

Original Research Article

Influence of Rheumatoid Factors and Bilirubin on the Sensitivity of Immunochromatographic Rapid Diagnostic Tests for HIV/AIDS and Hepatitis B in Cameroon

ABSTRACT:

Aims: This study aimed at identifying some **potential interference** factors in the immunochromatographic Rapid Diagnostic **Tests commonly** used in Cameroon for the diagnosis of Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) and Hepatitis B (HBV) which are major public health problems in the country.

Design and Methods: The sample population included patients referred to BETHANIE Laboratory for the accurate diagnosis of HIV/AIDS and HBV. RDTs were performed using FIRST RESPONSE HIV Card 1-2.0 and HEXAGON HBsAg. Tests results were confirmed using a high sensitivity 3rd generation ELISA for HIV and HBV, both from FORTRESS. Assays of Rheumatoid Factors and bilirubin were conducted on HIV and HBV samples respectively. Statistical analysis was done using the R software version 3.0.2; **the ANOVA was applied at** a threshold of 0.05.

Results: A total of 25 patients were included for the HIV study group and 30 for the HBV group. Test sensitivities of 14.28% and 92.85% for FIRST RESPONSE HIV Card 1-2.0 and HEXAGON HBsAg were found respectively. **Average blood levels of RF were 11.55 UI/L and 64.29 UI/L from FIRST RESPONSE HIV RDT positive and negative samples respectively. Blood levels of bilirubin were 88.63 mg/L and 131.66 mg/L from HEXAGON HBsAg RDT positive and negative samples respectively.**

Conclusion: Rheumatoid Factor (up to 238.8 UI/L) was not found to interfere with the performance of HIV FIRST RESPONSE RDT (*P-value* = 0.37) and there was no interference of bilirubin (up to **228.7 mg/L**) with the performance of HEXAGON HBsAg RDT (*P-value* = 0.25).

Key words: Interference, Rapid Diagnostic Test (RDT), HIV/AIDS, Hepatitis B (HBV), Rheumatoid Factor (RF), Bilirubin.

1. INTRODUCTION

Advances in Biotechnology have led to the development of rapid diagnostic tests (RDTs) for the early and rapid diagnosis of certain diseases which constitute a serious public health burden in resource-limited settings. An accurate diagnosis is very important because it would facilitate proper treatment and possibly lead to a total recovery of the patient [1]. Adequate diagnosis leads to the proper use of drugs and consequently avoids drug resistance. It may be useful in providing guidance

on the choice of drugs as in the case of antibiotics resistance testing. It is equally useful in guiding healthcare providers on when to stop and possibly change a particular treatment regimen. Thus, diagnosis occupies a primordial place in the health system.

Suitable **diagnosis requires** appropriate techniques which can lead to the early identification of an illness. In biological diagnosis, there are two kinds of tests: the direct test which detects some particles of the germ (antigens or nucleic acid material) and the indirect test which detects the presence or the previous existence of the germ in the body (antibodies).

Many biological diagnostic techniques exist worldwide, which require sophisticated equipment and skilled staff. These resources are **yet not** always available in developing countries (DCs). However, with the development of Biotechnology, RDTs have evolved and taken a leading position in applied Biology. In contrast to conventional diagnostic methods, RDTs can enable the diagnosis of an infectious disease within a short time [1]. They have in fact existed for the past three decades, especially for the detection of HIV/AIDS and malaria. Their use has gradually spread to other diseases [1].

More recently, single step rapid tests have been developed and marketed. More commonly called “soap tests” or “strip tests,” they are based on the principle of immunochromatography on a membrane or strip with pre-colored particles [2].

For the detection of antigens, the test sample is deposited at one end of a nitrocellulose membrane. If the desired antigen is present, it binds with specific antibodies labeled with colloidal gold. Under the effect of lysis-migration-buffer, the antigen-antibody complex migrates by capillarity and is arrested by a capture antibody attached to the membrane. A positive result is denoted by the appearance of a colored test line. An internal control is used to validate the test [3].

For the detection of antibodies, serum sample or whole blood is placed at one end and then migrates to an area where the antibodies bind to an antigen conjugated to a developer of selenium colloid type. This mixture continues to migrate towards the immobilized antigen on the membrane at the test window. The antibody-antigen **complex specifically** binds to the antigen and is immobilised, forming a colored line. In the absence of specific antibodies, the antigen-colloid conjugate migrates through the test zone area without producing a signal [4].

Nowadays, RDTs are widely used in DCs like Cameroon due to their potential comparative advantage created by the accrued lack of resources, though they also have the highest burden of most endemic and infectious diseases such as HIV/AIDS and Hepatitis B. The above-mentioned diseases are serious public health problems in Cameroon. RDTs are commonly used for the early diagnosis of these diseases throughout the national territory. However, their results often need to be confirmed by another method. A positive result does not exclude the presence of other pathogens that are not targeted by the test and a negative result does not exclude the presence of the target pathogen tested [4].

These RDTs are often subject to interference, leading to false results (false positives and/or false negatives). In this study, we aim to look for possible interferences on the RDTs for two viral diseases, one caused by an RNA virus (HIV/AIDS) and the caused by a DNA virus (Hepatitis B).

2. MATERIAL AND METHODS

2.1. Study design

Our study was designed as a cross-sectional and analytical study. (In my view, this whole thing must be deleted from here and incorporated into study population section. This will require that that sub-heading is changed.)

2.2. Study Area

The recruitment of subjects was conducted in the city of Yaounde, Cameroon; and the site of choice was the BETHANIE Laboratory for its proximity to some referral hospitals in the city and their affluence in patients. The study period ran from August to November 2013.

2.3. Study design and sample population

Patients visiting the BETHANIE Laboratory for the RF test or who had jaundice and agreed to participate in the study by signing the informed consent or assent forms after clear explanations and answers to their questions

2.4. Sample size

For all cases, we used a convenience sampling and thus recruited 25 patients for HIV/AIDS and 30 for Hepatitis B. (delete this and add it to the study design section)

2.5. Laboratory Investigations

2.5.1. Rapid Diagnostic Tests

The RDTs for Hepatitis B (HBsAg HEXAGON) and AIDS (FIRST RESPONSE HIV1-2.0) were performed using the sera of patients who came to the laboratory with jaundice or requested a RF examination respectively.

2.5.2. Confirmatory Tests

ELISA FORTRESS HIV 3rd generation (Fortress diagnostics ANTI-HIV 1+2+0) and high sensitivity HBV (Fortress diagnostics High Sensitivity HBsAg 3rd Generation) assays were used for the confirmation of HIV and HBV respectively.

2.5.3. Biochemistry assays

The determination of bilirubin (spectrophotometry) and the titrating of RF (turbidimetry) were performed on the sera of patients with jaundice and those who came for a RF examination respectively. BIOLABO reagents were used for the determination of bilirubin and FORTRESS DIAGNOSTIC for the determination of RF.

2.5.4. Quality Control (This could be part of the appropriate test. That is, the biochemistry assays or the ELISA tests to reduce the number of sub-headings. This is because information

provided is not tangible enough for a separate sub-heading. Please, delete and modify appropriately.)

Quality control was done for each assay. Positive and negative controls for the ELISA tests and the "BIOLABO EXATROL-N" (level I) REF: 95010 LOT: 111215C were included for the biochemistry assays. All the controls were in the range pre-established by the manufacturer.

2.6. Data collection

A questionnaire was used for data collection. Names, age, sex, address, neighborhood and number of sexual partners during the last six months were recorded. (I thought that this section was recommended for deletion. This is because: you seem to suggest that the ELISA and other laboratory results obtained are not part of your data. What you have presented here is just qualitative data. It cannot stand on its own as a sub-heading. It must be deleted and added to the study population.

2.7. Data Analysis

The results were analysed with the R version 3.0.2 software from which the sensitivities, specificities, efficiencies, positive and negative predictive values were calculated in addition to the determination of characteristics of the population such as average age and sex ratio. The ANOVA test was done at a threshold of 0.05.

3. RESULTS

3.1. Sample size and socio-demographic data

We collected specimens from 25 consenting patients referred to the BETHANIE laboratories to conduct a RF examination. Similarly, 30 specimens were collected from patients who presented with jaundice (bilirubin). Table 1 below presents the HIV and HBV infection distribution by gender. (NB: you need to give a bit more information on their demography. Such information must precede your table. So, your table 1 should be relocated appropriately.)

Table 1. HIV and HBV infection distribution by gender

Sex	HIV				HBV			
	Numbers	Percentages	Pos	Neg	Numbers	Percentages	Pos	Neg
Female	14	56.00	02	11	08	26.67	03	05
Male	11	44.00	05	06	22	73.33	11	11
Total	25	100	07	17	30	100	14	16

Pos: Positives

Neg: Negatives

The participants for the HIV/AIDS arm were of a mean age of 47.36 years, with the majority being of the 47.36±18.27 year interval. The youngest patient was 9 years old and the oldest 80 years. Of the 14 females, 2 (14.28%) were positive while 11 (78.57%) were negative and 1 (7.15%) was borderline. Out of the 11 males, 5 (45.45%) were positives while 6 (54.55%) were negatives.

For HBV participants the mean age was 39.83 years, with the majority found within the age interval of 39.83±18.33 years. The youngest patient was 13 years old and the oldest was 79. Of the 8

females, 3 (37.5%) were positive while 5 (62.5%) were negative, and 11 (50%) of the 22 males were positive while 11 (50 %) were negative.

3.2. RDTs and ELISA Results

A RDT was conducted on each sample and then confirmed by a high sensitivity ELISA. The results are presented in the Table 2 below.

Table 2. Cross tabulation of RDTs and ELISA Results

	HIV			HBV		
	RDT	Positives	Negatives	Positives	Negatives	Total
ELISA						
Positives		01 (TP)	06 (FN)	13 (TP)	01 (FN)	14
Negatives		01 (FP)	16 (TN)	00 (FP)	16 (TN)	16
Total		02	22	13	17	30

Where TP: True Positive, TN: True Negative, FP: False Positive and FN: False Negative

➤ HIV/AIDS

Of the 25 blood samples analysed for HIV, 2(8%) were positive and 23 (92%) were negative by RDT. However, ELISA results showed that 7(28%) of the total samples were positive, 17 (68%) were negative while 1(4%) was borderline.

We found one false positive case with a low level of RF (10.6 UI/l). However, the patient who had the highest level of RF (238.8 UI/l) had concordant results with both tests. The sensitivity, specificity, efficiency, PPV and NPV were 14.28 %, 94.11 %, 70.83 %, 50.00 % and 72.72 % respectively.

➤ HBV

Using the RDT, 13 (43.33%) of the 30 blood samples tested positive for HBV and 17 (56.67%) were negative. By the ELISA, 14 (46.67%) of the total samples were found positive and 16 (53.33%) were negative.

Only one case was a false negative with a high level of bilirubin (22.8 mg/dl) was recorded. The sensitivity, specificity, efficiency, PPV and NPV were 92.85%, 100.00%, 96.67%, 100.00% and 94.11% respectively.

3.3. Biochemistry assays Results

3.3.1. Rheumatoid Factors assays:

We determined the concentration of rheumatoid factor in each sample by turbidimetry. During the analysis, patients' samples that had values higher than the detection of the reagent were diluted and new values obtained. The latter values were multiplied by the dilutions factors to obtain the real

value of the samples concerned. Reference RF values for the reagents used in our laboratory are < 200 UI/L. (NB: this section is supposed to be for results presentation. It is NOT description of methodology. This write-up must be deleted from here and moved to the appropriate methods section. What needs to be presented here is the summarized results of the relevant assays not how the assay results were obtained.)

Table 3. Rheumatoid Factors assays results

Research Codes	01HIVWIPA	02HIVNGRA	03HIVONDE	04HIVMBSA	05HIVIBPR	06HIVNJYV
RF (UI/L)	9.2	24.8	27.7	9.9	16.7	234.6
Research Codes	07HIVOWEL	08HIVBICE	09HIVAKCH	10HIVNJMA	11HIVTUJE	12HIVNGBI
RF (UI/L)	14.3	20.7	12.8	19.1	119.4	21.0
Research Codes	13HIVNGRE	14HIVMAMA	15HIVKATH	16HIVBILY	17HIVTALE	18HIVMAMI
RF (UI/L)	220.3	154.0	238.8	159.3	80.3	24.2
Research Codes	19HIVDI	20HIVNGNI	21HIVNGMA	22HIVLAMO	23HIVCHMA	24HIVIYJE
RF (UI/L)	17.1	12.4	18.2	14.6	11.8	10.7
Research Codes	25HIVME					
RF (UI/L)	9.9					

[NB: You cannot publish this. This is the raw data. That is not what I asked for. What I requested is a summarized result of the assay in appropriate format. This is unacceptable. Delete and replace with appropriate table of result.]

Of the 25 samples analysed, 21 had RF values < 200UI/L while RF values >200UI/L were recorded from 4 samples.

3.3.2. Bilirubin assays:

We determined the concentration of bilirubin in each sample by spectrophotometry. In the determination of bilirubin level, all samples were subjected to procedure N°1 (with detection limit less than 200mg/L). Samples with values >200mg/L were subsequently subjected to procedure N°2 (with detection limit of 1000mg/L). Reference bilirubin values for adult in our laboratory are 3 – 12mg/L.

(NB: this section is supposed to be for results presentation. It is NOT description of methodology. This write-up must be deleted from here and moved to the appropriate methods section.

What needs to be presented here is the summarized results of the relevant assays not how the assay results were obtained.)

Table 4. Bilirubin assays results

Research Codes	01HBVTEGE	02HBVNYMA	03HBVSACL	04HBVNS1	05HBVKOST	06HBVNDGE
Bilirubin (mg/L)	228.7	290.6	91.8	31.8	164.9	294.1
Research Codes	07HBVLEAN	08HBVAVTI	09HBVMEBE	10HBVTCDA	11HBVMOAB	12HBVWOOD
Bilirubin (mg/L)	58.8	325.0	270.0	42.7	100.6	16.9
Research Codes	13HBVESCL	14HBVTEMA	15HBVBELA	16HBVSEIC	17HBVNGBE	18HBVOL
Bilirubin (mg/L)	33.5	210.0	176.7	21.3	127.5	219.7
Research Codes	19HBVNS2	20HBVMVNA	21HBVDAP1	22HBVMBOM	23HBVFO	24HBVPO
Bilirubin (mg/L)	217.7	23.9	77.6	24.1	20.1	65.2
Research Codes	25HBVNZBE	26HBVTO	27HBVAKJE	28HBVMAMA	29HBVKIKI	30HBVNE
Bilirubin (mg/L)	40.0	13.5	51.4	58.8	33.4	60.2

[NB: You cannot publish this. This is the raw data. That is not what I asked for. What I requested is a summarized result of the assay in appropriate format. This is unacceptable. Delete and replace with appropriate table of result.]

Of the 30 samples analysed, measured levels of bilirubin could be summarized as follows; 11 samples had bilirubin levels <50mg/L, 7 [50-100mg/L], 2 [100-150mg/L], 2 [150-200mg/L] and 8 samples with values >200mg/L.

3.4. Correlation between blood level of RF and false positive result with RDT.

The proportion of positive and negative RDT results together with their corresponding values of rheumatoid factors is summarized in Table 5.

Table 5. RF and RDT assays results

Proportions (%)	RDT HIV FIRST RESPONSE	
	Positives	Negatives
	08	92

Mean of RF (UI/L)	11.55	64.29
Overall mean of RF (UI/L)	60.07	

Legend: Overall mean of RF (UI/L) is the mean of RF in both positive and negative samples.

NB: This table is confusing. Are you talking about false positives or what? You indicated only 1 false positive result in table 2. Why are you now presenting 8 positives. Are they all false positive? I don't think so. I still have a lot of problems with your persistent use of ANOVA. I honestly believe that your kind of data is inappropriate for ANOVA. Now, you just had 25 samples that tested qualitatively for HIV. In this group, you measured RF values quantitatively. The samples were grouped into two ie positive (TP, FN) and negative (FP, TN). So, how did you correlate your RF values with your positives/negatives using ANOVA? This is statistically wrong. ANOVA is suitable when comparing more than three groups of variables with normally distributed samples. With a total sample size of 25, it is very difficult to correctly apply ANOVA to this. Please, consult a biostatistician who understands your work for the needed assistance on this.)

Considering our null hypothesis (Ho) that "RF had no effect on the performance of our RDT" and a threshold of 0.05, a one-factor ANNOVA test was used to see if RF had an effect on the RDT of HIV/AIDS. With a $P\text{-value} = 0.37$; the null hypothesis (Ho) could not be rejected.????? This is questionable.

3.5. Correlation between blood level of bilirubin and false negative result.

The proportion of positive and negative RDT results together with their corresponding values of bilirubin is summarized in Table 6.

Table 6. Bilirubin and RDT assays results

	RDT HIV FIRST RESPONSE	
	Positives	Negatives
Proportions (%)	43.33	56.67
Mean of Bilirubin (mg/L)	88.63	131.66
Overall mean of Bilirubin (mg/L)	113.01	

Legend: Overall mean of bilirubin (mg/L) is the mean of bilirubin values in both positive and negative samples.

NB: my concerns are the same as expressed for table 5. Check it and re-consider your line of statistical argument.

Setting out from the Ho hypothesis that "bilirubin has no effect on the RDT," at threshold of 0.05, we used a one-factor ANOVA test to see if bilirubin had an effect on the hepatitis B RDT. With a $P\text{-value} = 0.25$), this Ho hypothesis could not be rejected.??????????? As already indicated.

4. DISCUSSION:

4.1. HIV/AIDS

This study focused on comparing the performance (sensitivity, specificity, efficiency, PPV and NPV) of the HIV FIRST RESPONSE RDT with a 3rd generation ELISA which is the gold standard, and to evaluate the possible interference of rheumatoid factor with the RDT results. It must be recalled that this RDT has been approved for use in Cameroon by the Ministry of Public Health.

This part of the study involved 25 patients. This sample size was lower than in other studies comparing an HIV RDT with ELISA, as by OonTek *et al* in 2012 [5] with 994 patients for the OraQuick ADVANCE Rapid HIV 1/2 Antibody Test; Zachary *et al* in 2012 [6] with 4458 patients for the OraQuick[®] RDT; Kroidi *et al* in 2012 [7] with 15,000 patients for the Determine RDT. These studies had bigger sample sizes primarily because of their durations (years). Secondly, this was not an HIV prevalence testing study, and so our sample size was calculated based on the prevalence of RF which was our parameter of interest. The sample size was therefore adequate.

We found overall sensitivity, specificity, efficiency, PPV and NPV of 14.3%; 94.1%; 70.8%; 50.0%; 72.7% respectively. The sensitivity and specificity found were lower than for Determine RDT which had 100% and 97.9% respectively [7] and for STAT-PAK RDT with 99.7% and 99.3% respectively [7]. The sensitivity turned out to be very much lower than that specified in the reagent leaflet (100%), and was due to the great number of false negatives.

The only false positive case found had a low value of RF (10.7 UI/L). [However, our statistical analysis showed no effect of this parameter on the HIV FIRST RESPONSE RDT] this is questionable. Appropriate statistics must be applied before this conclusion can be made. As it stands, you cannot not say so. This result is plausible because the average blood RF level was smaller in the group with positive RDTs compared to the group with negative RDTs; it has been also shown that a concentration of RF up to 238.8 UI/L does not create cross reactions which would lead to false positive results (Avila *et al.*, 2002). Our results were in contrast with a study in Sao Paolo, Brazil, which showed that RF values greater than 160 IU/L would lead to cross-reactions on a RDT for malaria, resulting in false positives [8]. However, according to the ANOVA test used to find a possible effect of RF on our RDT, we found a P-value = 0.37 for a threshold of 0.05 so our hypothesis could not be rejected which stated that there is no effect of RF on the performance of our RDT. (problematic test?????)

4.2. Hepatitis B

This study focused on comparing the performance (sensitivity, specificity, efficiency, PPV and NPV) of the HEXAGON HBsAg RDT with a high sensitivity ELISA (the gold standard) and to evaluate the possibility of interference of bilirubin on the RDT results.

For this, the 30 samples used were lower in number than those for similar comparative studies. Franzeck *et al* in 2013 [9] used 272 samples for Determine HBsAg while Maity *et al* in 2012 [10] had used 100 samples for Crystal HBsAg and SD Bioline HBsAg comparisons with ELISA. However our sample size was not focused on HBV prevalence but on the prevalence of jaundice in HBsAg positive patients (high bilirubin blood level was our parameter of interest). On this basis, our sample size was higher than the required calculated minimal sample size of 16 samples.

We found overall sensitivity, specificity, efficiency, PPV and NPV of 92.8%, 100%; 96.6%; 100%; 94.1% respectively. The values of the sensitivity, specificity, PPV and NPV were lower than those found using Crystal HBsAg and SD Bioline HBsAg with 100% for both [10]. They were also

higher for Determine HBsAg, with 96%; 100%; 99.5% and 100% respectively [9]. Our sensitivity and specificity were also lower than those specified in the reagent leaflet (100% and 99.7% respectively). With a PPV of 100% (which is the best value for consulting health professionals as it best correlates the diagnosis with the patient's condition), HEXAGON HBsAg RDT is adequate for good diagnosis in our epidemiological setting.

One false negative was found among our specimens, with a high blood level of bilirubin (228.7 mg/L). It is possible that this high bilirubin level may be responsible for the false negative results (100 %) by decreasing the sensitivity of the test. This result correlates with that of Asanghanwa *et al* in 2012 [11] who found that bilirubin levels above 300 mg/L in the sample decreased the concentration of C-peptide by about 10% in the ELISA technique. However, according to the ANOVA test used to determine the possible effect of bilirubin on our RDT, we found a *P-value* =0.25 for a threshold of 0.05. Therefore we could not reject the hypothesis which stated that bilirubin has no effect on the performance of the HBsAg RDT despite the fact that blood bilirubin levels were higher in the group with negative RDTs compared to the group with positive RDTs.

5. CONCLUSION

From the demographical data and the results of our study, it was found that Rheumatoid Factor does not interfere with the performance of the HIV FIRST RESPONSE RDT for HIV detection (*P-value* = 0.37). However, the PPV and low sensitivity of the FIRST RESPONSE HIV Card 1-2.0 test brings to question the conditions under which it was approved for use in the country. Nonetheless, our sample size does not permit us to make a conclusive recommendation on this point. Also, the HEXAGON HBsAg RDT was found to be adequate for the diagnosis of viral Hepatitis B in our epidemiological setting as there was no recorded interference of bilirubin with its performance (*P-value* = 0.25). However, the many other factors that could contribute to the poor performance of these immunochromatographic RDTs need to be investigated and taken into account while they are being validated as vital tools for the diagnosis of many diseases in Cameroon. { Questionable and problematic conclusion at this stage due probably to inappropriate statistics}.

CONSENT

Informed consent was sought and obtained from each patient visiting the BETHANIE Laboratory, before his/her involvement in the study.

ETHICAL APPROVAL

Ethical clearance N°2013/11/379/L/CNERSH/SP was obtained from the National Ethics Committee on Research for Human Health (CNERSH) of Cameroon. Research Authorizations were obtained from the Directorate of the School of Health Sciences, Catholic University of Central Africa and the directorate of BETHANIE Laboratory.

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