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ORIGINAL RESEARCH

Development of a novel magnetic particle-based agglutination immunoassay for anticardiolipin antibody detection in syphilis

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Received 10 January 2020

Revised 4 March 2020

Accepted 17 March 2020

Published Online First

6 May 2020

ABSTRACT

Objectives Serological tests of non-treponemal and treponemal types are the most frequently used for syphilis diagnosis. Treponemal tests are available in wide variety of assay formats; however, limited advances have been made for the improvement of conventional non-treponemal tests. The objective of this work was to develop a novel non-treponemal magnetic particle-based agglutination assay (NT-MAA) and evaluate its feasibility for syphilis testing.

Methods Cardiolipin was modified and coupled to magnetic microbeads. Serum diluted in phosphate-buffered saline was mixed with cardiolipin-coupled beads and incubated in a round bottom microplate for 90–120 min followed by visual inspection. A panel of reported syphilis (n=127) and non-reactive (n=244) specimens was prepared to evaluate the NT-MAA performance in comparison to conventional rapid plasma reagin (RPR). *Treponema pallidum* particle agglutination (TP-PA) assay and enzyme immunoassay (EIA) were included. Analytical sensitivity and reproducibility of NT-MAA were also determined.

Results The non-treponemal NT-MAA and RPR showed sensitivity of 90.6% and 88.2% and specificity of 96.7% and 100%, respectively. The treponemal TP-PA and EIA yielded sensitivity of 100% and 99.2%, respectively, and 100% specificity by both assays. The per cent agreement between NT-MAA and RPR was 97% (kappa=0.931, 95% CI 0.891 to 0.971). Analytical sensitivity determined with IgM anticardiolipin antibody (ACA) was 2.6 µg/mL for both NT-MAA and RPR, while IgG ACA yielded 0.9 µg/mL and 1.7 µg/mL for NT-MAA and RPR, respectively. Qualitative results of intra-assay and interassay reproducibility revealed 100% consistency for NT-MAA.

Conclusion Preliminary evaluation of the novel NT-MAA validated proof of concept using laboratory-characterised syphilis sera and demonstrated performance comparable to RPR. Further validation of NT-MAA using additional specimens with better clinical staging may broaden the scope of developed test for syphilis diagnosis.

INTRODUCTION

The aetiological agent of syphilis, *Treponema pallidum* subspecies *pallidum*, can be transmitted through sexual contact, from an infected mother to her child, and through blood transfusion or other

non-sexual contact.¹ Untreated syphilis progresses through multiple stages—primary, secondary, latent and tertiary—progressing from infectious and symptomatic stages to a latent asymptomatic phase, with the tertiary stage affecting multiple body organs. A recent surveillance report from the Centers for Disease Control and Prevention (CDC) showed a rise in primary and secondary syphilis cases in the USA particularly among gay, bisexual and other men who have sex with men.^{2,3} A resurgence in syphilis cases globally has also been described in recent years.⁴

Due to a lack of sustainable in vitro culture techniques for *T. pallidum*, unavailability of Food and Drug Administration (FDA)-cleared molecular tests and limitations associated with direct detection tests such as dark field microscopy, indirect serological tests that detect non-treponemal and treponemal antibodies remain the mainstay for routine syphilis detection.^{5,6} Non-treponemal tests include Venereal Disease Research Laboratory (VDRL) slide test, rapid plasma reagin (RPR) and unheated serum reagin that detect antibodies to lipoidal moieties derived from damaged host cells and treponemes as a result of the host immune response.^{5,7,8} Owing to the non-specific nature of these tests, the traditional algorithm requires confirmation of a reactive non-treponemal result with a treponemal-specific test that includes *T. pallidum* particle agglutination (TP-PA), *T. pallidum* haemagglutination assay, fluorescent treponemal antibody absorption or other automated treponemal assays. Most treponemal tests use whole bacterial lysate or a combination of recombinant and/or purified peptides and proteins to capture the antibody. Treponemal tests have become more mainstream for syphilis screening due to ease of use, high throughput and ability to improve workflow efficiency using automated systems.^{9,10} A caveat is that treponemal antibodies persist in successfully treated patients which means the treponemal test cannot distinguish current infection from infections that have been successfully treated in the past.¹¹ Confirmatory testing with non-treponemal tests is recommended when using the reverse sequence screening algorithm, followed by reflex testing with another treponemal assay to verify discordant results if any.¹² None of the serological tests currently approved for use



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To cite: Shukla MR, Deutsch JW, Pereira LE, et al. *Sex Transm Infect* 2020;**96**:411–416.

is sufficient as a stand-alone assay for definitive diagnosis of syphilis.

The use of a manual non-treponemal test in current screening algorithms has numerous practical limitations that include labour-intensive testing procedures, subjectivity in results interpretation, the need for fresh reagents (as required in VDRL) and additional and/or expensive equipment(s). Automated versions of the RPR test have been recently introduced in the USA, with the AIX1000 (Gold Standard Diagnostics, Davis, California, USA), ASI Evolution (Arlington Scientific, Springville, Utah, USA) and BioPlex 2200 Syphilis Total and RPR (Bio-Rad Laboratories, Hercules, California, USA) receiving FDA clearance, providing alternative platforms that could overcome the limitations associated with manual tests. Given their relatively nascent release, more data are required to validate their application for routine syphilis testing.¹³ An updated manual non-treponemal test could serve as an alternative option that reduces current shortcomings of conventional non-treponemal tests.¹⁴ The conventional non-treponemal tests have undergone few modifications since their inception. A limited number of studies have described improvements or changes in test design over the decades where researchers tried to develop a non-treponemal enzyme immunoassay (EIA) by drying or evaporating alcoholic VDRL antigen directly on microtitre plates.^{15,16} This approach is, however, not currently integrated in any commercially available product for syphilis testing. The central component of these assays, non-treponemal antigen cardiolipin, is a phospholipid that is insoluble in aqueous solution which impairs strong attachment to polar immunoassay surfaces.¹⁷ Castro *et al* described a method to overcome this difficulty for cardiolipin, by chemical oxidation and subsequent attachment to assay surfaces.¹⁷ This method was applied in the current study, with the objective of improving the conventional non-treponemal test design to one that could be performed with a minimum reagent and/or equipment, while also being efficacious as a syphilis test. A non-treponemal magnetic particle-based agglutination assay (NT-MAA) was developed as a proof of concept, using cardiolipin modified through a chemical oxidation process¹⁷ to facilitate subsequent covalent coupling to magnetic microbeads. The method was optimised and validated using characterised syphilis sera to ensure the quality and consistency of results. The developed assay is simple and involves less subjectivity in reading results.

METHODS

Specimens

A panel of 371 archived, deidentified frozen serum specimens was prepared at the CDC (Atlanta, Georgia, USA). Of these, 127 syphilis specimens were obtained from the CDC's syphilis serum repository bank,¹⁸ originally procured from the Association of Public Health Laboratory (APHL, Silver Spring, Maryland, USA) through an ongoing collaborative agreement beginning in 2015. Syphilis stage of those specimens was reported by the participating local and state public health laboratories to the APHL from the test request form. Of the syphilis specimens, suspected disease stage was reported that includes 13 as primary, 43 as secondary, 28 as early latent and 43 as late latent. The remaining 244 specimens included in our testing were syphilis non-reactive (NR) collected from the Georgia Public Health Laboratory (Atlanta, Georgia, USA) in 2016. All specimens were deidentified of any personally identifiable information and tested at the CDC, independent of their previously reported results using RPR (Arlington Scientific, Springville, Utah, USA), TP-PA assay (Fuji-ribo Diagnostics, Malvern, Pennsylvania, USA) and Trep-Sure

EIA (Trinity Biotech USA, Jamestown, New York, USA) as per the respective manufacturer's package insert. These specimens were also tested using the NT-MAA prototype.

Affinity-purified anticardiolipin antibody (ACA) preparation

The ACAs of IgG and IgM classes were purified from a reactive human syphilitic serum specimen (RPR titre 1:128; Plasma Service Group, Huntingdon Valley, Pennsylvania, USA) using an in-house method of affinity chromatography. Briefly, RPR reactive serum was subjected to ammonium sulfate precipitation and dialysis followed by application of the dialysed fractionate to a cardiolipin-affinity column. The affinity column was prepared by linking cardiolipin to carboxyLink coupling resin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a method described by Castro *et al*.¹⁷ The affinity-purified fractionate was then subjected to Protein-A matrix separation (Affi-Gel Protein A Resin, Bio Rad Laboratories, Hercules, California, USA), which after column washing and elution, yielded separate distinct peaks for IgM and IgG ACA. [Deutsch JW, Castro AR, Shukla MR, et al. Development of an enzyme-linked immunosorbent assay (EIA) for the quantification of nontreponemal immunoglobulins IgG and IgM in the sera and plasma of patients with syphilis. 2018]. The ACA were concentrated by ultrafiltration using a 10 KDa membrane (Ultracel 10 kD, Millipore, Bedford, Massachusetts, USA). The purity of ACA was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and concentration determined using the radial immunodiffusion test (Kent Laboratories, Bellingham, Washington, USA) following the manufacturer's directions. The reactivity of purified ACA was confirmed by RPR testing, then prepared as aliquots of 0.25 mL volume per tube and stored at -30°C until use.

Cardiolipin modification method

Cardiolipin was oxidised by a method described by Castro *et al*.¹⁷ Briefly, 100 mg of cardiolipin powder (Avanti Polar Lipids, Alabaster, Alabama, USA) was dissolved in 2 mL of t-butanol (Sigma-Aldrich, St. Louis, Missouri, USA) followed by addition of 60 mg of sodium m-periodate (Sigma-Aldrich) and 8 mg of potassium permanganate (Thermo Fisher Scientific) salts in aqueous solution as described. The reaction was continued for 24–48 hours under inert argon gas atmosphere and then stopped with 80 mg of sodium bisulfite (Sigma-Aldrich). The solution was centrifuged at $1000\times g$ for 5 min and the t-butanol phase was then dialysed with 10 mM phosphate buffer, pH 8.0, followed by lyophilisation.

Cardiolipin coupling to magnetic microbeads

A 1 mL volume of aminated magnetic microbeads from the supplier (Chemicell, Berlin, Germany) was concentrated using a magnetic separator (Chemicell) followed by supernatant removal. Concentrated microbeads were suspended to a 1 mL total volume using 10 mM phosphate buffer, pH 5.6. Microbeads were again concentrated and resuspended using the phosphate buffer as described above (two washes total) to obtain a 1 mL volume that was coupled to 10 mg/mL of oxidised/activated cardiolipin. The functional carboxyl group of cardiolipin was activated using 10 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Sigma-Aldrich) and 10 mg of sulfo-N-hydroxysulfosuccinimide (Thermo Fisher Scientific) in a glass vial.¹⁷ The coupling was continued for 2 hours on a rocker at room temperature (21°C – 25°C). The beads were washed twice with phosphate buffer and then blocked with 10% bovine serum albumin (BSA, Sigma-Aldrich) prepared in deionised water. The

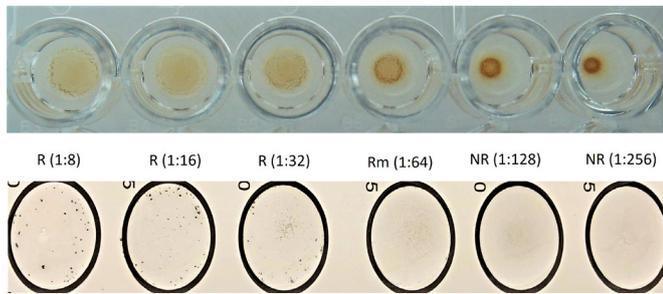


Figure 1 Representation of the reactivity patterns observed in NT-MAA (top) and RPR (bottom) tests using a higher reactive syphilis specimen diluted in normal human serum (1:8 to 1:256). NT-MAA, non-treponemal magnetic particle-based agglutination assay; RPR, rapid plasma reagin; R, reactive; Rm, minimally reactive; NR, non-reactive.

cardiolipin-coupled magnetic microbeads were stored at refrigerated temperature (2°C–8°C).

Test method for NT-MAA

For a given serum specimen, 25 µL of 1× phosphate-buffered saline (PBS) and 25 µL of serum (1:2) were dispensed in a single well of a clear round bottom 96-well microplate. In a second well, 50 µL of cardiolipin-coupled magnetic microbeads (prediluted to 1:30 in 1× PBS containing 1% BSA) was dispensed, followed by the addition of 10 µL of the 1:2 diluted serum specimen to the same well. The microplate was placed on a plate shaker at 1000 rpm for 1 min to mix, and then incubated on a vibration-free surface for 90–120 min at room temperature (21°C–25°C). Optionally, the room temperature incubation can be extended overnight and read thereafter. A test was interpreted as reactive (R) when visible clumps were formed as a result of agglutination lattice formation among ACAs and the cardiolipin-coupled magnetic microbeads. A diagram representing NT-MAA reactivity patterns is shown in figure 1. A nonreactive (NR) sample is defined as a compact button-shaped pattern in the centre of a microwell.

Data analysis

Sensitivity and specificity of tests were calculated using the following standard formula:⁷

$$\% \text{ Sensitivity} = [\text{True positive}/(\text{True positive} + \text{False negative})] \times 100$$

$$\% \text{ Specificity} = [\text{True negative}/(\text{True negative} + \text{False positive})] \times 100$$

The sensitivity of tests was calculated using reported syphilis staged specimens and were considered as true positive reference. The true positive number obtained by a respective test is divided by the same, plus false-negative results and were expressed as a percentage to determine sensitivity. For specificity calculation, syphilis NR specimens were included and considered as the true-negative reference, where the true-negative number obtained by a respective test is divided by the same, plus false-positive results followed by percentage expression as shown in the formula above. The CI was determined using Wilson interval estimates. The per cent agreement and kappa value between RPR and NT-MAA were determined using GraphPad web-based software (La Jolla, California, USA). Affinity-purified ACA of IgG and IgM classes were tested separately to determine analytical sensitivity; end-point titre was determined by twofold serial dilution and testing to determine lower limit of detection of both NT-MAA and RPR. The

reproducibility of NT-MAA was assessed using serial dilutions of a higher titre RPR reactive serum diluted in normal human serum (from 1:1 to 1:512); prepared dilutions represent specimens with variable reactivity. Intra-assay reproducibility of NT-MAA was performed by testing each dilution 10 different times in a single assay run, whereas interassay reproducibility was executed by testing all dilutions over five separate assay runs. Qualitative results defined as being R, minimally reactive (Rm) or NR were recorded during interpretation. Internal controls defined as syphilis R, Rm and NR were used at the time of testing for quality assurance.

RESULTS

The testing of cardiolipin-coated magnetic microbeads with previously laboratory-characterised syphilis specimens, NR specimens and purified ACA resulted in patterns as shown in figure 1. The reactivity patterns of RPR are also shown in figure 1 for comparison. The testing of 371 specimen yielded sensitivity/specificity data of NT-MAA, RPR, TP-PA and EIA as described in table 1. The sensitivity of NT-MAA and RPR in context to primary, secondary and early latent was found in the range of 95%–100%. Specimens from the late latent stage demonstrated sensitivity of 79% with NT-MAA and of 69.8% for RPR. Treponemal tests evaluated showed sensitivity in range of 92%–100% with staged specimens. With syphilis NR specimens, NT-MAA had a specificity of 96.7%, whereas RPR, TP-PA and EIA had a specificity of 100%. Qualitative test results of NT-MAA and RPR using syphilis staged and NR specimens were recorded and showed an agreement of 97% (kappa=0.931, 95% CI 0.891 to 0.971).

The analytical sensitivity of NT-MAA and RPR using purified ACA of IgM class showed an end-point titre or reactivity till 2.6 µg/mL of antibody concentration for both tests. ACA of IgG class showed reactivity till 0.9 µg/mL for NT-MAA and 1.7 µg/mL for RPR (table 2). Interassay and intra-assay reproducibility of NT-MAA revealed 100% consistency in measuring qualitative results during and among different assay runs (table 3).

DISCUSSION

We developed a method for covalently coupling cardiolipin to magnetic microbeads and optimised an assay to detect non-treponemal antibodies in suspected syphilis sera. The magnetic property of the microbeads allows quick purification of cardiolipin-coupled beads which reduces the time and cost of reagent recovery. The real-time/temperature stability testing of cardiolipin-coupled microbeads using syphilis R/NR sera demonstrated stability up to 1 year when stored at refrigerated temperature (data not shown). The testing of cardiolipin-coupled microbeads in the NT-MAA using a limited number of syphilis-staged and NR specimens demonstrated performance that is comparable with the RPR assay. The analytical sensitivity of both non-treponemal tests using purified ACA further confirmed a similar limit of detection with notably twofold higher sensitivity observed for NT-MAA with IgG class of ACA compared with RPR. Importantly, the agglutination patterns for weakly reactive specimens in the NT-MAA were observed to be better defined, making results interpretation less ambiguous when compared with RPR. In addition, the 96-well microtitre plate format of NT-MAA might be useful for laboratories that routinely test a large number of specimens and could be adapted for use in an automated system. The robust coupling chemistry of non-treponemal antigen with magnetic microbeads also has

Table 1 Sensitivity and specificity of RPR, NT-MAA, TP-PA and EIA

Specimens	Non-treponemal tests						Treponemal tests					
	NT-MAA			RPR			TP-PA			EIA		
	Sensitivity											
	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
Primary stage (n=13)	13	100	77.2 to 100	13	100	77.2 to 100	13	100	77.2 to 100	12	92.3	66.7 to 98.6
Secondary stage (n=43)	41	95.3	84.5 to 98.7	41	95.3	84.5 to 98.7	43	100	91.8 to 100	43	100	91.8 to 100
Early latent stage (n=28)	27	96.4	82.3 to 99.4	28	100	87.9 to 100	28	100	87.9 to 100	28	100	87.9 to 100
Late latent stage (n=43)	34	79.1	64.8 to 88.6	30	69.8	54.9 to 81.4	43	100	91.8 to 100	43	100	91.8 to 100
Overall sensitivity (N=127)	115	90.6	84.2 to 94.5	112	88.2	81.4 to 92.7	127	100	97.1 to 100	126	99.2	95.7 to 99.9
Specificity												
Non-reactive (n=244)	236	96.7	93.7–98.3	244	100	98.5–100	244	100	98.5–100	244	100	98.5–100

EIA=TrepSure Enzyme Immunoassay. NT-MAA, non-treponemal magnetic particle-based agglutination assay; RPR, rapid plasma reagin; TP-PA, *Treponema pallidum* particle agglutination assay.

potential applications in multiple assay platforms such as rapid test designs or autoanalysers, opening new avenues for syphilis diagnostic testing.

Using laboratory characterised syphilis specimens, both NT-MAA and RPR demonstrated similar sensitivity for primary, secondary and early latent staged specimens that were evaluated. The late latent staged specimens revealed reduced sensitivity by both non-treponemal tests and align with a prior report,¹⁸ though the NT-MAA was noted to have slightly more sensitivity than the RPR. The sensitivity of a non-treponemal test in the context to syphilis stage may vary and described in prior studies.^{12–19} The treponemal tests evaluated herein demonstrated higher sensitivity with the syphilis panel regardless of the disease stage and may be attributed to persistent treponemal antibodies, unlike non-treponemal antibodies that show decline in titre after succession of treatment or on progression of disease to late stage.^{6–7} The syphilis-staged specimens from secondary and latent stage that were not reactive by either of NT-MAA or RPR, and reactive by treponemal tests may suggestive of being collected from treated cases. However, CDC was not privy to any syphilis treatment history for the specimen panel used in this study. A possibility of prozone effect with those specimens was excluded by verifying results in semiquantitative RPR testing. The evaluation of the sensitivity of the NT-MAA and RPR assays in the context of disease stage is solely based on

the reported stage indicated on the panels' laboratory specimen submission forms and the accuracy of the disease stage could not be independently verified by the CDC laboratory, which is a limitation of this study.

Regardless of the limited information available for the specimen panels, the NT-MAA platform was shown to be consistent based on reproducibility testing, and agglutination patterns for reactive specimens were observed to be consistent from well to well. These findings also highlight the stability of the assay's cardiolipin-coupled microbead conjugate component, as the agglutination patterns remain stable for up to 24 hours past incubation, providing laboratorians with a more flexible timeframe for results interpretation. The agglutination patterns of reactive specimens in the NT-MAA could potentially be scored based on criteria similar to TP-PA test that includes NR (–), Rm/inconclusive (±) and R (+, ++ patterns). This approach could assist with quantifying non-treponemal antibody in a reactive specimen visually without the need for further titration as required in semiquantitative RPR. However, additional study is needed to accurately translate NT-MAA agglutination ratings to expected titres to advocate for use in semiquantitative test format. The results reading time of 90–120 min and/or overnight incubation may facilitate high-throughput laboratory testing but may not be conducive for point-of-care clinical settings. Hence, a future objective is to expand cardiolipin-coupled microbead use in a rapid lateral flow assay design that offers quick results.

Further improvement of the NT-MAA design is warranted, as demonstrated by the false-positive results obtained for 8/244 (3.3%) NR specimens. These false-positive specimens were NR by RPR and treponemal tests. A follow-up testing using a treponemal test could help to increase the specificity of the developed assay. Specimens with thorough clinical data would be needed to further address these findings, which could expand insight on NT-MAA performance. In the interim, syphilis serum bank is being continuously developed to obtain a greater number of characterised specimens for future validation of NT-MAA for syphilis.¹⁸ Of note, intrinsic differences in the underlying assay mechanism and its impact on test performance cannot be ruled out, as the standard RPR is based on the flocculation principle where activated charcoal particles facilitate visualisation of the antigen–antibody complexes in reactive specimens, while the NT-MAA involves visible agglutination as a result of a direct

Table 2 Analytical sensitivity of NT-MAA and RPR with purified anticardiolipin antibody

Anticardiolipin antibody (IgM class)			Anticardiolipin antibody (IgG class)		
Concentration (µg/mL)	NT-MAA	RPR	Concentration (µg/mL)	NT-MAA	RPR
83.8	R	R	54.5	R	R
41.9	R	R	27.3	R	R
20.9	R	R	13.6	R	R
10.5	R	R	6.8	R	R
5.2	R	R	3.4	R	R
2.6	Rm	Rm	1.7	R	Rm
1.3	N	N	0.9	Rm	N
0.7	N	N	0.4	N	N

N, non-reactive; NT-MAA, non-treponemal magnetic particle-based agglutination assay; R, reactive; Rm, minimally reactive; RPR, rapid plasma reagin.

Table 3 Interassay and intra-assay reproducibility of NT-MAA

Specimen	Dilutions	RPR ^a	NT-MAA																
			Intra-assay										Interassay						
			No of test repeats										No of assay repeats						
			1	2	3	4	5	6	7	8	9	10	1	2	3	4	5		
BS1505/RPR reactive specimen	Neat (1:1)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
	1:2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
	1:4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
	1:8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
	1:16	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
	1:32	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	1:64	Rm	Rm	>Rm	Rm	Rm	Rm	Rm	Rm	Rm	>Rm	Rm	Rm	Rm	Rm	Rm	Rm	Rm	Rm
	1:128	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	1:256	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1:512	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
RPR N control	Neat (1:1)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	

N, non-reactive; NT-MAA, non-treponemal magnetic particle-based agglutination assay; R, reactive; Rm, minimal reactivity; >Rm, slightly more reactivity than minimal reactive; RPR^a, rapid plasma reagin (semiquantitative).

binding between cardiolipin-coupled magnetic microbeads and the antibody. The suitability of the NT-MAA for other specimen types like cerebrospinal fluid (CSF) remains unexplored and could facilitate development of an alternative platform to CSF-VDRDL that is currently widely used for neurosyphilis diagnosis.

In summary, the performance of NT-MAA was found comparable to the conventional RPR with added advantages that include a lower volume requirement for both specimen and reagent, minimum equipment and a simpler reagent production method. The proof of concept demonstrated by our exploratory study will facilitate future validation studies for the NT-MAA using additional clinically defined specimens and may provide a novel alternative option to the existing array of non-treponemal assays.

Key messages

- ▶ The performance of developed non-treponemal magnetic particle-based agglutination assay (NT-MAA) showed 97% agreement with the conventional rapid plasma reagin (RPR).
- ▶ The robust covalent coupling of cardiolipin to magnetic microbeads and further purification by using a magnetic separator reduces the time and cost of reagent recovery.
- ▶ The agglutination patterns of NT-MAA are better defined, potentially making results interpretation less ambiguous when compared with RPR.
- ▶ Further evaluation of the NT-MAA is warranted with more clinically defined specimens to validate its use for syphilis testing.

Handling editor Federico Garcia

Acknowledgements The authors thank and acknowledge Dr Arnold Castro for providing training in the Laboratory Reference and Research Branch, CDC, Atlanta, Georgia, USA, on the cardiolipin modification and purification method. We greatly appreciate the contributions made by Dr Castro in the area of syphilis diagnosis. The authors also acknowledge Jaeyoung Hong and Joseph Kang of the Division of Sexually Transmitted Disease Prevention for their support with the statistical data analysis.

Contributors MRS and JWD participated in test concept design, specimen testing, validation and data analysis. All authors contributed equally for writing this manuscript. All authors read and approved the final manuscript.

Funding The study was funded by the CDC (Atlanta, Georgia, USA).

Disclaimer The opinions, interpretations and conclusions in this study are those of the author(s) and are not necessarily endorsed by the CDC. The use of trade names is for identification purposes only and does not constitute endorsement by the CDC or the US Department of Health and Human Services.

Competing interests None declared.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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