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ARTICLE

Application of a noninvasive oral fluid test for detection of treponemal IgG in a predominantly HIV-infected population

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Abstract The performance of a time-resolved fluorescence immunoassay (TRFIA) for detection of treponemal IgG from oral fluid specimens has been assessed in a predominantly HIV-infected population. Serological investigation is the method of choice for confirming clinical suspicion of syphilis; however, in the primary stage of disease, direct detection of treponemes in lesion fluid or *Treponema pallidum* DNA is recommended because of the reduced sensitivity of serological tests. There may be occasions when blood for serological investigation is difficult to obtain due to individual patient preference or logistical necessity to improve participation in screening initiatives, particularly in outreach situations. Collection of oral fluid for detection of treponemal antibody may prove an attractive alternative and, with this in mind, an oral fluid assay for detection of treponemal IgG was developed. Time-resolved fluorescence was used to detect treponemal IgG extracted from commercially available oral fluid collection devices. Paired serum and saliva samples were obtained from 210 individuals, 101 of whom were diagnosed with syphilis on the grounds of medical examination confirmed by serological testing. Oral fluid specimens from 14 subjects were rejected because they contained insufficient control antibody or were inhibitory. The population tested was predominantly men who have sex with men, many of whom were HIV infected. The overall sensitivity and specificity of the oral fluid assay was 95.8 and 86.1%, respectively, based on the 5th percentile of the positive results, and 93.7 and 91.1%, respectively, based on a cutoff derived by mixture model analysis. For individuals with primary syphilis, the optimum sensitivity of the oral fluid assay was 87.5%, whereas in those with disease classified as secondary syphilis and early latent syphilis, the sensitivity of the oral fluid assay was 100 and 94.7%, respectively. The oral fluid assay is a useful alternative to serological testing in certain situations, and further development of this technology to enable detection of treponemal IgM should increase its sensitivity for detecting cases of primary syphilis.

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Introduction

The incidence of syphilis in the UK increased by 1,290% between 1997 and 2004, an increase driven by a number of outbreaks [1]. This increase has challenged established public health intervention strategies, particularly the way in which cases are identified and managed. Syphilis detection

initiatives at social venues were undertaken in a number of outbreak areas to identify cases in groups at increased risk of infection. It has been shown that HIV-infected individuals have a higher risk of syphilis than non-HIV-infected individuals [2]. Pinprick blood tests are available, but the use of blood sampling in population screening is unlikely to be acceptable to many people, and, in view of the high HIV prevalence within the populations at increased risk of syphilis, is potentially hazardous. The use of oral fluid, instead of blood, as a means of detecting treponemal IgG to identify cases of syphilis is a potentially useful method for accessing individuals who prefer not to give blood or who do not wish to submit to medical examination. The benefits of this approach are that we enable healthcare workers to collect specimens in environments where phlebotomy is not possible, or desirable, and in a way that reduces the risk of blood exposure. The accuracy of such tests needs to be characterised further to determine the usefulness of this method for diagnosis of syphilis and for screening and population prevalence surveillance initiatives.

Oral fluid assays have been developed and applied to a number of antibody markers, such as HIV [3], measles, rubella, and hepatitis B [4]. Novel, highly sensitive immunoassays are needed [5] to detect past (IgG) or recent (IgM) infection because the levels of IgG and IgM found in saliva are approximately 1,000-fold less than those in plasma. We have used time-resolved fluorescence technology because of its high sensitivity and large linear dynamic range [6]. In order to enhance the amount of immunoglobulin obtained from saliva, specific oral fluid collection devices [7] are available. In this study, Oracol collection devices (Malvern Medical Developments, Worchester, UK), which are sponge-tipped swabs rubbed along the gum margins, were used to collect oral fluid specimens from all subjects.

Previously, we developed a time-resolved fluorescence immunoassay (TRFIA) for detection of treponemal IgG in oral fluid specimens and evaluated it in a predominantly heterosexual, non-HIV-infected population [8]. The oral fluid assay was 100% sensitive and 97.9% specific. Now, we report the performance of the oral fluid assay for syphilis in a larger, predominantly HIV-infected population.

Materials and methods

Patient recruitment and sampling

Ten genitourinary clinics in England that together had reported over 60% of the infectious syphilis cases in the UK during 2000–2001 took part in the study. Cases were patients attending the clinics with a clinical diagnosis of syphilis (primary, secondary, and early latent), including those known to be HIV positive. Controls were patients with no history of syphilis and who had negative syphilis serology. All patients were recruited by the lead clinician/research nurse/specialist registrar, who also completed a questionnaire that covered demographic, behavioural, and clinical data. Patients gave informed consent and were asked to provide blood (5 ml) and oral fluid samples. Oracol oral fluid collection devices were obtained from the manufacturer. The device comprises a sterile, polystyrene foam sponge at the end of a plastic stick that is stored in a plastic tube. Patients were advised to rub the sponge firmly along the gum (like a toothbrush), at the base of the teeth, if present, for approximately 1 min until it was wet. The swab was then placed back in the plastic tube, which was then capped, placed in appropriate transport packaging, and returned to a central laboratory for documentation processing. The samples were then forwarded to the testing laboratory.

All work undertaken in this study complied with the current laws of the UK to the best of our knowledge.

Processing of serum samples

Sera were tested by a commercial syphilis IgG EIA (Newmarket Laboratories, Newmarket, UK) and reactive sera confirmed by a *Treponema pallidum* particle agglutination assay (Fuji Rebio, Tokyo, Japan) and a quantitative Venereal Disease Research Laboratory (VDRL) assay (Syphscreen RPR; Shield Diagnostics, Dundee, UK). Treponemal IgM status was determined using the Pathozyme Syphilis M Capture EIA (Omega Diagnostics, Alloa, UK). Tests were performed and interpreted according to a standard algorithm [9].

Processing of oral fluid samples

On receipt by the testing laboratory, the oral fluid was extracted from each swab by the addition of 2.0 ml of transport medium (phosphate-buffered saline, pH 7.2, with 10% foetal calf serum, 0.2% Tween 20, 0.5% gentamicin, and 0.2% fungizone) followed by vortexing for 20–30 s. The transport medium was allowed to equilibrate with the oral fluid for at least 1 h at 4°C, after which the swab was removed from the plastic tube and the remaining oral fluid extracted from the sponge by squeezing/twisting the head of the swab against the side of the plastic tube as it was removed. The oral fluid was then filtered through a Celtron 30, 0.2-μm filter (Schleicher & Schuell MicroScience, Dassel, Germany) and collected in a sterile, 2.0-ml microtube (Sarstedt, Numbrecht, Germany). The oral fluid was then stored at −20°C until needed for testing.

Table 1 Characteristics of the syphilis-seronegative and syphilis-seropositive populations tested

Variable	Negative	Positive	Percent seropositive (%)	*p* value
Sex (*n*=191)				
Male	88	87	49.7	
Female	14	2	12.5	0.004[a]
Mean age in years±SD (*n*=192)	34±9.4	37±10.0		0.072[b]
Sexual preference (*n*=195)				
Heterosexual	34	12	26.1	
Men–men	65	75	53.6	
Bisexual	4	5	55.6	0.004[a]
HIV status (*n*=129)				
Negative	36	27	42.9	
Positive	26	40	60.6	0.044[c]

[a] Fisher's exact test
[b] Two-sample *t* test
[c] Chi-square test

Time-resolved fluorescence immunoassay for detection of *Treponema pallidum* IgG

Treponemal antigen (TpN15 kDa, TpN17 kDa, and TpN47 kDa recombinant antigens)-coated microtitre plates (Newmarket Laboratories) were loaded with 100-µl oral fluid specimens in duplicate. The plates were shaken at 100 rpm on a Stuart Mini-Orbital shaker (Bibby Sterilin, Stone, UK) for 2 h at room temperature and then washed four times with Delfia wash buffer (Perkin Elmer Life Sciences, Cambridge, UK) using a Delfia plate washer (Perkin Elmer). Europium-labelled anti-human IgG conjugate (Perkin Elmer) diluted 1:500 in Delfia assay buffer (Perkin Elmer) was added, at 100 µl per well, to all wells of the plates, which were then shaker incubated (100 rpm) for a further 2 h at room temperature. After washing four times with Delfia wash buffer, 150 µl of Delfia enhancement solution (Perkin Elmer) was added to all wells. The plates were put in a dark box and shaker incubated for 10 min and then read using a Wallac 1234 Delfia research fluorometer (Perkin Elmer).

Testing of oral fluid sample quality by determination of total IgG

The total IgG content of oral fluids was determined by radial immunodiffusion using Nanorid kits (The Binding Site, Birmingham, UK). Briefly, 20 µl of undiluted oral fluid, IgG calibrators, and control serum diluted 1:10 were added to designated wells of a pre-prepared radial immunodiffusion plate. The plate was incubated at 30°C in a humid chamber for 96 h, and then the diameter of the ring of precipitation around each well was read.

Testing of oral fluid sample quality by determination of Varicella Zoster Virus IgG

The assay for varicella zoster virus (VZV) was identical to that used for treponemal antibody except that Delfia microtitre plates (Perkin Elmer) coated with VZV EIA grade antigen (The Binding Site) were used instead of plates pre-coated with treponemal recombinant antigen (Newmarket Labs).

Statistical methods

The population characteristics were investigated in a descriptive way by tabulating the disease status (positive/negative) for age, sex, HIV status, and sexual preference using Pearson's chi-square test, Fisher's exact test, and Student's *t* test.

Separate positive/negative cutoff values were calculated from the quantitative results of seronegative subjects using log-transformed europium counts by different methods. Positive results included cases of primary, secondary, and early latent syphilis. The mean plus two or three standard deviations is often used to estimate a positive/negative cutoff value for serological assays [10]. However, for this study, the main interest was the sensitivity of the assay, and therefore the positive distribution was used instead of the negative. The 10th, 5th, and 2.5 percentiles were calculated instead of subtracting two or three standard deviations from the mean, since the distribution of the positive results was not normal.

For epidemiological purposes, a positive/negative cutoff value was required that would maximise both sensitivity and specificity, whereas for clinical diagnosis high sensitivity was a prerequisite. An "epidemiological cutoff value" was calculated using mixture modelling [11]. Data were assumed to follow two normal distributions, one for negative results and one for positive results. Using mixture modelling, the distribution estimates were calculated by maximum likelihood methods. The optimal cutoff point was estimated to minimise the discrepancy between the sensitivity and specificity, based on the resulting model estimates [11]. A receiver operating characteristic (ROC) analysis was also carried out as a non-parametric alternative to the mixture modelling. The under-the-curve area of the ROC curve provided a measurement of discrimination of the results.

The analysis was carried out using Microsoft Excel 2000. The ROC analysis was carried out using Stata 8.3 (StataCorp, College Station, TX, USA).

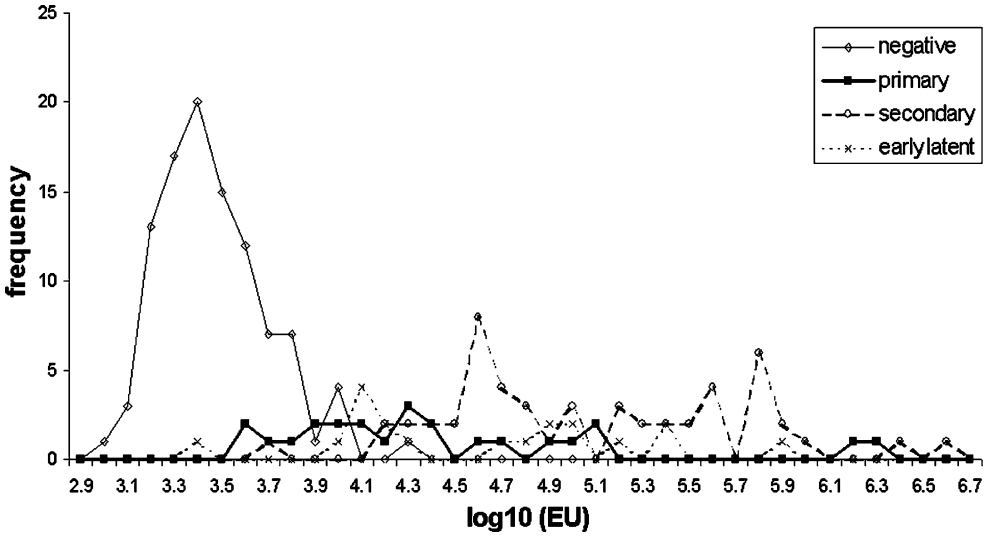

Fig. 1 Distribution, by disease status, of syphilis TRFIA europium counts for 196 evaluable oral fluid specimens

Results

Paired serum and oral fluid swabs were collected from 210 individuals. Following serological testing for syphilis, 101 individuals were seropositive and 109 individuals seronegative. Testing of Oracol-collected oral fluids for control antibody (VZV IgG) showed six specimens from seropositive individuals and eight specimens from seronegative individuals to be insufficient. After removing these specimens from the analysis, results from 95 syphilis-seropositive individuals and 101 syphilis-seronegative individuals were evaluable. The demographic characteristics of the seropositive and seronegative populations tested are shown in Table 1. More males were syphilis seropositive than females (49.7% and 12.5%, respectively; $p=0.004$). There was no significant difference in age, although individuals with syphilis were slightly older (average age, 37 years) than those without syphilis (average age, 34 years). Bisexuals and men having sex with men were more likely to have syphilis than heterosexuals (55.6, 52.1, and 26.1%, respectively; $p=0.004$).

The distribution of europium counts for the 196 evaluable oral fluid specimens is shown in Fig. 1. The counts have been categorised by disease status: 101 (51.5%) individuals had no evidence of syphilis, 24 (12.2%) had primary syphilis, 52 (26.5%) had secondary syphilis, and 19 (9.7%) were classified as having early latent syphilis. A number of methods were used to determine the cutoff value for syphilis seropositivity, depending on whether the assay was going to be used for diagnostic or epidemiological purposes. The estimated cutoff values and the values for sensitivity, specificity, positive predictive value, and negative predictive value are shown in Table 2.

The 10th, 5th, and 2.5 percentiles of europium counts from seropositive individuals were 9,020, 4,828, and 3,414, respectively (Table 2). Using a cutoff of the 5th percentile of the positive samples, the oral fluid time-resolved fluorescence immunoassay (TRFIA) was 95.8% sensitive and 86.1% specific. This approach resulted in high sensitivity values and reasonably high specificity values for the assay.

Using mixture modelling, the two-normal distribution provided a good fit (deviance =16.8 and 191 *df*; Fig. 2).The optimum cutoff was 5,660 europium counts, which gave a sensitivity of 93.7% and a specificity of 91.1%. Finally, an

Table 2 Estimated cutoff values as well as sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) for the syphilis oral fluid TRFIA

Method	Cutoff (log10)	Europium count	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Cutoff calculated by taking the 10th, 5th, and 2.5 percentile of the positive distribution						
Positive 10th percentile	3.955	9,020	90.5	97.0	96.6	91.6
Positive 5th percentile	3.684	4,828	95.8	86.1	86.7	95.6
Positive 2.5 percentile	3.533	3,414	97.9	73.3	77.5	97.4
Cutoff calculated using modelled data analyses						
Mixture modelling	3.753	5,660	93.7	86.0	85.6	93.9
ROC analysis	3.858	7212	92.6	89.7	88.9	93.2

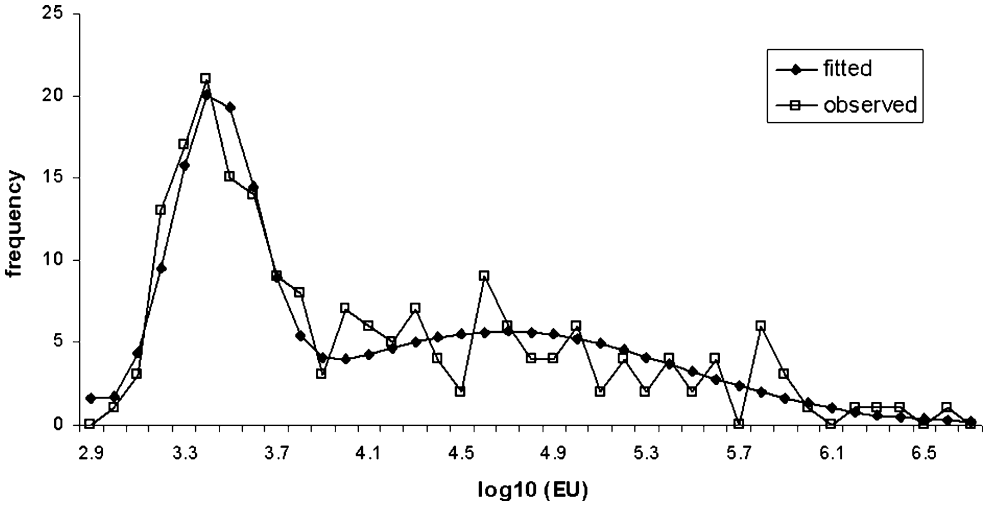

Fig. 2 Observed data and fitted mixture model distribution of the log-transformed europium counts in 196 individuals

ROC analysis was undertaken (Fig. 3), which showed a very high area under the ROC curve (AUC =0.97) that corresponds to a high probability of correct identification between the negative and positive distributions. The optimal cutoff value was estimated as 7,212 europium counts, which gave a sensitivity of 92.6% and a specificity of 94.1% (Table 2).

Discussion

A combination of dark-ground microscopy (DGM) with treponemal serology provides the highest sensitivity for the detection of early syphilis, principally because treponemal serology, compared to DGM, has reduced sensitivity in the detection of primary syphilis and greater sensitivity in the detection of secondary syphilis [12]. For individuals with primary syphilis, the sensitivity of the oral fluid assay was 87.5% if the 5th percentile of the positive results was used (europium count cutoff=4,828) and 83.3% if the mixture-modelled cutoff (europium count=5,660) was used. In patients with secondary syphilis, the sensitivity of the oral fluid assay in the population studied was 100% using the aforementioned cutoff values. Finally, in early latent syphilis, the sensitivity of the oral fluid assay was 94.7%, according to the aforementioned cutoff criteria.

In simple terms, the oral fluid assay we have used measures serum-derived IgG diluted in oral fluid secretions. Thus far, we have not developed a treponemal oral fluid IgM TRFIA, and such an assay used in conjunction with the syphilis oral fluid IgG TRFIA may yield increased

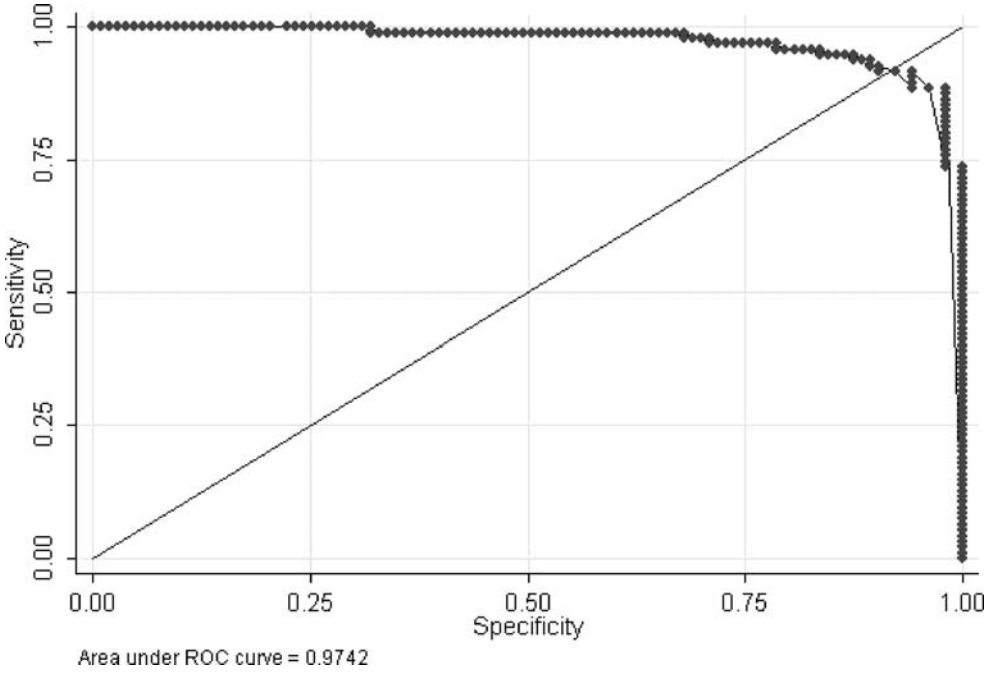

Fig. 3 Receiver operating characteristics (ROC) curve for the syphilis TRFIA

detection of primary syphilis. Our main consideration in the development of the syphilis oral fluid assay was not to replace traditional syphilis serology and microscopy but to make available an alternative in situations where these established methodologies cannot be used. Previously, it has been shown that sampling by oral fluid assay can increase client acceptance of the offer of syphilis testing in outreach situations [13]. Ward and Weber [14] have discussed the positive benefits of screening tests for control of sexually transmitted infection, using tests with sensitivity less than that of gold standard techniques, an example of which is the screening for infection by point-of-care devices. On the basis of our personal experience, the availability of the syphilis oral fluid screening test will enhance our ability to target socially isolated populations because sample collection is discrete and can be self-performed in a variety of environments.

Our findings derive from cases of early syphilis in individuals presenting to genitourinary medicine clinics, mainly in London. The syphilis-seropositive population we studied consisted predominantly of men who have sex with men, a population with a high incidence of HIV coinfection. This population is representative of that associated with the current syphilis epidemic in the UK, and it has been suggested [15] that regular syphilis screening for HIV outpatients is an effective means of detecting asymptomatic infection. Obviously, DGM has limited applicability in asymptomatic individuals. Previously, we reported sensitivity and specificity values of 100% for the syphilis oral fluid TRFIA in individuals with early untreated syphilis, although the population studied was small and mainly heterosexual, with very low levels of HIV coinfection [8]. The difference in sensitivity for early syphilis between the two studies may be due to a number of factors, including sample size, different study populations, and collection of oral fluids in different settings. In the previous study [8], a small number of dedicated staff collected oral fluids, and there was a short transit time (<48 h) between sample collection and processing in the laboratory. In the study described here, a large number of staff in various clinics collected oral fluid samples, and the time between sample collection and processing was on average 1–2 weeks, although in some instances it was as long as 1–2 months. Such extended periods of time between specimen sampling and processing may lead to sample/antibody degradation, and for this reason we used a control test for VZV antibody. Sample degradation [16] is of particular significance in cases of primary syphilis in which patients have only recently seroconverted or have received early treatment, since such individuals may exhibit low levels of serum antibody. In such instances, relatively low levels of degradation can lead to false-negative results in the oral fluid assay.

A number of oral fluid studies have relied on the measurement of total IgG [17, 18] as an indicator of specimen quality. We started using this approach but found that it was very difficult to assign a cutoff value for total IgG to discriminate between satisfactory and unsatisfactory specimens; moreover, such a cutoff may be dependent on the method as well as the collection device [7, 19]. To overcome these problems, we used detection of VZV IgG [20] as a test of specimen quality. This method was chosen because earlier we had developed and validated a VZV oral fluid TRFIA using Oracol collection devices and found 94.2% of adults tested to have VZV IgG. This corresponded with reported adult immunity to VZV of 94.4% from serological surveys [21]. Using this approach, we have to accept that oral fluid VZV IgG negativity may be a consequence of inadequate specimen collection or of patient VZV antibody seronegativity. In either case, additional follow-up of VZV IgG-negative oral fluids is worthwhile.

In this study, we have shown that oral fluid specimens collected using the Oracol collection device and analysed using highly sensitive detection technologies can be used for detection of treponemal antibody. This methodology had high sensitivity and specificity for identifying individuals with secondary and early latent syphilis. The sensitivity of the oral fluid assay (87.5%) was reduced in identifying individuals with primary syphilis, and the lack of sensitivity of antibody detection methods needs to be taken into consideration if these methods are to be used for detecting primary syphilis. Repeat sampling after 1 month, or regular sampling of high-risk individuals, has been suggested [9, 15] as a means of improving the rate of detection of primary syphilis by detection of treponemal-specific antibody. Further attention to the storage of samples to prevent degradation and the development of appropriate IgM assays may increase the sensitivity of the syphilis oral fluid TRFIA, particularly in the detection of primary syphilis. A number of different clinics and a variety of personnel collected the specimens, showing this specimen collection technique to be robust. The syphilis oral fluid TRFIA described should have high applicability as an alternative to traditional serology for diagnostic, epidemiological, and interventional studies where collection of blood is not feasible.

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