Rapid microfluidic immunoassay for surveillance and diagnosis of *Cryptosporidium* infection in human immunodeficiency virus-infected patients

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Cryptosporidiosis has been reported to be associated with HIV/acquired immune deficiency syndrome, which greatly reduces the quality of life and shortens the life expectancy of HIV-infected patients. In order to properly treat the infected patients, accurate and automatic diagnostic tools need to be developed. In this study, a novel microfluidic immunochip system was presented for the surveillance and the rapid detection of *Cryptosporidium* infection in 190 HIV-infected patients from Guangxi, China, using the P23 antigen of *Cryptosporidium*. The procedure of detection can be completed within 10 min with 2 μ l sample consumption. The system also was evaluated using the standard ELISA method. Among 190 HIV-infected individuals, the rate of P23 positivity was 13.7%. Seropositivity in HIV-infected individuals was higher in female patients. The seropositivity to P23 was higher in HIV-infected individuals with high viral load, although the difference was statistically insignificant. Significantly higher *Cryptosporidium* seropositivity was observed in HIV-infected individuals with a CD4⁺ T-cell count of <200 cells/ μ l than in those with $\geq$ 200 cells/ μ l. Our results also demonstrate that a lower CD4⁺ T-cell count may reflect an increased accumulated risk for cryptosporidiosis. The detection system was further validated using the standard ELISA method and good correlation between the two methods was found ($r = 0.80$). Under the same sensitivity, this new microfluidic chip device had a specificity of 98.2%. This developed system may provide a powerful platform for the fast screening of *Cryptosporidium* infection in HIV-infected patients. © 2015 AIP Publishing LLC.
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INTRODUCTION

The increase in awareness of acquired immune deficiency syndrome (AIDS) has led to extensive studies on opportunistic protozoan parasites, such as *Cryptosporidium* spp., *Isospora* spp., and *Microsporidia* spp.¹ *Cryptosporidium* is one of the most common enteric protozoan parasites in vertebrates with a wide range of hosts including humans and domestic animals.² Cryptosporidiosis may be a devastating disease in AIDS patients with severely impaired immunity; the disease not only causes chronic, severe, and intractable diarrhea, which greatly reduces the quality of life of patients, but also significantly shortens life expectancy.^{3–5} In China, about 780 000 individuals were infected with human immunodeficiency virus (HIV) as of December

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2011 in China and this number has increased rapidly in recent years.⁶ Investigation of the influence of HIV-induced immunodeficiency on the epidemiology and outcome of *Cryptosporidium* infection is therefore of significant public health interest. Unfortunately, limited information is available on the epidemiology of cryptosporidiosis among individuals infected with HIV in China. Data on correlations of CD4⁺ T-cell levels and HIV/AIDS status with the prevalence of this opportunistic protozoan parasite are also scant.

A major challenge in this effort has been the detection of *Cryptosporidium*. Several methods ranging from fecal examination with acid-fast stains to various staining techniques and other modifications are cumbersome and impractical for epidemiological studies because of the short duration of oocyst excretion and poor sensitivity of the procedure.^{7,8} Specific detection of *Cryptosporidium parvum* by polymerase chain reaction (PCR) features high sensitivity, but the approach is limited to well-equipped laboratories because of the high cost and advanced technique involved. Therefore, immunoassays with specific antigens of *C. parvum* may be viable alternative approaches for determining the cryptosporidiosis.

Immunoassays such as Western blotting and ELISA have been applicable for epidemiologic surveillance in recent years.^{9–12} In some immunoassays, P23 (also known as Cp23, p27, or 27-kDa antigen) was used as a captured antigen, which is a major target of antibody responses elicited by infective stages of the parasite, and many studies have verified its value in epidemiologic studies of *C. parvum* infections.^{11,13,14} However, immunoassays such as Western blotting and ELISA are always the drawbacks for the relatively time and labor-intensive protocols which require highly skilled operators. Recently, microfluidic system which manipulates small volumes of fluids within microscale channels has been demonstrated as an efficient detection platform for rapid and automatic diagnosis of samples. This platform could be programmed into either manual or automatic mode, requires very little professional knowledge or special skills to be operated, provides a possibility of rapid parallel automatic analysis for multiple samples with higher analysis speed and lower reagent/power consumption in comparison with the conventional ELISA kit.^{15,16} Due to its good performance, several microfluidics-based biosensing methods have recently been developed for the rapid and quantitative detection of microbes and large molecules.^{17–20} Furthermore, microfluidic chip devices performing high complexity assays may bring confirmatory testing from the specialized laboratories to the point of care (POC) setting.^{17,21–23}

In the previous work, we selected the *Entamoeba histolytica* as a model to prove the reliability and diagnostic value of this immune-microfluidic diagnostic device.²⁴ In this work, as the continuing study, the novel integrated microfluidics-based diagnostic device was used for surveillance and diagnosis of *Cryptosporidium* infection in HIV-infected patients. This is to our knowledge the first example of a diagnostic test for detecting the prevalence of *Cryptosporidium* and studying its association with the immune status of HIV/AIDS patients in China. Moreover, the detection system was validated using the standard ELISA method. Unlike the standard ELISA assay, however, the microfluidic chip can perform these procedures with minimal equipment, can be completed within 20 min and uses only several microliters of blood sample, which can be obtained on site from a needle prick.

MATERIALS AND METHODS

Study cases and data collection

This study was approved by the Medical Ethics Committee of Fudan University Shanghai Medical College. Serum samples from 190 known HIV-positive patients who either complained of diarrhea or had a history of diarrhea were obtained at the Third People's Hospital in Guilin, Guangxi Province. All serum samples were inactivated at 56 °C for 30 min and stored at –80 °C until use.

A standardized form was used to collect information, including the following: (1) socio-demographic characteristics (gender and age) and (2) clinical information, such as virus load and CD4⁺ T-cell counts. The HIV-1 DNA load in plasma was measured by the Human Immunodeficiency Virus Type 1 (HIV-1) Fluorescent PCR Diagnostic Kit (DAAN Gene Co., Ltd. of Sun Yat-sen University, China). Quantification of CD4⁺ lymphocytes in individuals

with HIV/AIDS were determined by flow cytometry (BD Biosciences, Franklin Lakes, NJ) according to the instructions provided by the manufacturer.

Preparation of recombinant P23

The P23 gene was amplified by PCR from *C. parvum* genomic DNA using primers 5'-CCCATATGATGGGTTGTTTCATCATCAAAGCC-3' (forward primer) and 5'-CCCTCGAGTTAGGCATCAGCTGGCTTG-3' (reverse primer) designed from Gen-Bank sequence U34390. The PCR product was digested with *Nde* I and *Xho* I, then purified and cloned into the expression vector pET19b (Novagen, Madison, WI). Plasmid containing the correct sequence was introduced into *Escherichia coli* BL21 Star (DE3) pLysS (Invitrogen, Carlsbad, CA). Bacteria were grown in Luria broth containing 100 µg/ml ampicillin. When the optical density at 600 nm (OD₆₀₀) of the culture reached 0.6, isopropyl thiogalactopyranoside was added to a final concentration of 1 mM. Cells were further incubated for 3 h and harvested by centrifugation at 10 000× g for 10 min at 4 °C. The P23 antigen was further purified from the soluble fraction using His•Bind resin (His•Bind Kit; Novagen) according to the manufacturer's instructions. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoassay process with microfluidic chip platform

As the continuing study of microfluidic device for diagnosis of *Entamoeba histolytica*,²⁴ the current microfluidic platform has similar structure, which consists of a core microfluidic chip, cartridges for reagents, an injection pump, a fluorescence microscopy, and a digital camera. The core microfluidic chip was designed by AutoCAD and fabricated from polydimethylsiloxane (PDMS) based on validated microfluidic components and engineering methods. The integrated microfluidic devices contain functional elements such as valves, pumps, and columns that allow the precise delivery, mixing, and movement of reagent. As shown in Fig. 1(A), half-closed sieve valve blocks off microspheres in the reaction channel to form an immuno-column. The microfluidic chip contains five microcolumns to perform 5 parallel analyses. Various samples were loaded from the top 5 parallel blue channels separately and reagents were loaded from the top channels and left blue channels. Each immune-column has dimensions of length

FIG. 1. Schematic diagram of the microfluidic device. (A) Illustration of the valve and sieve valve. (B) Progress of the immunofluorescence assay within the microfluidic chip.

5 mm, width 200 μm , and thickness 35 μm . The volume of the column is about 35 nl. The detailed operation mechanism of the various valves as well as the operation procedure of the microfluidic chip was described in detail in Ref. 24. Briefly, pressures in the different control channels are individually controlled by specific solenoid valves, which are digitally controlled by a computer through a program. During diagnosis using the microfluidic chip, the key components are the immune columns filled with P23-coated microspheres. Schematic diagrams of the indirect immunofluorescence assay process are shown in Fig. 1(B). Protein A-coated microspheres with a diameter of 9 μm could bind with anti-His monoclonal antibodies, and the microspheres could bind with P23 with a 6 $\times$ His tag.¹⁸ The microspheres with P23 are preloaded into the columns, ready for use in the rapid analysis of patient serum samples. 2 μl of the serum samples (50 times dilution) were pumped into the reaction column, and then the fluorescein isothiocyanate-conjugated goat anti-human polyclonal antibody was added into the column after the microspheres were washed by PBS-Tween. The reaction solution was pumped into the device using the syringe pump at a constant flow rate. Finally, the fluorescence image was collected by a fluorescence microscope. The results were analyzed by Image Pro Plus 6.0 (IPP6.0). The cut-off for a positive result was defined as a fluorescence intensity >3 SD above the mean for healthy negative controls.

ELISA

A 96-well ELISA plate (Corning Incorporated, USA) was coated with 100 ng/well recombinant P23 in 50 mM sodium bicarbonate buffer (pH 9.6) and incubated at 4 °C overnight. The plate was washed with PBS-Tween and then treated with PBS containing 1% skim milk for 1 h. A total of 100 μl of the serum samples diluted 1:400 with PBS was added to each well, followed by incubation for 1 h at room temperature. The wells were washed with PBS-Tween and incubated with Horse-Radish Peroxidase (HRP) conjugated goat antibody to human IgG (whole molecule; ICN Pharmaceuticals) for 1 h at room temperature. After being washed with PBS-Tween, the wells were incubated with 200 μl of substrate solution (0.4 mg per ml of o-phenylenediamine in citric acid-phosphate buffer [pH 5.0] containing 0.001% hydrogen peroxide). After 30 min of incubation, the reaction was terminated by the addition of 2.5 M H_2SO_4 and the OD490 values were read with a model 550 microplate reader (Bio-Rad, Hercules, Calif). The mean value of three replicates of each sample was calculated. Each sample was repeated in three wells and the mean value was calculated. Data were analyzed using the computer graphics software Prism version 4.0 (GraphPad, San Diego, CA). The cut-off for a positive result was defined as an ELISA score >3 SD above the mean for healthy negative controls.

Statistical analysis

Data entry and analysis were performed using SPSS version 13 software for Windows (SPSS, Chicago, IL). Data on qualitative variables were indicated by frequency and percentages, and quantitative variables were indicated by range and mean. Associations were verified by Pearson χ^2 test with a level of significance of $P < 0.05$.

RESULTS

Descriptive analysis

In total, 190 HIV-positive patient samples were used in the study. All patients were adults (age range = 20–74 years; mean age = 43.03 ± 13.51 years; median = 40 years), with CD4^+ T-cell counts ranging from 0 to 503 cells/ μl (mean = 136.55 ± 110.05 cells/ μl ; median = 124 cells/ μl) and HIV viral loads ranging from 0 to 5.79 $\log_{10}$ copies/ml (mean = 3.47 ± 1.59 $\log_{10}$ copies/ml; median = 3.97 $\log_{10}$ copies/ml). One hundred twenty-one patients were males (63.7%) while 69 were females (36.3%).

Immunoassay in the microfluidic device and the seroprevalence of *Cryptosporidium* infection in HIV-infected patient

Recombinant P23 was prepared in *E. coli* as a soluble protein. After expression, the recombinant protein was purified and analyzed by SDS-PAGE. The apparent molecular mass of the protein was found to be 27 kDa. The antigenicity of the recombinant P23 was confirmed by dot blot analysis using serum samples with cryptosporidiosis (data not shown). All the serum samples of patients with HIV/AIDS were analyzed in the microfluidic device. Protein A-coated microspheres with a diameter of 9 μm could bind with anti-His monoclonal antibodies, and the microspheres could bind with P23 with a 6 $\times$ His tag.¹⁸ The microspheres with P23 are pre-loaded into the columns, ready for used in the rapid analysis of patient serum samples. About 2 μl of the serum samples (50 times dilution) were pumped into the reaction column, and incubated at room temperature. Fluorescein isothiocyanate-conjugated goat anti-human polyclonal antibody was added into the column after the microspheres were washed by PBS-Tween. The reaction solution was pumped into the device using the syringe pump at a constant flow rate. The flow rate on microfluidic chip was constant (2 $\mu\text{l}/\text{min}$). Finally, the fluorescence image was collected by a fluorescence microscope and the results were transformed into digital data using IPP software. Different incubating time for the diagnosis has also been investigated and no significant difference has been found for various time periods. The total diagnosis time for washings, color development, and detection was about 5 min. Thus, the total assay time for our assay was calculated by adding the incubation time with 5 min.

In HIV-infected individuals, 26 serum samples reacted positively with P23. The cut-off value of fluorescence intensity was 52.7. The seroprevalence of P23 was 13.7%. P23 seroprevalence was higher in patients with a CD4⁺ T-cell count of <200 cells/ μl compared with those with a count of ≥ 200 cells/ μl ($P < 0.05$). Seropositivity to the P23 antigen was more prevalent among female subjects (20.3%) than male subjects (9.9%), and the difference was statistically significant ($P = 0.01$). The seropositivity to P23 was higher in HIV-infected individuals with a viral load of $\leq 4 \log_{10}$ copies/ml than in those with a viral load of $> 4 \log_{10}$ copies/ml, although the difference was statistically insignificant ($P = 0.439$). These findings are similar to those based on gender as described above. No statistically significant association was observed among different age groups and the presence of *Cryptosporidium* in HIV-infected patients (Table I).

Validation of the integrated microfluidic immunoassay

In order to evaluate the immune-assay microfluidic chip, all the serum samples of patients with HIV/AIDS were reconfirmed by the standard ELISA method. We compared our microfluidic chip immunoassay with conventional 96-well microplate ELISA, good correlation between

TABLE I. Seropositivity to *Cryptosporidium* of patients with HIV/AIDS in China. OR, odds ratio; CI, confidence interval.

Characteristics	n	Positive (%)	OR (95% CI)	p value
Sex	Total	190	26 (13.7)	0.010
	Female	69	14 (20.3)	
	Male	121	12 (9.9)	
Age	20–29 years	34	4 (11.8)	0.451
	30–39 years	56	8 (14.3)	
	40–49 years	43	3 (7.0)	
	50–59 years	27	6 (22.2)	
	≥ 60 years	30	5 (16.7)	
CD4 ⁺	$\geq 200 \text{ cell}/\text{mm}^3$	64	6 (9.4)	0.029
	$< 200 \text{ cell}/\text{mm}^3$	126	20 (15.9)	
HIV viral load	$\leq 4 \log_{10}$ copies/ml	95	15 (15.8)	0.439
	$> 4 \log_{10}$ copies/ml	95	11 (11.6)	

these methods was found ($r = 0.80$). Compared to the standard ELISA, the microfluidic-based diagnostic sensitivity and specificity was 100% and 98.2%, respectively (Fig. 2). As shown in Fig. 3, P23-positive serum samples were numbered 1 to 26. The detection result was marked by fluorescence intensity in the reaction column, with which the distinction between positive sample and negative sample was intuitively displayed in Fig. 3(A). One to one comparison between microfluidic-based method and standard ELISA for detecting the 26 microfluidic immunoassay-positive samples are shown in Fig. 3(B). In 26 P23-positive serum samples detected by microfluidic chip, 3 serum samples were detected negative by standard ELISA. However, the OD values of these 3 serum samples were just slightly lower than the cut-off value of ELISA.

DISCUSSION

This study is the first to report the high seroprevalence of *Cryptosporidium* infection in patients with HIV/AIDS in China. In other countries, such as India, Australia, and USA, *Cryptosporidium* infection is common among people with HIV/AIDS. Soave found *Cryptosporidium* antibodies using ELISA in 25 (20.3%) out of 137 HIV-infected individuals in New York City.²⁵ A similar trend was recently found in HIV-infected patients in San Francisco, with an estimated infection rate of 230 infections/1000 person-years.²⁶ However, little has been reported on *Cryptosporidium* infection among HIV-positive individuals in China. A survey of HIV/AIDS patients from several regions of Guangdong and Yunnan Provinces showed *Cryptosporidium* infection rates of 4% and 4.65%, respectively, by microscopic examination of stool samples.²⁷ One of the reasons is that microscopy has low sensitivity and specificity for the detection of these parasites. Another reason may be that the immune response to *Cryptosporidium* antigens will last for several years after infection, seroprevalence estimates may not only included recent infection but also the one that may have occurred in the distant past.^{14,26} In our current study, the stool samples of P23 positive HIV-infected patients were collected, only 2 samples were *Cryptosporidium* oocyst antigen positive (data not shown). It demonstrated that some of the P23 positive HIV-infected patients had been infected with *Cryptosporidium*. This may explain in part that why the seroprevalence of *Cryptosporidium*

FIG. 2. Results of the assay within the microfluidic chip: all the serum samples of patients ($N = 190$) with HIV/AIDS were detected by the microfluidic-based platform. The results were transformed into digital data by IPP software (Y-axis) and compared from standard ELISA (X-axis). Dashed lines indicate cut-off values.

infection in our study was much higher than the *Cryptosporidium* infection rates detected in stool samples in previous studies.

Many studies have focused on the importance of viral factors in the natural history of HIV, particularly the association between lymphoid tissues ($CD4^+$ cell count) and high levels of HIV viral loads in plasma and poorer prognosis. However, some controversies about the associations between the presence of *Cryptosporidium*, $CD4^+$ T-cell levels, and HIV viral loads among individuals with HIV/AIDS remain. Our results showed that patients with $CD4^+$ T-cell count less than 200 cells/ μ l of blood are at greater risk of acquiring *Cryptosporidium* infections, which is in agreement with some previous studies.^{28–30} This result may be due to the increased susceptibility of immunodeficient patients to acquire particular parasites and/or their inability to clear established infections.³¹ Interestingly, Schmidt reported that a rapid increase in mucosal $CD4^+$ T-cell count was followed by clearance of intestinal cryptosporidiosis in a patient with AIDS receiving Highly Active Antiretroviral Therapy.³² This result also highlights the relationship between *Cryptosporidium* infection and $CD4^+$ T-cell count in HIV-positive patients.

A previous study demonstrated an increase in HIV viral loads in patients with acute opportunistic diseases.³³ However, we found no significant association between HIV viral loads and the presence of *Cryptosporidium* in our study. Nevertheless, the seropositivity to P23 of HIV-infected individuals with viral loads $\leq 4 \log_{10}$ copies/ml was higher than of those with viral loads $> 4 \log_{10}$ copies/ml. Additionally, we found a significantly higher prevalence of serological responses in females than males among HIV-positive individuals. This observation is in accordance with the results from the National Health and Nutrition Examination Survey (NHANES), which demonstrated that females have higher seropositivity to the parasite, due perhaps to the higher involvement of women in taking care of children.³⁴ No statistically significant association was found between HIV viral loads and acquisition of Cryptosporidiosis in HIV-positive individuals. Further studies with a larger number of HIV-infected adults with extensive follow-up data must be carried out to further confirm these findings.

FIG. 3. One to one comparison between microfluidic-based method and standard ELISA for detecting P23-positive samples: (A) Fluorescent images of the columns inside the microfluidic chip for P23-positive serum samples were captured and (B) fluorescent intensity was analyzed. Sample No. C (median value of the results from P23-negative samples) was a negative control. In 26 P23-positive serum samples detected by microfluidic chip, 3 serum samples were detected negative by standard ELISA. However, the OD values of these 3 serum samples are just slightly lower than the cut-off value of ELISA (the straight lines represent the cut-off values of the two detections).

Rapid, sensitive, and cost-effective detection of *Cryptosporidium* from patients with HIV/AIDS is critical for effective treatment and to limit the spread of infection. Recently, the microfluidic systems currently under development to address a variety of medical problems^{17,35} have been used for the diagnosis of amoebiasis and malaria in our laboratory.²⁴ In this study, we miniaturized an immunoassay into a microfluidic chip to reduce the testing cost, decrease the turnaround time and move the immunoassay closer to true POC testing. The results were also confirmed by the standard ELISA. The results showed good correspondence with the traditional ELISA results. Although 3 microfluidic immunoassay positive serum samples were detected negative by standard ELISA, the OD values of these 3 serum samples are just slightly lower than the cut-off value of ELISA. The difference between positive samples and negative samples detected by microfluidic immunoassay was much higher than that detected by ELISA. This may be due to the detection of microfluidic immunoassay with the fluorescein isothiocyanate-conjugated antibody which is more sensitive than the HRP conjugated antibody. Compared with the traditional ELISA method, diagnosis using the microfluidic device took less than 20 min with only 2 μ l sample consumption and little reagent consumption. The high efficiency might be attributed to the high surface modification efficiency by using beads as well as the advantages from microfluidic device itself. The p23 modified microspheres can be easily prepared in 1 h and preloaded inside the microdevice for convenient application. The device is made from standard soft lithography by PDMS and its throughput can be easily improved by adding more micro columns into the microfluidic device in an economic manner, which is perfect for the onsite rapid and cheap diagnosis of Cryptosporidiosis. In particular, the high sensitivities of the microfluidic immunoassay for diagnosis of *Cryptosporidium* infection are useful for screening in remote settings and potentially infected patients can be diagnosed immediately. To further improve the sensitivity, better recombinant protein and better second fluorescent antibody shall be utilized. More dense and homogeneous surface modification techniques to immobilize the proteins onto the microsphere surface might also improve its sensitivity.

In conclusion, serological assays are important tools for monitoring infection, especially for organisms such as *Cryptosporidium* that may otherwise go undiagnosed.²⁶ Here, we used a novel microfluidic immunoassay to detect the seropositivity of *Cryptosporidium* infection. Compared with ELISA, the microfluidic immunoassay on a microfluidic chip has some advantages, particularly for the high speed, high sensitivity, automation, and integration. Further development of this system to allow on-chip sample processing could present a realistic technology for surveillance and diagnosis of *Cryptosporidium* infection at the site of patient care.

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¹H. L. Dupont and G. D. Marshall, "HIV-associated diarrhoea and wasting," *Lancet* **346**, 352–356 (1995).

²R. C. Thompson, M. E. Olson, G. Zhu, S. Enomoto, M. S. Abrahamsen *et al.*, "*Cryptosporidium* and cryptosporidiosis," *Adv. Parasitol.* **59**, 77–158 (2005).

³Y. C. Manabe, D. P. Clark, R. D. Moore, J. A. Lumadue, H. R. Dahlman *et al.*, "*Cryptosporidium* in patients with AIDS: Correlates of disease and survival," *Clin. Infect. Dis.* **27**, 536–542 (1998).

⁴F. Petry, V. Jakobi, and T. S. Tessema, "Host immune response to *Cryptosporidium parvum* infection," *Exp. Parasitol.* **126**, 304–309 (2010).

⁵V. McDonald, "Cryptosporidiosis: Host immune responses and the prospects for effective immunotherapies," *Expert Rev. Anti Infect. Ther.* **9**, 1077–1086 (2011).

⁶Ministry of Health of the People's Republic of China, 2012 China AIDS Response Progress Report (UNAIDS, 2012), <http://www.unaids.org.cn/pics/20130116094000.pdf> (accessed 2015-01-20).

⁷V. A. Cama, J. M. Ross, S. Crawford, V. Kawai, R. Chavez-Valdez *et al.*, "Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons," *J. Infect. Dis.* **196**, 684–691 (2007).

⁸R. Weber, R. T. Bryan, H. S. Bishop, S. P. Wahlquist, J. J. Sullivan *et al.*, "Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: Evidence for low sensitivity of current diagnostic methods," *J. Clin. Microbiol.* **29**, 1323–1327 (1991).

⁹S. O. Brockmann, C. Dreweck, C. Wagner-Wiening, R. M. Hagen, P. Kimmig *et al.*, "Serological and epidemiological analysis of an outbreak of gastroenteritis among military recruits in Germany caused by *Cryptosporidium parvum*," *Infection* **36**, 450–457 (2008).

- ¹⁰R. Sarkar, S. S. Ajampur, J. Muliyl, H. Ward, E. N. Naumova *et al.*, "Serum IgG responses and seroconversion patterns to *Cryptosporidium* gp15 among children in a birth cohort in south India," *Clin. Vaccine Immunol.* **19**, 849–854 (2012).
- ¹¹A. J. Borad, G. M. Allison, D. Wang, S. Ahmed, M. M. Karim *et al.*, "Systemic antibody responses to the immunodominant p23 antigen and p23 polymorphisms in children with cryptosporidiosis in Bangladesh," *Am. J. Trop. Med. Hyg.* **86**, 214–222 (2012).
- ¹²J. W. Priest, C. Bern, J. M. Roberts, J. P. Kwon, A. G. Lescano *et al.*, "Changes in serum immunoglobulin G levels as a marker for *Cryptosporidium* sp. infection in Peruvian children," *J. Clin. Microbiol.* **43**, 5298–5300 (2005).
- ¹³C. S. Ong, A. S. Li, J. W. Priest, R. Copes, M. Khan *et al.*, "Enzyme immunoassay of *Cryptosporidium*-specific immunoglobulin G antibodies to assess longitudinal infection trends in six communities in British Columbia, Canada," *Am. J. Trop. Med. Hyg.* **73**, 288–295 (2005).
- ¹⁴J. W. Priest, A. Li, M. Khan, M. J. Arrowood, P. J. Lammie *et al.*, "Enzyme immunoassay detection of antigen-specific immunoglobulin g antibodies in longitudinal serum samples from patients with cryptosporidiosis," *Clin. Diagn. Lab. Immunol.* **8**, 415–423 (2001).
- ¹⁵T. Thorsen, S. J. Maerkl, and S. R. Quake, "Microfluidic large-scale integration," *Science* **298**, 580–584 (2002).
- ¹⁶D. J. Beebe, G. A. Mensing, and G. M. Walker, "Physics and applications of microfluidics in biology," *Annu. Rev. Biomed. Eng.* **4**, 261–286 (2002).
- ¹⁷C. D. Chin, T. Laksanasopin, Y. K. Cheung, D. Steinmiller, V. Linder *et al.*, "Microfluidics-based diagnostics of infectious diseases in the developing world," *Nat. Med.* **17**, 1015–1019 (2011).
- ¹⁸J. Zhang, S. Liu, P. Yang, and G. Sui, "Rapid detection of algal toxins by microfluidic immunoassay," *Lab Chip* **11**, 3516–3522 (2011).
- ¹⁹Y. Zhang, L. Qiao, Y. Ren, X. Wang, M. Gao, Y. Tang, J. Jeff Xi, T. M. Fu, and X. Jiang, "Two dimensional barcode-inspired automatic analysis for arrayed microfluidic immunoassays," *Biomicrofluidics* **7**(3), 34110 (2013).
- ²⁰M. I. Mohammed and M. P. Desmulliez, "Planar lens integrated capillary action microfluidic immunoassay device for the optical detection of troponin I," *Biomicrofluidics* **7**(6), 64112 (2013).
- ²¹L. Bissonnette and M. G. Bergeron, "Diagnosing infections—current and anticipated technologies for point-of-care diagnostics and home-based testing," *Clin. Microbiol. Infect.* **16**(8), 1044–1053 (2010).
- ²²M. M. Gong, B. D. Macdonald, T. Vu Nguyen, K. Van Nguyen, and D. Sinton, "Field tested milliliter-scale blood filtration device for point-of-care applications," *Biomicrofluidics* **7**(4), 44111 (2013).
- ²³I. N. Katis, J. A. Holloway, J. Madsen, S. N. Faust, S. D. Garbis, P. J. Smith, D. Voegeli, D. L. Bader, R. W. Eason, and C. L. Sones, "Paper-based colorimetric enzyme linked immunosorbent assay fabricated by laser induced forward transfer," *Biomicrofluidics* **8**(3), 036502 (2014).
- ²⁴W. Zhao, L. Zhang, W. W. Jing, S. X. Liu, H. Tachibana, X. J. Cheng, and G. D. Sui, "An integrated microfluidic device for rapid serodiagnosis of amebiasis," *Biomicrofluidics* **7**(1), 011101 (2013).
- ²⁵K. L. Spencer, R. Soave, A. Acosta, B. Gellin, A. Prince *et al.*, "Cryptosporidiosis in HIV infected persons: Prevalence in a New York City population," *Int. J. Infect. Dis.* **1**, 217–221 (1997).
- ²⁶S. K. Sandhu, J. W. Priest, P. J. Lammie, A. Hubbard, J. M. Colford, Jr., and J. N. Eisenberg, "The natural history of antibody responses to *Cryptosporidium* parasites in men at high risk of HIV infection," *J. Infect. Dis.* **194**(10), 1428–1437 (2006).
- ²⁷X. H. Le, H. Wang, J. Z. Gou, X. C. Chen, G. L. Yang, Q. T. Yang, X. H. Li, B. P. Zhou, H. Q. Li, and W. P. Cai, "Detection of *Cryptosporidium* infection among AIDS patients in Guangdong and Yunnan," *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* **22**(5), 339–341 (2008).
- ²⁸D. Muthusamy, S. S. Rao, S. Ramani, B. Monica, I. Banerjee *et al.*, "Multilocus genotyping of *Cryptosporidium* sp. isolates from human immunodeficiency virus-infected individuals in South India," *J. Clin. Microbiol.* **44**, 632–634 (2006).
- ²⁹S. Gupta, S. Narang, V. Nunavath, and S. Singh, "Chronic diarrhoea in HIV patients: Prevalence of coccidian parasites," *Indian J. Med. Microbiol.* **26**, 172–175 (2008).
- ³⁰A. Kurniawan, T. Karyadi, S. W. Dwintari, I. P. Sari, E. Yuniastuti *et al.*, "Intestinal parasitic infections in HIV/AIDS patients presenting with diarrhoea in Jakarta, Indonesia," *Trans. R. Soc. Trop. Med. Hyg.* **103**, 892–898 (2009).
- ³¹S. Assefa, B. Erko, G. Medhin, Z. Assefa, and T. Shimelis, "Intestinal parasitic infections in relation to HIV/AIDS status, diarrhea and CD4 T-cell count," *BMC Infect. Dis.* **9**, 155 (2009).
- ³²W. Schmidt, U. Wahnschaffe, M. Schafer, T. Zippel, M. Arvand *et al.*, "Rapid increase of mucosal CD4 T cells followed by clearance of intestinal cryptosporidiosis in an AIDS patient receiving highly active antiretroviral therapy," *Gastroenterology* **120**, 984–987 (2001).
- ³³R. M. Donovan, C. E. Bush, N. P. Markowitz, D. M. Baxa, and L. D. Saravolatz, "Changes in virus load markers during AIDS-associated opportunistic diseases in human immunodeficiency virus-infected persons," *J. Infect. Dis.* **174**, 401–403 (1996).
- ³⁴F. J. Frost, T. B. Muller, R. L. Calderon, and G. F. Craun, "Analysis of serological responses to *Cryptosporidium* antigen among NHANES III participants," *Ann. Epidemiol.* **14**, 473–478 (2004).
- ³⁵P. Gascoyne, J. Satayavivad, and M. Ruchirawat, "Microfluidic approaches to malaria detection," *Acta Trop.* **89**(3), 357–369 (2004).