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Evaluation of immunological response to hepatitis B vaccine among healthcare workers at Rwanda Military Hospital

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ABSTRACT

INTRODUCTION: Healthcare workers (HCWs) are at high risk of acquiring hepatitis B viral (HBV) infection through occupational exposure to blood or body fluids. However, the rates of non-responders after HBV vaccination among HCWs are not well documented. Therefore, we aimed to determine the proportion of immunological non-responders among HCWs who received Hepatitis B vaccine at Rwanda Military Hospital (RMH) and characterize the memory T cell responses to the Hepatitis B vaccine.

METHODS: A cross sectional study was conducted at the RMH. HBV vaccinated HCWs were evaluated for immune response by measuring serum Hepatitis B surface antibody (anti-HBs) titers and levels of HB core antibodies (HBcAb) on COBAS e411 machine, 6 years post vaccination.

RESULTS: Initially 87 employees were included in the study. Four participants were excluded due to incomplete records. 52 (62.7%) participants were female and 31 (37.3%) were male. HWCs' age ranged between 23 and 66 years with a mean (±SD) age of 38.2 ± 7.3. Of 83 HCWs, 70 (84.3%) showed response to HBV vaccine, non-responders were 11 (13.3%) and 2 (2.4%) showed immunity due to natural infection. There was no significant difference in Th cell frequencies and function between responders and non-responders after stimulation with rHBsAg vaccine.

CONCLUSION: Immunological response six years post HBsAg vaccination was 84.3% in HCWs at RMH and similar to the global prevalence. Anti-HBs levels should be tested in all HCWs following HBsAg vaccination. Personal protective equipment, and a dose of Hepatitis B prophylaxis when exposed should be emphasized.

Keywords: Hepatitis B Vaccine, Immunological response, Healthcare Workers, Rwanda Military Hospital

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INTRODUCTION

Hepatitis B is an infection caused by the hepatitis B virus (HBV), transmitted through percutaneous or mucosal exposure to infectious blood or body fluids [1]. The virus is highly infectious for non-immune persons, and transmission from a needle stick exposure is up to 100 times more likely for exposure to hepatitis B e antigen (HBeAg)–positive blood than to HIV-positive blood [2]. Healthcare workers (HCWs) are at risk of acquiring blood-borne diseases, including HBV, due to potential occupational exposure to blood and body fluids. The World Health Organization (WHO) estimated that 35 million HCWs worldwide and 3 million experience percutaneous exposure to blood pathogens each year. Of these, 2 million are exposed to HBV [3].

Since the availability of the HBV vaccine in 1982, the decline in the incidence of HBV infection and associated morbidity and mortality was reported [4,5]. Therefore, in 1997 the US CDC recommended that all HCWs should be vaccinated against HBV [4]. Despite the recommendation and excellent protection profile among post-vaccinated personnel, compliance with this recommendation remained poor in various healthcare settings [4,6]. Immune response to HBV vaccine is assessed by measuring antibody levels after 6 weeks of completion of the 3 doses. The anti-HBs >10 IU/l is generally taken to be protective against HBV infection [7,8]. An anti-HBs titer of <10 mIU/ml is regarded as non-responsiveness to HBsAg vaccination [5]. Anti-HBs levels between 10 and 100 mIU/ml are regarded as hypo-responsiveness and levels >100 mIU/ml are taken as a high level of immunity.
Although the majority of persons vaccinated against hepatitis B successfully respond to vaccination, an estimated 5-15% of persons may not produce enough antibodies to mount adequate protection [9,10].

Factors like smoking, obesity, aging, chronic medical conditions, male sex, genetic factors, and immunosuppression are associated with a decrease in immune response [4,11]. The current study aimed to evaluate the immune response to HBsAg vaccination in HCWs and to assess the memory T cell immune responses in non-responders.

METHODS

Study design: A cross-sectional study was conducted from March to July 2017 among 850 HCWs at RMH who had received the complete standard course of intramuscular HBsAg vaccination. HCWs who did not complete 3 doses of the HBV vaccine and those who were not available during the study period were excluded from the study. With a 99% confidence level and an absolute allowable error of 5%, a sample size of 83 was attained. The sample size was calculated based on the prevalence of non-responsiveness to the HBV vaccine's initial 3-dose regimen (5-15 %) [9,10]. Eighty-seven potential participants were approached, and 83 participants were enrolled in the study. Of the 850 hospital personnel, 61 (73%) were healthcare providers, and 22 (27%) were other support staff.
Sample collection, preparation, and storage: Aseptically, 5ml of blood was collected in the serum separator tubes (SST) from each consented vaccinated 83 HCWs to detect anti-HBs and anti-HBc antibody levels. Samples were centrifuged for 15 minutes at 280 x g to obtain serum and analyzed for anti-HBs and anti-HBc using the COBAS e411 machine. All non-responders (anti-HBs <10 IU/l) were requested to give samples for PBMCs, and 20 ml of venous blood were collected into Acid Citrate Dextrose (ACD) tubes and transported to Projet San Francisco (PSF) in an immunology laboratory for peripheral blood mononuclear cell isolation and storage.
The Ficoll-Hypaque density gradient centrifugation standard was used to isolate PBMCs, cryopreserved in 90% heat-activated fetal calf serum (FCS, Invitrogen) plus 10% DMSO, and stored in liquid nitrogen. The cryopreserved PBMCs were transported to the Immunology laboratory of Uganda Virus Research Institute (UVRI), Kampala, Uganda, for T cell functional assays on flow cytometry.
Anti-HBs and anti-HBc antibody testing: Cobas e411 machine (Roche, Germany) was used for the quantitative detection of anti-HBs and Anti-HBc antibodies levels. Every Elecsys Anti-HBs reagent set has a barcoded label containing specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer using A HBS Cal1 and A HBS Cal2. PreciControl Anti HBs were run once per kit following each calibration. The vaccine

responders were participants whose anti-HBs were ≥ 10IU/l and anti-HBc negative.

PBMC stimulation: PBMCs were thawed and rested overnight prior to stimulation. And cell count was done to check cell viability. Cells (1 $\times 10^6$ cells/well) were seeded into 96-well cell culture plates in the presence of HBsAg, and both Phytohemagglutinin (PHA) and Staphylococcal Enterotoxin (SEB) were used as a positive control to stimulate cells, and mock was used as a negative control. We incubated the plate in a humidified atmosphere with 5% CO2 for 6 hours.

VIability staining: Viability staining is required to determine the viable cells prior to the fixation and permeabilization required for intracellular antibody staining. The plate was washed with pre-warmed PBS buffer prior addition of aqua amine dye (which can stain live cells and preserve that staining pattern after fixation is critical for intracellular immunophenotyping) diluted 1/40 and incubated in the dark at room temperature for 20min and then washed again with PBS.

Fixation: 100ul of cytofix-cytoperm were added to each well for fixation and permeabilization of the cells, which is necessary for intracellular cytokine staining with fluorochrome-conjugated anti-cytokine antibodies and incubated for 20min at 4°C and protected from direct light. Perm/wash buffer was used to dilute anti-cytokine antibodies for staining.

Intracellular cytokine staining and surface staining: 100ul of a cocktail of antibodies (anti-IL13, IL17, IL2, and IFN-γ for ICS; anti-CD3, CD4, CD8, CCR7, and CD45RA for surface staining) were added to each well and incubated in the dark at room temperature for 20min. 1x perm/wash was used to wash and re-suspend the cells. Samples were run on a LSRII flow cytometer after successfully running CST and compensation beads.

Flow cytometry gating strategy to identify key populations: PBMCs were stimulated with recombinant HBsAg for 6h in the presence of CD28/CD49 co-stimulators. BFA/ Golgi plus was also added as a protein transport inhibitor to block the escape of cytokines from the cell. Each sample had four conditions: Unstimulated (Negative control), stimulated, and positive control. A cocktail of fluorescence-labeled antibodies was added and incubated at 4°C in the dark to all binding, acquired on a flow cytometry machine. Forward and side scatter and marker expression gates were placed around populations of cells to distinguish populations of cells based on their properties (Figure 1).

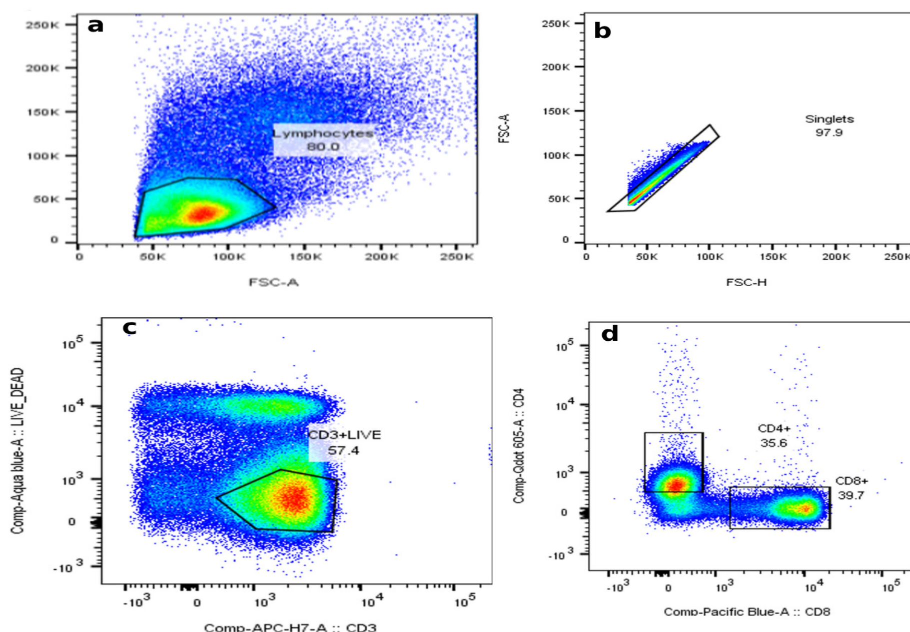

Figure 1: *Gating strategy for identification of cell populations.* ***a)*** *Gating on a population of lymphocytes based on side scatter and Forward Scatter area.* ***b)*** *Gating on a population of singlets based on forward scatter area and forward scatter height.* ***C)*** *Gating on a population of Live/ dead cells based on aqua amine dye and CD3+ APC-H7.* ***d)*** *Gating on CD4+ and CD8+ populations of based CD4+ Qdot and CD8+ Pacific.*

Approval to carry out the research was obtained from the Research and Ethics Committee of Rwanda Military Hospital (Ref.:EC/RMH/087/2016) and the Higher Degrees Research and Ethics Committee (SES-HDREC-417) of School of Biomedical Sciences, Makerere University College of Health Sciences. Laboratory numbers were used to identify blood samples, and consent from the participants was obtained before any sample was drawn.

RESULTS

Overall, 70 (84.3%) participants have anti-HBs antibodies titers >=10 IU/L (Table 1).

Table 1: The proportion of immune protection (anti-HBs antibodies titters >= 10 IU/L) in immunized healthcare workers

Type of response	n	%
Response to HB vaccine	70	84.34
Response due to natural infection	2	2.41
Non- Responders	11	13.25

Two (2.4%) had evidence of response due to naturally resolved infection, and eleven (13.3%) had anti-HBs antibodies titers <10 IU/L (non-responders). The highest level of response to HB vaccine was between 30-39 (Table 2).

Gender, sex, smoking, and alcohol consumption were the variables assessed for their contribution to the response to the vaccine. There was no statistical significance between the above variables and response to the HB vaccine (all p>0.05) (Table 3).

Frequency of t-cell responses to rHBsAg in responders and non-responders: The frequency of CD4+ and CD8+ T cells in the two populations had no statistical difference. However, responders had a higher percentage of CD8+ cells in response to PHA as a positive control (Figure 2).

Expression of memory markers in non-responders and responders: The expression of memory markers in responders and non-responders was assessed. CD45RA and CCR7 expression was determined in both CD4 and CD8

Table 2: The level of response to the vaccine by age group

		Age group				
Population	Anti HBs	20-29	30-39	40-49	>=50	Total
Group I	<10 IU/L	1 (20%)	6 (14%)	4 (15%)	0 (0%)	11 (13.6%)
Group II	10-100 IU/L	1 (20%)	3 (7%)	5 (19%)	0 (0%)	9 (11%)
Group III	>100 IU/L	3 (60%)	34 (79%)	18 (66%)	6 (0%)	61 (75.4%)
Total		5	43	27	6	81

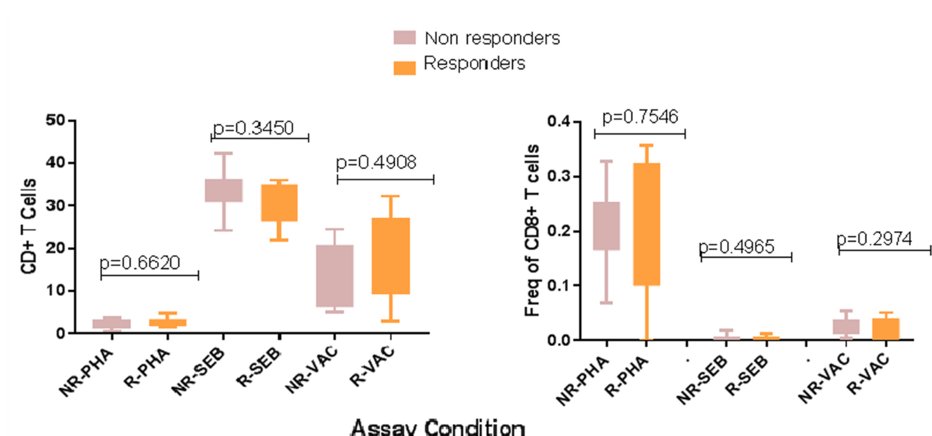

Figure 2: The percentage of CD4+ and CD8+ expression in responders and non-responders

populations of responders and non-responders. CD45RA as a memory marker and CCR7 to determine the homing capacities. There was no statistical difference between responders and non-responders in the expression of memory markers. Both central and effector memory markers were expressed at the same level in both populations (Figure 3).

The intracellular cytokine staining to assess the function of TH cells: To characterize CD4+ T cells for their specific functions in responders and non-responders, we stimulated the PBMCs with rHBsAg vaccine to differentiated memory and effector T-helper subtypes (TH) that include TH1, TH2, and TH17 subsets, all of which are present in PBMC. To characterize the above subtypes, IFNg, IL2, IL13, and IL17 were assessed for the production of these cytokines. There was no statistical difference in the production of IFNg, IL13and IL17 in responders and non-responders in response to the rHBsAg vaccine. However, there was a statistical difference in the production of IL2 (p=0.0293) and a big difference (p=0.07) in the production of IFNg in responders

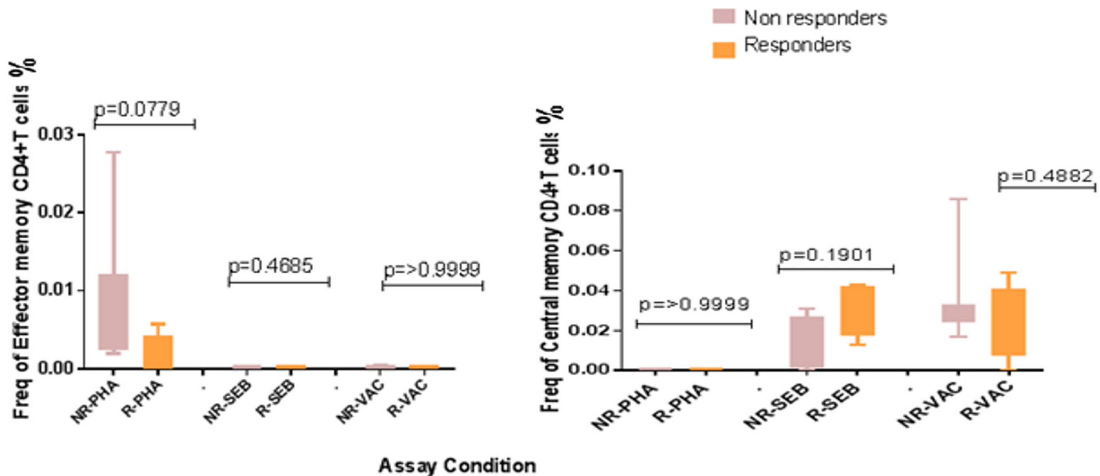

Figure 3: The expression of memory markers by CD4+ T cells

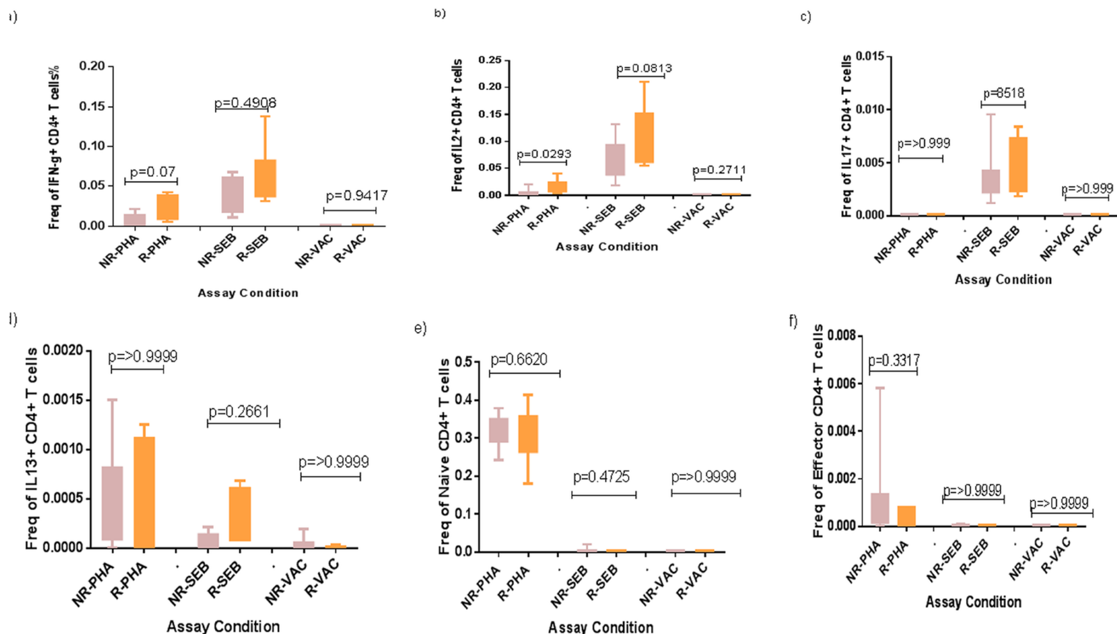

Figure 4: The production of type 1 and type 2 cytokines in response to rHBsAg vaccine and positive control

Table 3: Distribution of respondents by participant characteristics

Participant characteristics			Overall N=81	Responders 70 (86.4%)	Non-responders 11 (13.6%)	p-value
Sex	Female	50 (61.7)	41(82.0)	9 (18.0)	0.190*	
	Male	31 (38.3)	29 (93.6)	2 (6.5)		0.190
Age, median (IQR) years		38 (33-42)	38 (33-42)	37 (31-41)	0.423$^{\beta}$	0.423
Smoking	No	73 (90.1)	62 (84.9)	11 (15.1)	0.590*	0.590
	Yes	8 (9.9)	8 (100.0)	0 (0.0)		
Alcohol use	No	59 (72.8)	49 (83.1)	10 (17.0)	0.273*	0.273
	Yes	22 (27.2)	21 (30.0)	1 (9.1)		

compared to non-responders (Figure 4).

DISCUSSION

The risk of acquiring hepatitis B virus (HBV) infection is high among healthcare workers (HCWs) because of their high risk of exposure to blood or its products. CDC recommends that all HCWs get vaccinated against viral hepatitis [12] and thereafter tested for protective immune responses to the hepatitis B vaccine since a proportion of vaccines do not respond to the vaccine [4]. This study found that 86.4% of HCWs at RMH had an appropriate immune response after 6 years post-HB vaccination. This proportion is in line with what was seen by Mohammad et al. among HCWs in Pakistan [13].

These findings indicate that a significant proportion (13.6%) of RMH HCWs are still at risk of acquiring HBV. Thus, the hospital needs to provide extra protection to these workers to prevent infection. The non-responder rate of 13.6% is also way higher than the efficacy rates of 96% in adults provided by the Vaccine manufacturers [14]. Previous studies have suggested that age, gender, smoking, obesity, nutritional status, vaccine administration site, and genetic factors are probable reasons to account for the reduced immune response in non-responders. Some studies reported gender-based differences in immune responses to the hepatitis B vaccine; those females show better responses than males [15,13].

The development of a protective immune response to HBsAg is associated with the production of specific neutralizing antibodies. The antibody production process for this antigen is T cell-dependent and requires Th-CD4+ cell activation [16]. However, until now, the underlying causes of non-response to the rHBsAg vaccine are unknown [18]. Our characterization of the Th-CD4+ cell subsets in hepatitis B vaccine responders and non-responder participants found some differences in the proportion of Th1 (p=0.07) but not in Th2 (p=0.99) CD4+ T cells between the two groups using IFN_{γ} and IL-13 production as a surrogate for Th1 and Th2 cell population respectively. We equally did not find any significant differences in the frequency of CD4+ (p=0.4908) and CD8+ (p=0.2974) T cells among responders and non-responders.

Our efforts to perform in-vitro stimulation/activation of PBMCs with the rHBsAg present in the Angerix B vaccine were unsuccessful and in agreement with several studies [2,17,18]. As Larsen et al. (2000) reported, the in vitro cytokine response to HBsAg is very complex and subject-dependent [19]. Both types of cytokine patterns, Th1 and Th2 (IFN-g and IL-13, respectively) and additionally IL17 were produced at non-significantly lower levels in our responder and non-responders. However, the production of IL-2 was found to be high in responders than in non-responders (p=0.029). IFNg production was also slightly higher in responders than non-responders, though it was not statistically significant (p=0.07). All these differences were only observed in the cells stimulated with PHA. There was no difference in the production of cytokines by cells stimulated

by recombinant hepatitis B vaccine in both groups. Despite the failure of the rHBsAg vaccine to stimulate the cells, the higher production of IL2 in responders could explain its role in the proliferation and differentiation of T cells into T helper subsets which plays an important role in the production of cytokines that activate B cells to produce neutralizing antibodies and that was lacking in non-responders' group. However, we must consider previous events that anticipate proliferation, cytokine, and antibody production, which may be involved in the non-response phenomenon against rHBsAg. Sallusto et al. showed that once T cells are activated, they proliferate vigorously, generating effector cells that can migrate to B-cell areas or inflamed tissues. A fraction of primed T lymphocytes persist as circulating memory cells that can confer protection and give, upon secondary challenge, a qualitatively different and quantitatively enhanced response [20]. Our study assessed memory T cell subsets by measuring specific markers (CD45RA, CCR7) of memory T cells (effector memory and central memory) in both responders and non-responders. Our study did not agree with Tanja Bauer and Wolfgang Jilg's study, which found more effector memory T cells in responders than in non-responders. The possible reason is that the recombinant HBsAg vaccine that was used to stimulate PBMCs in vitro did not activate T cells to proliferate and differentiate into different subsets.

This study has some limitions to consider. It was not able to evaluate the association of decreased immune response with risk factors beyond age, gender, and smoking, leaving us unable to find other factors that could have been associated with non-responder status. The small sample size due to the delay of participants to consent for the study was also a limitation.
Recombinant hepatitis B surface antigen (rHBsAg vaccine) could not stimulate cells in vitro, resulting in failure to assay for differences in vaccine-specific immune responses between responders and non-responders. We were not able to obtain Recombinant HBsAg (rHBsAg; ayw subtype) that proved to effectively stimulate PBMCs in vitro in several other studies [18].

CONCLUSION

The study has demonstrated that the majority of RMH healthcare worker recipients of hepatitis B vaccines developed protective immune antibody titers and that there is a 13.6% of recipients that did not respond to the hepatitis B vaccine.
There was a correlation between the production of IL-2 in responders and the high production of anti-HBs antibody titers, which may explain the poor expression proliferation and activation markers in non-responders. The data demonstrated and reinforced that the rHBsAg-based vaccine (Engerix B™ B) did not stimulate PBMCs in vitro under laboratory experimentation settings. There is a need to introduce a policy on assessing immune responses after the completion of hepatitis B vaccine doses at RMH. HCWs with no or weak responses to the hepatitis B vaccine should be covered by a corrective action plan, such as being given a booster dose. Prophylaxis should be available at the hospital for HCWs who are non-responders to the vaccine and are exposed to known HBV-positive blood and other body fluids to prevent HBV infection. There is also a need for assessing the T cell function by using recombinant HBsAg (rHBsAg; ayw subtype) that is known to stimulate the cells in vitro effectively.

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