JCM Accepted Manuscript Posted Online 10 February 2021
J Clin Microbiol doi:10.1128/JCM.02509-20

Antibody responses to two recombinant treponemal antigens (rp17 and TmpA) before and after azithromycin treatment for yaws in Ghana and Papua New Guinea.

Running title: Change in yaws antibodies post-treatment.

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Abstract (226 words)

WHO and its partners aim to interrupt yaws transmission in endemic countries and to certify others as being yaws-free. Transmission can be assessed using rapid plasma reagin (RPR) tests, reflecting current or recent infection, but RPR is operationally impractical.

We evaluated changes in antibody levels against two recombinant treponemal antigens, rp17 (also known as Tp17) and TmpA, after antibiotic treatment given as part of a randomized controlled trial for yaws in Ghana and Papua New Guinea. Paired serum samples from children aged 6–15 years with confirmed yaws, collected before and after treatment, were tested for antibodies to rp17 and TmpA using a semi-quantitative bead-based immunoassay.

Of 344 baseline samples, 342 tested positive for anti-rp17 antibodies and 337 tested positive for anti-TmpA antibodies. Six months after treatment, the median decrease in anti-rp17 signal was 3.2%, whereas the median decrease in anti-TmpA was 53.8%. The magnitude of change in the anti-TmpA response increased with increasing RPR titer fold-change. These data demonstrate that responses to TmpA decrease markedly within 6 months of treatment whereas (as expected) those to rp17 do not.

Incorporating responses to TmpA as a marker of recent infection within an integrated sero-surveillance platform could provide a way to prioritize areas for yaws mapping.

Key words: Yaws, antibody, mass drug administration, serosurveillance

Introduction (manuscript: 2436 words)

Yaws, caused by *Treponema pallidum* subspecies *pertenue*, is a chronic relapsing and remitting condition of the skin, bones, and soft tissues. If left untreated, the disease may progress to its tertiary stage, in which irreversible destruction of soft tissue and bone occur. In 2012, WHO adopted a new approach to yaws eradication based on community mass treatment with azithromycin followed by active case finding to identify and treat remaining

cases (1). This is a highly effective intervention for reducing the community prevalence of both active and latent yaws (2-5).

A major barrier to yaws eradication efforts is the lack of adequate epidemiological data and therefore there is an urgent need to conduct surveys to identify communities in need of intervention (6). Based on mostly clinic-based data reported to WHO, 15 countries are known to be currently endemic, whilst another 79 countries that have previously reported cases have in the last ten years either reported only “suspected” yaws cases (3 countries) or provided no information on the current national status of the disease (76 countries) (7). Responses to a WHO questionnaire suggested that a number of these possibly-formerly-endemic countries may in fact still be endemic for yaws (7). In 2018, a modelling exercise identified a list of countries to prioritize for active surveillance: countries where, in the event of ongoing yaws transmission, passive surveillance by the health system was unlikely to detect it (8).

Large-scale surveillance for yaws is not an easy task. Active yaws prevalence is often low (<1% of the general population) and symptomatic cases are highly clustered at multiple scales (9, 10), making detection systems challenging to design and implement. Serology-based approaches may be useful because latent yaws is more prevalent (range 3–31%) than active yaws in endemic populations (11). Systems that detect latent yaws could decrease the number of people that need to be tested to conclude with reasonable confidence that infection was not being transmitted. Initial serological surveys could be used to select areas in which community mass treatment should be implemented followed by more detailed active case searches.

Serological evidence of yaws requires demonstrable reactivity using two distinct antibody tests: treponemal-specific tests such as the *T. pallidum* passive particle agglutination [TPPA] test that may remain positive for life, and non-treponemal tests such as the rapid plasma reagin (RPR) test that measures antibodies against lipoidal material from the bacterium and/or damaged host cells, and for which reactivity wanes after successful treatment. The use of the TPPA and RPR in sero-surveys for yaws is impractical because these tests require venipuncture, collection of whole blood, separation of serum samples and transport of samples using a cold-

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chain. They are also somewhat operator dependent. The Dual Path Platform (DPP) Syphilis Screen & Confirm Assay (Chembio Diagnostics, Medford, NY, USA) is a useful rapid point of care test able to separately detect both treponemal and non-treponemal antibodies using whole blood, but suboptimal for large surveillance programs because of its low throughput (12). Use of an approach such as the multiplex bead assay (MBA) would facilitate large-scale sampling by utilizing dried blood spots collected by finger prick and thereby encouraging integration with sero-surveys targeting other diseases.

We have previously adapted the recombinant treponemal antigens rp17 (13) and TmpA (14, 15) to the MBA and shown good correlation of anti-rp17 antibodies to other laboratory-based treponemal tests (measuring long-lived responses) and of anti-TmpA antibodies to non-treponemal tests (reflecting current or recent infection) (16). The ability of TmpA responses to track current infection potentially allows it to substitute for RPR testing in large-scale surveillance work. There is a need to assess the dynamics of this test before and after intervention to increase confidence that TmpA responses wane in the way that RPR titers do after infection has been cleared. In this study, we measured antibodies to rp17 and TmpA using MBA in dried blood spots taken from individuals before and after azithromycin treatment for active yaws. We nested our investigation within a trial for comparing different doses of azithromycin for yaws conducted in Ghana and Papua New Guinea.

Methods

Study Design and Sample Collection

Samples were collected in the context of a randomized, controlled, open-label non-inferiority trial of 20 mg/kg versus 30 mg/kg azithromycin for the treatment of yaws. Participant recruitment has been described previously (17). Briefly, children aged 6–15 years were invited to participate based on the following criteria: presence of a clinical lesion consistent with infectious primary or secondary yaws and the DPP syphilis screen and confirm assay that was positive for both treponemal and non-treponemal antibodies (18). Three children in the sub-study presented here were sampled out of protocol (age 5 years) but were included in the analysis. Children were seen at baseline for enrolment, initial data collection, and antibiotic treatment, and again for follow-up at

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4 weeks and 6 months. The current analysis used only blood samples collected at baseline and 6 months after treatment, at which time points venous blood samples were collected. Serum samples were sent to CDC for quantitative TPPA and RPR testing. Swabs were collected from lesions and tested at CDC for the presence of *T. pallidum* DNA by real-time PCR (TP-PCR); data from these investigations were previously published (17). For the current study we included individuals who had positive yaws serology at enrollment into the original trial, who had been seen for a 6-month follow-up visit, and for whom residual paired serum samples were available.

Multiplex Bead Assay

Serum samples were diluted in 1.5 mL tubes to a final concentration of 1:400 in Buffer B (1X PBS, 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone, 0.5% casein, 0.3% Tween-20, 0.02% sodium azide containing 3 µg/mL of *Escherichia coli* extract) and incubated overnight at 4°C.

Total IgG was detected by incubating diluted specimens in duplicate in a 96-well plate with beads coupled to rp17 or TmpA, and then detecting antigen-specific IgG with monoclonal anti-human IgG and IgG4 and streptavidin-linked R-phycoerythrin reporter, as described previously (16). Antibody binding was reported as median fluorescence intensity (MFI) using a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA) with the background fluorescence from a Buffer B-only blank subtracted (MFI-BG). Specimens for which the coefficient of variation (CV) was >15% between the MFI-BG of duplicate wells for any bead were repeated. Pre- and post-treatment samples from the same individual were run on the same plate to minimize variation.

Cutoffs for seropositivity were determined by receiver operating characteristic curves. The positive panel consisted of 18 samples from 4–15-year-old children from yaws-endemic areas who were positive by both TPPA and RPR, and the negative panel consisted of 162 samples from individuals living in yaws-endemic areas that were negative by both TPPA and RPR. The cutoff was set as the midpoint between the highest MFI-BG value in the negative panel and the lowest MFI-BG value in the positive panel.

Data analysis

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Data were analyzed using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA). Descriptive analyses were performed to compare the results of conventional serological testing performed in the original trial to Luminex-based serological analysis conducted in the current study. For various analyses, data were stratified by baseline TP-PCR positivity, RPR titer decline from baseline to 6 months after antibiotic treatment, or serological cure (defined as a 4-fold decline in RPR titer or seroreversion of the RPR test result by 6 months). Confidence intervals for the median percent changes in rp17 and TmpA MFI-BG were calculated using order statistics. The percent changes did not have a normal distribution, and therefore required the use of non-parametric methods.

Assay performance

A validation panel was run to determine the repeatability and dilutional linearity of the assays. Initially, six high-titer archived samples were run in single wells with two-fold dilutions ranging from 1:400 to 1:12,800 to establish a rough estimate of the range to be used for precision and dilutional linearity testing. Then, high titer sera from the study samples (n= 10) were selected and a three-point (1:400, 1:4000, 1:8000) dilution series run to identify ranges for validation testing (Figure 1). From these six samples, four were selected for validation testing. Samples were diluted serially at four-fold intervals ranging from 1:4000 to 1:65,536,000 and run in duplicate on five days over the course of two weeks by one technician. Only data through the 1:4,096,000 dilution are shown.

Ethics

Ethical approval for the trial and this sub-study was obtained from the London School of Hygiene & Tropical Medicine (Ref 8832), Ghana Health Service Ethical Review Committee (Ref GHS-ERC: 3), the Papua New Guinea Institute of Medical Research (IRB number 1504), the Papua New Guinea Medical Research Advisory Committee (MRAC No. 14.31), the WHO Ethics Review Committee (Protocol ID RPC720), and the Centers for Disease Control and Prevention (6474/7285).

Results

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Samples

In the original trial, 406 participants had positive yaws serology at baseline and had been seen for a 6-month follow-up visit. Of these participants, residual paired serum samples were available from 344 participants (84.7%) and were included in the current study. Demographic information is shown in Table 1. Study participants were Ghanaian and Papua New Guinean children aged 5–15 years. The proportion of the sample that was male (71.5%, n = 246) was similar to the proportion of males seen in the original study (71.8%). The median age of participants contributing sera to the current study was 11 years.

At baseline, 342 (99.4%) of 344 samples tested positive for antibodies against rp17, and 337 (98.0%) tested positive for antibodies against TmpA. Of samples from TP-PCR-positive children collected at baseline (n = 129, 37.5%), 127 (98.4%) tested positive for antibodies against rp17 and 128 (99.2%) tested positive for antibodies against TmpA.

Assay Performance Characteristics

Performance tests showed a high reactivity (>97%) to rp17 and TmpA antigens in samples from individuals testing positive by TPPA. Repeatability assessment showed that CVs from inter-plate replicates were consistently <10% across the linear range for rp17 and <15% for TmpA, as shown in Table 2. The dilutional linearity of each assay is shown in Figure 1. Dilution curves were run starting at 1:4000 (10-fold higher than run during the standard MBA protocol) using 4-fold dilutions. Signals associated with TmpA-specific responses were consistently lower than those against rp17 and showed signal reduction in a linear range at lower dilutions than rp17-specific responses. To assess inter-assay precision, we ran the two antigens as multiplex, so the MFI-BG for anti-TmpA fell below the cutoff in a few dilutions, resulting in fewer points in linear range. The Luminex-200 used for the analysis has a maximum channel range of 32,766, meaning that any signal stronger than that will saturate the system, potentially limiting our ability to get meaningful quantitative data from the rp17 antigen when MFI-BG >25,000.

Changes in anti-treponemal antibody levels after treatment

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The median rp17 change was a 3.2% reduction (Table 3) in MFI-BG after excluding an outlier sample that had a 7000% increase in MFI-BG (fold-change of 0.25 or 0.5 indicates an increase in RPR titer). Participants who achieved serological cure (i.e. ≥4-fold decrease in RPR titer or seroreversion) after 6 months had a median rp17 reduction of 4.8%, while for participants who did not achieve serological cure, the overall median change was a 1.9% reduction. The median TmpA change was a 53.8% reduction in MFI-BG after removing an outlier sample that had a 3800% increase in MFI-BG. Among participants who achieved serological cure after 6 months, the median TmpA change was a 68.3% reduction in MFI-BG, while for those who did not achieve serological cure, the median TmpA change was a 33.5% reduction in MFI-BG. Changes in TmpA responses stratified by RPR titer are shown in Table 3.

Individuals with TP-PCR-positive swabs showed greater percent change in MFI-BG after treatment for both antigens than those testing PCR-negative (Table 4).

Discussion

Multiplex-based serology has the potential to add value to surveys and public health programs by generating seroprevalence data on more than one disease of public health interest from a single sample set. A disease that would particularly benefit from this approach is yaws, which has a WHO eradication target by 2030 but little information available about where interventions are currently required and insufficient funding to generate epidemiological data through widespread stand-alone mapping efforts, as has been done for trachoma, as an example (19). Traditional yaws laboratory diagnosis is based on serology, but these tests utilize readouts such as particle agglutination or antibodies against lipid antigens that are not well suited to the multiplex bead-based platform that facilitates integrated surveillance. Here, we show good test performance for two treponemal antigens (rp17 and TmpA) that run on the MBA, with the expected downward trends in antibody signals from TmpA in children from Ghana and PNG following treatment of yaws with azithromycin. Responses to both antigens not only show good analytical performance against positive and negative control panels but also an

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excellent dilutional linearity and repeatability, particularly for rp17, which demonstrated higher inter-operator precision than TmpA.

While we noted an overall downward trend in TmpA-specific antibody levels following treatment, our 6-month time frame for follow-up may not have been long enough to detect large changes and was specifically not sufficiently long to observe seroreversion from positive to negative. This is also a limitation of the RPR test, for which TmpA testing is broadly analogous. Serological cure on standard non-treponemal serology is defined as either seroreversion or a 4-fold decrease in titer: current standards do not require undetectability of antibody. Our test performance data suggest that within the linear range, decreases of 30% or more are likely to be technically meaningful (in terms of the repeatability of the assay), and we found an association between the percentage change in TmpA and the RPR titer fold change. Additional data would be needed to define biologically meaningful decreases in the TmpA signal after treatment.

Our data support the idea that testing for antibodies to rp17 and TmpA could be informative for yaws surveillance in previously endemic countries for which the current yaws status is unknown. The slower decline in TmpA-specific antibody responses compared to RPR titers after treatment may have a programmatic advantage for this application because a negative TmpA response would indicate a more prolonged interval since the most recent infection in an individual. On the other hand, more than six months' follow-up of treated patients will be needed to determine whether TmpA will be useful for certifying a of lack of ongoing transmission in a recently endemic country that has implemented a yaws elimination program. Such studies would allow us to determine the time to seroreversion in cohorts with yaws and inform guidance on optimal timing for assessing evidence of interruption of transmission using TmpA.

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Footnotes

The authors alone are responsible for the views expressed in this article and they do not necessarily represent the views, decisions or policies of the institutions with which they are affiliated. The authors declare no conflict of interest. We thank the governments of Ghana and Papua New Guinea, community leaders, schoolteachers, and schoolchildren at the study sites for their cooperation and support. The parent study was funded by a grant from the Neglected Tropical Diseases Support Center to WHO (NTD-SC/NCT 053). Laboratory work in the current study was funded by the U.S. Centers for Disease Control and Prevention. The data shown herein have not been previously published or presented.

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231 **Table 1.** Demographic information on individuals providing samples in for this multiplex bead assay (MBA) sub-
232 analysis, compared to original study sample set.

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		MBA sub-analysis	Original sample set (17)
		n (%)	n (%)
Total		344 (100)	406 (100)
	Male	247 (71.8)	290 (71.4)
	Female	97 (28.2)	116 (28.6)
Age (years)			
	5	3 (0.9)	3 (0.7)
	6	24 (7.0)	32 (7.9)
	7	37 (10.8)	40 (9.9)
	8	42 (12.2)	49 (12.1)
	9	37 (10.8)	47 (11.6)
	10	43 (12.5)	57 (14.0)
	11	47 (13.7)	47 (11.6)
	12	38 (11.0)	47 (11.6)
	13	3 1 (9.0)	37 (9.1)
	14	22 (6.4)	25 (6.2)
	15	20 (5.8)	22 (5.4)

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236 **Table 2.** Inter-assay precision of multiplex bead assay to measure antibodies against treponemal antigens rp17

237 and TmpA. CV = coefficient of variation; MFI-BG = median fluorescence intensity with background subtracted.

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	sample 1		sample 2		sample 3		sample 4	
rp17								
Dilution Factor	MFI-BG	%CV	MFI-BG	%CV	MFI-BG	%CV	MFI-BG	%CV
4000	27788	5.7	26980	7.1	26663	6.6	27505	5.2
16000	15954	4.1	14752	4	8416	8.2	15056	4.4
64000	2382	5.9	2326	7.5	1238	6.7	2538	7.3
256000	426	7.6	405	3.1	233	9.4	439	7.9
1024000	107	6.8	92	2.4	62	7.8	93	10.8
4096000	25	8.6	205	2.4	16	25.9	19	16.6
TmpA								
Dilution Factor	MFI-BG	%CV	MFI-BG	%CV	MFI-BG	%CV	MFI-BG	%CV
4000	2103	11.3	3314	6.3	2755	7.3	834	4.4
16000	376	7.8	750	11.5	451	7.7	131	7.5
64000	64	12.6	139	9.8	80	16.9	24	12
256000	16	20.5	27.5	8.4	16	13.5	5.5	40
1024000	4.5	43.2	9	15.3	6	21.1	6	90
4096000	2	71.3	9.5	24.7	1.5	129.8	0.5	418

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242 **Table 3.** Median percent changes in antibody responses to treponemal antigens rp17 and TmpA in multiplex
243 bead assay, stratified by decrease in rapid plasma reagin (RPR) titer. 95% confidence intervals (CI) calculated
244 without normal distribution assumption.

	rp17 Median % Change		TmpA Median % Change	
RPR Titer Decline	Point Estimate	95% CI	Point Estimate	95% CI
0.25 (n=1)	-8.1	-	-35.9	-
0.5 (n=14)	-2	(-2.9, 0.3)	-20.7	(-33.5, 5.6)
1 (n=40)	-1.8	(-3.1, -1)	-32.7	(-41.4, -22.6)
2 (n=67)	-1.9	(-2.7, -1.2)	-35.1	(-43.1, -28.3)
4 (n=63)	-2.7	(-3.8, -2.1)	-53.1	(-61.7, -49.6)
8 (n=52)	-3.8	(-5.9, -2.8)	-59.4	(-70.8, -48.9)
16 (n=28)	-10.2	(-18.9, -6.4)	-77.2	(-85.3, -70.6)
32 (n=10)	-7.4	(-52.5, -1.9)	-78.8	(-91.9, -60.5)
64 (n=6)	-26	(-82.2, -8.4)	-91.3	(-96.5, -79)
128 (n=2)	-6.1	(-10.9, -1.3)	-75.5	(-75.8, -75.2)
Seroreversion (n=60)	-26.8	(-60.3, -4.6)	-83	(-89.2, -69.1)

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248 **Table 4.** Median Percent Changes in antibody responses to rp17 and TmpA in multiplex bead assay stratified by

249 PCR result. 95% Confidence Intervals calculated without normal distribution assumption.

	TmpA Median % Change		rp17 Median % Change	
	Point Estimate	95% CI	Point Estimate	95% CI
All (n=343)	-53.5	(-57.8, -50.2)	-3.1	(-4.1, -2.8)
PCR Negative (n=215)	-42.4	(-49.6, -37.3)	-2.6	(-3.1, -2.1)
PCR Positive (n=128)	-75.1	(-80.8. -68.3)	-7.8	(-12.3, -4.1)

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Figure 1. Test performance of multiplex bead assay to detect antibodies against rp17 (top) and TmpA (bottom). Each line represents a dilution curve for 4 different samples. Error bars are standard error of triplicate runs on 5 separate plates. S=sample, MFI-BG = median fluorescence intensity with background subtracted.

Figure 2. Percentage change in rp17 (left) and TmpA (right)-specific antibody responses following treatment, stratified by rapid plasma reagin (RPR) titer change. Seroreversion indicates RPR=0 at the 6-month timepoint. Five outliers for which >anti-TmpA responses increased by >100% were excluded from the graph. Boxes show median and inter-quartile range and whiskers show 1.5 x the inter-quartile range. MFI-BG = median fluorescence intensity with background subtracted.

Figure 1

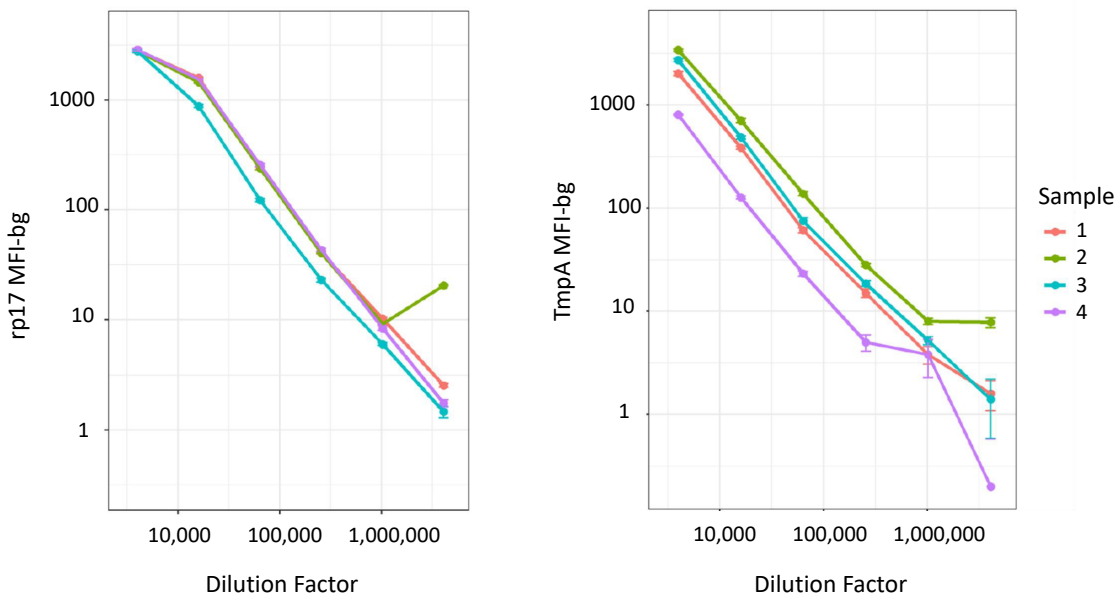

Figure 2