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Development and trial of a dried tube specimen (DTS) proficiency testing panel for dual HIV/syphilis rapid diagnostic tests

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ABSTRACT

Purpose: To develop and trial a dried tube specimen (DTS) panel for proficiency testing of dual HIV/syphilis rapid diagnostic tests (RDTs) at clinical sites.
Results: DTS panels were prepared using plasma samples with known HIV and syphilis results, to give varying reactivity for syphilis and HIV test lines on RDTs. Laboratory DTS panels were stable for a minimum 4-week period at ambient temperatures with no inter-reader variability of results. Field testing of panels with Standard Diagnostics Bioline HIV/Syphilis duo showed 100% correlation with laboratory results, and excellent mean pair agreement between the two clinical sites (k = 1.0). With Chembio Dual Path Platform HIV-Syphilis, there were two false negative results for HIV and syphilis, respectively, at one site; and good mean pair agreement between the two sites (k = 0.9).
Conclusion: It is feasible and practicable to incorporate DTS panels into a field proficiency testing scheme for dual HIV/syphilis RDTs.

1. Introduction

The World Health Organization recommends syphilis screening for pregnant women as part of their basic antenatal care (ANC) (World Health Organization, 2002). Women should be serologically screened to detect untreated syphilis infections which may result in miscarriage, stillbirth, preterm delivery, low birth weight and congenital syphilis. These adverse pregnancy outcomes may be experienced in up to 80% of untreated maternal syphilis (Wijesooriya et al., 2016). Screening and treatment of pregnant women and their partners is one of the pillars of the WHO strategy toward the global elimination of mother-to-child-transmission of syphilis (World Health Organization, 2007). WHO guidelines advise countries on the development of a screening strategy based on national antenatal syphilis seroprevalence as well as access to laboratory services (WHO, 2017).

The standard diagnostic tool that is used for syphilis screening is the serum non-treponemal Rapid Plasma Reagin (RPR) test in conjunction with a *Treponema pallidum* specific assay such as the *Treponema pallidum* haemagglutination assay or automated *Treponema pallidum* antibody chemiluminescence or enzyme immunoassays. These tests are laboratory-based and used in traditional or reverse-testing algorithms. They do not deliver same-day results and require women to return to the ante-natal care facility for results and treatment, if seropositive on both non-treponemal and treponemal assays. Syphilis rapid diagnostic tests (RDTs) are useful in settings where laboratory facilities are not easily accessible, patients do not reliably return for results, or where there are "late bookers" for ANC, leaving insufficient time for screening and treatment to prevent neonatal infection (Mabey et al., 2012). These RDTs have been found to fulfill the WHO REASSURED criteria, which stipulate the technical requirements for a true point of care test (Land et al., 2019). The WHO also recommends the use of HIV RDTs as screening assays in HIV testing algorithms at ANC settings (World Health Organization, 2019). Dual RDTs for syphilis and HIV facilitate the provision of integrated testing services for the elimination of mother to child transmission of both HIV and syphilis, and improved quality of antenatal care (World Health Organization, 2019). Examples of dual RDTs include the Standard Diagnostics (SD) Bioline HIV/Syphilis duo (Abbott Diagnostics; Princeton, NJ) and Chembio Dual Path Platform (DPP)® HIV-Syphilis Assay (Chembio Diagnostics Systems Inc; Medford, NY) for the detection of antibodies to HIV-1 and -2 and *Treponema pallidum* antigen (Van Den Heuvel et al., 2019).

The successful implementation of dual HIV/syphilis RDTs in clinical practice relies on their integration into sexual and reproductive healthcare programmes, as well as the existence of a sustainable external quality assurance (EQA) system and proficiency testing scheme (PTS) for healthcare facilities. The types of samples used in

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PTS should be acceptable to the users (i.e., healthcare workers on site) and the testing methodology should be technically simple. The Dried Tube Specimen (DTS) protocol is a cold chain independent method that is easy to prepare (using plasma/serum), store and transport to clinical sites. The specimens are stable at room temperature and can be easily reconstituted at the site before testing (Parekh et al., 2010).

The primary objective of our study was to develop and evaluate a DTS panel using clarified plasma for use in EQA of dual HIV/syphilis RDTs at clinical sites. Secondary objectives were (1) to assess inter-operator variability in panel reconstitution and interpretation of results, (2) to assess the stability of the panel over a period of 4 weeks, and (3) to conduct a preliminary clinic-based evaluation of its utility in proficiency testing.

2. Methodology

2.1. Plasma selection and preparation of trial DTS panel

For the preparation of a DTS panel for use in PTS for dual HIV/syphilis RDTs, we modified the DTS method for HIV RDTs described by Parekh et al. (2010). The DTS panel was prepared at the Centre for HIV and STIs of the National Institute for Communicable Diseases in Johannesburg, using well-characterized plasma samples. Syphilis and HIV negative plasma samples were sourced from the South African National Blood Service (SANBS) and clinical samples with high rapid plasma reagin (RPR) titres of ≥64 were requested from the National Health Laboratory Service laboratories. The HIV results for all specimens were confirmed using the Alere Determine™ HIV 1/2 (Alere Medical Co Ltd; Chiba, Japan) and the Uni-gold™ HIV (Trinity Biotech PLC.; Wicklow, Ireland) RDTs. The Immutrep® RPR (Omega Diagnostics LTD; Alva, UK) test kit and the Serodia® TPPA (Fujirebio Inc; Tokyo, Japan) were used to verify the RPR titres and treponemal-specific antibody results, respectively. High RPR titre samples, which inherently also have high treponemal titres, were pooled according to their HIV result (i.e., HIV reactive or non-reactive).

Two-fold serial dilutions were prepared from each of the two HIV pools using a volume of 200 μl of the dually HIV and syphilis sero-negative plasma as diluent, to give dilutions ranging from 2 to 2048 (Fig. 1). A volume of 20 μl per tube was then transferred from each dilution into four appropriately labelled tubes. The uncapped tubes were left to dry overnight in a level 2 biosafety (BSL-2) cabinet.

The following day, at least one dried tube specimen (DTS) from each dilution was reconstituted with 200 μl of Phosphate Buffered Saline (PBS) – 0.1% Tween Buffer, mixed by tapping or inverting the tube, and then re-incubated overnight prior to testing. The DTS tubes were mixed thoroughly by tapping or inversion of tubes before testing with two dual RDTs.

2.2. Test kit evaluation for trial DTS panel selection

Two dual RDTs that is the SD Bioline HIV/Syphilis duo (Abbott Diagnostics) and the Chembio DPP® HIV-Syphilis (Chembio Diagnostic Systems Inc) were tested with each DTS dilution as per manufacturers' instructions. At the time, these dual RDTs were undergoing WHO-facilitated clinic-based evaluations of performance and operational characteristics at ante-natal care facilities in South Africa (The ProSPeRo Network, 2020). Both tests are lateral flow immunochromatographic assays for the qualitative detection of HIV and specific treponemal antibodies in plasma, serum or capillary whole blood specimens. The Chembio DPP assay is equipped with an optional Micro Reader to aid the objective interpretation of test results. Each assay has a control line, and two test lines for HIV and syphilis, respectively. Results of testing are read visually as strong, medium or faint lines; even a faint line is interpreted as a positive result. Absence of a control line on testing indicates an invalid result. The results of each dilution by test kit were documented, together with the strength of reactivity for HIV and syphilis test lines. Four dilution tubes of differing reactivities (strong, medium, faint and negative) for HIV and syphilis were selected for the trial DTS panel.

A new identification number (A1–A4) was then assigned to each of the four specimens selected for the trial DTS panel (Fig. 2). For trial DTS pre-release & stability testing and field DTS panel preparation, a larger volume (500 μl aliquot) of each selected specimen was used. 5 μl (1:100 dilution) of 0.4% Trypan blue dye was then added to each aliquot. The mixture was vortexed and 20 μl aliquots of the final mix from each specimen transferred into 10 appropriately labelled tubes. The tubes were then left uncapped in a BSL-2 cabinet, and allowed to dry overnight. Once the samples had dried, they were capped

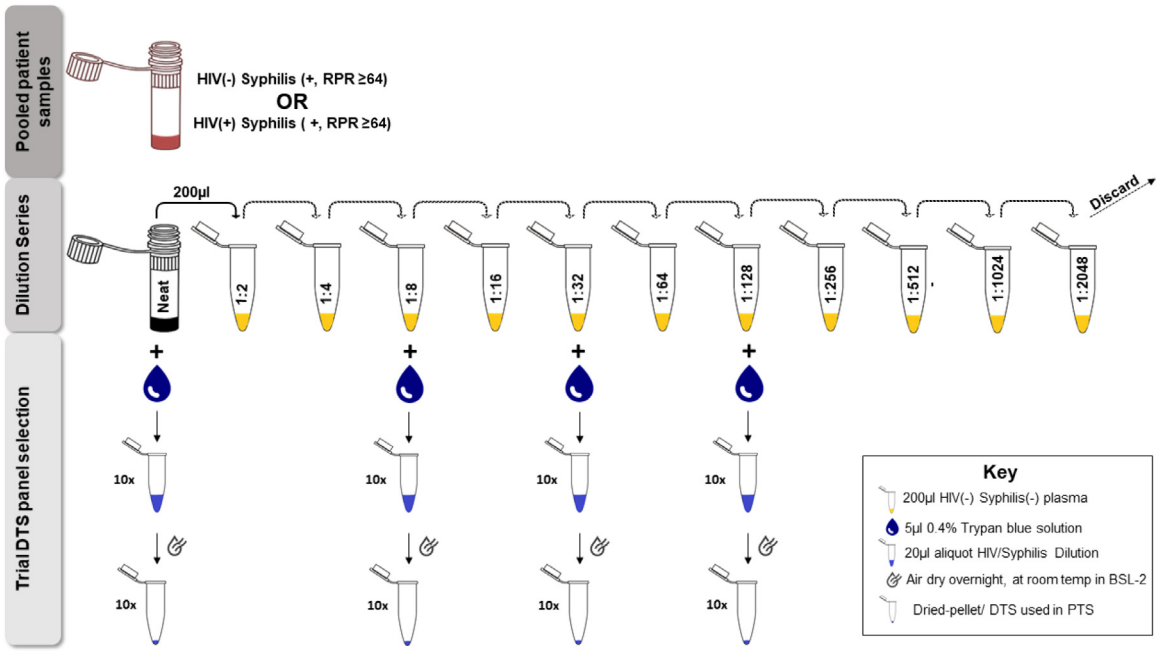

Fig. 1. Selection of trial DTS panel specimens from a pool of patient samples. BSL-2 = level 2 biosafety; DTS = dried tube specimen; PTS = proficiency testing scheme; RPR = rapid plasma reagin.

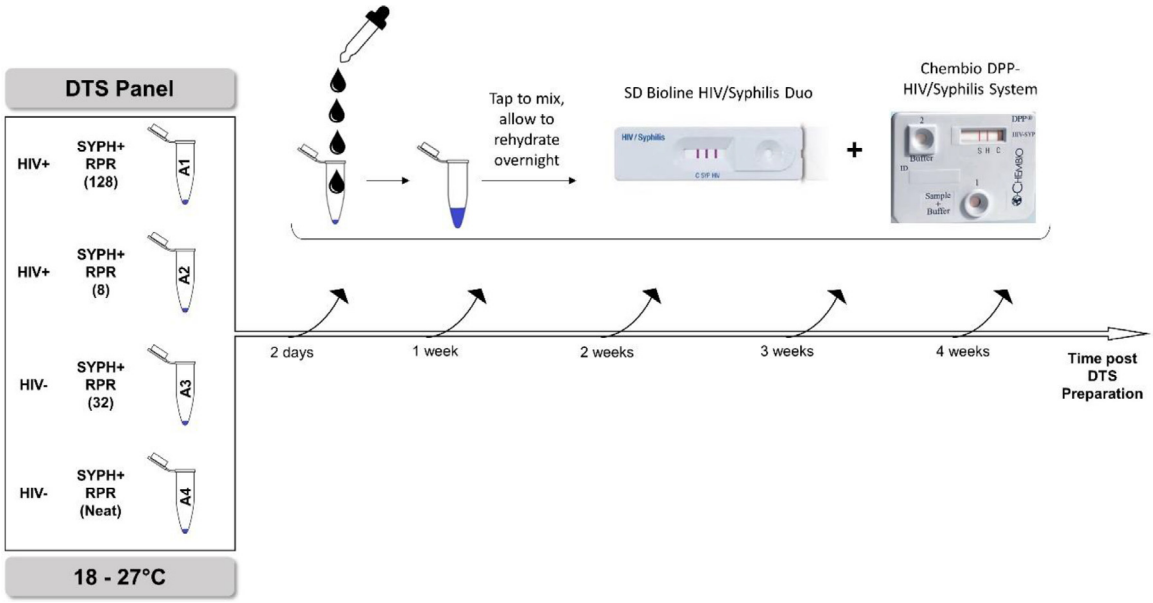

Fig. 2. DTS trial panel reconstitution and stability testing. DTS = dried tube specimen; RPR = rapid plasma regain; SD = standard diagnostics.

following verification that a blue pellet was visible at the bottom of each tube.

2.3. Trial DTS panel stability and pre-release testing

The trial DTS panels were kept at room temperatures ranging from 18 to 27 °C for up to 4 weeks, in order to simulate the temperatures likely to be encountered at field sites. Pre-release testing was initiated 2 days after the preparation of the DTS panel by adding 4 drops of PBS- Tween Buffer and incubating overnight prior to testing. This testing was then repeated weekly for a period of 4 weeks to assess panel stability and consistency of results with the SD Bioline and ChemBio DPP assays (Fig. 2). This period would represent the duration of time that the proficiency testing survey would be open for testing and resulting by healthcare workers in the field.

2.4. Inter-reader variability of trial DTS panel results

Following trial DTS panel preparation, two different operators reconstituted the DTS panels. Pre-release and stability testing were performed independently by the two operators, as outlined above, in order to assess inter reader-variability of results with the two dual HIV/syphilis RDTs.

2.5. Piloting of field DTS panels

To assess the acceptability and ease of use of the DTS panels, four field panels were piloted at each of the two sites participating in a WHO-facilitated clinic-based evaluation of dual SD Bioline and Chembio DPP® HIV/Syphilis assays in pregnant women (The ProSPeRo Network, 2020). Each field DTS panel comprised four samples of varying HIV/syphilis reactivity, ranging from faint to strong on dual RDTs, as well as one sample that was non-reactive for both HIV and syphilis antibodies (A1–A5). Field DTS panels were transported to sites at quarterly intervals (Q1–Q4) between November 2018 and November 2019. The panels were prepared as per the method used for the trial DTS panels and transported at ambient temperatures. A 2 ml vial of PBS- Tween Buffer, 3 ml plastic dropper pipettes, an instruction sheet for DTS reconstitution and testing, as well as a results documentation form were included in each field panel kit. Professional nurses in the ante-natal section of the two primary healthcare facilities located in Pietermaritzburg, Kwa Zulu Natal province and White River, Mpumalanga province participated in the field PTS. In total, 11 professional nurses had the opportunity to test and read the panels.

2.6. Statistical analysis

Correlation of qualitative results for DTS HIV and syphilis with RDTs in the field versus RDTs performed in the reference laboratory was calculated as follows:

$$\%\ \text{Positive Agreement (PPA)} = \frac{\text{number of positive HIV/Syphils results with field RDT} \times 100}{\text{number of positive HIV/Syphilis results with reference lab RDT}}$$

$$\%\ \text{Negative Agreement (NPA)} = \frac{\text{number of negative HIV/Syphilis results with field RDT} \times 100}{\text{number of negative HIV/Syphilis results with reference lab RDT}}$$

Inter-observer variability was determined using the Mean Pair Agreement Index to compare trial DTS panel results from the two clinical sites. This was calculated separately for each of the two dual HIV/syphilis RDTs.

$$\text{Mean Pair Agreement Index for RDT} = \frac{\text{number of results in agreement between the two trial sites} \times 100}{\text{number of results compared between the two trial sites}}$$

Additionally, the kappa statistic was calculated to determine the proportion of agreement beyond chance for results from the two sites.

$$\text{Kappa statistic (k) for RDT} = \frac{\text{Observed Agreement} - \text{Chance Agreement}}{1 - \text{Chance Agreement}}$$

2.7. Ethical approval

Ethics approval for use of plasma specimens from South African National Blood Service and National Health Laboratory Service

Table 1
Dilutions used in trial DTS panel preparation in the laboratory.

Reference testing of samples		REACTIVITY of test lines on two RDTs		NEW ID of panel tubes
HIV result	RPR result, titre	HIV	SYPHILIS	
Reactive	Positive, 128	S	S	A1
Reactive	Positive, 8	M	F	A2
Non-reactive	Positive, 32	N	S	A3
Non-reactive	Negative (TPPA positive)	N	F	A4

DTS = dried tube specimen; RPR = rapid plasma regain; RDTs = syphilis rapid diagnostic tests; S = Strong; M = Medium; F = Faint; N = Negative.

Table 2
Results of field DTS panel testing from the two study sites for the SD Bioline assay.

	SD Bioline HIV/Syphilis Duo			
Site 2	Site 1			
		Positive	Negative	Total
	Positive	20	0	20
	Negative	0	20	20
	TOTAL	20	20	40

DTS = dried tube specimen; SD = standard diagnostics.
Mean Pair Agreement Index = (40/40) × 100 = 100%.
k = 1.0.

Table 3
Results of field DTS panel testing from the two study sites for the Chembio DPP assay.

	Chembio DPP-HIV/Syphilis			
Site 2	Site 1			
		Positive	Negative	Total
	Positive	18	2	20
	Negative	0	20	20
	TOTAL	18	22	40

DTS = dried tube specimen.
Mean Pair Agreement Index = (38/40) × 100 = 95%.
k = 0.9.

laboratories was granted by the University of the Witwatersrand Human Research Ethics Committee.

3. Results

Pooling of RPR positive specimens based on HIV reactivity, and further dilution of these two pools, allowed for different combinations of HIV and syphilis results with test lines on RDTs ranging from negative to strong. These dilutions could then be used to prepare specimens for the trial DTS panel (Table 1). The plasma volume of 20 μl per tube in each trial DTS panel dried easily overnight and produced concordant results with the two dual HIV/syphilis RDTs. The trial DTS panels were stable at room temperature for a 4-week period, with no inter-operator variability in result interpretation between the two laboratory technologists who reconstituted the tubes and performed the tests. Additionally, the strength of test line reactivity in panel samples A1–A4 (Table 1) remained consistent for HIV and syphilis with both RDTs over the 4-week period.

Field DTS panels were received at sites within 2 days of shipping. PTS results from sites were sent to the reference laboratory within 4 weeks of receipt of the panels. Pilot testing conducted at the two clinical sites showed a high degree of correlation between field and reference laboratory results for HIV and syphilis with the two dual RDTs. With SD Bioline 100% of observed results were in accordance with expected results for both HIV and syphilis (PPA = 100%; NPA = 100%). The mean pair agreement index of 100% indicated high inter-observer reliability between the two sites, and the kappa statistic represented perfect agreement (Table 2). With Chembio DPP, there were two discordant results which were not in keeping with expected results. For HIV, the PPA was 85.7% (6/7); and the NPA was 100% (13/13). For syphilis, the PPA was 92.3% (12/13) and the NPA was 100% (7/7). Site 1 incorrectly reported a negative HIV result for Tube A5 (Quarter 1) and a negative syphilis result for Tube A3 (Quarter 2). Results for the same samples were correctly reported by Site 2. The mean pair agreement index of 95% indicated high inter-observer reliability between the two sites, and the kappa statistic of 0.9 represented very good agreement (Table 3).

4. Discussion

We describe in detail the method used for the development and pre-release testing of DTS proficiency testing panels for dual syphilis/HIV RDTs. Our evaluation and piloting of trial and field DTS panels for two commercial HIV/syphilis RDTs showed that these panels could be incorporated for use in future EQA schemes, nationally. The panels were stable at room temperature for a period of at least 4 weeks prior to reconstitution, and nurses at primary healthcare facilities were able to correctly reconstitute the tubes and perform testing.

In Sub-Saharan Africa, the laboratory-based RPR test is widely used as a screening tool in ANC; however, syphilis RDTs are seen as a viable option to increase testing coverage (Blandford et al., 2007; Kuznik et al., 2013). The use of RDTs in testing algorithms requires a sustainable and implementable EQA system and PTS (Kularatne, 2018). The PTS panels should be robust to withstand supply chain challenges such as delays in transportation to peripheral sites, storage at a range of ambient temperatures in the field, and fluctuating environmental conditions. Testing methodology used in PTS should be technically simple, feasible, and acceptable to end-users, that is busy clinical staff in resource-constrained settings. DTS panels using plasma or serum as the matrix have been evaluated, and found

to be a stable and acceptable PTS method for HIV and Syphilis RDTs in remote settings (Beber et al., 2015; Benzaken et al., 2014).

It is important that plasma specimens giving faint, borderline results on RDT or low positive results on reference testing for example low titre RPR (<16) are not used in initial panel preparation, since these would also have relatively low treponemal antibody titres. Hence further dilution and reconstitution of specimens used in field DTS panels would lead to loss of reactivity on RDT. A variety of treponemal test line results on RDTs can be obtained through pooling and dilution of high-titre RPR specimens. Moreover, the small volume that is utilized in the preparation of tubes in each panel facilitates preparation of multiple panels for clinical sites without the requirement for large volumes of RPR and HIV-reactive specimens. Our evaluation confirms that DTS panels are stable at temperatures of up to 26 °C over at least a 4-week period without loss of reactivity. Stability testing conducted to determine the reliability of DTS panels for EQA of HIV/Syphilis point of care test in the Brazilian Amazon, revealed that panels stored at 37 °C in a humid environment, or at 45 °C in dry and humid conditions, did not give the expected results for syphilis RDTs (Benzaken et al., 2014). This was attributed to the fact that the dried pellet did not dissolve in PBS following reconstitution under these conditions. This underscores the importance of proper transportation of the panels, and the provision of adequate instructions to healthcare workers to ensure panel storage and reconstitution in appropriate conditions.

The introduction of dual HIV/syphilis RDTs in antenatal care is likely to increase access to testing and treatment for pregnant women. It is important that the accuracy of results is ensured to avoid misdiagnosis. The implementation of EQA and PTS processes is an essential component for the successful and sustained implementation of these assays. Although dual syphilis/HIV RDTs are technically simple and require few steps, there are specific kit requirements with respect to the volume of blood tested, and time intervals for the addition of reagents and reading of results. A robust EQA programme will objectively evaluate factors in test performance (testing methods, staff training) through the quality of results obtained. The DTS protocol is a versatile technique for preparing PTS panels for HIV and Syphilis RDTs (Parekh et al., 2010). However, the selected field DTS panel should comprise specimens giving varying results (including faint lines) with RDTs that will challenge readers and test their interpretive skills.

The results from our clinical sites indicate that healthcare workers were able to reconstitute the DTS tubes and perform the assays as per kit instruction. The two false negative results reported for syphilis and HIV with Chembio DPP occurred at one site only, for two different samples sent in quarter 1 and 2, respectively. These results could not be attributed to faint test lines. They may be user-related, and associated with error in the testing or reading procedure specifically for the Chembio DPP assay, as results from the same samples were correctly interpreted at this site with the SD Bioline kit. Although the false negative result rate was low, there are significant implications for patient management and risk of mother-to-child transmission of HIV or syphilis if there is missed diagnosis in pregnancy. It is important to address potential sources of false negativity attributable to user error. A study in the Brazilian Amazon identified various operational challenges with an EQA system using DTS for syphilis RDTs (Beber et al., 2015). These included the availability of adequately trained professionals to correctly test and interpret RDT results. In a study of the integration of simultaneous triple point-of-care antenatal screening for syphilis, HIV and hepatitis B in Peru, fear of disclosure of positive results by healthcare workers led to weakly reactive results (faint lines) being reported as negative (Smith et al., 2015). Continuous training and monitoring is vital to ensure the accuracy of the RDT results, especially when there is high staff turnover at clinical sites. Our study did not investigate the acceptability of this PTS method to healthcare workers, or the protocol for implementing corrective actions when proficiency testing results prove unsatisfactory. Our evaluation of trial and field DTS panels in the laboratory and field, respectively, demonstrate that they may be used in future EQA schemes for dual HIV/syphilis RDTs.

Authors' contributions

Venessa Maseko: Methodology, Investigation, Validation, Writing – original draft preparation. Duduzile Valashiya: Methodology, Investigation, Validation, Writing – reviewing. Ranmini Kularatne: Conceptualization, Visualization, Supervision, Formal analysis, Writing – reviewing and editing.

Author statement

All authors have seen and approved the final version of the manuscript being submitted. The authors confirm that the article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

Declaration of competing interest

Authors state no conflicts of interest.

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