Table 1 | Primers used for loop-mediated isothermal amplification

Primer type		Sequence	Location of the target sequence on the complete genome sequence[a]
F3	Forward outer	5′ – CTGGCTCAGGACGAACG – 3′	1,487,551 – 1,487,563
B3	Backward outer	5′ – GCCCATCCCACACCGC – 3′	1,487,759 – 1,487,746
FIP	Forward inner primer	5′ – TGCCCACGTGTTACTCATGCAAGTCGAACGGAAAGGCCT – 3′	1,487,654 – 1,487,638 + 1,487,588–1,487,609
BIP	Backward inner primer	5′ – TCGGGATAAGCCTGGACCAGAAGACATGCGTCTTGA –3′	1,487,669 – 1,487,683 + 1,487,732–1,487,712
FL	Loop forward	5′ – GTTCGCCACTCGAGTACCTCCG – 3′	1,487,634 – 1,487,613
BL	Loop backward	5′ – GAAACTGGGTCTAATACCGG – 3′	1,487,684 – 1,487,703

[a]*M. avium* 104 (GenBank accession no. CP000479.1).

within 50 min. For further confirmation, the amplified products were examined by restriction analysis using *Taq*I enzyme, which was selected on the basis of the restriction maps of the target sequences of the LAMP product. Following overnight digestion at 37 °C, the digested products were analyzed by agarose gel electrophoresis using a 2% agarose gel, followed by staining with ethidium bromide. For further confirmation that the correct LAMP product was obtained, melting curve analysis was performed as follows. The LAMP reaction was carried out after addition of SYBR Green I (1:50,000; Molecular Probes Inc., Eugene, OR, USA), and the melting curves of LAMP amplicons were obtained over a temperatures range of from 64–95 °C using an Applied Biosystems 7500 fast real-time PCR system. The ROX reference dye was not used.

Strains and environmental samples

The specificity of the selected primer sets was examined by performing the LAMP method for DNA extracted from various bacterial strains: *M. tuberculosis* ATCC 25618, *M. bovis* Ravenel, *M. bovis* BCG Tokyo, *M. africanum* ATCC 25420, *M. microti* TC 77, *M. kansasii* ATCC 12476, *M. avium* ATCC 15769, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. intracellulare* ATCC 13950, *M. marinum* ATCC 927, *M. simiae* ATCC 12476, *M. shimoidei* ATCC 27962, *M. nonchromogenicum* ATCC 19530, *M. xenopi* ATCC 19250, *M. scrofulaceum* ATCC 19981, *M. gordonae* ATCC 14470, *M. chelonae* subsp. *abscessus* ATCC 19977, *M. fortuitum* ATCC 6841, *M. austroafricanum* ATCC 33464, *M. pulveris* ATCC 35154, *M. asiaticum* ATCC 25276, *M. tokaiense* ATCC 27282, *M. malmoense* ATCC 29571, *Achromobacter xylosoxidans*, *Acinetobacter haemolyticus*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Shigella boydii*, *Staphylococcus aureus*, and *Streptococcus haemolyticus*. Genomic DNA was prepared from the bacterial strains by mechanical disruption, as described previously (Suzuki *et al.* 1995), and dissolved in 300 μl of TE buffer containing 10 mmol l^{-1} Tris-HCl (pH 8.0) and 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA). All extracts were verified to contain DNA of >10 ng μl^{-1} concentration. Environmental samples collected by us previously (Nishiuchi *et al.* 2009) were also used in the present study. In brief, the samples collected on cotton swabs (scale and slime) were preincubated in 1 ml tryptic soy broth for 3 h at 25 °C and subjected to alkali treatment by the addition of 3 ml 2% sodium hydroxide solution and incubation for 10 min, followed by addition of 6 ml of phosphate buffer (PB) at pH 6.8 and centrifugation at 2270 × g for 15 min. The pellets were resuspended in 0.5 ml of PB, and 0.2 ml of these suspensions was used for culture while the remaining samples were frozen until DNA extraction for use in the LAMP method. The water samples (200 ml) were centrifuged and subjected to alkali treatment, as described above.

Comparison of various methods of DNA extraction for LAMP analysis

Five methods of DNA extraction were used for comparing DNA detection limits obtained by the LAMP method. The methods included the conventional phenol/chloroform/isopropanol extraction method (Suzuki *et al.* 1995), the

Puregene Yeast and Gram-positive Bacteria Kit (Gentra, Tokyo, Japan), the QIAamp DNA Micro Kit (Qiagen, GmbH, Hilden, Germany) after overnight treatment of samples with 2 mg ml^{-1} of lysozyme solution (1 mol l^{-1} NaCl, 0.1 mol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl (pH 8.0), 0.5% Brij-58, 0.2% deoxycholate, and 0.5% sarkosyl), the silica-based method, which is capable of detecting 1–10 mycobacteria in samples (Bahador *et al.* 2004), and the FTA elute card method (Whatman Inc.), for which 40 μl of cell suspension containing 1.0×10^2–10^5 colony forming units (CFU) 100 μl^{-1} was used. For the other four methods, 100 μl of the cell suspension was used. Extractions using kits were performed according to the manufacturers' instructions, and extracts were eluted with 30 μl of TE buffer.

When punching FTA elute cards for recovery of DNA templates, precautions had to be taken to exclude the risk of contamination with carryover DNA. Therefore, every time an elute card was punched, the puncher was decontaminated by subsequently punching a wet Kimwipes® containing 1000 ppm of sodium hypochlorite, which is a well-known chemical decontaminant for DNA (Prince & Andrus 1992). We confirmed the effectiveness of this hypochlorite system for *M. avium* bacilli (up to 4×10^8 cells) and *M. avium* DNA (up to 1.2 μg) on FTA elute cards (data not shown). Subsequently, the decontaminant and damaged DNA remaining on the puncher were removed by punching a clean Kimwipes® twice.

Extraction by the silica-based method was performed as described previously (Bahador *et al.* 2004). In brief, 500 μl of lysis buffer (1.2% guanidine thiocyanate (Fluka Chemie AAG, Switzerland) in 0.1 mol l^{-1} Tris-HCl (pH 6.4), 36 mmol l^{-1} EDTA, and 2% Triton X-100) was added to 100 μl of the cell suspension, followed by 20 μl of acid-washed silica. The suspension was mixed vigorously and incubated for 30 min at 60 °C, followed by centrifugation at 13,800 × *g* for 2 min. The pellet was washed twice with washing buffer containing 12% guanidine thiocyanate in 0.1 mol l^{-1} Tris-HCl (pH 6.4), twice with 70% ethanol, and once with acetone and then dissolved in 30 μl of TE buffer.

Extraction of DNA from environmental samples

The frozen samples were centrifuged at 13,800 × *g* for 10 min, and cell pellets were resuspended in 80 μl of PB (pH 6.8). Forty microliters of the concentrated environmental samples was applied to FTA elute cards, and DNA was extracted in 30 μl of TE buffer as described above.

RESULTS

Sensitivity and specificity of the LAMP method

We first examined the sensitivity of this method by monitoring the detection of serially diluted DNA extracted from *M. avium* (Figure 1). The results indicated that the DNA detection limit was 100 fg/reaction as opposed to the detection limit of 1 pg DNA/reaction obtained using the previously reported primer sets that targeted *gyr*B (Iwamoto *et al.* 2003).

The method of DNA extraction is also known to influence sensitivity because the recovery rate of DNA generally depends on both the method used and the skills of the researcher. Although many methods have been proposed, some require numerous steps and the use of corrosive reagents, such as phenol and chloroform, while others require a large number of bacilli in the starting material because of their poor recovery. Thus, we next examined how many bacilli were required in the sample for the successful detection of *M. avium*, using five different methods of DNA extraction. Among these methods (Table 2), the FTA elute card method was the most sensitive and suitable for subsequent DNA detection using the LAMP method, as it detected *M. avium* when a minimum of 400 bacilli were present in the volume (40 μl) applied to the FTA elute card. Moreover, this method only required only 2–3 h because of the simple procedure.

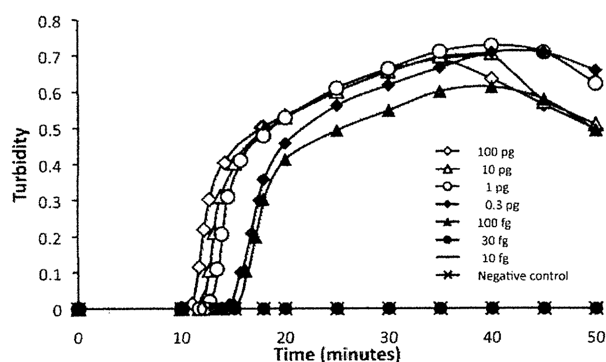

Figure 1 | Sensitivity of the LAMP method for *M. avium* detection.

Table 2 | Comparison of the DNA extraction methods[a]

Extraction method	Detection limit (CFU μl^{-1}) of sample	Processing time (h)	Use of corrosive reagent
Phenol/ chloroform/ isopropanol	$>10^5$ CFU 100 μl^{-1}	4	+
QIAamp DNA Micro Kit	10^3 CFU 100 μl^{-1}	25–26	–
Puregene yeast and Gram-Positive Bacteria Kit	10^5 CFU 100 μl^{-1}	2–3	–
Silica-based method	10^4 CFU 100 μl^{-1}	2–3	–
FTA elute card method	400 CFU 40 μl^{-1}	2–3	–

[a]DNA extracts were eluted with 30 µl of TE, and 4 µl of the extracted templates was used for the LAMP method.

The presence of contaminants such as dust, fungi, and other bacteria in the environmental samples necessitated the use of certain procedures, including pre-incubation for 3 h at 25 °C to bud fungi and spores, and the subsequent alkali-treatment to kill other microorganisms, to culture the organisms (Nishiuchi *et al.* 2009). We thus examined the effect of contaminants and the pretreatment procedures on the efficiency of DNA extraction. We used samples previously collected from the dust of air conditioners as a contaminant that contained many inorganic materials, bacteria, and fungi, but not mycobacteria (Nishiuchi *et al.* 2007). We pretreated the samples according to the previous method before extraction of DNA. Table 3 shows that both the presence of the dust and the pretreatment procedures hampered DNA extraction using the QIAamp DNA Micro Kit, but they had less effect on the efficiency of DNA extraction using the FTA elute card.

Table 3 | Effect of contaminants and pretreatment procedure on DNA extraction

	CFU of *M. avium* in sample ($\log_{10}$)								
	QIAamp DNA Micro Kit					FTA elute card method			
	6	5	4	3	2	4.6	3.6	2.6	1.6
Control	+	+	+	+	–	+	+	+	–
Added dust sample	+	+	–	–	–	+	+	–	–
Pretreatment[a]	+	+	–	–	–	+	+	+	–

[a]Pretreatment involved preculture for 3 h followed by alkali treatment.

We then evaluated the specificity of LAMP using genomic DNA from 23 different mycobacterial species and 10 other bacterial species. A successful LAMP reaction with species-specific primers caused turbidity in the reaction tubes. *M. avium* subsp. *paratuberculosis*, which causes Johne's disease, was also amplified using a *M. avium*-specific primer set and yielded a positive reaction. The specificity of amplification was further confirmed by restriction enzyme digestion of the LAMP products and melting curve analysis. As shown in Figure 2(a), restriction digestion yielded products that were in good agreement with the predicted sizes (171 and 163 bp). Furthermore, the peaks of the melting temperature curves were identical between *M. avium* genomic DNA and the environmental samples (Figure 2(b)).

Comparison between LAMP analysis and culture for the detection of *M. avium* in the environmental samples

In the present study, we used previously collected samples from bathrooms in the residences of 29 patients with pulmonary *M. avium* disease (Nishiuchi *et al.* 2009) and performed the DNA extraction followed by LAMP. The results were then compared with those obtained by culture (Table 4). Of a total

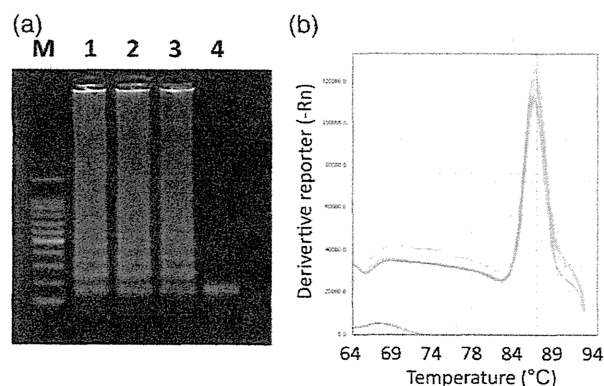

Figure 2 | The specificity of LAMP products. (a) LAMP products obtained from 10 pg of *M. avium* 104 DNA (Lane 1) and from nucleic acid extracts of pure cultures of *M. avium* 104 on the FTA elute cards (approximately 10^8 and 10^7 cells, Lanes 2 and 3, respectively), and restriction digestion of the products obtained from 10 pg of *M. avium* 104 DNA with *TaqI* (bands corresponding to 171 and 163 bp are expected, Lane 4). (b) The peaks of melting temperature curves of LAMP products amplified from control *M. avium* 104 DNA coincided with those from five isolates of environmental *M. avium*. Products from all isolates had a melting temperature of 87.3 °C.

Table 4 | Recovery and detection of *M. avium* from the residential bathrooms of patients with pulmonary *M. avium* disease using the culture and LAMP methods

Sampling site (sample type)	Number of samples: Surface of the shower head (scale)	Inside the shower head (scale)	Shower (water)	Bathtub inlet (scale)	Bathtub (water)	Drain (slime)	Total no. of samples[a]
No. of test samples	29	24	29	25	26	29	162 (29)
Culture positive	1	2	1	14	7	7	32 (15)
LAMP positive	3	0	3	13	6	6	27 (17)

[a]Numbers in parentheses represent the number of residences.
The result of the culture method is cited from the previous report (Nishiuchi *et al.* 2009).

of 162 samples *M. avium* was recovered from 32 samples (20%) by culture and detected in 28 samples (18%) by LAMP. Twenty samples (12%) were positive and 123 samples (76%) were negative for *M. avium* by both methods. The samples that tested positive by culture and/or the LAMP method are listed in Table 5. All samples that gave a *M. avium* recovery of >20 CFU/primary isolation plate by culture were also positive by LAMP.

DISCUSSION

Our data show that the combination of the FTA elute card (for DNA extraction) and the LAMP method is rapid and sensitive for detection of *M. avium* in environmental samples. It is advantageous over the culture method as it takes significantly less time and the entire procedure, from obtaining samples to *M. avium* detection, can be completed within 2–3 h. In general, the yields of DNA obtained from mycobacteria are low because of the presence of a robust, waxy cell wall that makes it difficult to lyse mycobacterial cells. Moreover, the samples examined in the present study, which were previously used for culturing the bacteria, had to be subjected to several procedures prior to DNA extraction, such as pre-culture to bud fungi, alkali-treatment to kill other microorganisms, freezing preservation, and concentration by centrifugation of the samples. These steps could decrease the recovery of DNA (Table 3) and the efficiency of NAA. Alkali-treatment may increase the concentration of alkali-soluble inhibitors such as humic substances that are widespread in the environment and are known to hamper PCR (Matheson *et al.* 2010). Although the presence of dust and pretreatment procedures hampered the efficiency of DNA extraction and the NAA method, they had minimal effect when the FTA elute card was employed. Therefore, use of the FTA elute card is likely to be suitable for examining the environmental samples containing mycobacteria, although it was originally developed to extract DNA from whole blood samples or buccal swabs (Tables 2 and 3).

Both the culture and LAMP methods yielded consistent results in 88% of the 162 examined environmental samples. In contrast, inconsistencies were observed with only 19 samples (12%; Table 5), which yielded <20 CFU/primary isolation plate by culture. It has been also reported that results obtained by NAA may show discrepancies with those obtained by culture (Iwamoto *et al.* 2003). This discrepancy is attributable to characteristic features of NAA, namely that it is capable of detecting DNA from dead cells. In addition, the NAA method is theoretically capable of detecting even a single copy of genomic DNA, but it is very susceptible to contamination with inhibitors and the efficiency of DNA extraction. Another possible reason for the discrepancy is the presence of viable but nonculturable (VNC) bacilli, although culture is theoretically capable of recovering a single viable bacterium. It has been recognized recently that the majority of bacteria in the environment enter a VNC state (Roszak & Colwell 1987). The pathogens in tap water (Moritz *et al.* 2010; Pawlowski *et al.* 2011) and in drinking water biofilms (Moritz *et al.* 2010) also enter the VNC state, as does *M. avium* in shower water or in the showerhead. This might also contribute to the discrepancy observed in the present study. However, whether *M. avium* can enter the VNC state remains uncertain. Further

Table 5 | List of all samples that tested positive by culture and/or LAMP methods

Participant no	Sampling site	Sample	Culture CFU/plate[a]	LAMP[b]
P-9	Bathtub inlet	Scale	>1,000	Positive
P-17	Bathtub inlet	Scale	>1,000	Positive
P-25	Bathtub inlet	Scale	>1,000	Positive
P-27	Bathtub inlet	Scale	>1,000	Positive
P-26	Bathtub inlet	Scale	>300	Positive
P-27	Bath drain	Slime	>300	Positive
P-8	Bathtub inlet	Scale	>100	Positive
P-12	Bath drain	Slime	>100	Positive
P-22	Bathtub inlet	Scale	>100	Positive
P-29	Bathtub inlet	Scale	>100	Positive
P-27	Bathtub	Water	47	Positive
P-12	Bathtub inlet	Scale	42	Positive
P-23	Bathtub inlet	Scale	20	Positive
P-29	Bath drain	Slime	13	–
P-17	Bath drain	Slime	9	Positive
P-8	Bath drain	Slime	6	Positive
P-28	Bathtub inlet	Scale	6	Positive
P-29	Bathtub	Water	6	Positive
P-9	Bathtub	Water	5	Positive
P-21	Bathtub inlet	Scale	4	Positive
P-26	Bathtub	Water	4	–
P-2	Bath drain	Slime	3	–
P-29	Showerhead inside	Scale	3	–
P-8	Bathtub	Water	1	–
P-9	Showerhead inside	Scale	1	–
P-9	Shower	Water	1	Positive
P-13	Bathtub inlet	Scale	1	–
P-15	Bathtub inlet	Scale	1	–
P-22	Bathtub	Water	1	–
P-23	Bath drain	Slime	1	–
P-25	Bathtub	Water	1	–
P-27	Showerhead surface	Scale	1	–
P-6	Shower	Water	–	Positive
P-9	Showerhead surface	Scale	–	Positive
P-9	Bath drain	Slime	–	Positive
P-11	Bathtub	Water	–	Positive
P-16	Showerhead surface	Scale	–	Positive
P-33	Showerhead surface	Scale	–	Positive
P-33	Shower	Water	–	Positive

[a]CFU/primary isolation plate where 200 μl of the sample was inoculated.
[b]The LAMP method was performed with 4 μl of the template in an assay mixture. The template was eluted from an FTA elute card with 30 μl of TE where 40 μl of the concentrated sample was originally applied to the FTA elute card.

studies are required to clarify this issue. In summary, all these facts should be taken into account when we assess environmental samples.

CONCLUSIONS

We demonstrated the utility of the LAMP method for the direct detection of *M. avium* in environmental samples by employing a novel set of six specific primers. Furthermore, we demonstrated that the FTA elute card is useful for DNA extraction from environmental *M. avium* without resorting to the use of harmful reagents. Thus, use of the LAMP method in combination with an FTA elute card for DNA extraction may facilitate the direct detection of environmental *M. avium* within a short period.

ACKNOWLEDGEMENTS

We are grateful to all participants who donated samples and to the hospital nurses who took part in our study. This research was supported by a Grant-in-Aid from the Institute for Fermentation, Osaka (IFO). It was also supported in part by the Global Center of Excellence (COE) Program; 'Establishment of International Collaboration Centers for Zoonosis Control', Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; Grants-in-Aid for the Program of Founding Research Center for Emerging and Re-emerging Infectious Diseases from MEXT (Y.S.); the Japan Health Sciences Foundation; and the Ministry of Health, Labor and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research grants). We would like to thank Dr Tomotada Iwamoto for designing and providing us with the primer sets for *M. avium*. We would also like to thank Dr Eiichi Momodani for providing us with *M. avium* subsp. *paratuberculosis* samples.

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First received 4 January 2013; accepted in revised form 12 October 2013. Available online 25 November 2013

Serological Surveillance Development for Tropical Infectious Diseases Using Simultaneous Microsphere-Based Multiplex Assays and Finite Mixture Models

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Abstract

Background: A strategy to combat infectious diseases, including neglected tropical diseases (NTDs), will depend on the development of reliable epidemiological surveillance methods. To establish a simple and practical seroprevalence detection system, we developed a microsphere-based multiplex immunoassay system and evaluated utility using samples obtained in Kenya.

Methods: We developed a microsphere-based immuno-assay system to simultaneously measure the individual levels of plasma antibody (IgG) against 8 antigens derived from 6 pathogens: *Entamoeba histolytica* (C-IgL), *Leishmania donovani* (KRP42), *Toxoplasma gondii* (SAG1), *Wuchereria bancrofti* (SXP1), HIV (gag, gp120 and gp41), and *Vibrio cholerae* (cholera toxin). The assay system was validated using appropriate control samples. The assay system was applied for 3411 blood samples collected from the general population randomly selected from two health and demographic surveillance system (HDSS) cohorts in the coastal and western regions of Kenya. The immunoassay values distribution for each antigen was mathematically defined by a finite mixture model, and cut-off values were optimized.

Findings: Sensitivities and specificities for each antigen ranged between 71 and 100%. Seroprevalences for each pathogen from the Kwale and Mbita HDSS sites (respectively) were as follows: HIV, 3.0% and 20.1%; *L. donovani*, 12.6% and 17.3%; *E. histolytica*, 12.8% and 16.6%; and *T. gondii*, 30.9% and 28.2%. Seroprevalences of *W. bancrofti* and *V. cholerae* showed relatively high figures, especially among children. The results might be affected by immunological cross reactions between *W. bancrofti*-SXP1 and other parasitic infections; and cholera toxin and the enterotoxigenic *E. coli* (ETEC), respectively.

Interpretation: A microsphere-based multi-serological assay system can provide an opportunity to comprehensively grasp epidemiological features for NTDs. By adding pathogens and antigens of interest, optimized made-to-order high-quality programs can be established to utilize limited resources to effectively control NTDs in Africa.

Citation: Fujii Y, Kaneko S, Nzou SM, Mwau M, Njenga SM, et al. (2014) Serological Surveillance Development for Tropical Infectious Diseases Using Simultaneous Microsphere-Based Multiplex Assays and Finite Mixture Models. PLoS Negl Trop Dis 8(7): e3040. doi:10.1371/journal.pntd.0003040

Editor: Patrick J. Lammie, Centers for Disease Control and Prevention, United States of America

Received October 25, 2013; **Accepted** June 10, 2014; **Published** July 31, 2014

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Funding: This work was, in part, supported by Asia-Africa Science & Technology Strategic Cooperation Promotion Program of Special Coordination Funds for Promoting Science and Technology (SCF); funds for integrated promotion of social system reform and research and development, by The Ministry of Education, Culture, Sports, Science, and Technology (MEXT); and by the Global Center of Excellence (GCOE) Program at Nagasaki University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Combating infectious diseases, including neglected tropical diseases (NTDs), among the poorest segment of the population is a major concern of the international community. In sub-Saharan Africa, the impact of NTDs as a group is comparable to that of malaria and tuberculosis; however, NTDs collectively receive less attention and research funding than human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), tuberculosis, or malaria, despite the fact that NTDs affect an estimated one billion people in tropical and subtropical climates [1]. The poor can be afflicted by more than one NTD. According to the World Health Organization, approximately 74% of the affected population simultaneously harbors two NTDs; 18% three; 13% four; 9% five; 11% six; and 8% seven or more NTDs [2]. Furthermore, most NTDs have a geographical distribution similar to that of HIV, malaria, and tuberculosis [3]. The population at risk for NTDs also is at risk for these life-threatening infectious diseases.

Although prevalence surveys of NTDs used for monitoring temporal and geographical distributions of each disease are important from a public health point of view, such surveys are limited by technical issues. For instance, diagnosis must be performed independently for each disease. If infectious diseases could be concurrently detected, the cost and effectiveness of diagnosis may improve; and timely data on the prevalence of NTDs and other infectious diseases may become more readily available [4].

Microsphere-based multi-serological assays permit the simultaneous measurement of many different analytes in a small sample volume [5]. Technically, such assay systems apply the same concepts as that of flow cytometry, but unlike flow cytometry, the microsphere-based assays use 500 unique dye mixtures to identify individual microspheres. Theoretically, a multiplexed technique can concurrently run up to 500 different assays. In practice, such methods already are being used for screening different serotypes of a single pathogen [5–9] and for serological assays against several mixed pathogens [10,11]. If the systems were optimized to couple microspheres to antigens of selected NTDs in sub-Saharan Africa, the prevalence survey or routine surveillance of NTDs could be managed easily, effectively, and in a timely manner.

We have been developing a new assay to apply this technology as part of a large-scale surveillance program to detect several infectious agents simultaneously and efficiently. As we report here, we evaluated the developed assay system, and used the system to survey seroprevalence in Kenya. We go on to discuss the possibility of large-scale comprehensive surveillance programs for NTDs and other infectious diseases in Africa.

Methods

Determination of antigen structures and antigen preparations

We chose cholera toxin (CTX), as well as 7 recombinant antigens, to measure IgG antibodies to each antigen assayed in this study (Table 1). CTX was obtained as purified native *Vibrio cholerae* CTX subunit A plus B purchased from Sigma-Aldrich (#C8052, MO, USA). The recombinant antigens were as follows: C-terminal part of the *Entamoeba histolytica* intermediate subunit (C-IgL) of galactose- and N-acetyl-D-galactosamine-inhibitable lectin for amebiasis [12]; *Leishmaniasis donovani* kinesin-related protein KRP42 for visceral leishmaniasis [13]; *Toxoplasma gondii* surface antigen 1 (SAG1) for toxoplasmosis [14]; *Wuchereria bancrofti* SXP1 for lymphatic filariasis [15]; and *HIV1* gag(MA+CA), gp41 ectodomain, and gp120 for HIV [16].

Antigens of *Mycobacterium tuberculosis*; CFP10 and ESAT6 [17], were part of the initial panel in the simultaneous microsphere-based multiplex assays. The descriptions for these antigens are retained in the methods section; however, they are omitted from the results and discussion due to poor the sensitivity.

The recombinant antigens were expressed in *Escherichia coli*. DNA encoding the recombinant antigens were amplified by polymerase chain reaction (PCR) and cloned into pET52b vectors or (for the C-IgL-encoding fragment only) pET19b. The resulting expression vectors encoded the respective epitopes as antigenic regions with fusion tags. Accession numbers for the respective antigens are provided in Table 1. The construction of the C-IgL expression vector has been described previously [12].

Antigen purification

Each expression plasmid was transformed into BL21Star (DE3) pLysS chemically competent cells (Invitrogen, Carlsbad, CA, USA) and grown in LB broth containing 50 µg/ml carbenicillin and 34 µg/ml chloramphenicol. At an approximate density of 1.0 A_{600}, protein expression was induced with 1 mM IPTG for 2–3 h at 37°C. After harvesting cells by centrifugation, the pellet was suspended in BugBuster (MERCK-Millipore, Darmstadt, Germany) containing 25 unit/ml Benzonase Nuclease. Protein solubilization and nucleotide degradation was performed in a rotating tube at room temperature for 20 min. Soluble and insoluble materials were separated by centrifugation at 14000×*g* at 4°C for 10 min.

HIV1 gag, KRP42, and CFP10 were purified from soluble fractions by two-step affinity purification on COSMOGEL His-Accept resin (NACALAI TESQUE, Kyoto, Japan) and Strep-Tactin Superflow Plus resin (QIAGEN, Hilden, Germany) according to each manufacturer's instructions, with modifications as follows. In brief, the soluble fraction was loaded on His-Accept resin equilibrated with BugBuster, then washed once with BugBuster. After further washing twice with wash buffer (50 mM phosphate buffer, 0.5 M NaCl, 0.01% Tween 20, pH 8.0), antigen was eluted with elution buffer (50 mM phosphate buffer, 0.5 M NaCl, 0.01% Tween 20, 500 mM imidazole, pH 8.0). The eluate was diluted twice with wash buffer to reduce the imidazole concentration, then directly applied to Strep-Tactin resin. After washing the resin twice with wash buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0), the antigen was eluted with elution buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8.0). The purified

Author Summary

Monitoring the distribution of neglected tropical diseases (NTDs) is a key to controlling their spread in Africa. Currently, such surveillance is conducted independently for each NTD. To tackle this problem, we developed a microsphere-based system to permit simultaneous measurement of IgG antibody levels for antigens from six infectious diseases: *Entamoeba histolytica, Leishmania donovani, Toxoplasma gondii, Wuchereria bancrofti,* HIV, and *Vibrio cholerae.* Using this system, we conducted a serological survey using two health and demographic surveillance system (HDSS) areas in coastal and western Kenya. We randomly selected 4,600 individuals according to sex and age group, of whom 3411 agreed to participate in the study. Mathematical analyses of the distributions of the participants' reactivity to each antigen and the reactivity of the sero-positive and -negative controls indicated that this system could be used to monitor infections, especially, those associated with HIV, filariasis, toxoplasmosis, leishmaniasis, and amebiasis. For the practical development and eventual implementation of actual programs in Africa, pathogens and antigens of interest can be added to optimize made-to-order monitoring programs.

antigen then was dialyzed in phosphate-buffered saline (PBS) without calcium and magnesium[PBS(-)]. *HIV1* gp120, *HIV1* gp41 ectodomain, IgL, SAG1, SXP1, and ESAT6 were purified from the insoluble fractions. Preparation of inclusion bodies was performed with BugBuster protein extraction reagent according to the protocol for the protein refolding kit (TB234 12/98, Novagen, Inc, WI, USA). The prepared inclusion bodies (which primarily contained expressed antigens) were suspended in 0.3% N-lauroylsarcosine in CAPS buffer (pH 11), and then rotated for 15 min at room temperature. Insoluble materials were removed by centrifugation at 14000×*g* for 10 min at 4°C. Solubilized samples were dialyzed in 0.3% N-lauroylsarcosine/PBS(−) and purified by His-Accept resin as described previously, with the modification that His-Accept resin was equilibrated with 0.3% N-lauroylsarcosine/PBS(−) instead of BugBuster. Purified antigens were dialyzed in 0.3% N-lauroylsarcosine/PBS(−).

The protein concentrations of soluble antigens were determined with a Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, CA, USA) and concentrations of insoluble antigens were determined using a Pierce 660-nm Protein Assay Kit with Ionic Detergent Compatibility Reagent (Thermo Scientific, Rockford, IL, USA).

Each of the purified proteins (5.0 μg/lane) was separated on NuPAGE Novex 4%–12% Bis-Tris Gel in 1× MES SDS Running Buffer (Life Technologies Corporation, Carlsbad, CA, USA) under reducing conditions. The gel was stained with Coomassie Brilliant Blue.

DNA and protein data were analyzed to predict the molecular weights of antigens by CLC Main Workbench 6 software (CLC bio, Aarhus, Denmark). Antigen homologies also were analyzed with the National Center for Biotechnology Information (NCBI) Protein Basic Local Alignment Search Tool (BLAST).

Coupling antigens with microspheres

Following purification, the individual antigens were coupled with microspheres (MagPlex) using a separate color for each antigen. Two types of microspheres made from different materials are commercially available from Luminex Corporation: MicroPlex microspheres and MagPlex microspheres (Luminex Corporation, Austin, TX, USA). A high nonspecific immunological background of MicroPlex microspheres in serological assays has been reported [18,19]. For immunoreactions, filter-bottom plates (MultiScreenHTS -BV, MERCK-Millipore) washed using an ELx405 microplate washer with a vacuum system were used for Microplex, and Bio-Plex Pro flat-bottom plates (Bio-Rad) and an ELx405 microplate washer (BioTek, Winooski, VT, USA) with magnetic plates were used for MagPlex. After comparing two types of non-treated microspheres for nonspecific immunological background

Table 1. Structure of recombinant antigens.

Pathogen	Antigen	Vector	Fusion tag on N-terminus	Antigen Region	Fusion tag on C-terminus	GenBank #
Entamoeba histolytica	C-IgL	pET19b	MGHHHHHHHHHHSSGHIDDDDKHMLE	603–1088		AF337950
Leishmania donovani	KRP42	pET52b	MASWSHPQFEKGALEVLFQGPGYQDP	1–337	IEFHHHHHHVDAAAELALVPRGSSAHHHHHHHHHH	BAF34578
Toxoplasma gondii	SAG1	pET52b	MASWSHPQFEKGALEVLFQGPGYQDP	61–300	LEVDAAAELALVPRGSSAHHHHHHHHHH	AAO61460
Wuchereria bancrofti	SXP1	pET52b			MASWSHPQFEKGALEVLFQGPGYQDPVTSSLNLTK	1–153
					IEFHHHHHHLQVDAAAELALVPRGSSAHHHHHHHHHH	AAC17637
Human Immunodeficiency Virus type 1	gag	pET52b	*MASWSHPQFEKGALEVLFQGPGYQDP*	*1–363*	*VDAAAELALVPRGSSAHHHHHHHHHH*	AAB50258
	gp120	pET52b	MASWSHPQFEKGALEVLFQGPGYQDP	34–511	VDAAAELALVPRGSSAHHHHHHHHHH	AAB50262
	gp41	pET52b	MASWSHPQFEKGALEVLFQGPGYQ	512–683	ELALVPRGSSAHHHHHHHHHH	AAB50262
Mycobacterium tuberculosis	CFP10	pET52b	MASWSHPQFEKGALEVLFQGPGYQDP	1–100	AAAELALVPRGSSAHHHHHHHHHH	ZP_04982462
	ESAT6	pET52b	MASWSHPQFEKGALEVLFQGPGYQDP	1–95	AAAELALVPRGSSAHHHHHHHHHH	NP_338543

Note: For *Vibrio cholerae*, cholera toxin subunit A and B, which were not recombinant protein, were used in this study.
doi:10.1371/journal.pntd.0003040.t001

in a single-plex format for 148 serum samples collected in the field survey in Kenya, MagPlex microspheres were chosen for our assay development.

Anti-human IgG antibody and CTX were dialyzed in PBS (−) before the coupling reaction.

Each antigen, the anti-human IgG antibody, and CTX were coupled with a different "color" of MagPlex microspheres in a one-to-one pairing following the manufacturer's instructions, with modifications as follows. In brief, carboxyl groups on the microspheres were activated with EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, Thermo Scientific Inc.) and S-NHS (N-hydroxysulfosuccinimide, Thermo Scientific Inc.) in activation buffer (0.1 M NaH_2PO_4, pH 6.2) for 30 min at room temperature. After incubation, the microspheres were washed with PBS (−), pH 7.4. Antigen then was coupled to the microspheres for 2 hours at room temperature with gentle agitation. Antigen amounts in the coupling reaction for 1.25 million microspheres were determined after a titration experiment, and were as follows: 3 μg of KRP42; 10 μg of gag; cholera toxin, and 30 μg of C-IgL, SAG1, CFP10, and ESAT6. After reaction, the coupled microspheres were washed with PBS (−), then free carboxyl groups were blocked by 50 mM ethanolamine pH 8.5 (Wako, Osaka, Japan) for 30 min at room temperature. The microspheres were washed twice with StabilGuard (SurModics, Eden Prairie, MN, USA), and the concentration was adjusted to 1000 microspheres/μl in StabilGuard and stored at 4°C. To determine serum reaction during the assay, 100 μg of mouse monoclonal antibody raised against a human IgG Fab fragment (Clone 4A11, EXBIO, Vestec, Czech Republic) was coupled to microspheres and served as a positive control.

Multiplex assays and evaluations

Two microliters of serum was diluted with 98.0 μl of staining buffer [0.1% bovine serum albumin, 0.05% Tween20, 0.05% sodium azide in PBS (−), pH 7.5]. In total, 16 different-colored microspheres were used for the assays, as follows: 15 colors corresponding to 14 antigens (6 antigens were not included in this paper) and one anti-human IgG antibody and one color corresponding to non-coupled microspheres (used to monitor the level of nonspecific reactions with the microspheres). Suspensions (1.0 μl at 1000 microspheres/μl) of each microsphere species were added sequentially to 84 μl of staining buffer. The resulting 100-μl suspensions, which included 15 different types of coupled microspheres and a non-coupled microsphere, were added to the wells containing the diluted serum.

A binding reaction was performed for 30 min at room temperature while shaking at 750 rpm in the dark. The plate then was transferred to an ELx405 microplate washer (BioTek, Winooski, VT, USA) with a magnetic plate for three washing steps with washing buffer [0.05% Tween 20, 0.05% sodium azide in PBS(−), pH 7.5]. While the binding reactions were in progress, phycoerythrin-conjugated goat anti-human IgG F(c) $F(ab')_2$ fragment (detection antibody; #709-1817, Rockland Inc., Gilbertsville, PA, USA) was diluted to a phycobiliprotein concentration of 2.0 μg/ml in staining buffer. The diluted detection antibody was added to the wells and incubated for 30 min at room temperature with shaking at 750 rpm in the dark. After incubation, the wells were washed as described previously. The microspheres were suspended on a shaker at 750 rpm for 5 min after addition of 125 μl of wash buffer to each well. Fluorescence was monitored using a Bio-Plex200 system (Luminex Corporation, Austin, TX, USA).

Table 2. Summary of serum reactivity from positive and negative controls.

Pathogen	Antigen	Cut-off value	Mean of MFI*	S.D.	Positive control			Negative control			Sensitivity		Specificity	
					Total	Positives	False negatives	Total	Positives	False positives	%	95%CI	%	95%CI
HIV1	gp41	171.16	104.48	22.23	50	50	0	40	0	0	100.0%	(92.9–100)	100.0%	(91.2–100)
	gag	193.66	95.15	32.84	50	40	10	40	2	2	80.0%	(66.3–90)	95.0%	(83.1–99.4)
	gp120	213.34	133.75	26.53	50	40	10	40	0	0	80.0%	(66.3–90)	100.0%	(91.2–100)
V. cholerae	CTX	224.51	95.5	43	7	5	2	40	1	1	71.4%	(29–96.3)	97.5%	(86.8–99.9)
E. histolytica	C-IgL	144.22	83.43	20.27	20	20	0	40	0	0	100.0%	(83.2–100)	100.0%	(91.2–100)
L. donovani	KRP42	277.12	87.48	63.21	16	15	1	40	1	1	93.8%	(69.8–99.8)	97.5%	(86.8–99.9)
W. bancrofti	SXP1	485.07	176.68	102.8	20	19	1	40	1	1	95.0%	(75.1–99.9)	97.5%	(86.8–99.9)
T. gondii	SAG1	435.15	91.48	114.56	19	18	1	40	2	2	94.7%	(74–99.9)	95.0%	(83.1–99.4)

*Means of the distributions of median fluorescence intensity (MFI) among 40 negative control serum samples measured by the multiplex system

doi:10.1371/journal.pntd.0003040.t002

Stability of the antigen-coupled microspheres during storage

Following coupling, microspheres were stored at 4°C; all assays were conducted within 26 days of the coupling reaction. The stability of the coupled microspheres was confirmed by combining coupled microspheres with a mixed positive control serum (in triplicate) for all antigens and monitoring the assay values for all antigens regularly (approximately 4–5 times/week) over 26 days.

Evaluation of the antigen-coupled microspheres using sera of patients and healthy Japanese

The evaluation of the microsphere-based multiplex assay system itself was performed using sera from infected patients (as positive controls) and sera from healthy Japanese (as negative controls). The immune status of the positive control sera was confirmed by standardized methods or routine clinical processes for the target infection and not clinically confirmed for more than two simultaneous infections. For HIV, sera were collected at the Kitale District Hospital, Saboti County, Kenya. These sera were screened using the following diagnostic kits; Determine HIV 1/2 tests (Abbott Diagnostic Division, Hoofddorp, Netherlands) and Uni-Gold HIV Kits (Trinity Biotech, Bray, Ireland). Positive control sera for cholera were collected from the Machakos Provincial General Hospital, Machakos County, Kenya. For *V. cholerae*, sera were obtained from a routine cholera surveillance program in Kenya; all were confirmed by PCR using isolates obtained following selection from patient stool specimens using TCBS medium. For *T. gondii*, we had difficulty finding symptomatic toxoplasmosis patients in a hospital setting. Therefore, we used sera obtained as part of the field survey used in this study; these sera were used after confirmation of *Toxoplasma* status by RDT Kits (OnSite Toxo IgG/IgM Rapid Test-Cassette, CTK Biotech, CA, USA). For *W. bancrofti*, sera were obtained from Kenya Medical Research Institute, Kenya; diagnoses were confirmed parasitologically by identifying microfilariae in blood smears drawn at night. For *E. histolytica*, sera were obtained from the International Center for Diarrheal Disease Research, Bangladesh; diagnoses were confirmed by detection of *E. histolytica*-specific DNA in liver abscess pus specimens. For *L. donovani*, sera were obtained from the Rajshahi Medical College in Bangladesh; diagnoses were parasitologically confirmed by microscopic examination of spleen aspirates. As negative controls, serum samples from 40 healthy Japanese individuals were used to calculate cut-off values for each antigen for the purpose of evaluation; seronegativity of these samples was assumed based on the rarity of infection in Japan for the pathogens examined for this study; infections by HIV, *W. bancrofti*, *E. histolytica*, *V. cholerae* cases are quite rare and *L. donovani* infection has not been reported according to the National surveillance in Japan and other research reports [20–23]. The seropositivity of *T. gondii* is reported as 10.3% in pregnant women in Japan [24]. The cut-off values for the evaluation were calculated as the means plus three standard deviations (SD) of the distributions of median fluorescence intensity (MFI) values from two independent assays. The details of the number of positive and negative control sera are provided in Table 2.

Population-based serological survey: Study sites and selection of individuals for blood sampling

For the serological survey, a database corresponding to two Health and Demographic Surveillance System (HDSS) sites managed by the Institute of Tropical Medicine, Nagasaki University, and the Kenya Medical Research Institute (KEMRI) was used for blood sampling. As of 2011, we have established and implemented two HDSS programs in the western (Mbita site) and coastal (Kwale site) areas of Kenya, where there are distinct disease burdens and cultures as described in detail elsewhere [25]. Of 77,887 individuals recorded in the HDSS database, 4,600 individuals were randomly selected by HDSS site, sex, and age group. A total of 10 age groups were divided by five-year intervals up to the age of 45 years; individuals older than 45 years of age were consolidated as a group. As a result, all individuals were categorized into 40 groups, and 115 individuals were randomly selected from each group anticipating a 15% loss of participation.

Blood sampling

Between July 2011 and August 2011, a blood sampling survey was conducted at the Kwale and Mbita HDSS sites according to the list of 4,600 randomly selected individuals. Each site was divided into 20 wards according to the HDSS address system. Blood samples were collected in each ward on a separate day. On the day prior to blood sampling, village volunteers visited the selected households, explained the purpose of the study, and invited individuals to the place where blood sampling would be performed. Blood sampling sites typically consisted of elementary schools and health centers. For those who did not come to the sampling place, village volunteers took the selected individuals to the site using project vehicles. For those who were not available on the scheduled day, additional days for blood sampling were scheduled. After re-explanation of the study and obtaining informed consent (confirmed by participant signature), blood samples were drawn using 3-ml sampling tubes for those who were more than five years of age and 0.5-ml tubes for children under 5 years of age. For children less than 18 years of age, the purpose of the study was explained to a parent or a guardian and a written agreement to participate was obtained. Following collection, blood samples were stored in a refrigerator and then sent to the main laboratory of the project (in Nairobi) by shipping in a mobile refrigerator or courier package service with dry ice. Microsphere-based multi-serological assays for all samples were conducted in the laboratory.

Mathematical models for cut-off values

High immunological backgrounds have been reported among African populations [26,27]; therefore, the cut-off values defined by Japanese serum samples could have been too sensitive, which would lead to false positives. Additionally, from public health point of view, populations currently infected (high antibody titer) and those infected in the past but treated (low or intermediate antigen titer) must be differentiated. For this purpose, the cut-off values using Japanese or negative control sera would not be suitable.

To optimize cut-off values for the study sample population, we applied the concept of finite mixture distribution, which is used to model data from populations known or suspected to contain hidden separated subpopulations [28]. We assumed that this population could be separated into several subpopulations with regard to immune status. For example, a population might be split into "immunity," "modified immunity," and "without immunity" categories if one assumed that there were three hidden subpopulations; and in some infectious agents, it might be separated into more than three immune statuses [29]. The finite mixture model has been used in past studies to optimize cut-off points from survey data from populations for several infectious diseases [29,30]. In the present study, a distribution of all assay values (MIF values) for each antigen was separated into components using the "fmm" (finite mixture model) command [31] of Stata statistical software

(version 12.1; Stata Corporation, TX, USA). To select the best model, which also returns the component number, we applied the theory of Bayesian Information Criterion (BIC). To build the model, individual data were weighted according to the proportions of site, sex, and age group that were obtained from the population data of HDSS. After separating the distribution of assay values, a cut-off point for each antigen was optimized as the index maximum value [32], which was calculated as sensitivity-(1-specificity) obtained from the distribution separated by a finite mixture model. Sensitivity and specificity were defined as a proportion of two distributions beyond the Youden index value. For the antigens in which values were separated into four or five normal distributions, two cut-off values were set for evaluation of seroprevalence: one cut-off value with high sensitivity, and a second with high specificity. For HIV, we defined seropositivity as two or more positives among the three antigens tested.

Ethical considerations

The survey protocol was approved by the Ethical Review Committee of Kenya Medical Research Institute (KEMRI SSC No. 1934) and the Ethical Committee of the Institute of Tropical Medicine, Nagasaki University (10061550 and 10122261-2). Prior to the survey, we sensitized the communities in the HDSS sites by inviting village elders and community members to learn about the project. For blood collection, we explained the project in advance and collected blood samples from those who agreed to participate and signed consent forms. For children <18 years of age, we explained the project to their parent or guardian and collected blood samples after obtaining the parent/guardian agreement and signature. This procedure (to obtain consent from the parent or guardian of children <18 years of age) was approved by the both ethical committees of Kenya Medical Research Institute and the Institute of Tropical Medicine, Nagasaki University.

Accession numbers/ID numbers for genes and proteins

GenBank accession numbers for each antigen were as follows; *Entamoeba histolytica* (C-IgL): AF337950, *Leishmania donovani* (KRP42): BAF34578, *Toxoplasma gondii* (SAG1): AAO61460, *Wuchereria bancrofti* (SXP1): AAC17637, Human Immunodeficiency Virus type 1 (gag): AAB50258, Human Immunodeficiency Virus type 1 (gp120): AAB50262, Human Immunodeficiency Virus type 1 (gp41): AAB50262, *Mycobacterium tuberculosis* (CFP10): ZP_04982462, and *Mycobacterium tuberculosis* (ESAT6): NP_338543.

Results

Evaluation of antigen purification

Proteins prepared for coupling on microspheres were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis

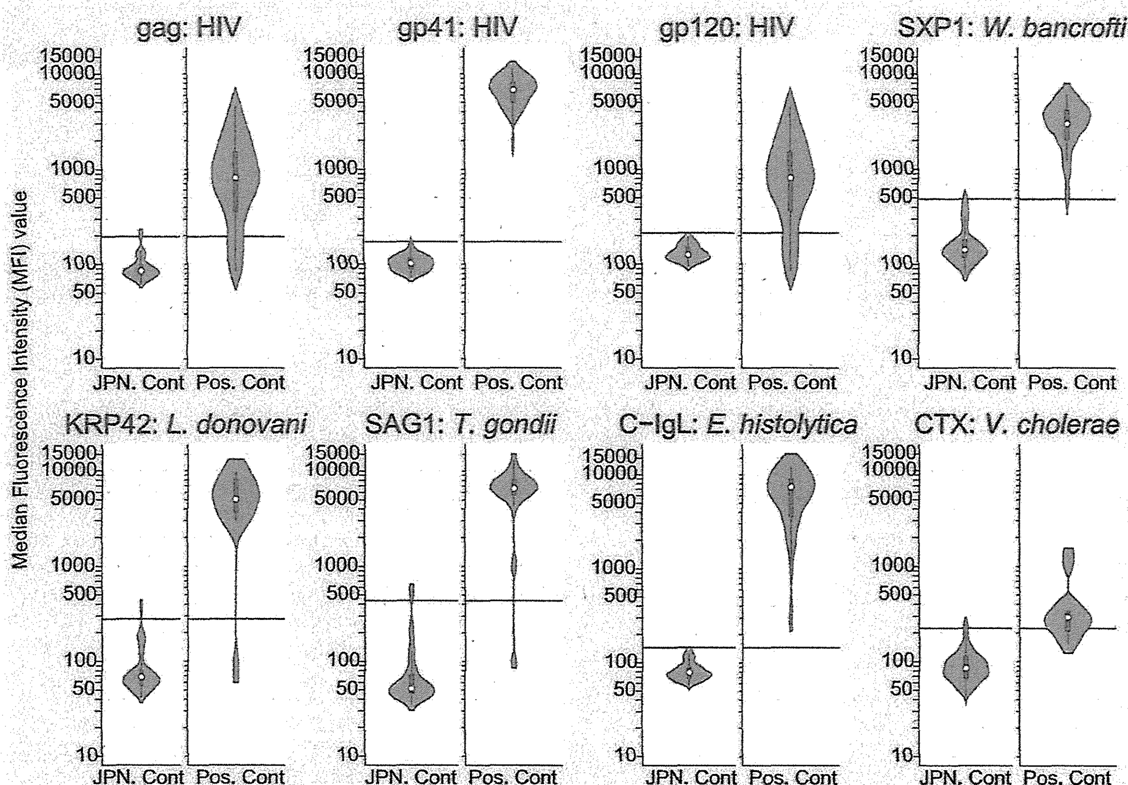

Figure 1. Serum reactivity by the multiplex assays for 8 antigens among healthy Japanese and clinically diagnosed positive sera: HIV (gag, gp41and gp120), *W. bancrofti* (SXP1), *L. donovani* (KRP42), *T. gondii* (SAG1), *E. histolytica* (C-IgL), and *V. cholerae* (CTX) antigens. Red horizontal lines represent cut-off values calculated as the means plus three standard deviations (SDs) of the distributions of median fluorescence intensity (MFI) values of 40 healthy Japanese serum samples.
doi:10.1371/journal.pntd.0003040.g001

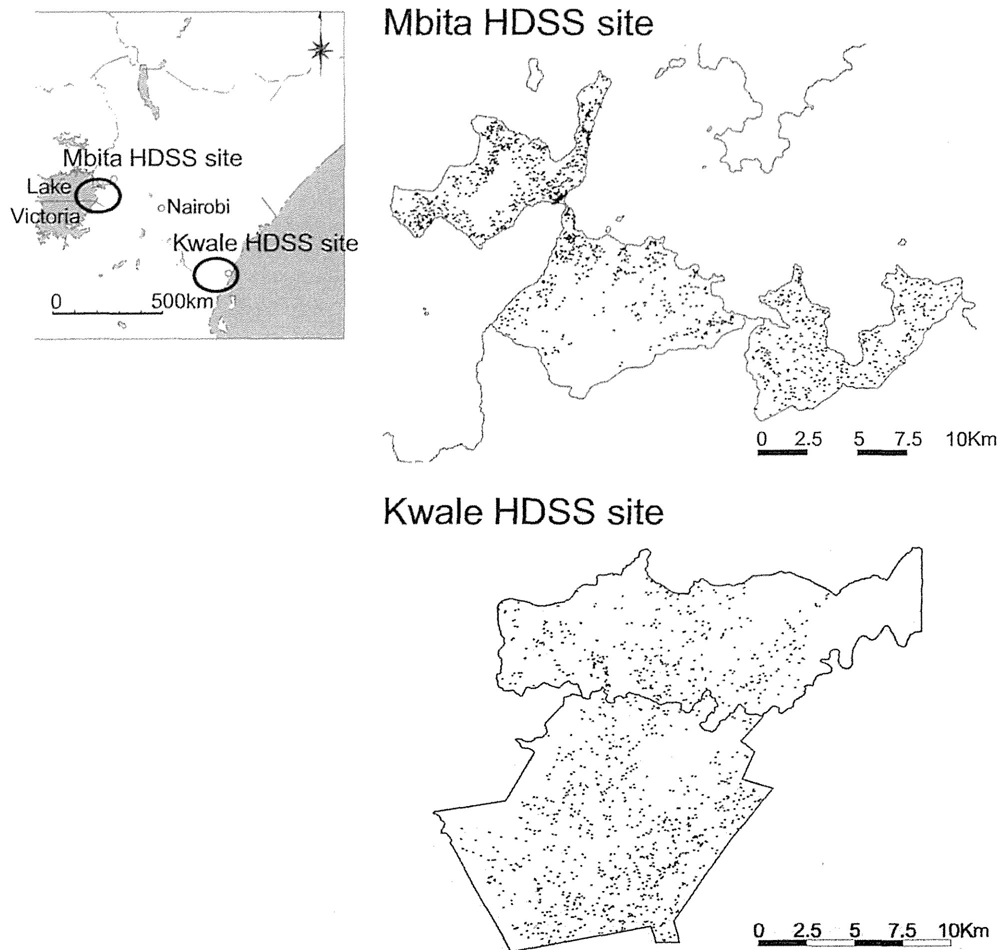

Figure 2. Geographic distribution of sampled populations from the two HDSS sites. Each dot represents individuals selected for the survey. From each HDSS site, 2200 residents were selected by sex and age group. At the Kwale HDSS site, 1453 individuals agreed and participated in blood sample collection. At the Mbita HDSS site, 1958 individuals agreed and participated.
doi:10.1371/journal.pntd.0003040.g002

(SDS-PAGE). All antigens were of good purity as shown in Figure S1. All recombinant antigens except gp41 ectodomain were detected at the predicted molecular weight calculated based on their amino acid sequences. Regarding gp41 ectodomain, the predicted molecular weight was 24.9 kDa; however, the expressed protein ran as two major bands of approximately 24 kDa and 48 kDa. The bigger size band was presumed to be the dimer of the recombinant antigen. For CTX, two major bands were detected, consistent with the expected presence of a single A-subunit (approximately 27.2 kDa) and five B-subunits (approximately 11.6 kDa) in each CTX molecule. Two bands also were detected in the anti-human IgG antibody, consistent with the expected tertiary structure of light chains (approximately 25 kDa) and heavy chains (approximately 50 kDa).

Evaluation of the microsphere-based multiplex assay

The ranges of MFI values for each antigen are shown in Figure 1 for negative and positive controls (separately) using a violin plot format that shows the median, a box indicating the interquartile range, spikes extending to the upper- and lower-adjacent values, and kernel density estimation. Means, SDs, and calculated cut-off values for each antigen for validation are shown in Table 2 along with seropositive numbers for positive and Japanese controls. The cut-off values varied by antigen, ranging from 144.2 for C-IgL to 485.1 for SXP1. Sensitivities and specificities (respectively) for each antigen were as follows: 100.0% (95%CI: 92.9–100.0) and 100.0% (95%CI: 91.2–100.0) for gp41 (HIV1), 80.0% (95%CI: 66.3–90.0) and 95.0% (95%CI: 83.1–99.4) for gag (HIV1), 80.0% (95%CI: 66.3–90.0) and 100.0% (95%CI: 91.2–100.0) for gp120 (HIV1), 71.4% (95%CI: 29.0–96.3) and 97.5% (95%CI: 86.8–99.9) for CTX (*V. cholerae*), 100.0% (95%CI: 83.2–100.0) and 100.0% (95%CI: 91.2–100.0) for C–IgL (*E. histolytica*), 93.8% (95%CI:69.8–99.8) and 97.5 (95%CI: 86.8–99.9) for KRP42 (*L. donovani*), 95.0% (95%CI: 75.1–99.9) and 97.5 (95%CI: 86.8–99.9) for SXP1 (*W. bancrofti*), 94.7% (95%CI: 74.0–99.9) and 95.0% (95%CI: 83.1–99.4) for SAG1 (*T. gondii*).

Temporal stability of stored microspheres coupled with antigens

The MFI values of mixed positive sera were measured in the assay processes. The period ranged from one storage day after the microspheres were coupled with antigens to 26 storage days. The

Table 3. Age and sex distribution of serological survey participants at the two HDSS sites.

years of age	Kwale site							
	Females			Males			Total	
	Sample No.	Population	Weight*	Sample No.	Population	Weight*	Sample No.	Population
0–4	91	2707	30	89	2910	33	180	5617
5–9	82	2874	35	88	3012	34	170	5886
10–14	88	2565	29	84	2835	34	172	5400
15–19	66	1969	30	76	2166	29	142	4135
20–24	58	1685	29	56	1373	25	114	3058
25–29	59	1320	22	55	1078	20	114	2398
30–34	77	1109	14	52	866	17	129	1975
35–39	79	777	10	55	645	12	134	1422
40–44	79	651	8	67	545	8	146	1196
≥45	81	2179	27	71	2004	28	152	4183
Total	760	17836		693	17434		1453	35270
years of age	**Mbita site**							
	Females			**Males**			**Total**	
	Sample No.	**Population**	**Weight***	**Sample No.**	**Population**	**Weight***	**Sample No.**	**Population**
0–4	102	3343	33	97	3353	35	199	6696
5–9	106	3763	36	107	3806	36	213	7569
10–14	105	3108	30	105	3105	30	210	6213
15–19	99	1998	20	101	2137	21	200	4135
20–24	86	2186	25	85	1690	20	171	3876
25–29	100	1983	20	93	1643	18	193	3626
30–34	97	1335	14	92	1455	16	189	2790
35–39	102	871	9	87	870	10	189	1741
40–44	103	723	7	91	539	6	194	1262
≥45	108	2911	27	92	1798	20	200	4709
Total	1008	22221		950	20396		1958	42617
years of age	**Total**							
	Females			**Males**			**Total**	
	Sample No.	**Population**		**Sample No.**	**Population**		**Sample No.**	**Population**
0–4	193	6050		186	6263		379	12313
5–9	188	6637		195	6818		383	13455
10–14	193	5673		189	5940		382	11613

Table 3. Cont.

years of age	Total					
	Females		Males		Total	
	Sample No.	Population	Sample No.	Population	Sample No.	Population
15–19	165	3967	177	4303	342	8270
20–24	144	3871	141	3063	285	6934
25–29	159	3303	148	2721	307	6024
30–34	174	2444	144	2321	318	4765
35–39	181	1648	142	1515	323	3163
40–44	182	1374	158	1084	340	2458
≥45	189	5090	163	3802	352	8892
Total	1768	40057	1643	37830	3411	77887

*Weights represent the probability that an individual was selected into the sample from a population. The weights are calculated by taking the inverse of the sampling fraction; and used for finite mixture models.

doi:10.1371/journal.pntd.0003040.t003

assays were not conducted every day, so that the measurement of MFI of the mixed positive serum was not performed on a daily basis. The coefficients of variation of the MFI measurements were <13%, indicating that the assay should be stable for at least 26 days (Figure S2).

Population-based serological survey

A total of 3411 individuals agreed to blood collection, including 1453 individuals from the Kwale HDSS and 1958 individuals from the Mbita HDSS. The geographical distributions of the sampled populations are shown in Figure 2, and sex and age group distributions of the participating individuals are shown in Table 3. Participation proportions from the two sites were 63.2% (1,453/2,300) from Kwale and 85.1% (1,958/2,300) from Mbita.

The distributions of immunoassay values for each antigen are shown in Figure 3 along with separated subpopulations obtained by the finite mixture model; cut-off values were obtained from the distribution of assay values among Japanese populations as well as from the Youden index calculation. According to finite mixture models, the distribution of immunoassay values (MFI values) were separated into two to five components, with specific numbers as follows: HIV (gp41), four; HIV (gp120), four; HIV (gag), five; *W. bancrofti* (SXP1), three; *L. donovani* (KRP42), three; *E. histolytica* (C-IgL), three; cholera toxin (CTX), two; *T. gondii* (SAG1), five. For each antigen, the model with the lowest BIC value was selected. The BIC results are shown in Table S1.

From the distributions separated by the model, optimized cut-off values for the immunoassays were calculated. For some antigens, the distributions were separated into four or five components; two cut-off value points were calculated to compare the differences of the cut-off values, although the third and fourth distributions were used for HIV because there were apparent separations between those distributions. Using the two separated distributions, we optimized cut-off values for each antigen by calculating the Youden index. The optimized cut-off value was defined as the maximum value of the Youden index for each antigen. The distributions of antibody MFI values to *T. gondii* were separated into four or five normal distributions. For *T. gondii*, only the cut-off value with the high-specificity setting was used because of the separable shape of the distribution. Sensitivities and specificities were obtained using the above-calculated cut-off values (using the same positive and negative control sera as used for the validation process); the results are shown in Table S2.

In Table 4, seropositivities simultaneously measured by the microsphere-based multi-serological assay are shown according to pathogens from the Kwale and Mbita sites, respectively. Seropositivities of each pathogen for the whole population were as follows for the Kwale and Mbita HDSS sites (respectively): HIV, 3.0% and 20.1%; *L. donovani*, 12.6% and 17.3%; *E. histolytica*, 12.8% and 16.6%; *T. gondii*, 30.9% and 28.2%. Seropositivities for *W. bancrofti* were 21.8% and 13.5% and those for *V. cholerae* were 31.7% and 24.9%. These results might be carefully interpreted taking immunological cross reactions into account between *W.* bancrofti-SXP1 antigen and other parasitic infection; and between cholera toxin and enterotoxigenic *E. coli* (ETEC). The values of seropositivities by sex, age, and site are shown in Table S3and S4.

Discussion

The aim of this study was to develop and evaluate a microsphere-based multiplexed serological assay for infectious diseases that makes it possible to conduct immunological assay for several NTDs concurrently. This assay system enabled us to

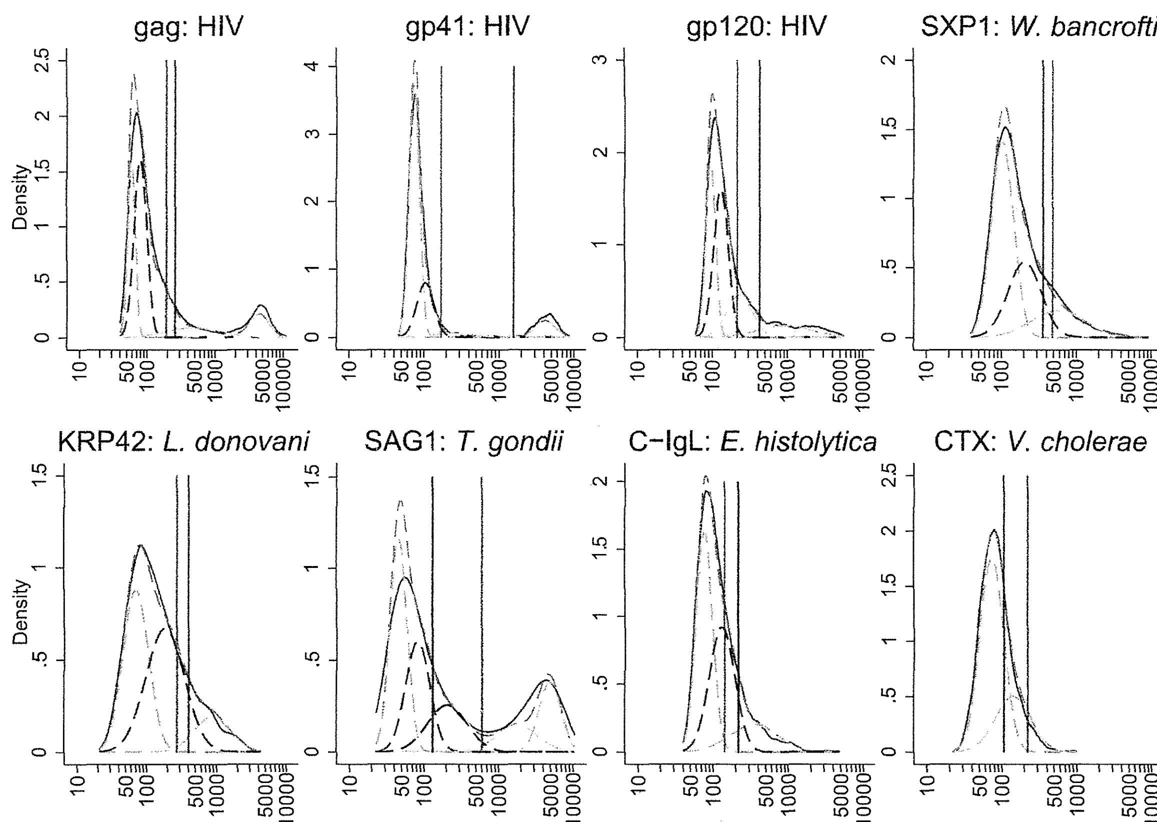

Figure 3. Distribution of median fluorescence intensity (MFI) for antigens of HIV (gag, gp41, and gp120), *W. bancrofti* (SXP1), *L. donovani* (KRP42), *T. gondii* (SAG1), *E. histolytica* (C-IgL), and *V. cholerae* (CTX). Solid lines: Kernel density of the distribution. Actual distributions are expressed as histograms. Dotted lines: Normal distribution separated by mathematical models (finite mixture models) used to calculate cut-off values for each antigen. Vertical lines: Green represents cut-off values calculated using Japanese volunteers; red represents those calculated by two normal distributions obtained by mathematical models. MFI: outcome value of the microsphere-based assay system; these values are roughly equivalent to antibody titer.
doi:10.1371/journal.pntd.0003040.g003

determine the epidemiological and geographical distributions of these diseases,and to monitor the effects of control programs of NTDs and other infectious diseases, particularly those with similar geographical patterns [4]. For development of the system, we applied IgG antibody measurement. Such antibody titering is generally considered unsuitable for clinical diagnosis of infectious diseases, because the technique does not permit distinguishing between present and past infections. However, from an epidemiological point of view, IgG antibody prevalence to target antigens is effective for long-term monitoring and surveillance for chronic infectious diseases like NTDs in communities [4].

During the development of the assay system, two major issues had to be addressed prior to applying this system for our field survey. The first issue was antigen production. Antigens suitable for antibody detection had to be selected and prepared by coupling with microspheres. In the development process, we focused primarily on recombinant antigens; recombinant antigens were expected to provide higher specificity than crude antigens, which were expected to induce non-specific antibody reactions in serum. Purity of recombinant antigens is essential to avoid unintended binding of antibodies to non-specific bacterial antigens. We purified soluble antigens (derived by expression from the pET52b or pET19b expression vector) by two-step affinity chromatography using N-terminal StrepTag II- and polyhistidine-tag. In our hands, this two-step process was effective for obtaining highly purified antigens, particularly for antigens with low expression levels.

The second issue was that of cut-off value evaluations. We tried to set cut-off values for each antigen based on assay value (MFI value) distribution among infection-negative groups (specifically, in the sera obtained from a Japanese population). However, a high immunological background had been reported in African populations [26,27]. As the result, cut-offs determined using the serum of a Japanese population or from developed countries may correspond to values lower than those expected in infected populations, leading to elevated frequencies of false positives in an African population.

Additionally, the sampled population might have different immune status groups; for example, populations currently infected (high antibody titer); those infected in the past but subsequently treated or lacking active infection (low or middle antigen titer); and those never infected (lower antibody titer). For public health intervention to control infectious diseases, the surveillance should incorporate the currently infected population at the community

Table 4. Sero-positive proportions measured by multiplex assays and prevalence ratio at the two study sites.

	Female		Male		Total		Prevalence Ratio	
Pathogen	**Positive**	**95%CI**	**Positive**	**95%CI**	**Positive**	**95%CI**	**Ratio**	**95%CI**
1) Kwale site								
HIV	3.70%	(2.3–5.0%)	2.30%	(1.2–3.4%)	3.00%	(2.1–3.9%)	ref	
W. bancrofti	19.20%	(16.4–22.0%)	24.70%	(21.5–27.9%)	21.80%	(19.7–23.9%)		
L. donovani	11.10%	(8.8–13.3%)	14.30%	(11.7–16.9%)	12.60%	(10.9–14.3%)		
E. histolytica	12.50%	(10.1–14.9%)	13.10%	(10.6–15.6%)	12.80%	(11.1–14.5%)		
V. Cholerae	29.60%	(26.4–32.9%)	33.90%	(30.4–37.4%)	31.70%	(29.3–34.1%)		
T. gondii	31.80%	(28.5–35.2%)	29.90%	(26.5–33.3%)	30.90%	(28.5–33.3%)		
2) Mbita site								
HIV	24.10%	(21.5–26.7%)	15.80%	(13.5–18.1%)	20.10%	(18.3–21.8%)	6.63	(6.32–6.93)
W. bancrofti	12.60%	(10.6–14.6%)	14.50%	(12.3–16.8%)	13.50%	(12.0–15.0%)	0.62	(0.47–0.77)
L. donovani	17.30%	(14.9–19.6%)	17.40%	(15.0–19.8%)	17.30%	(15.6–19.0%)	1.37	(1.21–1.54)
E. histolytica	17.30%	(14.9–19.6%)	15.90%	(13.6–18.2%)	16.60%	(15.0–18.2%)	1.3	(1.13–1.46)
V. Cholerae	24.20%	(21.6–26.9%)	25.70%	(22.9–28.5%)	24.90%	(23.0–26.8%)	0.79	(0.68–0.90)
T. gondii	31.30%	(28.5–34.2%)	24.90%	(22.2–27.7%)	28.20%	(26.2–30.2%)	0.91	(0.81–1.02)

doi:10.1371/journal.pntd.0003040.t004

level. To avoid such false results and to determine the mixed distribution of different immune statuses among sampled population, we applied finite mixture models to identify hidden population groups in the distribution of assay values within the sampled population [29,30].

Distinct immune distributions in the assay values can be identified by including different geographical areas with distinct endemic for each pathogen. Ideally, if we could cover three geographical areas with different endemicity (e.g., non-endemic areas, middle endemic areas, and highly endemic areas), the ideal cut-off values for each pathogen would be obtained. We covered two distinct areas with different endemicity of infectious diseases in Kenya for this study, although endemicity did not differ for all of the examined pathogens.

Regarding interpretation of the results from the microsphere-based multiplex serological assays, the theoretical principle behind the technology for multiplex assays is the same as that for ELISA; therefore, the interpretation for each assay is expected to be similar to that for the interpretation of ELISA results. The MFI distribution for HIV1 antigens was sufficient to permit distinguishing between samples that were negative and positive for infection, as mentioned in the supplementary text. In terms of geographical differences, seropositivities for HIV antigens in Mbita were higher than those in Kwale, both for males and females. Sorting by age group made the difference in seropositivities much clearer. Particularly in age groups older than the twenties, seropositivities rapidly increased up to approximately 50% in Mbita, but such trends were not observed in Kwale. This trend may reflect differences in HIV infection risk factors between the sites. Lower rates of seropositivity among the young population (<20 years old) may be atributed to the current situation of HIV control; however, results will need to be carefully monitored among all of these age groups.

Seropositivities for other pathogens can be interpreted in a fashion similar to that for HIV. Regarding toxoplasmosis, seropositivities to *T. gondii* increased linearly according to age both in Kwale and Mbita, meaning that exposure to *T. gondii* exists in both areas with similar probabilities for all age groups. Toxoplasmosis is a zoonotic parasitosis, and the risk of human infection in rural areas of Africa is common, since sheep and goats likely have been infected by *T. gondii*. Based upon our results, the risk of infection may be the same for all age groups, given that the prevalence of seropositivity increased linearly from the young age group to the elder age groups. *T. gondii* infection is reported as one of the risk factors for convulsive epilepsy in Africa [33]; thus, more active surveillance and control programs for *T. gondii* may be necessary for Africa women of child-bearing age.

Regarding the serological results for *L. donovani*, there was a report of an outbreak in northeastern Kenya in 2000 [34]. Although no similar report has been published regarding other regions, there may be undetectable sporadic outbreaks in other areas of Kenya; the seropositivity observed in the present study may reflect one or more such sporadic outbreaks. The seroprevalence was higher in the population <20 years old from the Mbita site (Figure 4). The cause for this higher positivity for *L. donovani* in the younger population of the Mbita site is unknown, although the trend might reflect cross-reactivity to other pathogens [35]. Further investigations are required to clarify this high frequency of seropositivity for *L. donovani*.

For the assay for *E. histolytica*, the recombinant surface antigen C-IgL was previously validated [12] and was used for seroepidemiology of *E. histolytica* in Chinese populations [36]. Seropositivities in all age groups were higher in our study areas compared with the Chinese population, in which seropositivities ranged from 3–8% [36]. Comparing the seropositivities among children from our two study sites, the rates for children from Mbita were generally higher than those for children from Kwale. This trend may be due to the difference in the source of drinking water and latrine distributions for both sites. In Mbita, most of the households use water directly fetched from Lake Victoria and there is low latrine usage; on the other hand, in Kwale, many households use water collected from water pipes and latrine distribution has improved in recent years [25].

For *W. bancrofti*, seropositivity should be interpreted carefully, though the seropositive proportion in Kwale was higher in those > 20 years of age compared with the same cohort in Mbita and this result may reflect the past endemic status of filariasis and the effect of elimination programs for filariasis with mass drug administration (MDA) in recent years in the coastal area of Kenya, historically a region where filariasis was highly endemic [37,38]. However, the seropositive rate in the young population in Mbita was approximately 10%, meaning that there might be some cross reactions between SXP1 and other parasitic diseases [39], because Mbita region is recognized as a non-endemic area of filariasis. Basically, for sero-diagnosis to SXP1 antigen, IgG4 subclass is used; however, our microsphere-based multiplex assay is to detect IgG antibody titers for multiple pathogens, simultaneously; therefore, our system might be capturing other cross-reactive IgG subclasses to other pathogens. To reduce the effect of the cross-reaction on prevalence estimation, further study must be done to add different type of antigens on our assay system.

Cholera seropositivity must be also interpreted with care, because cholera toxin and the enterotoxigenic *E. coli* (ETEC) are immunologically related and ETEC infection is common in developing countries [40]. Furthermore, in a case-control study on young child death in another area of Kenya, there was no case of cholera, though deaths due to ETEC were detected [41]. Therefore, the positive prevalence of cholera in the present study may reflect infection by ETEC in the study area. The decreasing trends of positivity are also consistent with the decreasing ETEC infection after five years of age in other countries [42]. Therefore, the detection of CTX by microsphere-based serological assay may not be practical.

Beyond the specific interpretations of the results of our study for individual pathogens, the largest advantage of the microsphere-based multi-serological assay is to facilitate comprehensive and simultaneous monitoring for multiple pathogens. Establishing seroepidemiological surveillance programs in different and larger regions is expected to provide a monitoring system to determine geographic and temporal trends for several infectious diseases. Such a multiplexed assay also is expected to permit the evaluation of control programs such as MDA, and to facilitate monitoring for re-emergence of infectious diseases of interest once it is decided that such diseases have been successfully controlled.

Selection of target pathogens and antigens are essential for any such surveillance program. The pathogens surveyed in this study were selected on a somewhat ad hoc basis, depending on antigen availability and comments from disease specialists. However, realistically pathogens must be selected according to the demand from the field and policy in the affected areas, as well as support from a large number of researchers working in the area. Further development would be enhanced by a platform to share information on pathogens; such a platform should be targeted and should incorporate antigens suitable for surveillance programs. By doing so, made-to-order surveillance programs suitable for specific areas with different situations and disease burdens can be devised. Furthermore, multiplexed assays should include several antigens for each single pathogen, to provide detection with high

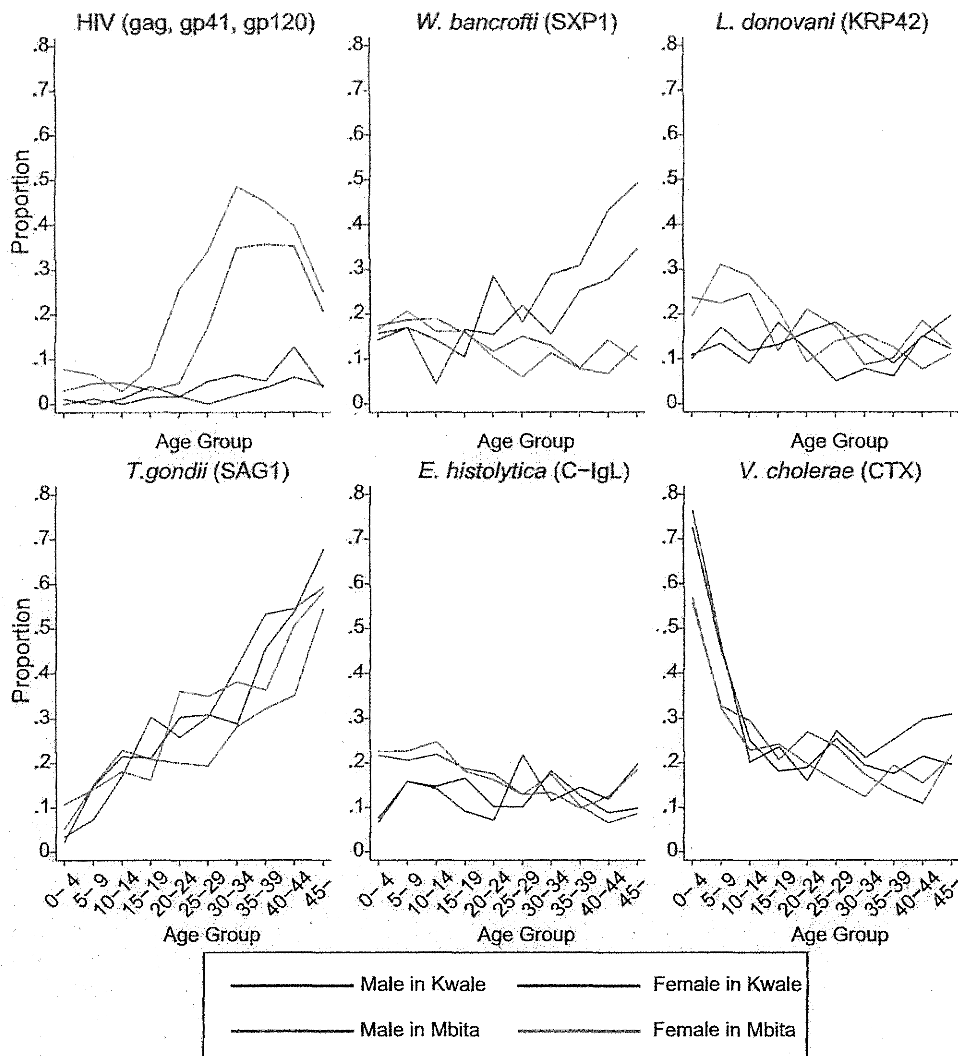

Figure 4. Age- and sex-specific prevalence of serological positives among sampled populations from Kwale and Mbita sites for HIV, *W. bancrofti*, *L. donovani*, *T. gondii*, *E. histolytica*, and *V. cholerae*. HIV positivity defined as at least two serological positives among three antigens tested (gag, gp41, and gp120).
doi:10.1371/journal.pntd.0003040.g004

sensitivity and specificity. In Kenya, the national multi-year strategic plan for control of NTDs was published by the government in 2012 [43]. The target NTDs are schistosomiasis (bilharziasis), soil transmitted helminthiasis, lymphatic filariasis (elephantiasis), trachoma, leishmaniasis (Kala-azar), and hydatid disease (echinococcal disease). At minimum, those targeted diseases would have to be covered by our system.

Moreover, the advantage of the microsphere-based multiplex serological assays is the cost of the raw material expenses for production of microspheres coupled with antigens; in this study, the cost for 9 antigens per sample was about one US dollar including the cost for antigen preparations, purifications, coupling antigens with microspheres, and microsphere purchases. The low running cost for assays would be benefit to continue and geographically expand the surveillance activities, although we cannot conclude that the same cost can be applied if the manufacturing processes were transferred to a commercial basis. In contrast, the equipment (assay machine) for the microsphere-based multiplex serological assay is not inexpensive (about US$ 80,000). Because the manipulation of the device is simple and easy, we would encourage the establishment of a centralized system of the assays with a blood sampling framework from geographically wider areas; filter paper sampling or dried blood spot sampling would be a good option for such centralized surveillance system [44].

In summary, we report the development of a microsphere-based multiplex serological assay to simultaneously measure IgG against several antigens. This work is just the first step in the practical development and implementation of actual future programs. By adding pathogens and antigens of interest as well as geographically expanding the covered areas, we would be able to establish and optimize made-to-order high-quality programs with the ability to choose suitable pathogens and antigens according to the actual situations. This new assay system is expected to enable us to provide a monitoring framework that