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

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors SDK and PMN designed and financed the study, as well as wrote the protocol and first draft of the manuscript. Author SDK performed the sample collection and laboratory assays under the supervision of Authors PMN and JD respectively. Authors TP and DSOT provided technical guidance on research design and data analysis. Authors MK and BAT corrected the protocol and did a thorough review of the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** We aimed at studying the influence of some potential interference factors on 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 of this cross-sectional study included patients referred to the 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 Chi-square test with continuity correction 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 IU / L and 64.29 IU / 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:** HIV FIRST RESPONSE RDT results were independent (*P-value* = 1.00) of Rheumatoid Factor values (up to 238.8 IU / L). However, we found that HEXAGON HBsAg RDT results were not independent of bilirubin values (*P-value* = 0.01547), suggesting that the latter could potentially have an influence on the former.


*Keywords: Influence; 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]. Apart from mutations and low titer of the target antigen, inappropriate manipulation, poor quality as well as poor conservation of the RDTs, numerous factors, classified as endogenous or exogenous may alter the performance of Rapid Diagnostic Tests (RDTs). Endogenous interference originates from substances present in the patient's own specimen while exogenous interferences are substances introduced into the patient's specimen [5]. The performance of a RDT could be influenced by endogenous interfering factors such as rheumatoid factor (RF), bilirubin (up to 10 mg/dL), hemoglobin (up to 0.5 g/dL) and triglycerides (up to 300 mg/dL) [6]. Such interfering factors may constitute the basis for the false-positive results observed in patients with rheumatoid factor (RF), hepatitis C, toxoplasmosis, human African trypanosomiasis, dengue, leishmaniasis, Chagas disease, and schistosomiasis [7]. Conflicting observations have however been reported about the potential influence of these interfering factors in the performance of RDTs. Maltha et al. [8] reported that high RF levels were associated with malaria RDT false positivity while Iqbal et al. [9] showed that 33 of 35 false-positive specimens were negative when the RF was absorbed in the immunochromatographic test. Cha el al. in 2013 [6] on the other hand observed that neither bilirubin (up to 10 mg/dL) and hemoglobin (0.5 g/dL) nor triglycerides (up to 300 mg/dL) showed any interference with the OraQuick HCV test results.

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 other caused by a DNA virus (Hepatitis B). We thus sought to investigate the effect of Rheumatoid Factors (RFs) and Bilirubin as possible causes of interference in the performance of RDTs for HIV and HBV respectively. Our choice of investigating the potential role of RF to influence HIV RTD was guided by the fact that the RDT been tested targets antibodies, and RFs (which can behave like heterophillic antibodies) could bind competitively and lead to cross-reactions resulting in false positive results. Also, our research team at the Bethanie laboratory encourages physicians to communicate any clinical suspicion of discordance between the clinical and the laboratory data. In the establishment of such an on-going laboratory-physician contact (essential to the continuing awareness of wrong patient results due possibly to interference), we realized that there were complains about the HBV results in icterus patients. These observations [coupled with reports that bilirubin tend to interfere with biochemistry assays [10] and immunoassays [11], motivated our choice to explore a possible interference caused by bilirubin on an HBV RDT widely used in Cameroon.

## 2. MATERIALS AND METHODS

### 2.1 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.

### 2.2 Study Design and Sample Population

Our study was designed as a cross-sectional study. Sample population included 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. A questionnaire was used for data collection. Initials of names, age, sex, address, neighborhood and number of sexual partners during the last six months were recorded. For all cases, we used a convenience sampling and thus recruited 25 patients for HIV/AIDS and 30 for Hepatitis B study arms respectively.

## 2.3 Laboratory Investigations

### 2.3.1 Rapid diagnostic tests

The RDTs for Hepatitis B (HBsAg HEXAGON) and HIV/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. Tests were performed following manufacturers' instruction.

### 2.3.2 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.

Positive and negative controls were used during the assays for quality control. Their values were in the range pre-established by the manufacturer.

### 2.3.3 Biochemistry assays

The determination of bilirubin (spectrophotometry) and the tittering 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 DIAGNOSTICS for the determination of RF.

During the RF analysis, patients' samples that had values higher than the detection limit (200 IU/L) of the reagent were diluted. 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 IU/L.

In the determination of bilirubin levels, all samples were subjecting to procedure N° 1 (with detection limit less than 200 mg/L). Samples with values ≥ 200 mg/L were subsequently subjected to procedure N° 2 (with detection limit of 1,000 mg/L). The "BIOLABO EXATROL-N" (level I) REF: 95010 LOT: 111215C were included for quality control of all biochemistry assays. All the controls were in the range pre-established by the manufacturer. Reference bilirubin values for adult and children in our laboratory are 3 – 12 mg/L.

## 2.4 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 Chi-square test with continuity correction was performed at a threshold of 0.05 to analyse the influence of blood levels of RF and bilirubin on result of RDTs of HIV and HBV respectively. To do this, we categorized RF results into two categories (RF < 200 IU/L and RF ≥ 200 IU/L) while bilirubin results were classified into three categories (Bilirubin levels < 100 mg/L; between 100 - 200 mg/L and ≥ 200 mg/L).

# 3. RESULTS

## 3.1 Socio-demographic Data

We collected specimens from 25 consenting patients referred to the BETHANIE laboratory to conduct a RF examination. Similarly, 30 specimens were collected from patients who presented with jaundice (bilirubin). The mean age of participants for the HIV/AIDS arm was 47.36 years with a standard deviation of 18.27. The youngest HIV participant was 9 years old and the oldest 80; while HBV participants' mean age was 39.83 years (SD = 18.33). The youngest participant on the HBV arm was 13 years old and the oldest was 79. All participants were heterosexual with 32% (8/25) of participants for HIV/AIDS arm having more than one sexual partner while 68% (17/25) of participants had none or a maximum of one partner. For the HBV arm, 43.33% (13/30) of participants had more than one sexual partner while 56.67% (17/30) of participants had 0 or a maximum of one partner. Table 1 below presents the HIV and HBV infection distribution by gender.

For the HIV/AIDS arm, 2 (14.28%) of the 14 females, 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.

**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*

Of the 8 females recruited in the HBV arm, 3 (37.5%) were positive while 5 (62.5%) were negative, while 11 (50%) of the 22 males were positive and 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.

## 3.3 HIV/AIDS

Of the 25 blood samples analysed for HIV, 2 (08%) 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 IU/L). However, the patient who had the highest level of RF (238.8 IU/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.

## 3.4 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 (228.7 mg/L) recorded.

The sensitivity, specificity, efficiency, PPV and NPV were 92.85%, 100.00%, 96.67%, 100.00% and 94.11% respectively.

## 3.5 Biochemistry Assays Results

### 3.5.1 Rheumatoid factors assays results

The results of Rheumatoid Factors assays results arranged in two groups (< 200 IU/L and ≥ 200 IU/L) with frequency and mean of RF are summarized in Table 3.

Of the 25 samples analysed, 22 had RF values < 200 IU/L with a mean of 36.73 IU/L while RF values ≥ 200 IU/L were recorded from 03 samples with a mean of 231.23 IU/L.

### 3.5.2 Bilirubin assays

The results of bilirubin assays arranged in three groups (< 100 mg/L; [100 – 200 [mg/L and ≥ 200 mg/L) with frequency and mean of bilirubin are summarized in Table 4.

Of the 30 samples analysed, 18 had bilirubin values < 100 mg/L with a mean of 42.5 mg/L; bilirubin values in range of [100 – 200] mg/L were recorded from 04 samples with a mean of 142.4 mg/L and 08 samples had bilirubin values ≥ 200 mg/L with a mean of 256.9 mg/L.

## 3.6 Analysis of the Influence of Blood Levels of RF on RDT Result for HIV

The proportion of positives and negatives RDT results together with their corresponding group values of rheumatoid factors is summarized in Table 5.

Considering our null hypothesis (Ho) that "RF values and RDT results are independent", suggesting that RF does not have any influence on the RDT results, our Chi-square test with continuity correction (at a threshold of 0.05) gave a *P-value = 1.00.* Thus, the null hypothesis (Ho) could not be rejected. Therefore, RF values and RDT results are independent signifying that RF values do not interfere with the RDT results for HIV.

## 3.7 Analysis of the Influence of Blood Levels of Bilirubin on RDT Result for HBV

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

**Table 2. Cross tabulation of RDTs and ELISA results**

| RDT ELISA | HBV | | | HBV | | |
|---|---|---|---|---|---|---|
| | **Positives** | **Negatives** | **Total** | **Positives** | **Negatives** | **Total** |
| Positives | 01 (TP) | 06 (FN) | 07 | 13 (TP) | 01 (FN) | 14 |
| Negatives | 01 (FP) | 16 (TN) | 17 | 00 (FP) | 16 (TN) | 16 |
| Total | 02 | 22 | 24 | 13 | 17 | 30 |

*Where TP: True Positive; TN: True Negative; FP: False Positive and FN: False Negative*

**Table 3. Rheumatoid factors assays results**

| RF values (IU / L) | < 200 | ≥ 200 |
|---|---|---|
| Participants | 22 | 03 |
| Frequency (%) | 88 | 12 |
| Mean of RF | 36.73 | 231.23 |

**Table 4. Bilirubin assays results**

| Bilirubin values (mg/L) | < 100 | [100 – 200] | ≥ 200 |
|---|---|---|---|
| Participants | 18 | 04 | 08 |
| Frequency (%) | 60.00 | 13.33 | 26.67 |
| Mean of RF | 42.5 | 142.4 | 256.9 |

**Table 5. RF and RDT assays results**

| RDT RF (IU/L) | Negative | Positive |
|---|---|---|
| < 200 | 21 | 01 |
| ≥ 200 | 03 | 00 |

**Table 6. Bilirubin and RDT assays results**

| RDT Bilirubin (mg/L) | Negative | Positive |
|---|---|---|
| < 100 | 10 | 08 |
| [100 – 200] | 00 | 04 |
| ≥ 200 | 07 | 01 |

Considering our null hypothesis (Ho) that "bilirubin values and RDT results for HBV are independent", suggesting that bilirubin does not have any influence on the RDT results of HBV, our Chi-square test (at a threshold of 0.05) gave a *P-value = 0.01547. Thus,* the null hypothesis (Ho) could be rejected. This suggests that bilirubin levels and RDT results for HBV are not independent signifying that blood bilirubin values interfere with the RDT results for HBV and could influence the later.

## 4. DISCUSSION

### 4.1 HIV/AIDS

This arm of our study focused on comparing the performance (sensitivity, specificity, efficiency, PPV and NPV) of the HIV FIRST RESPONSE RDT with a 3$^{rd}$ generation ELISA which is the gold standard, and to evaluate the possible influence 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 that used in other studies comparing an HIV RDT with ELISA, such as the study by OonTek et al. [12] with 994 patients for the OraQuick ADVANCE Rapid HIV 1/2 Antibody Test; Zachary et al. [13] with 4458 patients for the OraQuick® RDT; Kroidi et al. [14] with 15,000 patients for the Determine RDT. These studies had bigger sample sizes primarily because of their durations (years). Besides, our study was not an HIV prevalence testing study. Therefore, our sample size was calculated based on the prevalence of RF which was the parameter of interest for this study. The sample size was therefore considered 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 [14] and for STAT-PAK RDT with 99.7% and 99.3% respectively [14]. The sensitivity turned out to be very much lower than that specified in the reagent leaflet (100%). This was mainly due to the great number of false negatives 6 (24%) out of 25 samples. The only false positive case had a low value of blood RF (10.7 IU/L). This was not surprising as it has been shown that a concentration of RF up to 238.8 IU/L does not create cross reactions which would lead to false positive results [15].

Our results are however opposing to results obtained in 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 [15]. Nonetheless, according to the Chi-square test used to analyse if there was a possible influence of RF values on HIV RDT results, we found a P-*value* = 1.00 for a threshold of 0.05. This implies that, our null hypothesis (Ho) which states that "RF values and RDT results are independent", could not be rejected. Therefore, RF values and RDT results are independent signifying that blood RF values do not interfere with and possibly do not influence the HIV FIRST RESPONSE RDT results. This observation is plausible because the average blood RF level was smaller in the group with positive RDTs compared to the group with negative RDTs. However, this result should be interpreted with caution as this is simply an approximation of the Chi-square because one cell on the contingency table generated in this study has no participant. This aspect constitutes a limitation of our study.

## 4.2 Hepatitis B

This arm of the study focused on comparing the performance (sensitivity, specificity, efficiency, PPV and NPV) of the HEXAGON HBs Ag RDT with a highly 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. For example, Franzeck et al. [16] used 272 samples for Determine HBsAg while Maity et al. [17] had used 100 samples for Crystal HBsAg and SD Bioline HBsAg to compare with ELISA. However, our sample size was not focused on HBV prevalence but rather 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 [17]. 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 could be considered adequate for good diagnosis of HBV 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 the cause of the false negative result (100% of false negative case) which was the result of the decreased sensitivity (92.8%) of the HEXAGON HBsAg RDT. This result correlates with that of Asanghanwa et al. [18] who found that bilirubin levels above 300 mg/L in samples decreased the ability of ELISA technique to detect C-peptide concentrations by about 10%. The latter is possibly due to interference of bilirubin on the ELISA. Besides, the Chi-square test used to determine if there was a possible dependence of bilirubin values on our RDT results, gave a *P-value* = 0.01547 for a threshold of 0.05. This implies that our null hypothesis (Ho) could be rejected. This suggests that bilirubin levels and RDT results for HBV are not independent; signifying that blood bilirubin values could interfere with the RDT results for HBV and could influence the later. Nevertheless, this is just an approximation of the Chi-square given that some cells on the contingency table have small numbers of or no participants. Thus, our result should be interpreted with caution. Though this may constitute a limitation of this study, this observation can be confirmed by 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 the performance of the HIV FIRST RESPONSE RDT for HIV detection is independent of Rheumatoid Factor values (*P-value* = 1.00). 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 though we recorded a dependence of the performance of the test (*P-value* = 0.01547) with bilirubin values. Our findings suggest that many other factors that could contribute to the poor performance of these immunochromatographic RDTs and need to be investigated and taken into account while they are being validated as vital tools for the diagnosis of many diseases in Cameroon and beyond.

## 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.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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