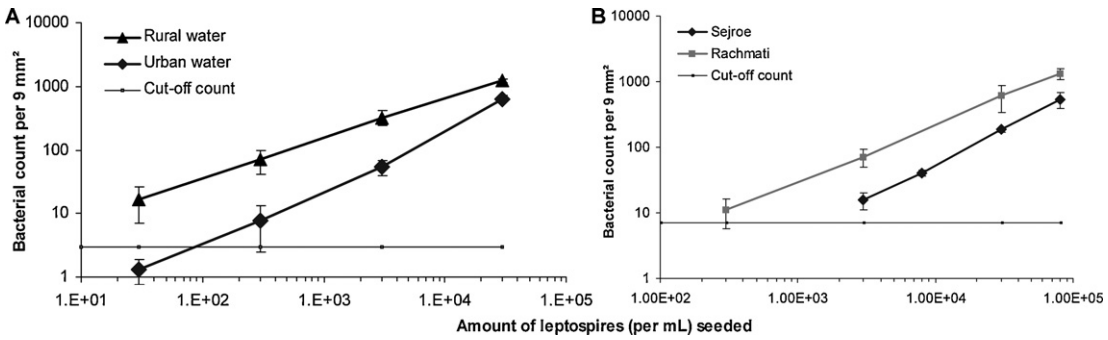

Fig. 4. The detection limits of the assay. (A) IMS-DFA assay; the inoculum concentration of *Leptospira interrogans* serovar Sejroe in two different sources of water – RN (▲), and CS water (♦) – was increased tenfold from 3×10^1 to 3×10^4 per mL. (B) mIMS-DAOS assay; 1 mL of CS water that was inoculated with 3×10^2 to 8×10^4 cells per mL of serovar Rachmati (■) and Sejroe (♦). The sample was incubated with a mixture of 6 individual beads that were reactive against serovar Canicola, Rachmati, Sejroe, Bratislava, Ranarum, and Shermani. The data are the cell counts for a chamber slide area of 9 mm² and represent the mean of 3 independent experiments.

materials in the urban samples, which may prevent the binding leptospires to the immuno-particles.

To determine the detection limit of the multiplexed IMS system, 6-fold multiplexed immuno-magnetic beads were used to analyse CS water samples inoculated with Rachmati and Sejroe serovar (Fig. 4B). The LLOD was 100-fold higher for Sejroe detection and 10-fold higher for Rachmati detection compared to the LLOD of the IMS-DFA assay (Fig. 4A). This increase in the LLOD is associated with a marked decrease in the cell count with the 6-fold mIMS (Fig. 3). As was mentioned earlier, our data indicate that it is essential to use beads with a CE greater than 97% for the multiplexed system to keep the LLOD low.

The specificity of the Sejroe, Bratislava and Canicola serovar IMS-DFA and mIMS-DAOS assay was evaluated on sterile PBS that was inoculated with the non-target serovar Patoc at a concentration of 2×10^4 cells per mL. A low level of nonspecific binding was observed, numbering 2 ± 1 cells per field in the IMS-DFA test and a maximum number of 6 ± 1 cells per field were obtained in the mIMS-DAOS test. To control false positivity, we defined a cut-off count of 3 leptospires per field for environmental water samples in the IMS-DFA assay and 7 leptospires per field in the mIMS-DAOS assay, respectively. We observe a slightly lower specificity compared to IMS-DFA because the mIMS-DAOS does not use secondary antibody staining for identification and is therefore less specific.

3.5. Assay evaluation

Based on the initial encouraging results, we performed a short pilot study on 8 different quality water samples (Table 3). The natural untreated water samples were analysed by DFM, IMS-DFA (against serovar Canicola), mIMS-DAOS, and *LipL32* real-time PCR. DFM analysis indicated the presence of leptospires at a concentration of approximately 2×10^4 cells per mL in the CS water and 5×10^3 cells per mL in the CA water. However, DFM does not yield any information regarding pathogenic leptospires, because pathogenic and non-pathogenic leptospires are indistinguishable by morphology. It is likely that these samples contain only non-pathogenic leptospires. All the water samples to be tested were negative according to pathogen-specific PCR and both types of immunoassay when the previously defined cut-off count criteria for unknown samples were applied (Fig. 4A and B).

To analyse the assay sensitivity, sterile PBS, tap water, and six different environmental water samples were experimentally inoculated with the serovar Canicola at serial tenfold dilutions ranging from 2×10^4 to 2×10^1 cells per mL. Both the PCR and the immunoassays were repeated at least three times for each sample. IMS-DFA assay and PCR were positive over the entire concentration range in PBS. In contrast to IMS-DFA, PCR could not detect the lowest inoculation concentration in 50% of the tests. The lowest detectable concentration by both techniques was 20 cells per mL with the experimental settings used in this study. For PCR, this minimum detection limit corresponds to 1 genomic copy in the PCR mixture. The mIMS-DAOS assay did not detect the lowest inoculation concentration, which agrees with the previously defined LLOD values. The PCR assay and both immunoassays were positive for concentrations greater than 2×10^3 cells per mL for all of the water samples. PCR and immunoassay detection was decreased in the water samples that had debris and other microbes, such as the CS and CA samples (Table 3). The data indicate that PCR is more influenced by the environmental sample composition than the immunoassay, as the lowest detectable concentration was increased by at least one order of magnitude for all of the fresh water samples. PCR may be inhibited by the presence of inhibitory substances in the samples that affect DNA template preparation and amplification. For the immunoassays, our data have indicated that water quality reduces the counts per field and increases the

Table 3
Assay results of evaluation study.

Spiking conc. per mL		PBS	Tap water	CS-type water	CA-type water	RS-type water	LN-type water	RC-type water	KN-type water	Positive samples
		pos.(28.3)	pos.(29.9)	pos.(29.5)	pos.(29.4)	pos.(31.1)	pos.(29.8)	pos.(30)	pos.(30.7)	
2.0E+4		pos.(968)	pos.(719)	pos.(378)	pos.(396)	pos.(981)	pos.(846)	pos.(540)	pos.(1080)	100%
		pos.(621)	pos.(225)	pos.(99)	pos.(212)	pos.(153)	pos.(639)	pos.(198)	pos.(846)	
		pos.(30.3)	pos.(33.2)	pos.(31.4)	pos.(32.2/50%)	pos.(34.6)	pos.(34)	pos.(34.1)	pos.(34)	
2.0E+3		pos.(162)	pos.(189)	pos.(99)	pos.(23)	pos.(108)	pos.(86)	pos.(108)	pos.(108)	100%
		pos.(63)	pos.(72)	pos.(18)	pos.(7)	pos.(54)	pos.(36)	pos.(54)	pos.(54)	
	PCR[a] (Ct-value)	pos.(33.8)	pos.(37.8/80%)	neg.	neg.	pos.(37.7/50%)	neg.	neg.	pos.(36.8/60%)	50%
2.0E+2	IMS-DFA[b] (counts/field)	pos.(9)	pos.(16)	pos.(6)	pos.(3)	pos.(9)	pos.(5)	pos.(11)	pos.(18)	100%
	mIMS-DAOS[c] (counts/field)	pos.(9)	pos.(9)	neg.(2)	neg.(1)	pos.(7)	pos.(2)	pos.(4)	pos.(13)	75%
		pos.(36.5/50%)	neg.	neg.	neg.	neg.	neg.	neg.	neg.	12.5%
2.0E+1		pos.(2)	pos.(1)	neg.(0)	neg.(0)	pos.(1)	pos.(1)	pos.(4)	pos.(1)	75%
		neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	0%
		neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	
No spiking		neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.(0)	neg.
		neg.(0)	neg.(0)	neg.(2)	neg.(1)	neg.(2)	neg.(0)	neg.(2)	neg.(0)	

[a] PCR values are occasionally presented with adjacent percentage stating the fraction of positive runs.
[b] Conjugated antibodies reactive against serovar Canicola.
[c] 6-Fold multiplexed IMS.

immunoassay background signal, especially with AO staining and mIMS.

4. Conclusions

Few studies have described the distribution of leptospires in the environment, potentially due to a lack of accurate, simple, and inexpensive techniques for monitoring contamination. Thus, new assays are needed to detect leptospire contamination of environmental water sources. In this study, we modified a practical detection method for environmental sample analysis by combining traditional fluorescence microscopy with microparticle technology. This combination enables the detection of pathogenic leptospires in natural water samples at concentrations of at least 10^1 cells per mL within a short time. Two variations of the fluorescence assay can be applied to target an individual or multiple leptospiral serovar simultaneously; however, it should be noted that the multiple detection approach has a lower accuracy. Based on our results, we believe that the modified immunoassay can be utilised to monitor leptospire contamination of environmental water sources. Future studies are needed to further evaluate the assay by comparing this new approach to conventional cultivation and PCR analysis in the framework of an environmental field study.

Conflict of interest

The authors declare no conflicts of interest.

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