Roche LightCycler 480 system (Roche, Indianapolis, IN, USA) using Taqman primers and probes; 5′- GCGACGTCCGT GGAAAGAA-3′, 5′-GGCGGGTACACATTAGCAGAA-3′ and reporter (FAM): 5′-CAACGCGTATTCCC-3′ (Applied Biosystems Inc., Foster City, CA, USA). The custom gene assay was developed using ABI FileBuilder 3.1 (open selection) from the conserved region of *Leishmania* REPL repeats (L42486.1), which can be detected in *L. donovani* and *Leishmania infantum* [18,19]. For standard curves, *L. donovani* promastigotes were spiked into uninfected human blood/paxgene mixtures to provide a concentration of 1000 parasites/mL in duplicate, allowed to incubate at room temperature for 4 h, and then serially diluted with human blood/paxgene mixture to provide a range of $1000–10^{-1}$ parasites/mL blood. The dilution set was frozen at −20°C overnight, then DNA was extracted using the QIAamp DNA Mini Kit. No template controls or blood from healthy US donors was included in each assay. Samples were initially examined in duplicate; for those samples that did not amplify, or that had very late amplification (≥40 cycles) the assay was repeated in triplicate. Mean Cp values of amplification were extrapolated against the standard curve to enumerate the number of parasites per mL of blood. Samples that displayed no products at >40 cycles were considered negative. A two-tailed, paired *t*-test was performed to determine correlations between the circulating parasite DNA at baseline and follow up.

Results

Quantitative PCR as a measure of circulating parasite DNA

The presence of *L. donovani* DNA was assessed by quantitative PCR, which is sensitive enough to detect the DNA equivalent of 0.01 *L. donovani*/mL of blood and can be accurately extrapolated to a standard curve to measure the presence of parasite DNA equivalents in the blood of study subjects. The number of PCR-positives fell from 47 of the 56 study subjects (84%) at baseline to 16 (28%) at 12-month follow up (Table 1). One new PCR positive emerged at follow up while 40 positives at intake became PCR negative. The predicted parasite burden in these samples was very low, the median being 0.1 *L. donovani* DNA equivalents/mL of blood. A plot of individual trends of PCR status through the study of the 40 study subjects who became negative at follow up illustrates a highly significant drop in PCR positivity at follow up, with a *p*-value of $2.5*e^{-9}$ by a paired, two-tailed Student's *t*-test (Fig. 2a). The 16 who remained positive at follow up showed similar parasite DNA contents at baseline and follow up with one negative gaining detectable parasite DNA at follow up (Fig. 2b). The data suggest that the presence of *L. donovani* DNA is transient, consistent with results obtained in a larger survey of asymptomatic children in Brazil [20].

TABLE 1. Summary of study outcome. Number and percentages of study subjects who tested positive by the indicated tests at baseline and 12-month follow up including those that became negative and new positives detected at follow up

Diagnostic test	Positive at baseline (%)	Positive at 12-month follow up (%)	Became negative at follow up	Became positive at follow up
Direct agglutination test	32 (57)	32 (57)	2	2
Leishmania donovani whole cell lysate ELISA	46 (82)	42 (75)	8	4
rk39 ELISA	50 (89)	51 (92)	1	2
Quantitative PCR	47 (84)	16 (28)	32	1

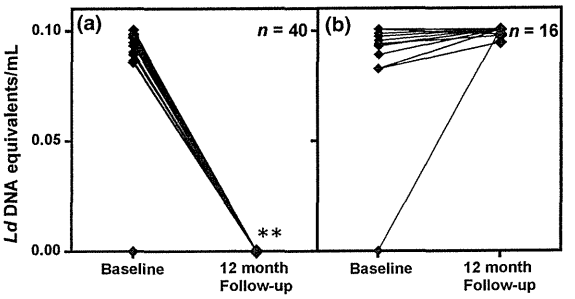

FIG. 2. *Leishmania donovani* DNA equivalents measured by quantitative PCR, quantified by extrapolating to a standard curve with known numbers of parasite. (a) Progression of *L. donovani* DNA loads of the 40 study subjects who tested PCR negative at 12-month follow up. (b) Progression of *L. donovani* DNA loads of the 16 study subjects who tested PCR positive at 12-month follow up. Each line represents an individual tested at baseline and follow up and *n* represents the number of individuals. ***L. donovani* DNA equivalents at baseline and follow up were significantly different as measured by a p value of $2.5*e^{-9}$ by a paired, two-tailed *t*-test.

DAT provides an incomplete indication of asymptomatic infection

DAT is a standard test, developed primarily for serological confirmation of VL. When applied to sero-evaluation of asymptomatic individuals, DAT positivity was 57%, both at baseline and at 12-month follow up (Table 1). Thirty-two study subjects were DAT positive at baseline with 30 of the 32 remaining positive at follow up (Table 1). At follow up, two new positives emerged. At baseline and follow up, the median dilution at which agglutination was observed remained 3200 (Fig. 3a). Correlation of DAT with PCR at baseline was about

64% whereas two PCR negative samples were DAT positive (Fig. 4a). We then compared DAT and PCR profiles at follow up to see how the dynamics of *Leishmania*-specific antibodies correlates to parasite DNA. DAT profiles at follow up revealed poor correlation to PCR. Twenty-two of the 40 PCR negatives at follow up (55%) remained DAT positive, two among them being new seroconverts (Fig. 4a). DAT profiles of the 16 PCR positives at follow up reveal a 63% correlation, with ten also remaining DAT positive (Fig. 4b).

ELISAs indicate persistent presence of antibodies

To provide comparability between DAT and ELISA, we used *L. donovani* WCL as the coating antigen to detect anti-leishmania antibodies. At baseline, 46 individuals were positive for antibodies against WCL (82%) of which 38 remained positive at the 12-month follow up, with four new seroconverts (75%) (Table 1). Similar to the results obtained with DAT, the median OD was relatively unchanged over time, being 2.5 at baseline and 2.0 at follow up (Fig. 3b). At baseline, *L. donovani* WCL positivity correlated well with PCR positivity, with 40 of the 47 PCR positives also testing positive for *L. donovani* WCL antibodies (85% correlation) but seven of the nine PCR negatives also testing positive for *L. donovani* WCL antibodies (78%) (Fig. 4a). At follow up, correlation between PCR and the *L. donovani* WCL ELISA was higher than with DAT, with 75% testing positive or 12 out of 16 PCR positives (Fig. 4b). But 80% of PCR negatives at follow up (32 out of 40 individuals) also remained positive by *L. donovani* WCL ELISA (Fig. 4b).

Recombinant rk39 is a well-defined diagnostic antigen that has been used in both ELISA and RDT [21,22]. However, tests composed of the rk39 antigen have also been used, in an off-label manner, for epidemiological screening, as opposed to only for diagnosis. Consistent with initial inclusion of asymptomatic individuals based on rk39 RDT positivity, antibodies against rk39 were detected in sera of most study subjects at both baseline and follow up by ELISA. At baseline, 50 (89%) tested positive by rk39 ELISA, of whom 49 remained positive at follow up with two new seroconverts (92%) (Table 1). The median OD remained relatively unchanged at 2.8 and 2.9, respectively, between these times (Fig. 3c). Though rk39 positivity correlated well with PCR positivity at baseline, with 89% or 42 of the 47 PCR positives also being rk39 positive, similar to the *L. donovani* WCL ELISA, eight of the nine PCR negatives at baseline (89%) remained rk39 positive (Fig. 4, top). Among the 16 PCR positives at follow up, 75% or 12 remained

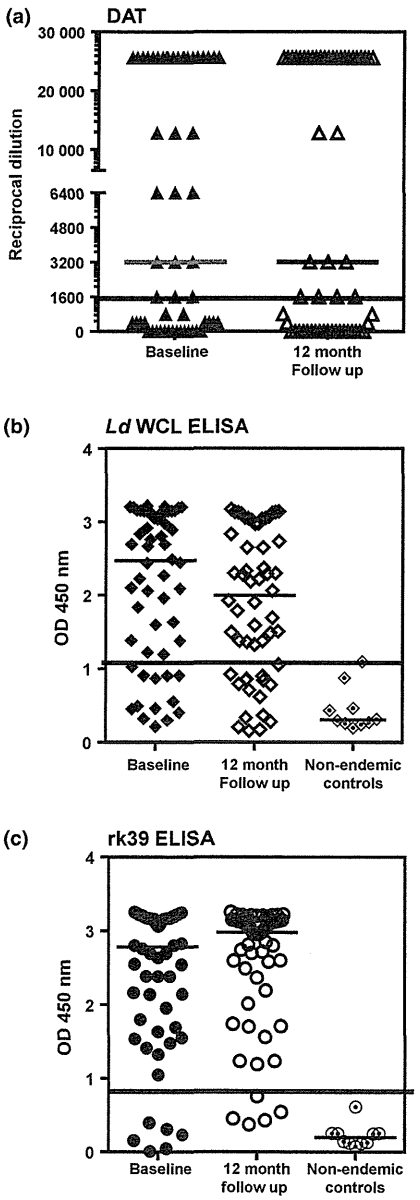

FIG. 3. Distribution of seroreactivity of asymptomatic individuals at baseline and follow up. (a) Comparison of the reciprocal serum dilution at which agglutination was observed by direct agglutination test of individuals at baseline (closed triangles) and follow up (open triangles). Black line represents cut-off for positivity at 1600. (b) Comparison of optical density (OD) at 450 nm of individuals at baseline and follow up by antibody detection ELISA against *Leishmania donovani* whole cell lysate (WCL; closed and open diamonds) along with non-endemic controls (dotted diamonds). Black line represents cut-off OD for positivity at 1.05. (c) Comparison of OD at 450 nm of individuals at baseline and follow up by antibody detection ELISA against rk39 (closed circles) along with non-endemic controls (dotted circles). Black line represents cut-off OD for positivity at 0.89. Medians for each distribution are represented by black bars. No significant differences between baseline and follow-up measurements were seen for any of the serological tests.

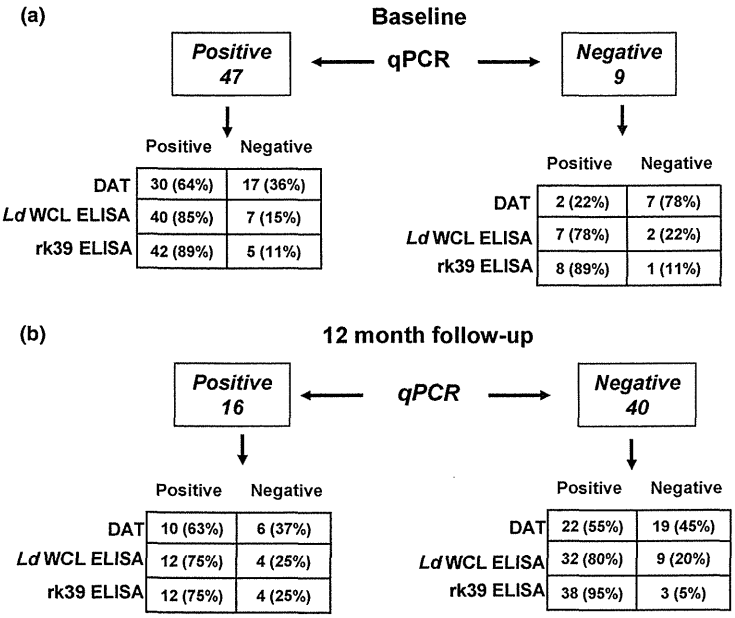

FIG. 4. Correlation between quantitative PCR (qPCR) and serological tests in asymptomatic individuals. The distribution of qPCR positive and negative individuals as detected by direct agglutination test (DAT), *Leishmania donovani* whole cell lysate (WCL) and rk39 ELISA has been depicted. (a) Correlation of qPCR positivity and negativity with DAT, *L. donovani* WCL and rk39 ELISA at baseline. Number of individuals positive or negative followed by percent positive or negative in parenthesis. (b) Correlation of qPCR positivity and negativity with DAT *L. donovani* WCL and rk39 ELISA at 12-month follow up.

rk39 ELISA positive. But 95% of PCR negatives at follow up (38 out of 40) still remained rk39 ELISA positive attesting to a poor correlation between rk39 ELISA and PCR, very similar to the other serological tests used in this study (Fig. 4, bottom). Hence, a persistent antibody response to *L. donovani* WCL and rk39 was seen at baseline and at 12-month follow up. The serological results of all study subjects at baseline and follow up relative to PCR are represented in Fig. 6.

Serological tests are complementary

We then compared the complementation among the three serological tests. The *L. donovani* WCL ELISA detected five out of six rk39 ELISA-negative individuals (83% complementarity) at baseline whereas DAT could detect three of six (50% complementarity) (Fig. 5a, left). With two individuals being positive by both tests, one was detected only by DAT and three were detected only by *L. donovani* WCL ELISA (Fig. 6). At follow up, *L. donovani* WCL ELISA detected three out of five rk39-negative individuals (60% complementarity) compared with two by DAT (40% complementarity) (Fig. 5a, right). Because DAT positivity was lower than ELISA positivity in this study and DAT is a widely used diagnostic test in the Indian sub-continent we asked whether ELISA could complement DAT. At baseline, 21 of the 24 DAT negatives were positive by rk39 ELISA with the trend remaining the same at follow up (Fig. 5b). Of the 24 DAT negatives at baseline, 20 were positive by *L. donovani* WCL ELISA and 18 of the 24 DAT negatives at follow up were positive by *L. donovani* WCL ELISA (Fig. 5b). Both DAT seroconverts at follow up were positive by rk39 ELISA whereas one was positive by *L. donovani* WCL (Fig. 6).

Discussion

Disease intervention programmes, particularly disease elimination programmes, will benefit from proactive identification of high-risk individuals. A comprehensive application of biomarker-based tests for diseases in which humans are the primary or only source of infection can make such identification possible. An excellent example of a disease to which this strategy may be applied is VL. Because VL in the Indian subcontinent is anthroponotic, identifying asymptomatic infected individuals should be considered a priority within kala-azar elimination programmes [23]. Developing tools that can predict progression to VL disease will lead to early intervention and treatment, two goals of the elimination programme [6]. In previous studies to identify such tools in

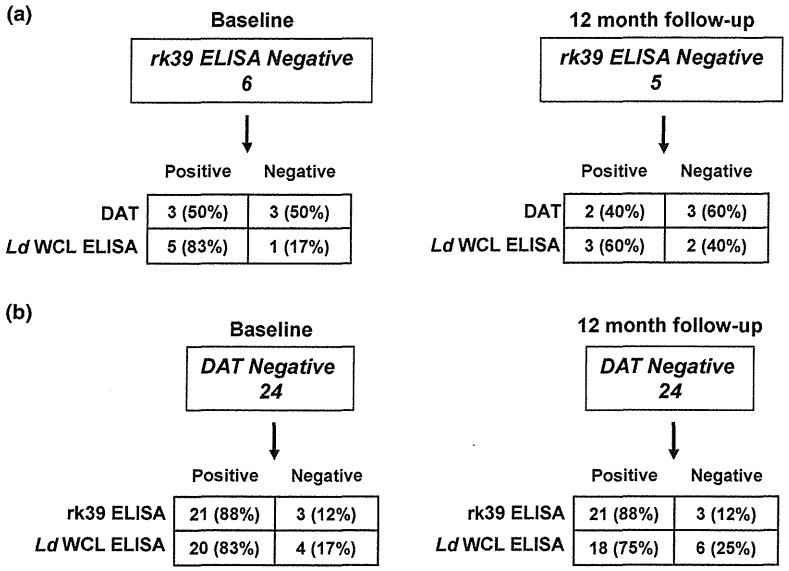

FIG. 5. Complementation of serological tests in asymptomatic individuals. Detection ofrk39 and direct agglutination test (DAT) -negative individuals. (a) Complementation of rk39 ELISA negativity by DAT and *Leishmania donovani* whole cell lysate (WCL) ELISA at baseline and 12-month follow up. (b) Complementation of DAT negativity by rk39 and *L. donovani* WCL ELISA at baseline and 12-month follow up.

India, the rk39 RDT and DAT have proven to be of limited utility in predicting progression to disease, because the tests did not correlate with each other nor did seroconversion correlate with disease progression [11,23,24]. To develop better tools to reflect the dynamics of asymptomatic infection, we wanted to determine the utility of a direct method (quantitative PCR) and indirect methods (serology) for detecting asymptomatic *L. donovani* infection. We conducted a study on 56 rk39 RDT-positive individuals in an area of VL endemicity in Bangladesh using DAT and ELISA to detect antibodies and quantitative PCR to detect DNA over 24 months. Fifty-four of the 56 enrolees at baseline and 55 of the 56 enrolees at follow up tested positive by any of the serological tests used (Fig. 6). This indicates that in endemic areas, antibodies to *Leishmania* antigens can be encountered in the absence of disease, mirroring the observation that circulating antibodies are observed over extended periods after cure [13,14]. At baseline, the PCR results agreed closely with serological tests, but at the 12-month follow up the number of PCR positives was significantly decreased while serology positives remained relatively unchanged (Table 1 and Fig. 6). However, given the low parasite burden of asymptomatic infections, it is unclear whether the results reflect the actual number of individuals with active disease. Studies are underway to determine the robustness of PCR methods to detect low parasite burdens.

Though all 56 study subjects tested positive by the rk39 RDT at study intake, six baseline samples tested negative by ELISA against rk39 (Fig. 5, top). This discrepancy can be attributed to both differences in the sensitivity of the rapid test between whole blood and serum and the use of a quantitative ELISA with stringent cut-offs to identify positives, highlighting the utility of standardized tests with defined cut-offs for sensitivity when screening populations. Rapid tests may be more suitable to confirm infection in the presence of symptoms. The complementation of the few rk39-negative sera by the other serological tests underlines the utility of a broader panel of antigens provided by both lysed or freeze-dried promastigotes to detect early infection (Figs 5 and 6). Our data indicated that *L. donovani* WCL ELISA complemented rk39 better than DAT. A quantitative test such as ELISA may be more sensitive in detection compared with DAT, in which agglutination is visually assessed.

DAT is used extensively in the Indian subcontinent for both screening and detection [11,25,26]. Similar to other reports, our DAT results did not correlate with PCR [15,27]. A study conducted in the VL-endemic Terai region of Nepal found PCR positivity among healthy individuals with no history of past VL to be 17.6% and among treated VL patients to be 25.6%, while DAT positivity in these groups was 5.5% and 95%, respectively [27]. In the same study, DAT positivity was higher and correlated well with PCR in healthy household contacts of persons with present or past VL disease, confirming previous observations that constant exposure to VL patients increases the risk of acquiring infection [10,23–25].

Among the 56 study subjects, only three went on to develop VL within the 24-month study period, about 5%. Of those, two individuals remained consistently positive by PCR

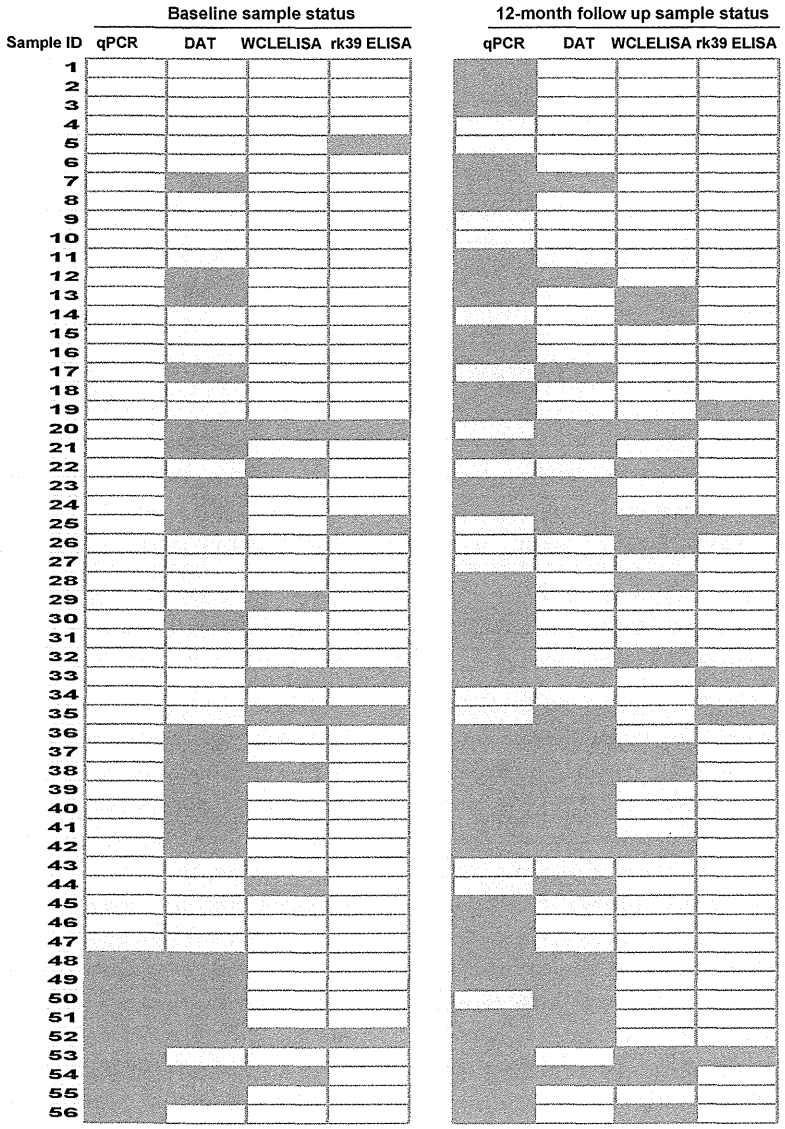

FIG. 6. Serological and PCR test status of all samples at baseline and 12-month follow up, compared side-by-side. Light grey shaded boxes indicate positive status and dark grey boxes indicate negative status

and serology, while the other was PCR positive at baseline and follow up but was consistently negative by all serological tests at baseline and positive by only rk39 ELISA at follow up. DNA has a shorter half-life than antibodies, and its presence in blood constitutes direct evidence of ongoing infection [14,28]. Appearance of DNA could also precede the appearance of detectable antibodies, signalling recent infections. In a canine model of VL, circulating parasite DNA was detected by PCR 4 months after infection, while *Leishmania*-specific antibodies were detected by ELISA 6 months after infection, reflecting the different kinetics of these biomarkers [29]. Because PCR positivity was a shared feature of those individuals that progressed to disease, sensitive and standardized DNA amplification platforms will be useful tools in active surveillance for asymptomatic infections and effectively complement standardized point-of-care serological tests. The small number of individuals who are both serology and PCR positive may represent clusters or hyperendemic foci within regions where *Leishmania* is endemic that are at the highest risk of transmitting the parasite or progressing to disease. Hence, tests that detect the presence of parasite DNA and *Leishmania*-specific antibodies will identify those individuals likely to benefit most from a vaccine or other intervention [30]. Because vaccines against VL are in development, the tests used together could

help to set enrolment criteria for trials as well as defining end-points to assess vaccine efficacy.

A limitation of this study was the small sample size, which may be the reason for lower disease conversion rates (5%) than previously reported [11,23]. We are also aware that, because positivity in the rk39 RDT was the criterion for initial screening in this study, we may have preselected individuals who were already high in antibody titres against *Leishmania*. This study was undertaken to identify tools that can reveal how asymptomatic infection emerges and contributes to VL incidence and how to design vaccine trials [29]. To clearly understand the utility of the tools we generated, they should be used in longitudinal studies on large populations within endemic regions with no preselection except for past VL disease. Such a study will help us better use these biomarkers in the detection of asymptomatic infection and design effective programmes for disease control.

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Transparency Declaration

The authors declare that they have no conflicts of interest in relation to this work.

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Research Article Open Access

Relationship between *Mycobacterium Tuberculosis* and Hookworm Infections among School Children in Mbita, Kenya

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Abstract

Tuberculosis (TB) remains a serious threat for human health. The majority of TB cases arise from latent *Mycobacterium tuberculosis* infection (LTBI). Therefore, latent *M. tuberculosis* infection (LTBI) is a major reservoir of the pathogen, and every effort thus should be made to diagnose LTBI to ensure completion of the treatment of it. TB is endemic throughout most of the tropics, in which parasitic infections are prevalent as well. It was reported that Helminth infection, including hookworm, is a risk of active TB, but its effect on the establishment of LTBI is unknown. In this study, we conducted a cross-sectional survey of LTBI and parasitic infections among 240 children from schools situated along the shores of Lake Victoria in Mbita district, Kenya. Blood samples were analyzed for LTBI and enteric parasite infections. Among the 240 children examined, 75 (31.3 %) were found to have LTBI. Of the 75 children with LTBI, 10 children (13.3%) were found to be positive for hookworm eggs (odds ratio: 3.02; 95% confidence interval: 1.14-7.99). Our study suggests for the first time that hookworm infection is associated with not only active TB but also LTBI.

Keywords: Kenya; Hookworm; Neglected tropical diseases; Latent mycobacterium tuberculosis

List of abbreviations

LTBI: Latent *Mycobacterium Tuberculosis* Infection; TB: Tuberculosis; STH: Soil-Transmitted Helminthiasis; BCG: Bacille de Calmette et Guérin; MCV: Mean Cell Volume; MCH: Mean Cell Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; PBMCs: Preparation of peripheral Blood Mononuclear Cells; ESAT-6: Early Secreted Antigenic Target 6 kDa protein; CFP10: 10kDa Culture Filtrate Protein; PPD: Purified Protein Derivative; ConA: Concavalin A; IFN-γ: Interferon γ; Th2: T helper 2; Th1: T helper 1; IL-10: Interleukin-10

Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis*, an acid-fast bacillus and is a serious threat for human health. In 2010, 8.8 million people newly developed TB and 1.1 million people died from TB [1]. After the infection is established, the majority (~95%) of individuals does not develop the disease, but instead maintain long-term latent infection. Five to 10 percent of asymptomatically infected persons develop TB during their lifetime by endogenous reactivation. Therefore, Latent *M. Tuberculosis* Infection (LTBI) is a major reservoir of the pathogen, and every effort thus should be made to diagnose LTBI to begin treatment of it in order to reduce TB burden [2].

Parasitic infections are common in TB endemic areas and are likely to impact on the high burden of TB [3]. Among parasitic infections, neglected tropical diseases caused by parasites, such as filariasis, onchocerciasis, schistosomiasis, and Soil-Transmitted Helminthiasis (STH), are still serious problem for human health. A high prevalence of STH and schistosomiasis are closely related to poverty, poor environmental hygiene, and lifestyle [4]. It is estimated that 1.4, 1, and 1.3 billion people globally suffer from *Ascaris lumbricoides, Trichuris trichiura*, and hookworm, respectively. Seven hundred twenty million people infected with STH are estimated to have clinical symptoms, and approximately 135,000 people die from complications per year [5]. Recent studies also suggest that chronic helminthic infection may reduce the efficacy of Bacille de Calmette et Guérin (BCG) vaccination, a live attenuated vaccine against TB [5,6]. Furthermore, regions with a high burden of helminthiasis are correlated with a high TB burden, implying that helminthic infection is a potential risk for TB development and vice versa [7]. However, the relationship between establishment of LTBI and each parasitic infection is unknown.

TB in children is public health problems with special significance, because children are more likely to progress life-threatening forms of the disease than adults. Besides, TB in children is a marker of recent transmission. However, information has been lacking about LTBI and

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its risk among children in TB endemic area, so far. Kenya has a large and rising TB burden [8] and it ranks 13th among the 22 high-TB burden countries in the world. The incidence of TB in Kenya was reported as 29,800 per 100,000 people in 2010 by the World Bank report released in 2011. A high prevalence of other infectious diseases, including parasitic infections, has been reported in Kenya as well [9-11]. Mbita is located on the lakeshore of Lake Victoria, Nyanza province, Western Kenya and is endemic area of several life threatening infectious diseases. In this study, we investigated the relationship between LTBI and parasitic infection among school-aged children in Mbita, Kenya.

Materials and Methods

Ethical considerations

Study procedures followed were in accordance with the ethical standards of the Helsinki Declaration and approved by the Scientific Steering and Ethical Review Committees of the Kenya Medical Research Institute (SSC No. 2084). All data collection activities were careful explained, and oral consent was obtained from the district authority and other opinion leaders (health personnel and education officials), the headmasters, and the representatives of the pupils' parents. Informed consent for the participation of children to the study was obtained from the parents/guardians. The consent form was written in English and translated into the local languages (Kiswahili and Luo) to obtain understanding for the study. Pupils whose parents/guardians had given consent were provided with an explanation of the data collection activities and were allowed to not participate or drop out of the study if they chose. The parents or guardians of the children enrolled in the study did not incur any cost for the transport or processing of the samples.

Study area, design and population

This cross-sectional study was conducted in Mbita district located around Lake Victoria in Nyanza Province, western Kenya. The population in Mbita district is 55,929 [12]. The study target population comprised primary school children in standard four, aged 9 to 19 years. There are 64 primary schools in Mbita district. The total number of school children in standard four in the year 2011 was 1,747 (859 males and 888 females, District Education office; described in research proposal SSC1088). About aqueous supplies, 84% of the household use water of the lake. But particularly 53.2% of the students in Kombe who go to school in the city use the tap water. In the hygienic status, there were some differences between schools at the diffusion rate of the restroom.

Cluster sampling was used with primary schools as the clusters. Prior to randomization, information on the number and size of the schools in the study area was obtained from the district education authority and entered in a computerized database. Information of the longitude and latitude of each school was added to the database using mapping data, obtained using global positioning system hardware. Schools were ranked by geographical location to allow for equal distribution of the schools over the study area. Seven primary schools, including Kombe, Wanga, Nyawiya, Usungu, Ngodhe SDA, Wasaria, and Kamasengre, were randomly selected using R version 2. 14. 0 software. Pupils in standard four in the selected schools, whose parents/guardians agreed to the study, were enrolled in the study and assessed for BCG vaccination status and a history of ever being exposed to TB at home. Stool, urine and blood samples were collected from all registered individuals for diagnosis of the various infections.

Exclusion criteria

In this study, children were excluded if they did not complete an examination or the number of CD4-positive T cell counts was under 500 cells/ml. The reductions of CD4-positive T cell counts are more likely to indicate the infection of HIV.

BCG vaccine inoculation career

To know the status of previous vaccination with BCG, we checked the presence of BCG scars on the skins of the children.

Detection of intestinal helminthic infection

The children were appropriately instructed about the procedure for the collection of stool, after which they were given labeled specimen cups on the day before the test to collect stool in the next morning. The stool specimens for 3 consecutive days were transferred to a field lab and examined for hookworm eggs within an hour and intestinal helminthic infections by the Kato-Katz thick smear technique on the next day [13].

Detection of Intestinal Protozoan Infection

Intestinal protozoan infection was examined by the thin smear method using specimens collected as described above.

Detection of *Plasmodium falciparum, P. malariae, P. ovale, and P. vivax*

Plasmodium infection was identified by microscopic examination of a thin blood smear after staining with Giemsa. Four types of malaria parasites were distinguished by their morphological characteristics.

Measurement of hematological indicators

Blood samples were analyzed by using BM6050 (JEOL, Japan) to examine the amount of the hemoglobin, the number of red blood cells, and hematocrit. Mean Cell Volume (MCV), Cell Hemoglobin (MCH), and Cell Hemoglobin Concentration (MCHC) were calculated.

Detection of LTBI

PBMCs were purified from heparinized blood samples using BD Vacutainer cell preparation tubes (Becton, Dickinson and Company, Franklin Lakes, NJ USA 07417) according to the manufacturer's instructions. After blood drawing and inversion, cell preparation tubes were centrifuged at 1,800 rcf for 20 min at room temperature. PBMCs were then collected, washed twice in Roswell Park Memorial Institute (RPMI) medium, and resuspended in 2.5 ml of RPMI medium containing 10% FCS and 50 μM 2-mercaptoethanol (RPMI complete medium).

M. tuberculosis infection was assessed by monitoring T cell responses to ESAT-6 and CFP10, which are produced by *M. tuberculosis* complex but not by *Mycobacterium avium*-intracellular complex and BCG. We used overlapping synthetic peptides to detect T cell responses to ESAT6 and CFP10, because using these peptides can prevent the effect of nonspecific reactions due to contaminations of mitogens, such as LPS, and shorten the time to detect a reaction compared with use of recombinant proteins. The amino acid sequences of the synthetic peptides are as follows below.

ESAT-6 (1-20) (H-MTEQQWNFAGIEAAASAIQG-OH), ESAT-6 (11-30) (H-IEAAASAIQGNVTSIHSLLD-OH), ESAT-6 (21-40) (H-NVTSIHSLLDEGKQSLTKLA-OH), ESAT-6(31-50) (H-EGKQSLTKLAAAWGGSGSEA-OH), ESAT-6 (41-60) (H-AAWGGSGSEAYQGVQQKWDA-OH), ESAT-6 (51-70) (H-YQGVQQKWDATATELNNALQ-OH), ESAT-6 (61-80) (H-TATELNNALQNLARTISEAG-OH), ESAT-6

(71-90) (H-NLARTISEAGQAMASTEGNV-OH), ESAT-6 (76-95) (H-ISEAGQAMASTEGNVTGMFA-OH), CFP10 (1-20) (H-MAEMKTDAATLAQEAGNFER-OH), CFP10 (11-30) (H-LAQEAGNFERISGDLKTQID-OH), CFP10 (21-40) (H-ISGDLKTQIDQVESTAGSLQ-OH), CFP10 (31-50) (H-GQVESTAGSLQGQWRGAAGTA-OH), CFP10 (41-60) (H-GQWRGAAGTAAQAAVVRFQE-OH), CFP10 (51-70) (H-AQAAVVRFQEAANKGKQELD-OH), CFP10 (61-80) (H-AANKGKQELDEISTNIRQAG-OH), CFP10 (71-90) (H-EISTNIRQAGVQYSRADEEQ-OH), and CFP10 (81-100) (H-VQYSRADEEQQQALSSQMGF-OH) were synthesized. After purification with HPLC with over 95% purity, peptides were dissolved with DMSO and mixed together to a final volume of 1 mg/ml for each peptide in each antigen.

PBMCs were cultured with PPD purchased from Japan BCG Laboratory, ConA was purchased from Sigma-Aldrich (Organic Materials and Fiber Sigma-Aldrich Corporation, St Louis, MO), and the synthetic peptides mixture was obtained according to standard procedures or without stimulants as negative controls. In brief, PBMCs derived from 250 µl of blood were suspended in a half volume (125 µl) of RPMI complete medium and seeded into the wells of 96-well round-bottomed tissue culture plates (Becton, Dickinson and Company, Franklin Lakes, NJ 07417). Another 125 µl of RPMI complete medium, which contained each antigen peptide at a final concentration of 5 µg/ml for ESAT6 and CFP10, and 10 µg/ml for ConA and PPD, was then added to the wells. The plates were incubated overnight at 37°C in a humidified atmosphere under 5% CO_2. The supernatants were collected and frozen at -30°C until assay.

The amount of IFN-γ in the supernatants was assessed by using the immune-chromatographic test, which was a house-made for research use in Otsuka (Paper preparation, Fukushima et al., Usefulness of the measurement of interferon-gamma in tuberculous pleural effusion and quantiferon samples by the immunochromatography test). Briefly, 50 µl of culture supernatant was added to the reaction tube and the test stick, in which anti-IFN-γ antibody was embedded, was soaked in the supernatant. After 15 minutes, the test stick was transferred to the well of a plate containing 100 µl of washing buffer. After the color of the background of the test stick disappeared, a positive line was visualized when over 125 pg/ml of IFN-γ was contained in the supernatants.

Data analysis

Data were analyzed with Mann-Whiney test, Wilcoxon rank sum test, chi-square test and odds ratio by using IBM SPSS 20.0 software.

Results

In this study, we analyzed data from 240 school children. The demographic characteristics of the study population are shown in Table 1. One hundred sixty-seven students (53.8%) were vaccinated with BCG by confirming BCG scars on their skins.

We evaluated LTBI in a comprehensive manner with conventional health assessment and in vitro-examination of T cell immune responses to ESAT6 and CFP10 that are unique protein antigens expressed by *M. tuberculosis*, but not from BCG vaccine strains or the majority of non-tuberculosis mycobacteria. By examining culture supernatants of PBMCs derived from 240 students, who did not show fever or prolonged cough over 2 weeks by a health assessment, we detected more than 125 pg/ml of IFN-γ in 191 children (79.6%) when their PBMCs were stimulated with PPD and more than 125 pg/ml of IFN-γ in 75 children (31.3%) when stimulated with ESAT-6 and CFP10 (39 females and 36 males) (Figure 1). All responders to ESAT-6 and CFP10 were included in the PPD responders. As predicted, BCG vaccination did not show a correlation with responses to ESAT6 and/or CFP10 (p=0.11). Based on these results, the children were assigned as individuals with LTBI when they did not show any symptom of TB and their PBMCs produced IFN-γ upon being stimulated with ESAT6 and CFP10.

We simultaneously assessed infections with various parasites. There were no differences among schools at the prevalence of protozoal and parasitic infection except for *S. mansoni*. Table 2 summarizes the number of students found to be positive for hookworm, *A. lumbricoides, T. trichiura, Schistosoma mansoni, Plasmodium falciparum, P. malariae, P. ovale, P. vivax, Entamoeba coli, E. histolytica/E. dispar/E. moshkovskii, Iodamoeba, Giardia intestinalis, Balantidium, Coccidia, and Blastocystis.*

The association between LTBI and other parasitic infections was examined using the Mann-Whitney U-test, the Wilcoxon rank sum test, and the chi-square test. We found a significant association between LTBI and hookworm infection (p=0.02). By contrast, we did not find any association between LTBI and other parasite infections. The p values for comparison between LTBI and each parasite infection were p=0.61 for *A. lumbricoides*, p=0.22 for *T. trichiura*, p=0.30 for *S. mansoni*, p=0.16 for *P. falciparum*, p=0.50 for *P. malariae*, p=1.00 for *P. ovale*, p=1.00 for *P. vivax*, p=0.71 for *E. coli*, p=0.86 for *E. histolytica/E. dispar E. moshkovskii*, p=0.22 for *Iodamoeba*, p=0.28 for *G. intestinalis*, p=0.18 for *Balantidium*, p=0.21 for *Coccidia*, and p=0.68 for *Blastocystis* (Table 2).

The numbers of individuals positive for hookworm and LTBI are shown in Table 2 together with their odds ratios with 95% confidence intervals. The odds ratio of individuals infected with hookworm for LTBI was 3.019 times that in individuals without hookworm (95% confidence interval: 1.14-7.99), indicating a possible association between hookworm infection with LTBI among school children.

Discussion

In this study, 75 out of 240 (31.3 %) students were defined as LTBI among the students of standard 4 in Mbita, Kenya. Ten students were infected with hookworm out of 75 individuals with LTBI, while there were eight students with hookworm out of 165 individuals without LTBI. The odds ratio of hookworm infection in the LTBI population was 3.019 times as high as that in the non-LTBI population.

Many parasitic infections, including helminthiasis, are called neglected tropical diseases, which are specially endemic in low-income population in the developing countries but affect over one billion people [14]. The prevalence of hookworm infections, such as by *Ancylostoma duodenale* and *Necator americanus*, are estimated to

Characteristics	n	Kombe (n=26)	Wanga (n=25)	Nyawiya (n=40)	Usungu (n=39)	Ngodhe SDA(n=25)	Wasaria (n=57)	Kamasengre (n=28)
Males	105	14	12	19	16	9	24	11
Females	135	12	13	21	23	16	33	17
Mean age	240	11.8 ± 1.27	12.2 ± 1.33	11.8 ± 1.77	10.7 ± 1.16	11.4 ± 1.60	12.0 ± 1.66	12.3 ± 2.44

Table 1: Number of school children in standard 4 examined in each school in Mbita, Kenya.

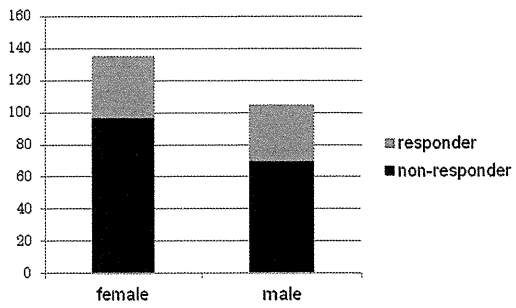

Figure 1: Number of asymptomatic ESAT6/CFP10 responders (LTBI population) and non-responders in male and female study subjects. The vertical axis indicates the number of students.

be frequent in developing countries, such as Asia, Africa, and Latin America, and approximately 740 million people are infected with these parasites in worldwide [14]. Infections with helminths, including hookworm, cause distinct immune responses. It is generally accepted that helminthic infection causes T helper 2 (Th2) immune responses. For example, Th2-dependent eosinophilic pneumonia is frequently observed after infection with a significant number of helminths, such as filaria, hookworm, or *A. lumbricoides*. However, some reports have shown that T helper 1 (Th1), rather than Th2 cytokines, are produced from PBMCs derived from hookworm infected-individuals in endemic areas [15-17]. In endemic areas, people are frequently exposed to pathogens that induce a Th1 response before or after they are infected with helminths, which may be the reason why Th2 immune responses appear dampened, even in those co-infected with helminthes. Infection with protozoa or bacteria that can stimulate a Th1 response results in suppression of the Th2 response induced by helminthic infection in mouse models [15,18,19].

On the other hand, it was reported that co-infection of both helminths and *M. tuberculosis* results in the suppression of IFN-γ production [20]. The Th2 response caused by hookworm infections may suppress a protective Th1 response against *M. tuberculosis* infection, and therefore, hookworm infection may allow the survival of *M. tuberculosis* and vice versa. Because we detected LTBI by measuring IFN-γ, an indicator of Th1 response, we cannot deny the possibility that LTBI-negative 8 children who infected with hookworm are false-negative for LTBI. Taking into account this possibility, our data indicates all the more significant association between LTBI and hookworm.

In our study, only hookworm infection was correlated with LTBI, in spite of the fact that other helminths, such as *A. lumbricoides* or *T. trichiura*, can evoke robust Th2 responses, as well as in the hookworm infection. In addition, we did not find the significant increase of the level of peripheral eosinophils, which are index of the Th2 immune response compared with those in uninfected children in this study. It is likely that the shift of an immune response from Th1 to Th2 caused by helminthic infections is not a sole reason to explain higher ratio of LTBI in hookworm-infected students.

Immunosuppressive T cells, such as regulatory T cells (Treg), suppress cellular immune responses through direct contact with immune effector cells and by the production of regulatory cytokines, including TGF-β and IL-10 [21,22]. It has been reported that hookworm infection causes induction of $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ regulatory T cells [23] and repeated infection with hookworm stimulates production of high levels of IL-10 [24], which inhibits host protective immunity against *M. tuberculosis*. Accordingly, there is a possibility that hookworm-induced IL-10 impacts on the sensibility to *M. tuberculosis* infection.

It is generally known that iron-deficiency anemia is caused by heavy infection with adult hookworms in the intestinal tract. It is estimated that 36 million out of 1,300 million people infected with hookworm have iron-deficiency anemia, and 65,000 people annually die from hookworm-induced anemia. Iron-deficiency anemia is a typical symptom observed in individuals heavily infected with hookworm, but rarely seen in those with other helminths. Iron status also affects TB disease; for example, iron overload is reported to be a risk for TB progression [25]. In this study, the MCV and MCHC of the students, which are indicators of iron-deficiency anemia, were lower than the standard values generally, but there was no difference between hookworm-infected and non-infected children (MCV: p=0.35; MCHC: p=0.47), and there was no difference in iron status between responders to ESAT6 and/or CFP10 and non-responders (p=0.16). Accordingly we did not observe obvious effects of anemia caused by helminth infection on the establishment of LTBI in this study.

The current study found that hookworm infection was associated with LTBI. As discussed above, the exact reason of this finding is unclear. In mice model, recent report showed that *Mycobacterium*-specific both Th1 and Th17 cells by hookworm infection enhances establishment of LTBI [26], supporting our human study. We consider that next studies should examine whether this association can be observed in other area and adults as well. Addictively, the studies should be conducted to examine whether hookworm infection causes expansion of the LTBI population and/or directly contributes to TB progression in addition to establishment of infection because there are several reports indicating that helminthic infection is a risk factor for developing TB [27-29]. Our study suggests the necessity of the deep studies to understand the relationship between *M. tuberculosis* and hookworm infection on the prevalence and progression of disease in high-burden countries, including tropical and subtropical areas.

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ソフトコンタクトレンズ消毒剤の汚染状況

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Incidence of Soft Contact Lens Disinfectant Bottle Contamination

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ソフトコンタクトレンズ（SCL）消毒剤の病原菌汚染を調査するために183例のSCL装用者から使用中のSCL消毒剤を回収した。SCL消毒剤の容器内液の6例（3%）から細菌が検出された。容器出口部擦過検体の58例（32%）から細菌あるいは真菌が検出された。調査期間前期（平均最高気温約27℃）と後期（平均最高気温約11℃）において検出率に差はなかった。消毒剤開封後使用期間の差による検出率の違いは認められなかった。多目的用剤使用群は過酸化水素使用群より有意に検出率が高かった（34%対6%）。容器出口部から検出された菌種はグラム陰性桿菌30例，グラム陽性桿菌25例，グラム陽性球菌19例，真菌1例だった。SCL消毒剤容器出口は高率に汚染されており，SCLケース汚染の菌叢とも類似している。SCL消毒剤容器は清潔に取り扱うよう注意する必要がある。（日コレ誌 55：109−113，2013）

キーワード：消毒，汚染，コンタクトレンズケア，細菌，アカントアメーバ

We investigated the incidence of contamination of soft contact lens (SCL) disinfectant solution bottles collected from 183 SCL users. Bacteria were found in 6 (3%) of the solutions and bacteria and fungi were found in 58 (32%) of scrapings from bottle nozzles. The contamination rates did not differ for bottles inspected earlier in the investigation period (mean highest temperature about 27℃) compared to later in the study (mean highest temperature about 11℃) and did not correlate with elapsed time after opening the bottle. However, the contamination rate was much higher (34% versus 6%) for multipurpose solutions than for hydrogen peroxide solutions. The organisms identified on disinfectant bottle nozzles included 30 Gram-negative rod species, 25 Gram-positive rod species, 19 Gram-positive cocci, and 1 fungus and were similar to organisms detected in our earlier study of contamination of SCL storage cases. This study shows that SCL users need to handle disinfectant solution bottles with greater care to prevent contamination.
(J Jpn CL Soc 55：109−113，2013)

Key Words : Disinfection, Contamination, Contact Lens Care, Bacteria, *Acanthamoeba*

緒 言

ソフトコンタクトレンズ（以下 SCL）装用に伴う角膜感染症は，重症化すれば入院加療が必要となり，治療を行っても矯正視力の低下を残すことがあるため，医学的にも社会的にも重大な問題となっている[1-3]。角膜感染症の病原菌は細菌，真菌，アカントアメーバなどと多彩であるが，ケア不良などが原因となってSCLケースの病原菌汚染が生じ，これがSCL装用時に眼表面に移動して感染を起こすと考えられる[4]。SCL消毒剤やケア用剤自体にも細菌などによる汚染が生じることが報告[5-10]されており，その頻度およびSCLケース汚染との関連を明らかにする必要がある。そこで一般的なSCL装用者が使用しているSCL消毒剤の細菌，真菌，アカントアメーバによる汚染

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の発生率およびその菌種を調査した。

実験方法ならびに対象

全国５箇所の施設（表１）を初めて訪れた，SCL消毒剤を使用しているSCL装用者とする。

方法は，SCL消毒剤回収用封筒およびSCL装用状況調査のためのアンケート用紙（表２），謝礼（クオカード500円分）を新品のSCL消毒剤とともに用意し，各施設を初めて受診した，SCL消毒剤を使用しているSCL装用者に配布した。装用者には帰宅後，使用中のSCL消毒剤と，記入したアンケート用紙を回収用封筒に入れて調査施設に返送させた。調査施設は返送された封筒を冷蔵保存し，１週間ごとにまとめて菌検査施設に送付した。菌検査は愛媛大学医学部附属病院臨床検査部が表３の手順に従って容器内液および容器出口部擦過検体に対する半定量検査を行った。容器出口部擦過検体については鳥取大学において，Ikedaら[11]の方法に従ってアカントアメーバのreal-time polymerase chain reaction（以下　PCR）法試験を行った。その際，プライマーは*Acanthamoeba*の18S rDNA領域を特異的に認識，forward:5'-CGACCAGCGATTAGGAGACG-3'，reverse:5'-CCGACGCCAAGGACGAC-3'とし，プローブは5'-FAM-TGAATACAAAACACCACCATCGGCGC-BHQとした。

気温による汚染度の違いをみるため，調査は前期（2011年９～10月。東京における平均最高気温，約27℃）と後期（2012年１～３月。東京における平均最高気温，約11℃）に分けて行った。

結　　果

前期98例，後期85例，計183例の検体が回収された。容器内液については前期の４例（４％），後期の２例（２％），全期合計の６例（３％）から細菌が検出された。容器出口部擦過検体については前期の30例（31％），後期の28例（33％），全期合計の58例（32％）から細菌または真菌が検出された。容器内液の検出率が低かったため，アカントアメーバに対するreal-time PCR法試験は容器出口部検体のみを対象とすることとした。結果は前期では200copies/mℓの１例，後期では1,850copies/mℓ，2,835copies/mℓ，3,125copies/mℓの各１例ずつ，計４例からアカントアメーバDNA断片が検出された。前期後期間で検出率に有意な差がなかったため，以後のデータは前期後期をあわせた全183例を対象として，集計，解析を行った。また，容器内液の汚染例は少なかったため，容器出口部擦過検体のみを

表１　調査施設一覧

施設名	所在地	調査担当医師
さど眼科	宮城県	佐渡　一成
道玄坂糸井眼科医院	東京都	糸井　素純
水谷眼科診療所	愛知県	水谷　　聡
稲葉眼科	大阪府	稲葉　昌丸
ウエダ眼科	山口県	植田　喜一

表２　ソフトコンタクトレンズ（SCL）装用者に記入させたアンケート用紙の項目

年齢
性別
使用SCLの種別 （１週間交換，２週間交換，１カ月交換，３カ月交換，その他）
SCLの使用頻度 （週６～７日，週３～５日，週１～２日，それ以下，その他）
使用中のSCL消毒剤の開封後経過日数 （３日以内，１週間以内，２週間以内，１カ月以内，２カ月以内，２カ月をこえる，不明）

表３　SCL消毒剤汚染調査の菌検査手順

１．SCL消毒剤内容液をピペットで抽出。同時に消毒剤容器出口をスワブで拭き，スワブを１mℓの滅菌生理食塩水を入れてミキサーで混ぜる。
２．液のうち，約50μℓを血液寒天培地/ブロムチモールブルー（BTB）寒天培地に広げ，35℃48時間培養する。
３．残った液はアカントアメーバPCR検査（表４）に使用する
４．48時間後，２．の培地でコロニーのグラム染色を行い，以下の手順で染色性および形態で区別し，性状を検査し同定を行う。
　Ⅰ．グラム陽性球菌
　　①カタラーゼ試験陽性の場合，結合型コアグラーゼ試験を行い，黄色ブドウ球菌（陽性）とコアグラーゼ陰性ブドウ球菌（CNS）に分類する。
　　②陰性の場合，EF寒天培地に接種し発育が認められた場合は腸球菌と同定する。
　　③EF寒天培地に発育しない場合，血液寒天培地でのβ溶血性を観察し，溶連菌（陽性），他のレンサ球菌（陰性）に分類する。溶連菌は群別を行う（A群からF群）。
　　④α溶血を示した場合，オプトヒン感受性試験を行い，感受性であれば*Streptococcus pneumoniae*と同定し，耐性であればα-*Streptococcus*とする。
　　⑤溶血のない場合はγ-*Streptococcus*とする。
　Ⅱ．グラム陽性桿菌
　　①芽胞陽性の場合*Bacillus*属と同定する。
　　②芽胞陰性の場合，カタラーゼ試験陽性は*Corynebacterium*属，陰性は通性嫌気性の*Lactobacillus*属とする。
　Ⅲ．グラム陰性桿菌
　　クリグラー寒天培地でグルコースの発酵性の確認およびオキシダーゼ試験を行う。
　　①グルコース発酵，オキシダーゼ陽性菌
　　*Vibrio*科，*Aeromonas*科，*Plesiomonas*属として同定を進める。
　　②グルコース発酵，オキシダーゼ陰性菌
　　腸内細菌科として，クリグラー寒天培地，リジン鉄寒天培地，シモンズ・クエン酸ナトリウム培地，DNA培地，SIM培地，VP半流動培地の性状により同定を進める。
　　③グルコース非発酵，オキシダーゼ陽性菌
　　*Pseudomonas*属，*Burkholderia*属，*Alcaligenes*属，*Achromobacter*属，*Chryseobacterium*属として同定を進める。
　　④グルコース非発酵，オキシダーゼ陰性菌
　　*Stenotrophomonas*属，*Acinetobacter*属として同定を進める。
　　⑤グルコース非発酵（上記の③。④以外）
　　IDテストNF-18を使用して，菌名を同定する。
５．菌量はおよそ次のような基準で簡易定量を行う（単位はCFU/mℓ）。
（±）10^3以下，（＋）$10^{4\sim5}$，（２＋）10^6，（３＋）10^7以上

PCR：polymerase chain reaction，SIM：sulfide-indole-motility，VP：Voges-Pronskauer

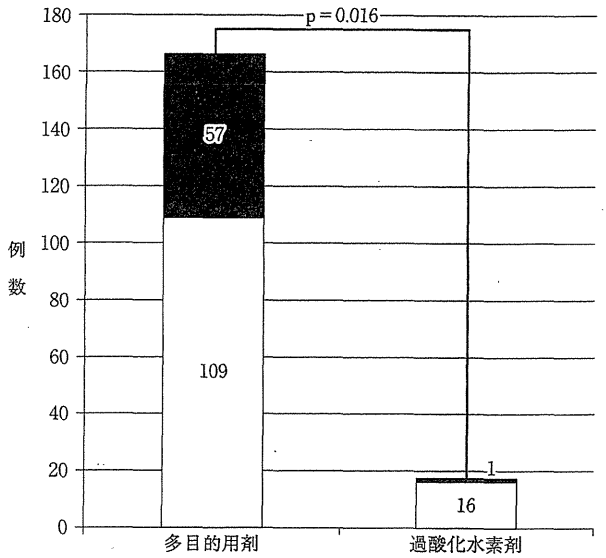

図１　ソフトコンタクトレンズ（SCL）消毒剤容器出口部擦過検体の汚染状況とSCL消毒剤の種類との関係（χ^2検定にて有意差あり）
■：汚染あり，□：汚染なし

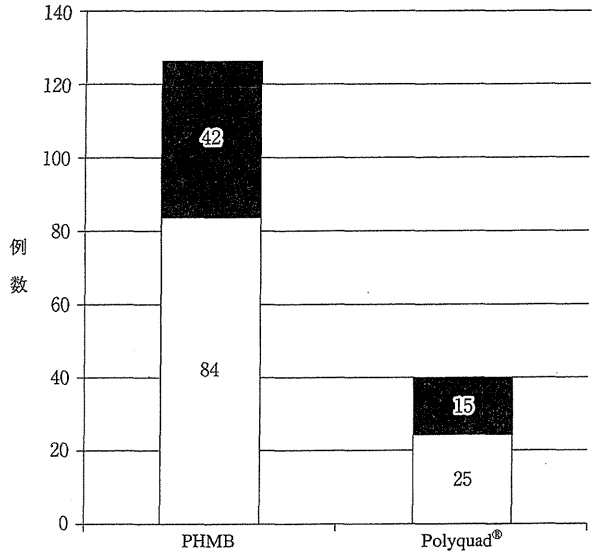

図２　SCL消毒剤容器出口部擦過検体の汚染と多目的用剤の種類との関係（χ^2検定にて有意差なし）
■：汚染あり，□：汚染なし
PHMB：塩酸ポリヘキサニド

表４　SCL装用者アンケート集計結果

年齢	16～54歳，平均年齢28±７歳（標準偏差）	
性別	女：150例　男：33例	
使用SCL種別	１週間交換SCL	2
	２週間交換SCL	149
	１カ月交換SCL	19
	３カ月交換SCL	1
	その他	12
SCL使用頻度	週６～７日	162
	週３～５日	9
	週１～２日	5
	それ以下	7
開封後使用日数	３日以内	2
	１週間以内	6
	２週間以内	19
	１カ月以内	54
	２カ月以内	52
	２カ月をこえる	36
	不明	13
	記入なし	1

表５　SCL消毒剤の開封後使用期間と容器出口部汚染状況の関連

開封後使用期間	SCL消毒剤容器出口の汚染状況	統計的有意差（χ^2検定）
２週間以内	汚染あり：８例　汚染なし：19例	なし（p＝0.778）
それ以上	汚染あり：46例　汚染なし：96例	
１カ月以内	汚染あり：28例　汚染なし：53例	なし（p＝0.484）
それ以上	汚染あり：26例　汚染なし：62例	
２カ月以内	汚染あり：46例　汚染なし：87例	なし（p＝0.158）
それ以上	汚染あり：８例　汚染なし：28例	

対象とした。

全症例中166例が多目的用剤（以下　MPS），17例が過酸化水素剤を使用しており，容器出口部擦過検体についてMPSの57例（34％），過酸化水素剤の１例（６％）に汚染が認められ，両者の差は統計的に有意であった（図１）。MPS使用者の内訳は塩酸ポリヘキサニド（以下　PHMB）剤使用者が126例，Polyquad®使用者が40例であったが，それぞれについて汚染例は42例（33％），15例（38％）であり，MPSの消毒成分の違いによる検出率の差は認められなかった（図２）。

装用者に記入させたアンケートの集計結果を表４に示す。検出率は女性では32％（150例中48例），男性では30％（33例中10例）であり，性別による差は認められなかった。使用SCL種別，SCL使用頻度についてはそれぞれ「２週間交換SCL」，「週６～７日の使用」が大多数であるため，SCL種別，使用頻度の違いについての統計的検討は行わなかった。SCL消毒剤の開封後の使用期間については開封後３日以内，１週以内は例数が少なかったため，開封後２週以内，１カ月以内，２カ月以内をそれぞれ基準として開封後使用期間についての検討を行った。「不明」および「記入なし」は対象外とした。結果は表５に示したとおり，いずれにおいても開封後使用期間の違いによる汚染状況の差は認められなかった。

容器出口部擦過検体から検出された菌種と菌の概要と詳細をそれぞれ表６および表７に示す。菌種はグラム陰性桿

表6 SCL消毒剤容器出口部擦過検体から検出された菌種

検出菌種	検出例数
グラム陰性桿菌	30
グラム陽性桿菌	25
グラム陽性球菌	19
真菌	1

菌が最も多く，次いでグラム陽性桿菌，グラム陽性球菌の順となり，菌では*Bacillus subtilis*とcoagulase-negative *Staphylococcus*（以下 CNS）が目立つ環境菌，体表常在菌主体の汚染パターンが認められた。

考　　察

SCLの消毒に用いる消毒剤自体が細菌，真菌，アカントアメーバによって汚染されている状態は問題である。しかし1987年，Donzisら[5]は支障なくCLを使用している100名の装用者から回収した126本のCLケア用剤内容液の13％に汚染が生じていたことを報告した。その後，Kanpolatら[6]はSCL装用者30例を対象としてMPS使用者（23例），過酸化水素剤使用者（6例）のそれぞれ17％において，消毒剤容器から滴下したサンプルの汚染を検出している。検出菌種は*Pseudomonas*をはじめとするグラム陰性桿菌と*Staphylococcus epidermidis*をはじめとするグラム陽性球菌がほぼ同数であった。Collins[7]らは44例のSCL装用者が2週間使用したSCL消毒剤の滴下サンプルを対象に，Polyquad®剤の14％，PHMB剤の3％，過酸化水素剤の4％から細菌汚染を検出しており，そのすべてがグラム陰性桿菌であった。また，Yungら[8]は101例のSCL装用者を対象とした調査において，SCL消毒剤容器から滴下したサンプルの11％から細菌を検出している。検出菌は*Serratia*，*Staphylococcus aureus*，CNSなどが主であった。容器出口（ノズル部）の汚染についてはLipenerら[9]が生理食塩水容器出口の60％から細菌を検出している。検出菌は緑膿菌をはじめとするグラム陰性桿菌が最も多く，次いで*S. aureus*などのグラム陽性球菌となっている。Sweeneyら[10]は40例のSCL装用者を対象にソルビン酸，ethylene diamine tetraacetic acid（EDTA）などの防腐剤を含有する生理食塩水一瓶をそれぞれ1週間，2週間，3週間，4週間使用させると同時に，別の一瓶についてはキャップの開け閉め操作だけ行わせて検出率の違いを調査している。その結果，内容液の26％，容器出口の55％から細菌あるいは真菌を検出したが，使用期間による違い，あるいは実際に使用した容器とキャップの開け閉めだけ行った容器の間に差を認めなかった。検出菌はCNSをはじめとするグラム陽性球菌が最も多く，次いで*Bacillus*属などのグラム陽性桿菌と報告している。

表7 SCL消毒剤容器出口部擦過検体から検出された菌

検出菌	検出例数
Bacillus subtilis	20
coagulase-negative *Staphylococcus*	16
Serratia marcescens	9
Achromobacter xylosoxidans	8
Bacillus spp.	5
Stenotrophomonas maltophilia	5
Comamonas acidovorans	3
Pseudomonas putida	3
Micrococcus spp.	2
Aspergillus niger	1
Staphylococcus aureus	1
Pseudomonas aeruginosa	1
Sphingomonas parapaucimobilis	1

今回の試験はSCL消毒剤を対象とし，容器内液の3％において汚染を検出した。この結果はCollinsら[7]がSCL消毒用PHMB剤，過酸化水素剤で得た結果とよく一致しているが，容器出口部擦過検体については，Collinsらの示したMPSの消毒成分の違いによる検出率の差は認められなかった。また容器出口部の検出率は34％であり，Lipenerら[9]，Sweeneyら[10]が生理食塩水の容器出口で得た55～60％という結果より低い。この差がSCL消毒剤と生理食塩水の違いに基づくものか，装用者のコンプライアンスなどの違いによるものかは不明であるが，今回，容器出口部の検出率が，より消毒力が強いと考えられる過酸化水素剤において有意に低かったことを考えると，SCL消毒剤の消毒力によって検出率が低くなった可能性がある。そうであれば，SCLケアにおいてすすぎなどに生理食塩水や防腐剤入りすすぎ液を使用する際には，容器出口，容器内液の汚染に十分注意する必要があることになる。ただし，MPSと過酸化水素剤では使用方法が大きく異なるため，今回の検出率の差が消毒力以外の要因によって生じた可能性も考慮する必要がある。

今回の検出菌種はグラム陰性桿菌が最も多く，その点ではSCL消毒剤を対象としたCollinsら[7]の結果と類似しているが，グラム陽性桿菌，グラム陽性球菌もある程度検出されている点で異なる。逆にグラム陽性球菌は少なく，SCL消毒剤についてのKanpolatら[6]，Yungら[8]の結果，生理食塩水についてのLipenerら[9]，Sweeneyら[10]の結果とは異なっている。このような差が湿度温度などの気候の違いによるものか，家屋構造や生活習慣の違いによるものか，あるいは水質やコンプライアンスの差などによるものかは不明だが，SCLケア用剤，SCL消毒剤から検出される細菌叢にバリエーションがあることは確かである。アカントアメーバについてはreal-time PCR法試験で4例においてアカントアメーバDNAの断片が検出されたが，実際

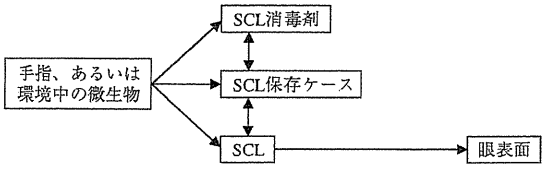

図3　SCL 保存ケース，SCL 消毒剤汚染と眼表面のあり得る関係

に生きたアカントアメーバが居たか，感染源になり得る状態だったかは不明である。

以前，我々が SCL 保存ケースの汚染状況について調査した際[12]，グラム陰性桿菌が最も多く検出され，次いでグラム陽性球菌，グラム陽性桿菌の順であった。今回はグラム陰性桿菌，グラム陽性桿菌，グラム陽性球菌の順であったが，ともに CNS と *Bacillus subtilis* が首位と 2 位を占めており，パターンは比較的相似している。このことから，SCL 保存ケースの汚染源と SCL 消毒剤の汚染源に共通性があるため，これらになんらかの交通があることが推測される。SCL 消毒剤内液より容器出口部の汚染が強いことから，手指や周囲との接触によって容器出口部が汚染され，それが SCL 保存ケース内液の汚染につながることが考えられる。逆に SCL 消毒剤を SCL 保存ケースに注出する際に，SCL 保存ケース内の液に接触することによって容器出口部が汚染される，あるいは両者間に交通が生じる可能性もあるが，Lipener ら[9]の結果のようにキャップの開閉しか行わなかった容器にも同様の汚染が生じることから考えると，SCL 保存ケースと消毒剤容器間の交通は少ないのかもしれない。一方，宇野ら[2]による重症 CL 関連角膜感染症全国調査において，CL 保存ケース内汚染と角膜感染症病巣との関連が示唆されている。これらをあわせると，図 3 のように手指あるいは環境からの微生物汚染が，SCL 保存ケース，SCL 消毒剤，SCL 自体を通して相互に関係しながら眼表面への感染へと発展する経路が推測できる。そのなかで SCL 消毒剤の汚染が占める役割が従属的なものなのか，主たる原因の一部なのかは不明であるが，眼表面への感染を防ぐために SCL 消毒剤を清潔に保つことは重要と考えられる。

容器出口部の汚染が主であり，内容液自体はほとんど汚染されていないことから，消毒剤を十分な量流して使用すれば汚染はある程度抑制できると考えられる。今回の結果では表 6 に示したように，開封後使用期間による差は明らかでなかった。ただし開封後使用期間の長さが CL 関連角膜感染症発症のリスクファクターであるか否かは，今回の結果だけでは判断できない。MPS の場合は大量に流して使用するとともに，短期間で使い切るよう指導する方が安全であろう。過酸化水素剤の場合は 1 回の使用量が専用保存ケースによって一定となっているから，短期間で使い切る指導が主となる。今回は開封後 2 週間以内とそれ以上との検出率までを比較したが，開封後 1 週間ではどうなのか，また通常は開封後どの程度の期間使用されているのかといった点も知る必要がある。ただ，Sweeney ら[10]の結果から考えると，開封後の使用期間と SCL 消毒剤からの細菌検出率は無関係であり，汚染例では使用開始直後から汚染が発生している可能性もある。消毒剤容器出口が手指にふれないよう注意する，使用後は直ちにキャップを閉める，SCL 保存ケースに消毒剤を注出する際には容器出口が液面に接触しないようにする，などの指導が必要である。

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原著論文 臨床研究

感染性角膜炎におけるグラム・ファンギフローラY®二重染色の有用性

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要 約

目 的：Impression法によるグラム染色・ファンギフローラY®二重染色の有用性を検討する．

対象と方法：感染性角膜炎を疑って二重染色を行った192例213眼について，臨床的な最終診断をもとにその特異性と感受性をレトロスペクティブに検討した．

結 果：感染性角膜炎165眼の内訳は，細菌性107眼，真菌性54眼，アカントアメーバ性15眼であり，二重染色にていずれかが陽性であった症例は147眼であった〔感度89.1%(95%信頼区間83.1%～93.2%)，特異度79.1%(64.6%～89.0%)〕．二重染色の感度/特異度は，細菌性角膜炎において88.8%/82.1%，真菌性角膜炎において81.4%/98.1%，アカントアメーバ角膜炎において80.0%/97.0%となった．

結 論：Impression法による二重染色は，少量のサンプルで感度の高い結果を得ることができ，特に真菌・アカントアメーバ感染症に関して特異度が高く有用性が高い．(日眼会誌117：351-356，2013)

キーワード：グラム染色，ファンギフローラY®染色，アカントアメーバ角膜炎，真菌性角膜炎

Original Article Clinical Science

Efficacy of Gram-Fungiflora Y® Double Staining in Diagnosing Infectious Keratitis

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Abstract

Purpose：To evaluate the efficacy of Gram-Fungiflora Y® double staining for corneal scraping samples collected by impression in diagnosing infectious keratitis.

Subjects and methods：Two hundred and thirteen eyes of 192 patients suspected of having infectious keratitis were retrospectively studied for the sensitivity and specificity of Gram-Fungiflora Y® based on final diagnosis.

Results：Of 165 infectious keratitis eyes, 107 had bacterial keratitis, 54 had fungal keratitis, and 15 had *Acanthamoeba* keratitis. Of these, 147 eyes were positive for one or other of the pathogens by the double staining method (overall sensitivity/specificity was 89.1% (95% confidence interval 83.1%-93.2%)/79.1% (64.6%-89.0%)). Sensitivity/specificity of the double staining for each of the pathogens was 88.8%/82.1% for bacterial keratitis, 81.4%/98.1% for fungal keratitis and 80.0%/97.0% for *Acanthamoeba* keratitis.

Conclusion：The double staining method for impression specimens was effective in diagnosing infectious keratitis with high sensitivity, and was especially useful for the diagnosis of fungal or *Acanthamoeba* keratitis.

Nippon Ganka Gakkai Zasshi (J Jpn Ophthalmol Soc) 117：351-356, 2013.

Key words：Gram staining, Fungiflora Y® staining, *Acanthamoeba* keratitis, Fungal keratitis

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I 緒 言

感染性角膜炎の診断において，起炎菌の速やかな同定は必須のステップである．培養による同定に先立ち，病巣擦過の染色標本による起炎菌の迅速評価の有用性は最近認知されつつある．一方，感染性角膜炎の病巣に対してその検体を培養に供出し，かつ，直接検鏡に供する場合，十分な量の検体が採取できない場合も多い．つまり，培養と検鏡の両方を行う場合，結果として検出力の低下につながる可能性がある．さらに，塗抹検鏡のみ行う場合であっても，その同定対象菌種に対してそれぞれ別の染色法を用いるのもあまり現実的ではない．

ファンギフローラY®染色液(バイオメイト，東京)は，β構造を有する糖鎖に染着し，セルロースとキチンに高い親和性を持つ．このため，真菌・アカントアメーバの細胞壁を特異的に染色する[1)2)]．また，染色後の保存性も高い．一方，塗抹標本のグラム染色は，標準的な細菌検査として認知されている[1)]．

これらの染色により，細菌および真菌類，アカントアメーバは，比較的信頼性が高いレベルで検出可能となる．しかしながら，小さい病巣から二つの染色を行うために適切な塗抹標本を2枚用意することは実際的には容易ではない．また，病巣組織をスパーテルでスライドグラスに塗布する場合，サンプルロスを免れることができない．このため，病巣からサンプルを直接スライドグラスに移す採取法(impression法)を考案した．

今回我々は，細菌，真菌，アカントアメーバの同時検出のため，impression法によりサンプル採取を行い，グラム染色，ファンギフローラY®染色の二重染色を行う評価法の臨床的有用性を検討したので報告する．

II 対象と方法

2004年1月1日から2011年9月30日までの間に当院で感染性角膜炎を疑い，インフォームドコンセントを得た後，診断目的にて採取した検体に対してグラム染色・ファンギフローラY®(ファンギフローラ)二重染色(以下，二重染色)を行った192例213眼について，臨床的な最終診断をもとにその感度と特異性をレトロスペクティブに検討した．細菌性，真菌性，アカントアメーバ性角膜炎の最終診断は，所見の特徴，治療への反応性を含めた経過，培養，病巣サンプルのreal-time polymerase chain reaction(PCR)による細菌数，真菌数(ユニバーサルプライマー)，アカントアメーバ数の定量に基づき行った[3)~5)]．感度，特異度は，最終診断に基づき，二重染色陽性および陰性のサンプル数よりVassarstatsプログラムを用いて算出した(http://vassarstats.net)．

病巣部は27ゲージ針にて擦過，切除し，あらかじめオートクレーブで滅菌したスライドグラスを病巣に直接押し当て(impression法)検体を採取した(図1)．検体を

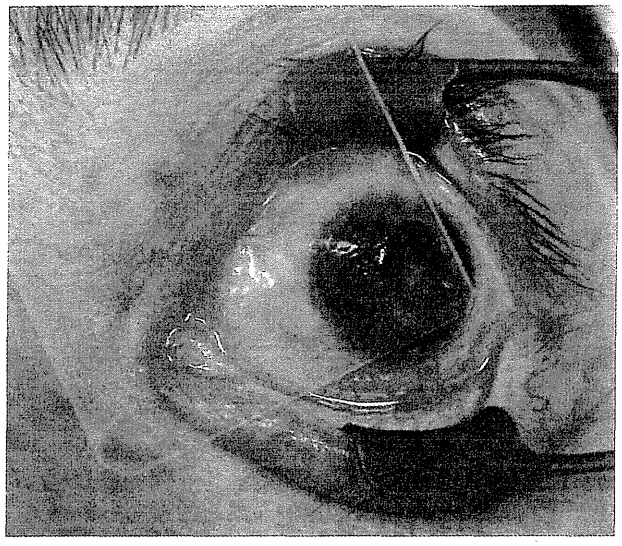

図1 Impression法による検体採取法．
27ゲージ針で病巣を擦過して擦過物を集めた後，オートクレーブにより滅菌したスライドグラスで押し当てることにより標本を採取する．

乾燥させた後，メタノールを滴下し乾燥させ固定した．次に，常法どおりにグラム染色をneo-B＆Mワコー®(和光純薬，大阪)を用いて施行し，洗浄した．ファンギフローラA液は用いず，引き続いてB液を用いて2分染色し，洗浄後乾燥させて標本とした．封入は行わず，UV励起蛍光顕微鏡(BX40，オリンパス，東京)を用いて直接観察した．

III 結 果

二重染色の有用性を検討するため，まず培養による検出率との比較を行った．感染性角膜炎が疑われ二重染色を行った症例は213眼であった．このうち治療経過，臨床所見も含めて最終的に感染性角膜炎として診断された症例は165眼であり，二重染色にて，病原体の種類にかかわらず何らかの病原体が認められたのは165眼中147眼(89.1%)であった．一方，培養にて何らかの病原体が検出された症例は165眼中75眼(45.5%)であり，二重染色は培養よりも有意に高い検出率を示した($p<0.0001$)．

165眼の感染性角膜炎のうち，細菌性は107眼(64.8%)，真菌性は54眼(32.7%)，アカントアメーバ性は15眼(9.1%)であり，その中で混合感染眼は，細菌・真菌混合感染9眼(5.5%)，細菌・アカントアメーバ混合感染が2眼(1.2%)であった．次に，二重染色の有用性の特徴を検討するため，起炎病原体別にその検出感度を検討した(図2)．二重染色は，細菌，真菌，アカントアメーバのいずれに対しても良好な感度を示し，細菌に対して88.8%(95%信頼区間80.9%～93.8%)，真菌に対して81.4%(95%信頼区間68.1%～90.3%)，アカントアメーバに対して80.0%(95%信頼区間51.4%～94.7%)となった．一方，培養の場合，感度は細菌に対して

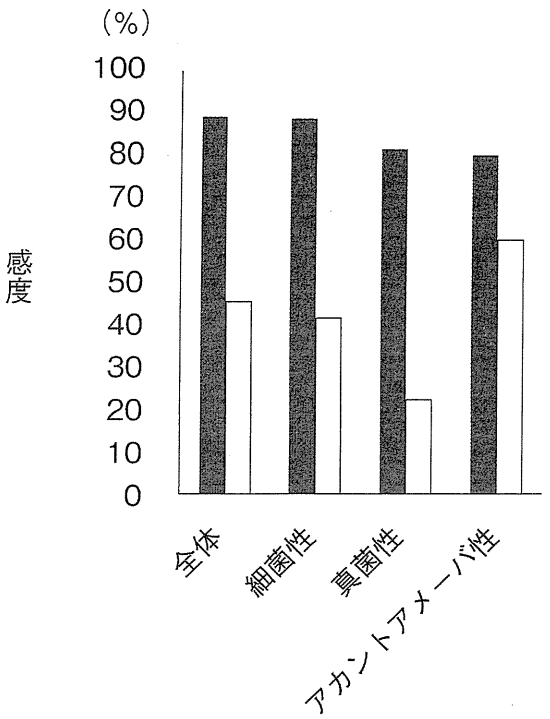

図2　最終診断に対する二重染色・培養の感度比較.
■：二重染色，□：培養.

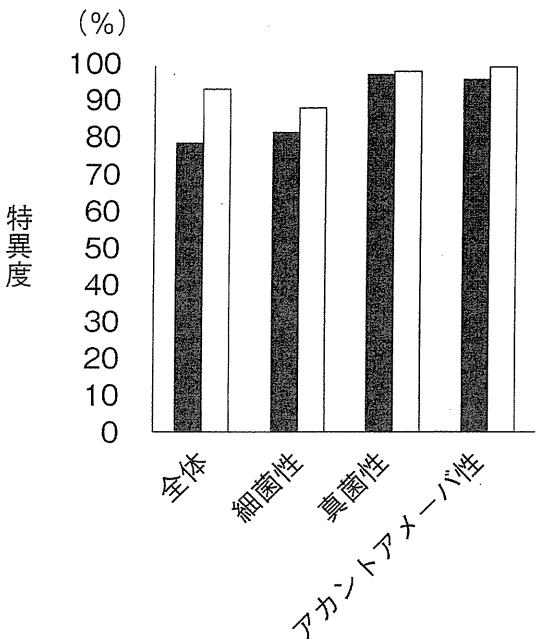

図3　最終診断に対する二重染色・培養の特異度比較.
■：二重染色，□：培養.

41.1%(95% 信頼区間 31.8%～51.1%)，真菌に対して22.2% (95% 信頼区間 12.5%～36.0%)，アカントアメーバに対して60.0%(95% 信頼区間 32.9%～82.5%)となり，特に真菌や細菌の検出に対して感度が低い傾向を示した.

次に二重染色および培養の特異度を病原体別に検討した．二重染色の特異度は，細菌に対して82.1%(95% 信頼区間 73.2%～88.6%)，真菌に対して98.1%(95% 信頼区間 94.2%～99.5%)，アカントアメーバに対して97.0%(95% 信頼区間 93.2%～98.8%)となり，特に真菌およびアカントアメーバに対して高い特異度を示した．一方，培養の場合，特異度は細菌に対して88.7% (95% 信頼区間 80.7%～93.8%)，真菌に対して98.7% (95% 信頼区間 95.1%～99.8%)，アカントアメーバに対して100.0% (95% 信頼区間 97.6%～100.0%)となり，二重染色は，培養に比べて遜色のないレベルの特異度を示した(図3).

図4に二重染色の代表的な染色像を示した．アカントアメーバ角膜炎の病巣の場合，励起光をカットするとグラム染色のみの画像となる(図4A左)．シストがぼんやりと観察できるが，この場合熟練した観察者でも同定は困難と想定される．一方，まったく同一視野を蛍光照明で観察した場合(図4A中，右)，アカントアメーバのシストが明瞭に観察される.

次に糸状菌による真菌性角膜炎の病巣部の二重染色を図4Bに示した．糸状菌は，グラム染色によりグラム陽性に染色される(図4B左)．一方，蛍光照明下では，さらに菌糸の詳細な構造が観察でき，真菌は容易に同定できる(図4B中，右)．同一患者の細菌と真菌が混在する爪白鮮検体の染色像を図4Cに示した．グラム染色では，多数の陽性球菌が認められるが，蛍光照明なしでは，グラム陽性像を示す病原体が真菌であるのかははっきりしない．しかしながら，蛍光照明下のイメージと比較すると，糸状真菌と細菌像は明瞭に区別可能となる.

Ⅳ　考　　按

Impression法による二重染色は，簡便であり，細菌，真菌，アカントアメーバなど病原体の有無に関して優れた感度を示した．スワブを介さず直接スライドグラスにとるため，検体のロスが少なく，さらに，グラム染色とファンギフローラ染色用に分割する必要がないことがその理由の一つになっていると考えられた．一方，培養による同定は，感度は低いものの二重染色より高い特異度を示し，互いに補完する役割を持つと想定された.

角膜潰瘍の病巣からは，一般に少量の検体しか採取することができない．また，病巣を表層や深層などレベル別に考えた場合，特定の部位のみに病原体が多い場合がある．この場合，感度を上げるためには，多いと想定される部位のサンプルを検鏡する必要があるが，現実的には，あらかじめの想定とは異なる分布を示す場合もある．そこで，例えば表層のみならず深層のサンプルを分割することなくまとめて1つの塗抹標本で評価する方法のメリットは高いと考える．これまで，標本採取は，スパーテルなどを用いてスライドグラスに塗抹する方法が