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Evaluation of a field test for antibodies against *Chlamydia trachomatis* during trachoma surveillance in Nepal

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Abstract

Purpose: Testing for antibodies to *Chlamydia trachomatis* has potential as a surveillance tool. Our evaluation compares lateral flow assays (LFAs) during surveillance surveys in Nepal with Multiplex bead array (MBA). Fifty children were randomly sampled from each of 15 random clusters in two districts of Nepal. Finger prick blood samples were collected from 1509 children and tested onsite for anti-Pgp3 antibodies by LFA. The LFA was read at 30 min as negative, positive, or invalid. Tests results were also rated as difficult to read ("equivocal"). Blood was processed at Johns Hopkins University using the MBA.

Results: The LFA had agreement of 40.0% for MBA-positive samples and 99.3% for MBA-negative samples. Inter-reader reliability was kappa = 0.65 (95% CI = 0.56–0.74). If the equivocal results (7%) could be decreased, reliability could be improved.

Conclusions: Further optimization and testing of the LFA test are needed to improve agreement with MBA and the interpretation of the results.

Keywords

Trachoma; *C. trachomatis* ; Antibodies; Nepal; Population surveillance

1. Introduction

Trachoma, the leading infectious cause of blindness worldwide, is the result of repeated ocular infection by *Chlamydia trachomatis* (CT) (Pascolini and Mariotti, 2012). The World Health Organization (WHO) has set a goal for the global elimination of trachoma as a public health problem by 2020 and, towards this end, recommends for endemic countries

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a multi-pronged control strategy of antibiotic treatment, facial cleanliness, environmental improvements, and surgery for trachomatous trichiasis (TT) (World_Health_Organization, 1998). The current targets for each endemic district are to achieve a prevalence of TT <0.2% in adults age 15+ years (excluding the TT cases known to the local health system) and to achieve a prevalence of trachomatous inflammation-follicular (TF) <5% in children ages 1 to 9 years (World_Health_Organization, 2015).

Districts that have reached these disease elimination targets are faced with the need to implement post-MDA surveillance to ensure that trachoma has not re-emerged. Currently, WHO recommends surveillance be undertaken with a district-wide, population-based survey administered two years after cessation of MDA in order to demonstrate that TF continues to remain less than 5% (World_Health_Organization, 2015). Such a survey would provide reassurance that trachoma has not re-emerged at that point. Whereas other neglected tropical diseases, such as onchocerciasis, have an elimination goal defined as interruption of transmission, trachoma has no marker for interruption of transmission, as TF (and markers of infection) is a cross-sectional prevalence measure. Recent studies have suggested that antibody responses to the Pgp3 antigen from CT may provide serologic evidence of cumulative exposure, suggesting the absence of antibody or low levels in young children born after program cessation could provide a marker for interruption/reduction of transmission (Goodhew et al., 2012, 2014; West et al., 2016). To date, studies evaluating antibody-based surveillance have used the multiplex bead array (MBA) platform, which requires reliable laboratory infrastructure and a high degree of technical expertise. If antibody-based surveillance for trachoma is to be implemented in previously-endemic countries, a test that is easier, less costly and does not require advanced laboratory equipment would be beneficial.

In this study, we undertook the first field-test of a rapid, inexpensive lateral flow assay (LFA) recently developed for the detection of anti-Pgp3 antibodies in a field setting (Gwyn et al., 2016). We compared the results to the results obtained from the MBA.

2. Materials and methods

2.1. Ethical statement

The study was conducted with approval from the Johns Hopkins University Institutional Review Board and the Nepal National Health Research Council and was in accordance with the Declaration of Helsinki. All parents/guardians gave written informed consent for study procedures and study procedures were completed between January 2016 and April 2016. CDC co-investigators were determined to be non-engaged and had no interaction with study participants.

2.2. Population and setting

This study was conducted in a random sample of 15 clusters (Village Development Committees) in each of two districts in Nepal, Kanchanpur and Surkhet, as part of Nepal's surveillance program for trachoma. Households were randomly selected for participation and approximately 50 children ages 1–9 years were included from each cluster. In

Kanchanpur, the last impact surveys and program activities were conducted 10 years ago, and TF prevalence was 4.5%. In Surkhet, the last impact survey was 8 years ago, and TF prevalence was 3.0%.

2.3. Sample collection

A new sterile retractable lancet was utilized for each child to collect blood onto six extensions of a circular filter paper, each calibrated to absorb 10 μL (TropBio Pty Ltd., Townsville, Queensland, Australia). Filter papers were dried, stored in airtight plastic bags, stored at −20 °C until being transported to The Johns Hopkins University (JHU). Dried blood spot samples were tested at the International Chlamydia Laboratory at JHU for IgG antibodies to the Pgp3 antigen on the Luminex platform (West et al., 2016)

2.4. Lateral flow assay

The LFA consisted of a nitrocellulose membrane with a test line containing Pgp3 antigen and a control line with biotinylated bovine serum albumin, as well as a conjugate pad treated with a Pgp3-gold conjugate and streptavidin-gold conjugate (Gwyn et al., 2016). We acquired a disposable microcapillary tube that transferred 10 μL of blood in the LFA sample port from local pharmacies. Two hundred microliters of 1× PBS pH 7.4 (ion free) + 0.3% Tween-20 chase buffer was added to the buffer port using a dropper bottle. The LFA was read for a positive test line 30 min after the addition of the chase buffer. The result was read again by 2 independent readers at 6 h after the buffer was added. Each test was recorded as negative or positive. The result was recorded as invalid when tests had missing control lines or partial control or test lines. Negative and positive tests could also be designated as “equivocal,” indicating difficulty in determining the presence of the positive test line (Fig. 1). For comparison with the MBA, the results from the 30-min reading were used. For reliability testing, the 6 hour readings were used. Because the individual readers changed over the course of the study, the reliability analyses were confined to comparisons where the readers were consistently the same person designated as Reader 1, 2, and 3 at the 6-h time period.

2.5. Pgp3 multiplex bead array

The MBA antibody assay was performed on dried bloodspot specimens tested with Pgp3 antigen-coupled beads on a Luminex platform as previously described (Goodhew et al., 2012). Total IgG was detected using biotinylated mouse anti-human total IgG (clone H2; Southern Biotech, Birmingham, AL, USA) and IgG4 (clone HP6025; Invitrogen, South San Francisco, CA, USA), and R-phycoerythrin-labeled streptavidin (Invitrogen, South San Francisco, CA, USA). After washing, beads were read on a BioPlex 100 instrument (Bio-Rad, Hercules, CA, USA) using Bio-Plex Manager 6.0 software (Bio-Rad). The level of fluorescence from each sample was reported as the median fluorescence intensity minus background intensity (MFI-BG). The cut-off value for positivity was 869.2 MFI-BG (log = 2.92).

2.6. Data analysis

All field data were recorded on paper forms and entered into a customized database. The prevalence of antibody positivity in the two districts was calculated and 95% confidence intervals calculated using Poisson approximation because there was no evidence of clustering at the community level. We calculated agreement between the results of the valid LFA tests read at 30 min and the results from the MBA. Inter-reader reliability was measured using the kappa statistic to compare readings between Readers 2 and 3, who read the test at the same 6 hour time point. Using MBA as a reference, we evaluated the sensitivity, specificity, positive predictive value and negative predictive value of the LFA test. A linear regression model with the logarithm of MFI-BG as dependent variable and group as the independent predictor was used. Depending on the analyses, the reference group was either the MBA−, LFA− group or the MBA+, LFA− group. Data analysis was performed using STATA version 14 (STATA Corp., College Station, TX, USA) and SAS (SAS Institute, Raleigh NC, USA).

3. Results

A total of 1509 children were examined, 755 in Kanchanpur and 754 in Surkhet. The prevalence of anti-Pgp3 antibodies determined by MBA testing was 1.46% (95% CI = 0.73–2.61) in Kanchanpur and 1.89% (95% CI = 1.11–3.28) in Surkhet. Of the 1509 LFAs completed, 29 (2%) were invalid and excluded from the analysis. Of the remaining 1480 LFAs, 20 were positive (1.35%). There were 1445 specimens negative on both tests, 10 were positive on both MBA and LFA assays, 15 were positive only by MBA, and 10 were positive only by LFA (Table 1). Sensitivity of the LFA was 40.0% and specificity was 99.3%. We compared the MBA MFI-BG values to LFA results and found that positives identified by both tests (MBA+/LFA+) were significantly higher than those by MBA alone (MBA+/LFA−) (P b 0.001) (Fig. 2). Those positive by LFA only had values that were slightly but statistically significantly higher from those who were negative on both tests (P = 0.04).

We excluded 476 records where the reader assignation was not the same person for the 30 minute or 6 hour readings as for the majority of the other readings, leaving 1004 records with the same person for each of Reader 1 or 2 or 3. An increase in positivity with time from test was observed when comparing results from the first reader (30 min) to the second (6 h), and the first reader to the third reader (6 h) (Tables 2a and 2b). At 30 min, Reader 1 graded 11 tests positive, 978 negative, and 15 invalid. At 6 h, reader 2 graded the same tests as 52 positive, 933 negative, and 19 invalid. Reader 3 graded 51 tests positive, 933 tests negative, and 20 tests invalid.

For inter-reader reliability, we compared the results from readers 2 and 3 at 6 h to remove the factor of time since test acquisition (Table 3). The kappa was 0.65 (95% CI = 0.56–0.74) between them. A total of 71 tests (7.1%) were judged equivocal by one or both readers. In order to determine if the low reliability was largely driven by the difficulty of grading these equivocal tests, we removed them from the analysis and the kappa improved to 0.74 (95% CI = 0.62–0.86).

4. Discussion

This was the first true field evaluation of an LFA for antibodies to the Pgp3 antigen from CT, conducted in a low trachoma prevalence setting where it would be most likely utilized. While the overall prevalence of antibody positivity was low using either test, the LFA results in the field did not compare favorably to the results obtained using MBA. Only 40% of the tests that were MBA positive were also LFA positive, and this agreement was lower than what was found in prior laboratory studies (Gwyn et al., 2016). The latter found agreement of 96% using serum samples and 81.5% using whole blood. In the current study the agreement among negative samples in the two assays was 99.3%, similar to the 100% specificity of the LFA, although we expected high specificity given the low seropositivity in the districts. While on a population level, the LFA and MBA results both showed <2% seropositivity in the districts surveyed, the lack of agreement between the two tests needs resolution.

While the use of a rapid test with a simple test line read-out is very appealing for testing in previously trachoma-endemic countries, the performance of this test was less than optimal. The use of blood samples from finger pricks in the field may have played a role in the lower agreement between the LFA and MBA observed. We attempted to place a drop of finger-prick blood directly on the sample port of the test. However, the blood pooled on the plastic cartridge encasing the sample port, forcing the field team to source locally available microcapillary tubes to transfer blood from the finger prick to the sample port. Reported values from laboratory testing of the Pgp3 LFA of whole blood used red blood cells spiked with serum from known Pgp3-positive samples (Gwyn et al., 2016) and the reduction of agreement between tests seen even between these whole blood samples and the serum samples run in the laboratory suggest that the performance of the LFA is not as good with use of whole blood samples. In addition, there were LFA positive samples that were not detected using the MBA; this may be due to the ability of the LFA to detect all antibody isotypes, whereas MBA only detects total IgG (Gwyn et al., 2016). Another possibility is that there may be false positives among the small number of positive specimens in this study, which would influence the agreement as it would not necessarily be expected that the same specimens would test false positive on both the 2 tests. Further testing in low prevalence areas where there is still some infection and disease is warranted.

The prevalence of antibody positivity by either LFA or MBA was slightly lower than prevalences reported in a district in Tanzania which undertook surveillance (West et al., 2016) and in a sub-village in Tanzania where infection was low (Martin et al., 2015). Our values are reasonable in light of our finding virtually no infection in these two districts in Nepal. The rates are comparable to another study evaluated at impact assessment post MDA in a community in Nepal, although the number of subjects evaluated for antibody testing was low in that study (Pant et al., 2016).

We found an overall lower level of reliability among readers than was reported previously (Gwyn et al., 2016), although the inter-rater agreement in that study was done at 30 min, whereas in this study it was done at 6 h. Unlike what was previously observed (Gwyn et al., 2016), the stability of the test line over time in the current study was poor. The second and

third readers found a higher number of positive readings after 6 hours compared with the first reader at 30 min, suggesting that previously negative tests could appear positive over time and that the test should be read at 30 min. A total of 71 of the 1004 LFA tests (7.1%) were judged to be difficult to read by the readers. The equivocals clearly contributed to the lack of agreement, because agreement between the graders improved when the equivocal results were excluded. These findings suggest that efforts to reduce the rate of equivocals observed in low prevalence settings would contribute greatly to improving the reliability of reading the test among various graders. In addition, the lower MFI-BG values seen in the LFA negative and MBA positive tests compared to tests identified as positive by both the LFA and MBA platforms suggests that further work is needed to optimize detection levels and distinguish positive from negative LFAs. An automated reader may also be useful to eliminate subjectivity from the determination of the LFA reading.

The LFA assessed is potentially inexpensive and was simple to use in the field, and thus holds promise as a potential tool for the detection of trachoma antibodies in low-resource settings. However, the low agreement between the LFA and MBA suggests that further optimization and field testing of the LFA may be needed to validate the usability of this test in post-elimination settings for trachoma surveillance.

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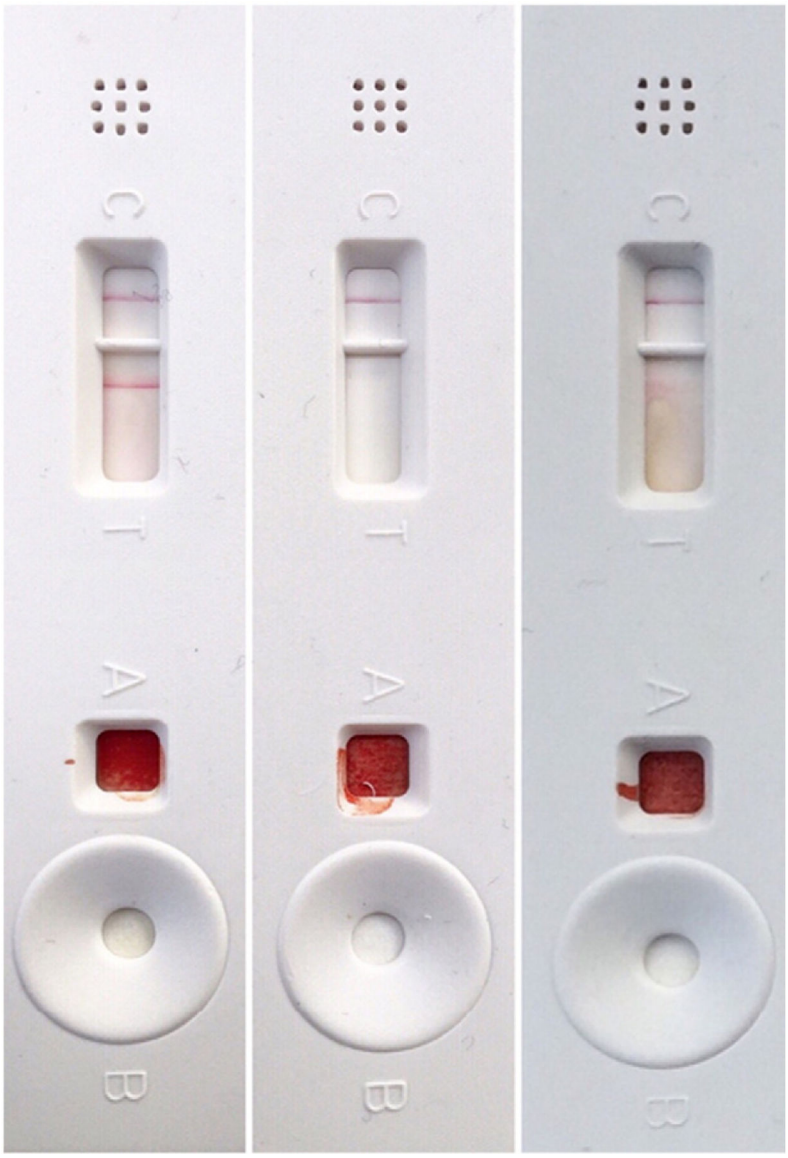

Fig. 1.
Example of lateral flow assays graded as positive, negative, and equivocal (respectively, from left to right).

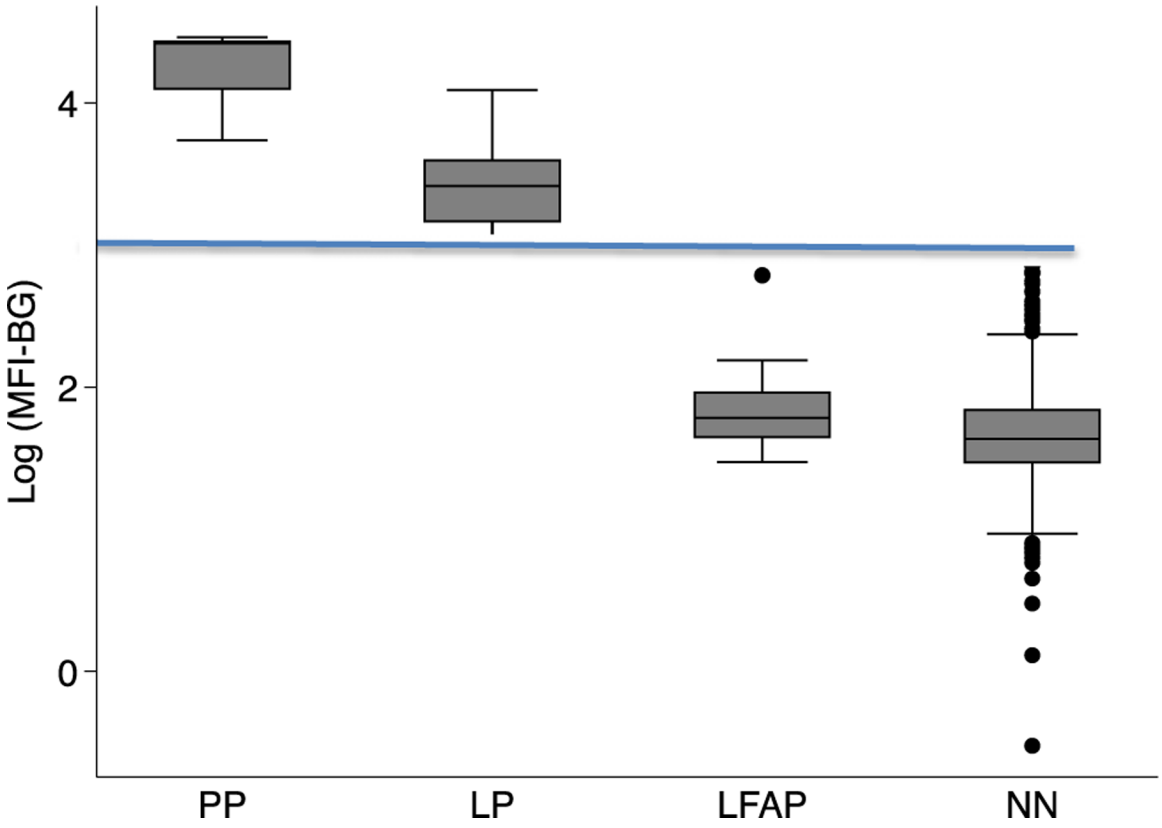

Fig. 2.

Multiplex bead array median fluorescence intensity minus background intensity (MFI-BG) results versus lateral flow assay (LFA) results. PP: MBA and LFA positive (n = 10), LP: MBA positive only (N = 15), LFAP: LFA positive only (N = 10), NN: MBA and LFA negative (N = 1445). Blue line is the positivity cut off.

Table 1

Comparison of lateral flow assay (LFA) results to the Pgp3 antibody multiplex bead array (MBA) results, Kanchanpur and Surkhet districts[*].

	MBA antibody test		
	Positive	**Negative**	**Total**
LFA positive	10	10	20
LFA negative	15	1445	1460
Total	25	1455	1480

Sensitivity: 40.0%; Specificity: MBA: 99.3%; positive predictive value: 50%; negative predictive value: 99.0%.

[*] Excludes 29 invalid LFA tests.

Table 2a

Comparison of positive and negative readings on the lateral flow assay for Reader 1 and 2.

		Grader one			
		Positive	**Negative**	**Invalid**	**Total**
Grader two	Positive	9	43	0	52
	Negative	2	927	4	933
	Invalid	0	8	11	19
	Total	11	978	15	1004

Table 2b

Comparison of positive and negative readings on the lateral flow assay for Reader 1 and 3.

		Grader 1			
		Positive	**Negative**	**Invalid**	**Total**
Grader 3	Positive	8	43	0	51
	Negative	3	928	2	933
	Invalid	0	7	13	20
	Total	11	978	15	1004

Table 3

Inter-reader reliability for the lateral flow assay for readers 2 and 3*.

		Grader 2			
		Positive	**Negative**	**Invalid**	**Total**
Grader 3	Positive	34	16	1	51
	Negative	18	910	5	931
	Invalid	0	7	13	20
	Total	52	933	19	1004

kappa (95%CI) = 0.65 (0.56–0.74).