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Noninvasive Sampling of Saliva as an Alternative Way for Malaria Diagnosis: A Systematic Review

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Abstract: To describe and evaluate the efficacy of different methods based on human saliva for malaria diagnosis compared with methods based on blood. A comprehensive search about malaria diagnosis based on saliva was undertaken from PubMed, China National Knowledge Infrastructure (CNKI), VIP and Wanfang databases with strict inclusion and exclusion criteria. Methodological quality was assessed by using the quality assessment of diagnostic studies (QUADAS) tool. Meta-Disc version 1.4 was used to analyze the studies and methods for sensitivity, specificity, and area under the summary receiver operating characteristic (sROC) curves. In result, nine studies (n=9) were enrolled from 150 studies retrieved only in the PubMed. Methodological quality was moderate. The pooled sensitivity and specificity with corresponding 95% confidence interval (CI) of all enrolled studies were 0.68 (0.65 to 0.71) and 0.91 (0.89 to 0.93) respectively. AUC of sROC was 0.8303. In ELISA subgroup of studies, pooled sensitivity and specificity were 0.61 (0.55 to 0.65) and 0.87 (0.84 to 0.90), and AUC was 0.7227 respectively. And 0.75 (0.71 to 0.80), 0.94 (0.92 to 0.95) and 0.9012 were corresponding in PCR subgroup of studies. The methods based on saliva displayed good prospects to be an alternative ways for malaria diagnosis and, attributing to their noninvasive sampling, will be welcome worldwide particularly in the large epidemiological surveillance of community populations or asymptomatic people as malaria declined significantly. However, the techniques with much better efficacy and a standardized procedure of sampling and concentration are encouraged to be developed.

Keywords: Noninvasive; saliva; malaria diagnosis; PCR; ELISA; systematic review

Introduction

The estimated incidence of malaria globally and malaria-specific mortality rates since 2000 have declined significantly which should be attributed to the strengthening of control efforts, but they are still unsatisfactory with the internationally agreed targets for 2010. Malaria is an important public health problem as before causing an estimated 216 million episodes of malaria in 2010, and an estimated 655000 malaria deaths, of which approximately 86% were children under 5 years old (WHO, 2011).

Early and accurate diagnosis is one of the core components for malaria control strategies as it is essential for the proper management of infected individuals, the avoidance of unnecessary presumptive treatment, and disease control and so on. In present, in order to control the continued spread of antimalarial drug resistance, only those with confirmed Plasmodium infection after a diagnostic test can receive antimalarial treatment.

Microscopy, RDTs and PCRs based on blood samples are the most popular laboratory diagnostics for malaria confirmation. Microscopic examination of blood smears remains the gold standard for malaria diagnosis. However, this procedure is often used by skilled and experienced microscopists, otherwise the inaccurate evaluation of smears will result in misdiagnosis and misclassification of malaria severity. Immunoassays such as rapid diagnostic tests (RDTs) provide an alternative method to improve malaria diagnosis with some advantages (Morankar, *et al.*, 2011; Osman, *et al.*, 2010; Wongsrichanalai, *et al.*, 2007). Furthermore, PCR is a reliable and sensitive technique which has been applied widely to detect and diagnose malaria and further characterize the *Plasmodium* species from blood (Hoyer, *et al.*, 2012; Mens, *et al.*, 2012; Johnston, et al., 2006; Berry, *et al.*, 2005; Di, *et al.*, 2004; Shehata, *et al.*, 2004; Van, *et al.*, 2002; Hanscheid and Grobusch, 2002).

In fact, aside from besides the accuracy, the patients' convenience and compliance, and sampling feasibility and safety are also essential for the diagnosis. Notwithstanding the benefits of the above mentioned blood-based tests, the increased risks of needle injuries and infectious diseases transmission, the fear of small children and some adults from blood collection, and the limitations of repeated measurement during post-treatment follow-up as well as cultural objections (Formenty, *et al.*, 2006; Spielberg, et al., 2000) often become obstacles. Thus, non-invasive, simple, practical, and applicable methods of malaria diagnosis using some materials other than blood are needed in the healthcare delivery system.

Saliva collection, a painless and simple alternative to blood sampling, meets these requirements. Moreover, compared to the training required for blood sampling, saliva collection can be easily performed by health care staff following relative instructions with little previous experience. Given the limited knowledge about the sensitivity and specificity of the diagnostic methods for malaria based on human saliva, this evaluation was carried out on their efficacies through a systematic review with the attempt to establish the laboratory and field applicable methods.

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Materials and Methods

Search strategy and data source We searched tThe PubMed was searched for articles with ("malaria"[MeSH Terms] OR "malaria"[All Fields] OR "Plasmodium"[MeSH Terms] OR "*Plasmodium*"[All Fields]) AND ("saliva"[MeSH Terms] OR "saliva"[All Fields]). And the keywords "(malaria/Plasmodium) and saliva" in Chinese were used to jointly search the China National Knowledge Infrastructure (CNKI), VIP and Wanfang databases, which are considered to be the most comprehensive and widely used electronic databases in China covering the majority of Chinese biomedical literatures. The final formal search was undertaken October 19, 2012.

Selection of studies

Articles were included in this systematic review if they met all of the following criteria: (1) saliva was one of the materials for malaria detection at least; (2) Sensitivity and specificity were reported or can be calculated; and (3) saliva detection efficacy was compared with other paired sources detection for malaria. Two authors were responsible independently for articles review and selection. A primary selection was performed based on the title and abstract. Studies that were not regarding malaria detection/diagnosis in saliva were removed. Then the screened articles were reviewed in detail. All potential publications were obtained in full text format and scrutinized for duplication.

Quality assessment of studies

Methodological quality of enrolled studies was assessed by two authors independently following the quality assessment of diagnostic studies (QUADAS) instrument, which is a tool designed specifically for quality assessment of studies of diagnostic accuracy included in systematic reviews (Whiting, et al., 2003). The studies design-related issues, and the external and internal validity of the results were scored as a result of 'yes' or 'no' or 'unclear' according to the 14 items of QUADAS tool (Whiting, et al., 2003).

For each article, two authors worked together to extract information illustrating the characteristics of the studies. The authors, year of publication, reference standard method, country where the study was conducted, samples size, diagnosis methods of the included studies, were all retrieved. The "sensitivity/specificity", or "true positive (TP)/true negative (TN)/false positive (FP)/false negative (FN)" were also extracted or calculated according to the published data. Then the pooled sensitivity, pooled specificity, pooled positive LR, pooled negative LR and summary receiver operating characteristics (sROC) curve were performed by Meta-Disc version 1.4 (Zamora, *et al*., 2006).

Results

Selection of studies Only the search in PubMed retrieved 150 studies, and no articles about the relative topics were in all the three Chinese databases. The flow diagram in Figure 1 shows the simple steps of the process for the study selection. Seventeen articles were selected after the primary selection based on the title and abstract according to the topic of malaria detection/diagnosis in saliva. Full texts of them were retrieved from both free and commercial databases. From these 17 articles, eight were excluded for the following reasons: 5 articles (Akinyinka, et al., 2000; Gordi, et al., 2000 and 2002; Rault, et al., 1996; Sowunmi, et al., 2013) focused on the drug pharmacokinetic investigations and 1 was about identification of biomarkers in saliva from patients with uncomplicated P. f malaria for diagnostic exploration (Huang, et al., 2012) and 1 was an editorial commentary (Sutherland and Hallett, 2009) about a major article which was enrolled in the present study, and one can't be satisfied with the 2nd inclusion criteria (SM, *et al.*, 2010). In addition, there were 4 articles (Buppan, et al., 2010; Mharakurwa, et al., 2006; Nwakanma, et al., 2009; Putapomtip, et al., 2011) relative to Plasmodium DNA detection/diagnosis and four (Chidi, *et al*., 2011; Estevez, *et al*., 2011; Fung, *et al*., 2012; Wilson, *et al.*, 2008) regarding the *Plasmodium* antibodies/antigen detection and one (Gbotosho, *et al*., 2010) about both the parasite antigen detection and DNA detection respectively (Table 1).

Characteristics of included studies

The details of the characteristics of the individual studies included in the review are presented in Table 1. The table shows the characteristics of all the included studies in terms of the malaria diagnosis based on human saliva rather than blood. Within the table, the studies were arranged according to the method applied in the publications. A summary of the information presented in Table 1 is shown below. Target molecules The antigens of PfHRP ?and pLDH, antibodies against MSP-119, AMA-1 or whole, asexual stage P.f antigens were selected in 3 immunoassays in the enrolled studies. Furthermore, five genes of *pfcrt,* MSP2, DHFR, Cytb and SSU rRNA were used for PCR amplification.

Samples

The studies enrolled in this report were conducted in 8 countries, one in each of Sudan, Nigeria, Tanzania, Ghana and Philippines respectively, and two in each of Zambia, Gambia and Thailand separately. At least 2 kinds of samples, paired blood and saliva, from patients in clinics or field survey participants were collected in the studies. Besides, other noninvasive materials including urine and buccal mucosa were also collected in some studies for exploring the malarial detective techniques. The sample size ranged from 24 to 386. Two studies recruited were more than 200 subjects while 6 studies involved less than 100 subjects.

Malaria detection/diagnosis methods

The studies included in this report contained four kinds of methods of ELISA, EIA, Optimal-IT dipsticks and PCR. Most of them only focused on the detection of *Plasmodium falciparum* infection (7 out of 9), especially all of the methods based on the antibodies or antigens. In particular, the results of these four methods based on saliva were equally compared with the results by other methods such as microscopy. PCR based on different genes is the most easy way to identify species of this parasite. The efficacy of all

methods varied. The sensitivity of immunoassays ranged from 43% to 100%, and the specificity from 90% to 100%.

Quality assessment

Results of distribution of study design characteristics in 9 studies according to QUADAS items are shown in table 2 as follows: patient spectrum (7/9 for "yes" responses to question 1), reference standard classification (7/9 for "yes" responses to question 3), the description of the execution of the index test (8/9 for "yes" responses to question 8), the interpretation of the index test results without knowledge of the reference standard results (6/9 for "no" responses to question 10), and clinical data availability (5/9 for "unclear" and 2/9 for "no" responses to question 12).

Systematic analysis

The forest plots of sensitivities and specificities from all studies are shown in Figure 2A. A homogeneity test of sensitivity and specificity shows Q = 103.93 (P< 0.0001), I^2 = 84.6% and Q = 198.86 (P< 0.0001), I^2 = 92.0% using the random effect model, respectively. Obviously, heterogeneities are detected. The pooled +LR = 8.13 (95% CI: 3.80 to 17.43) and the pooled -LR = 0.39 (95% CI: 0.30 to 0.49) are shown in Figure 2B. In addition, AUC = 0.8303 of sROC curve is shown in Figure 2C. The heterogeneities are possible for the different detective capability of ELISA and PCR. Then, further analysis was done in the subgroup as ELISA and PCR respectively. However, heterogeneities of pooled sensitivity and pooled specificity were also detected in both subgroups: pooled sensitivity = 0.61 (95% CI: 0.55 to 0.65), Q = 39.93 (P< 0.0001), I^2 = 77.5% and pooled specificity = 0.87 (95% CI: 0.84 to 0.90), Q = 151.47 (P< 0.0001), I^2 = 94.1%, pooled +LR = 9.10 (95% CI: 2.52 to 32.92) and the pooled -LR = 0.46 (95% CI: 0.34 to 0.62), AUC=0.7227 in ELISA group (Figure 3A-C); pooled sensitivity = 0.75 (95% CI: 0.71 to 0.80), Q = 43.49 (P< 0.0001), I^2 = 86.2% and pooled specificity = 0.94 (95% CI: 0.92 to 0.95), Q = 34.98 (P< 0.0001), I^2 = 82.8%, pooled +LR = 7.15 (95% CI: 3.37 to 15.18) and the pooled -LR = 0.31 (95% CI: 0.22 to 0.42), AUC = 0.9012 in PCR group (Figure 4A-C). This result suggests that the reference standard methods to be the potential factors to influence the heterogeneities.

Discussion

Prompt, accurate diagnosis before proper treatment is an essential component of malaria control and elimination strategies. The process of diagnosis is initiated by a suspicion of malaria on the basis of a defined set of clinical criteria such as fever which is one of the least specific for all major diseases ranging from viral infections to serious, life-threatening ones; this is then confirmed by a parasitological test which is the only way to diagnose malaria: either a blood film for microscopy or a rapid diagnostic test (RDT) or PCR (WHO, 2011). However, these methods are mainly based on the peripheral blood samples. Although they are generally acceptable for case management in the formal health care sector, there are still some challenges at the community level and in some private sector settings, particularly with regard to the potential risks of blood-borne infection and when taking samples from asymptomatic individuals and when needing repeated sampling for curative effect observation, which could diminish access to malaria diagnosis, treatment, and surveillance (The malERA Consultative Group on Diagnosis and Diagnostics, 2011). Especially surveillance continues going on in countries such as China, which has been in the age of malaria elimination, most areas are free of malaria or in the very low prevalence.

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Fortunately, noninvasive samples such as urine or saliva have the potential to overcome the above difficulties, although the limitations of sensitivity of non-blood sampling are even greater than the limitations of blood sampling for screening and surveillance due to lower mean DNA yield and protein contamination (Mharakurwa, et al., 2006; Nwakanma, et al., 2009; Wilson, et al., 2008). Therefore, effective saliva sampling procedures and concentration methods are needed eagerly. In order to improve the quality of DNA extracted from saliva, there have been some commercial kits or devices about saliva collection such as Oragene DNA Self-Collection kit (Abraham, et al., 2012; Nunes, et al., 2012), Oracol (Malvern medical developments limited, Worcester, U.K.) and OraSure-Intercept (Orasure Technologies, Inc. Bethlehem, PA, US) to collect and extract DNA from saliva (Estevez, *et al*., 2011; Nokes, *et al*., 1998; Nurkka, *et al*., 2000; Vyse, *et al*., 2001), but no practical and effective techniques for saliva concentration are currently available. What's more, the refinement of DNA extraction techniques are important as well (Mharakurwa, *et al*., 2006). Even so, noninvasive sampling of saliva collection compared with blood sampling has the advantages of lower cost, lower infection risk, increased convenience and acceptability for both patients and clinicians. has been used as a diagnostic fluid to evaluate immunity status against some infectious agents such as HIV and Dengue virus as well as for DNA extraction for detection (Haque, *et al*., 2010; Poloni, *et al*., 2010; AIDS Policy Law, 2012; Belza, *et al*., 2012; Carvalho, *et al*., 2010; Hu, *et al*., 2012).

Furthermore, an appropriate parasitological test based on human saliva is also important for malaria diagnosis, compared with the prompt parasitological confirmation based on blood films examined by microscopy or detection of parasite antigens with RDTs. Nucleic acid detection techniques such as PCR have been recognized as reliable and sensitive methods for malaria diagnosis, and it can identify morphologically similar species (Humar, *et al*., 1997) and outperformed in detection of mixed species infection. As a result, PCR amplification based on DNA extracted from saliva has been gradually used in clinical trials and cohort studies, malaria diagnosed by PCR was already in the initial stage. But still, the efficacy of both immunoassays and PCR are various and unstable (The malERA Consultative Group on Diagnosis and Diagnostics, 2011). In addition, metabonomics studies and proteomic techniques are improving at rapid, exponential rates, and salivary secretions are a highly complex and valuable mixture of proteins, . Huang *et al* (2012) attempted to identify some potential biomarkers with diagnostic values for malaria diagnosis by a proteomic analysis, and found some parasite proteins and several potential biomarkers in patients with malaria but not in patients with other causes of fever. It is concluded that

malaria diagnosis based on noninvasive sampling of saliva will be welcome worldwide as malaria declined significantly and in the large epidemiological surveillance of community populations or asymptomatic people with a sufficient sample size to have an adequately powered study. What's more, the techniques with much better efficacy and a standardized procedure of sampling and concentration are encouraged to develop.

Competing interests

The author(s) declare that they have no conflict of interests.

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Table 1 Sensitivity and specificity of methods based on human saliva and other information in the enrolled studies

Publication	Method	Target molecules	Samples (pairs)	Sensitivity (%)	Specificity (%)	Reference standard method
Wilson *et al*. 2008	ELISA	*Pf*HRP Ⅱ	40	43	100	Microscopy (thick film)
Estevez *et al*. 2011	ELISA	IgG against *P.f* MSP-1_{19}	253	64-77	91-100	ELISA (plasma antibody)
		IgG against *P.f* AMA-1		47-67	90-97	
Fung *et al*. 2012	ELISA	*Pf*HRP Ⅱ	24	100	100	Microscopy (thick film)
Chidi *et al*. 2011	EIA	Antibodies to whole, asexual stage *P.f* antigens	53	100	100	EIA (dried blood spots antibodies)
Gnotosho *et al*. 2010	Optimal-IT dipsticks	pLDH	78 (whole saliva) 72 (Supernatant of spun saliva)	77.9 (whole saliva) 48.4 (Supernatant of spun saliva)	100	Microscopy (thin and thick films)
	PCR	*Pfcrt* K76T	37	91	50	
Mharakurwa *et al*. 2006	Nested PCR	*Pf*MSP2	51	100	100	Microscopy (thick film)
		*Pf*DHFR				
Buppan *et al*. 2010	Nested PCR	SSU rRNA	120	74.1 (P. f) 84 (P. v)	100	Nested PCR based on SSU rRNA of blood
Putaporntip *et al*. 2011	Nested PCR	*Cytb*	157	74.2 (P. f) 79.2 (P. v)	100 (P. f) 98.7 (P. v)	Nested PCR targeting *Cytb* of blood
Nwakanma *et al*. 2009	Nested PCR	SSU rRNA	386	73	97	Microscopy (thick film)

Note: ELISA: enzyme linked immunosorbent assay; EIA: enzyme immunoassay; PCR: polymerase chain reaction;

PfHRP Ⅱ: *Plasmodium falciparum* Histidine-rich Protein Ⅱ; AMA: apical membrane antigen; pLDH: *Plasmodium falciparum* Lactate Dehydrogenase; *Pf*DHFR: *Plasmodium falciparum* dihydrofolate reductase; SSU rRNA: small subunit rRNA; *Cytb*: mitochondrial cytochrome *b*;

Table 2 Evaluation of quality of included studies using the QUADAS tool

Reference	QUADAS item													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Wilson *et al.*	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	No	Yes	Unclear	Yes	Yes
Estevez *et al.*	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes
Fung *et al.*	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Chidi *et al.*	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Unclear	Yes	Yes
Gnotosho *et al.*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Unclear	Yes	Yes
Mharakurwa *et al.*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes
Buppan *et al.*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes
Putaporntip *et al.*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Nwakanma *et al.*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes

Figure legends:

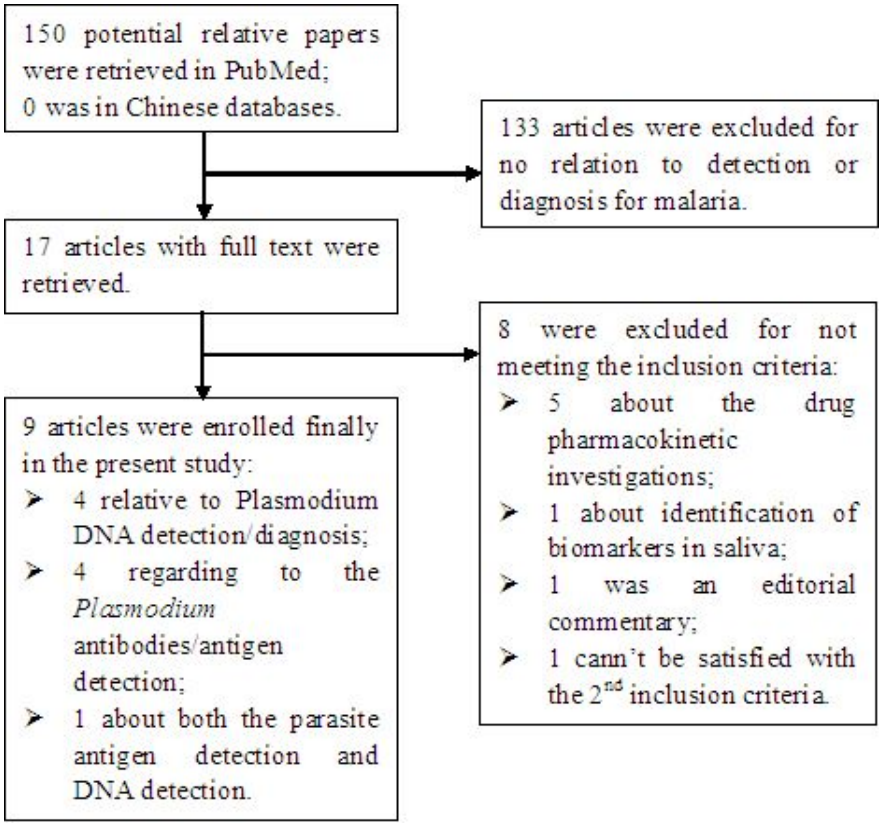

Figure 1 The flow diagram of the process of study selection

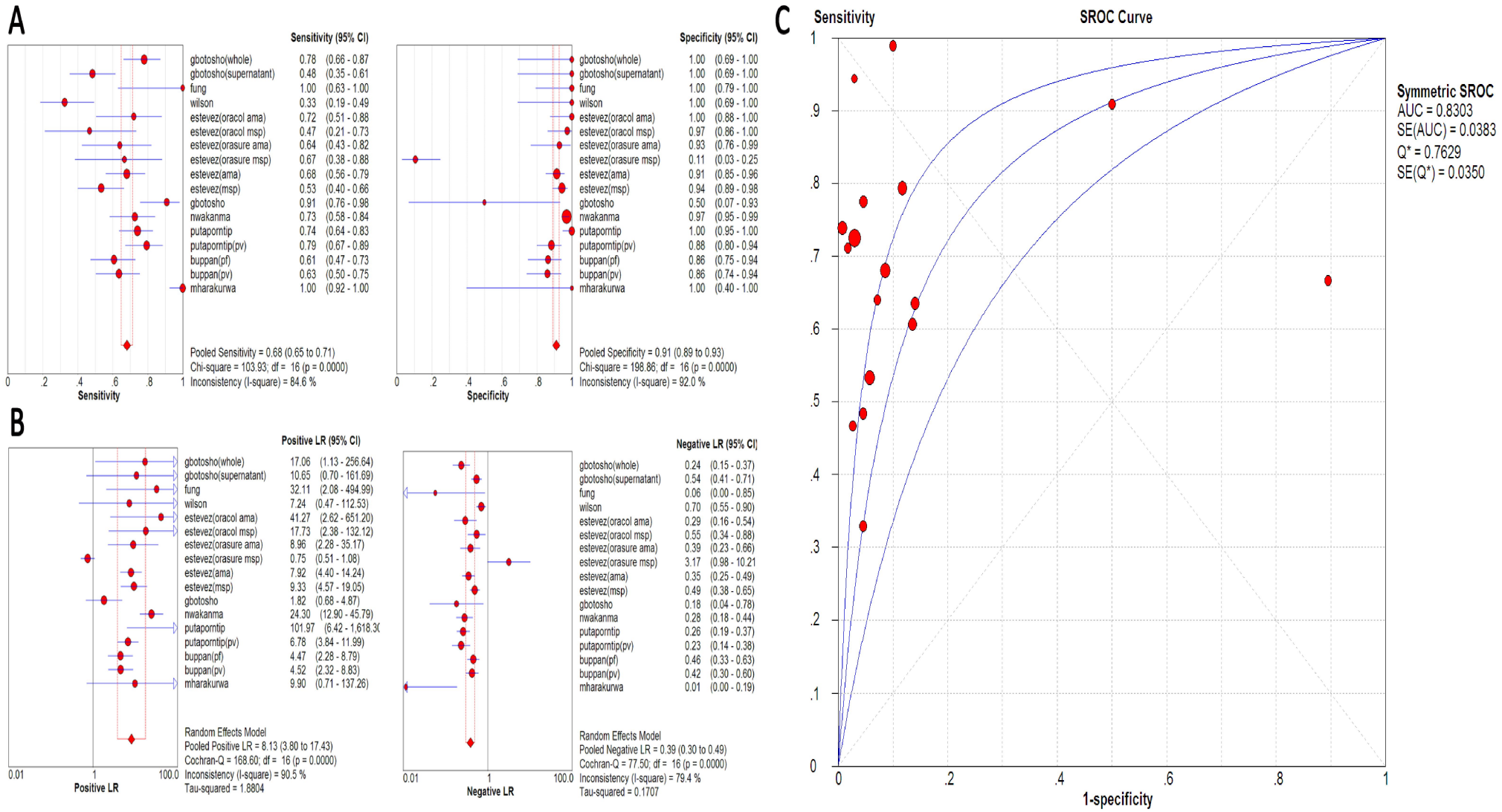

Figure 2 Analysis of all enrolled studies. A. Forest plots of sensitivity and specificity with corresponding 95% CIs; B. Forest plots of likelihood ratios for positive and negative test results; C. sROC curve.

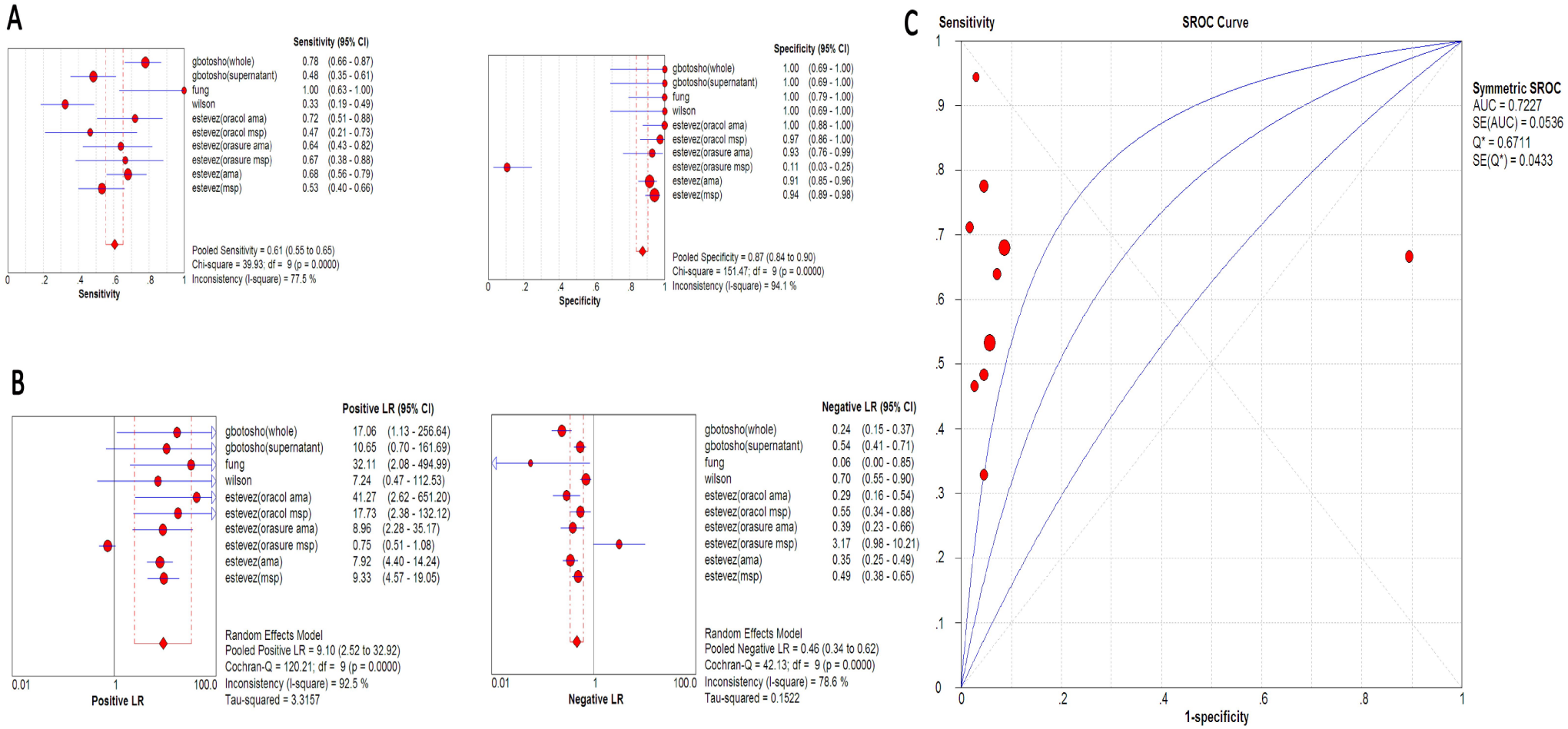

Figure 3 Analysis of studies in a subgroup of ELISA . A. Forest plots of sensitivity and specificity with corresponding 95% CIs; B. Forest plots of likelihood ratios for positive and negative test results; C. sROC curve.

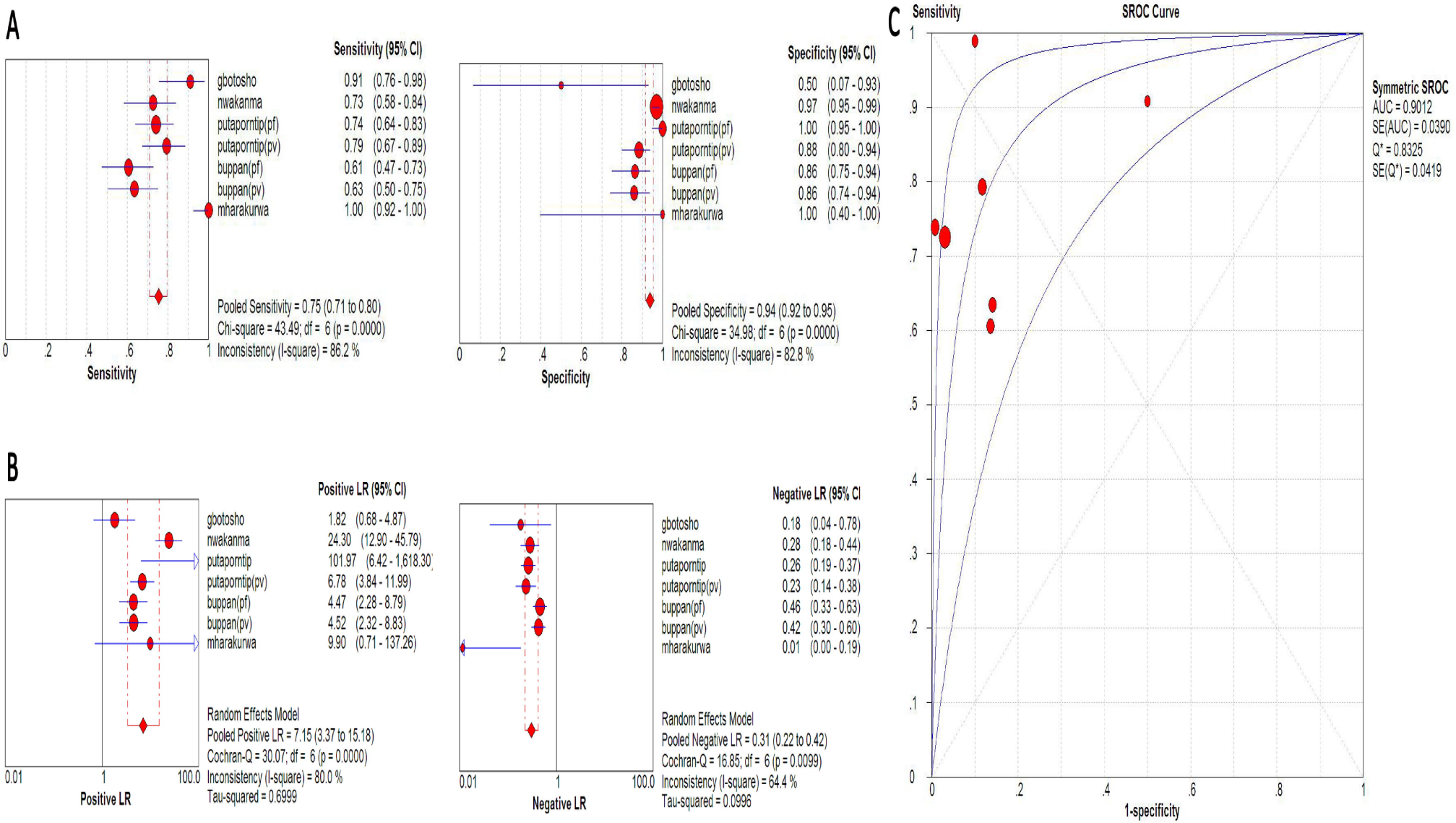

Figure 4 Analysis of studies in a subgroup of PCR. A. Forest plots of sensitivity and specificity with corresponding 95% CIs; B. Forest plots of likelihood ratios for positive and negative test results; C. sROC curve.