

Serological and molecular markers for Hepatitis B virus in university students

Henry Bautista Amorochio,¹ Yeny Zulay Castellanos Domínguez,² Ana Elvira Farfán García.³

¹ Clinical Laboratory Bacteriologist, MSc in Biomedicine, Researcher in the Research Group of Manejo Clínico – CliniUDES, Professor in the Bacteriology and Clinical Laboratory Program Clínico at the Universidad de Santander (UDES) Campus Universitario Lagos del Cacique in Bucaramanga, Colombia

² Clinical Laboratory Bacteriologist, MSc in Epidemiology, Researcher in the Research Group of Manejo Clínico – CliniUDES, at the Universidad de Santander (UDES) Campus Universitario Lagos del Cacique in Bucaramanga, Colombia

³ Clinical Laboratory Bacteriologist, MSc in Biomedicine, specialist in university teaching, Researcher and Group Leader of the Research Group of Manejo Clínico – CliniUDES, at the Universidad de Santander (UDES) Campus Universitario Lagos del Cacique in Bucaramanga, Colombia

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Abstract

Introduction: Reports from the World Health Organization (WHO) show that the prevalence of hepatitis B virus (HBV) infections varies by geographical region and risk group.

Purpose: The purpose of this study was to determine the prevalence of HBV infections, as well as the vaccination status, among university students from Bucaramanga.

Methodology: This was a cross sectional study conducted in 2010 which included 1298 students from five universities. Serological markers for HBV infection were detected using ELISA. Viral genomes were detected with nested polymerase chain reaction (PCR).

Results: Active infections were established in 0.15% of the study population, and this finding was confirmed by PCR. Resolved infections were identified in 0.60% of the population. Isolated anti-HBc antibodies were found, 30.2% of vaccinated individuals. 67.9% of the study population was susceptible. No occult HBV was detected.

Conclusions: The low prevalence of HBV infections reported in this study contrasts with the intermediate epidemiological pattern described in the region. We found poor vaccination coverage and absence of occult hepatitis B among these university students.

Key words

Hepatitis B virus, prevalence, serology, nested PCR, Colombia, students..

INTRODUCTION

Hepatitis B virus (HBV) infections are a global public health problem. It is currently estimated that 2 billion individuals have been in contact with the virus. Of those, about 350 million are chronically infected. Each year there are between 600,000 and 1.2 million deaths due to complications of acute or chronic hepatitis. About 25% of chronic cases develop cirrhosis and hepatocellular carcinoma (HCC) (1) of which HBV is considered to be the main agent (2,3). This chronic phase is characterized by

the persistence of serological markers, envelope antigens (HBsAg) and IgG class antibodies, against the core or core protein of HBV (anti-HBc) (4).

The World Health Organization (WHO) has established three categories to define the epidemiological endemicity pattern for HBV in the world based on the prevalence of the HBsAg marker in the general population. Thus, we have high (greater than 8% HBsAg), intermediate (2 to 7%) and low (below 2%) prevalence regions (5-7).

Knowledge of HBV's epidemiological pattern in each country is vital for establishing the risk of the population

for acquiring the infection and for defining the most common transmission route (8, 9). For example, in areas of high prevalence the risk is greater than 60% and most cases occur vertically during pregnancy or in early childhood. In contrast, in areas with low endemicity the risk of acquiring the virus is less than 20%, and it is acquired primarily in adulthood through sexual transmission (8, 10).

Within some Latin American countries there is a wide variation in the distribution of HBV (9, 11), a complex behavior that has been described especially in Colombia. In 2008, departments with high prevalences included Amazonas, Guainía, Guaviare and Tolima. Departments with intermediate levels of prevalence included Huila, Arauca, Norte de Santander, Casanare, Santander, Caldas, Guajira, Vichada Cesar, Boyacá, Magdalena, Cundinamarca, Antioquia, Meta Risaralda, Bolívar and Bogotá D.C. The rest of the departments have low HBV prevalences (12).

In addition, the prevalence of infection determined by HBsAg positivity in Colombia differs by risk group. A study in Medellin of patients infected with human immunodeficiency virus (HIV) showed an HBV prevalence of 2.1 while another study of HIV infected patients in Barranquilla showed an HBV prevalence of 2.9% (13,14). A study of patients who had undergone multiple transfusions in Bogota and Medellin found an HBV prevalence of 2.6% (15) while in a multicenter study conducted between 1992 and 1994 with blood donors from 10 Colombian cities the percentage varied between 0.04% and 4.05%. In this study Bucaramanga and Valledupar showed the highest prevalence values (1.12% and 4.05% respectively) (16). In a study conducted among the general populations of four Colombian departments between 1.97% and 8.39% positive cases of hepatitis B were identified (17).

In 2002 the Department of Santander showed a hepatitis B incidence rate between 2.16 and 3.9 per 100,000 people with a high risk of acquiring the infection during this period (18). Subsequently, in 2009 the incidence of hepatitis B increased to 4.6 per 100,000 people (19).

Screening studies for HBV serologic markers in different populations demonstrate that anti-HBc prevalence is greater than HBsAg prevalence (20-22). Similarly, isolated anti-HBc status in the absence of HBsAg is being found more frequently (22-26). In "isolated anti-HBc" cases we have identified the presence of viral genomes by molecular testing in blood or liver tissue with varying frequency depending on the risk group, the sensitivity of molecular testing, the local epidemiological pattern and the prevalence of different genotypes in the population (22, 24, 27-36). Detection of viral DNA in the liver, in the presence or absence of the virus in the peripheral blood of subjects who are negative for HBsAg, is called occult HBV infection

and may occur even in the absence all serological markers (25, 31, 32).

Knowing about HBV's epidemiological behavior in different risk groups allows for the design of specific programs such as vaccination and prevention campaigns for susceptible populations and clinical intervention for patients who are chronic carriers at risk of developing cirrhosis or HCC. This optimizes resources and efforts of public health surveillance institutions. To further this aim, this study was proposed to determine the prevalence of HBV infection, OHB and vaccination status against the virus among university students in the city of Bucaramanga.

MATERIALS AND METHODS

Design

A cross-sectional descriptive study was conducted between July and November 2010 in five universities in the urban and metropolitan area of Bucaramanga in the department of Santander, Colombia. We included state and private higher education institutions.

For convenience, sampling was not probabilistic. Sample size calculation was based on an expected 2% prevalence given the intermediate epidemiological pattern defined by WHO for Colombia (5) with a 5% α error and a 95% confidence interval. According to this information we estimated a sample size of 1,250 participants which is equivalent to 250 students per institution.

Recruitment of Participants

Researchers requested authorization from the school administration of each participating university. After obtaining permission leaflets and posters informed the student of basic information about HBV liver disease associated with infections, transmission mechanisms and prevention strategies. When interested students inquired about volunteering, the objectives of the study were personally explained and those who chose to participate signed an informed consent form. Volunteers between 18 and 40 years of age were included because this range represents previously described risky behaviors that facilitate transmission of the virus such as alcoholism, drug addiction and sexual promiscuity (37, 38). In addition, we required the presentation of a card that identified the participant as an active student and a membership card in an EPS (Entidad Promotora de Salud - Healthcare Promotion Entity). Hepatitis B vaccination documents were not part of the inclusion criteria. Socio-demographic information collected for each participant included full names, identification card number, age in years, gender and name of the EPS. This information

was used solely for the purpose of preparing laboratory test result reports which were confidentially given to each student at University Student Welfare Offices.

Sample Collection

After the interview 5 ml of venous blood was extracted from each student's forearm and placed in sterile vacuum tubes without anticoagulants using the BD Vacutainer[®] system. Samples identified with a consecutive code were transported to the Laboratory of Biomedical Research and Biotechnology (Libb) of the Bacteriology and Clinical Laboratory program of UDES, where the serum was separated by centrifugation at 3,500 rpm for 10 minutes. Aliquot samples were placed in 1.5 mL vials and stored at -80 ° C until processing.

Detection of HBV serological markers by enzyme immunoassay

All samples were analyzed in duplicate by "Enzyme-Linked Immunoabsorbent Assay (ELISA) for detection of the following HBV infection markers: HBsAg, anti-HBs and total antibodies against HBV core protein (total anti-HBc). Bioelisa[®] brand kits were used (Biokit, SA - 08,186 Lliçà d'Amunt - Barcelona, Spain) with semi-automated equipment. Internal quality control was performed with negative and positive control sera for each marker which had been donated by the Public Health Department Laboratory of Santander. In addition, we performed external control tests by sending 10% of the positive and negative sera to a reference laboratory in the city of Bogotá, Colombia. Figure 1 shows the way results of tests for serological markers for HBV

infection interpreted as active infections, resolved infections, susceptible stage, vaccinated or isolated anti-HBc.

Molecular tests for detection of HBV genome

The HBV genome was detected by using two protocol chain reactions (PCR) which amplify two different regions of the viral genome. The first PCR was the protocol previously described by Zeng et al. (39) while the second was a standardized test developed at LIBB. At 200 ul of serum samples that were HBsAg (+)/anti-HBc (+) (active infection) or HBsAg(-)/anti-HBc(+)/anti-HBs(-) (isolated anti-HBc), and at 10% of negatives for the three serological markers evaluated, deoxyribonucleic acid (DNA) was extracted using the silica gel column method (Blood Mini, QIAGEN[®]) according to the protocol described by the manufacturer.

The design of primers for the LIBB PCR test was performed with prototype sequences of 8 HBV genotypes obtained from Genbank which were aligned using the Clustal Wallis program. The conserved regions of the virus were edited with EditSeq and used to develop two sets of primers with PrimerSelect which amplified a fragment of the viral polymerase and precore/core. All bioinformatic analyses were done with Lasergene 8.1 software (DNASTAR Inc., Madison, WI, USA).

The PCR mixture was prepared in a final volume of 50 µl with the addition of the primers at a concentration of 200 nM, 5 U of Platinum High Fidelity Taq Polymerase (Invitrogen[®]), 200 µM of each dNTP (Invitrogen[®]), 1X of high fidelity PCR Buffer (Invitrogen[®]) and 8 ul of DNA. In the first round HB-F1 forward primers (1100-1126 5'-> TTCGCCAACTTACAAGGCCTTTCTCT-3'>) and

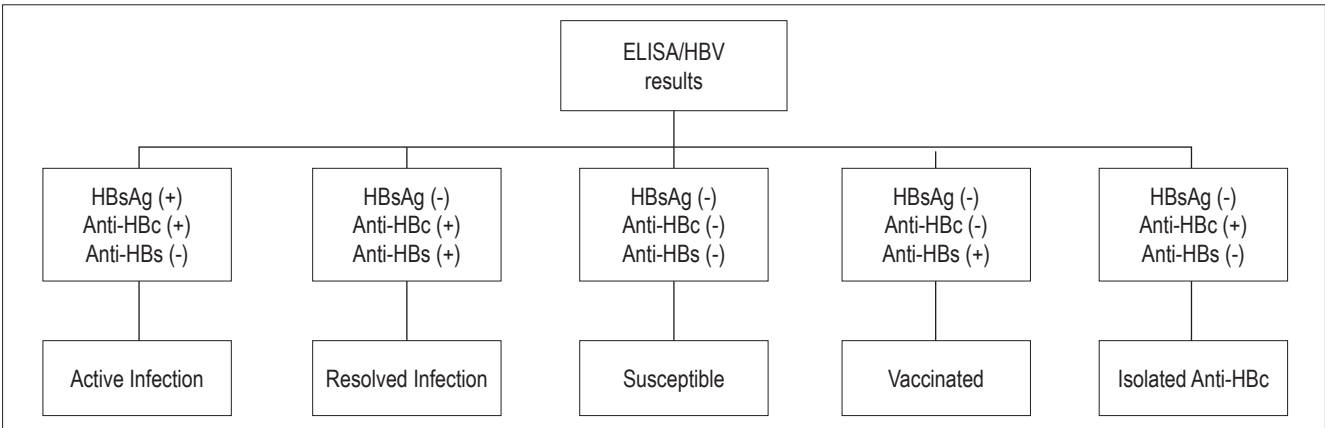


Figura 1. Algorithm of HBV infection stages according to serological markers. ELISA: Enzyme-Linked Immunoabsorbent Assay. HBV: Hepatitis B Virus. HBsAg: HBV surface antigen. Anti-HBc: total antibodies against HBV core protein. Anti-HBs: antibodies against HBV surface antigen.

HB-R1 reverse primers (2326-2304 5'-GAGTGC GAATCCACACTCCAAA-3') were used for an expected product of 1226 bp. The amplified program consisted of an initial cycle of denaturation at 94°C for 2 minutes, followed by 40 cycles at 94°C for one minute, alignment at 57°C for 30 seconds and extension at 68°C for 2 minutes with a final cycle at 68°C for 5 minutes. During the second round of PCR primers, forward inner HB F2 primer (1228-1247 5'-ATCAGCGCATGCGTGGAACC-3') and reverse inner HB R2 (1854-1877 5'-TTGGAGGCTTG AACAGTGGGACAT-3') were used with the same cycling conditions as in the first round except that alignment was at 61°C. This was amplified to 649 base pairs.

The PCR product was resolved in agarose gel at 1.3% and visualized by UV light after samples had been stained with ethidium bromide. We employed the same positive and negative control sera mentioned above as controls for all PCR stages. The detection limit of viral DNA using in-house nested PCR corresponded to 120 copies/ml of serum provided by the COBAS® AmpliPrep / COBAS TaqMan® HBV® Test, v2.0 kit.

Ethical Issues

Students were invited to participate voluntarily in the study and were clearly informed of its objectives, risks, benefits and the confidentiality of results. Each participant was registered with a code with names omitted. Permission to use information for research purposes in this and subsequent studies related to the issue was registered by signing the informed consent form. The research protocol and informed consent form were reviewed and approved by the Ethics Committee of the University of Santander in accordance with resolution No. 008430 of October 4, 1993 of the Ministry of Health of Colombia.

Data Entry and Analysis

All data obtained was registered in an Excel database (Microsoft Office, 2007) and the analysis was performed

using Stata 10.0 software (Stata Corp. 2007. Stata Statistical Software: Release 10. University Station, TX: StataCorp LP). We calculated the proportions of the frequencies obtained for demographic and laboratory data. The frequencies for each stage of infection were established according to the serological interpretation previously described. For each frequency observed we established a 95% confidence interval.

RESULTS

The final sample consisted of 1,298 students proportional to each of the universities. The female: male ratios were 1.57 at the Universidad Santo Tomás de Aquino; 0.47 at the Universidad Industrial de Santander, 1.07 at the Universidad Autónoma de Bucaramanga; 1.58 at the Universidad de Santander, and at the 0.41 Unidades Tecnológicas de Santander. In total 47.3% were women and 52.7% were men. Participants ages ranged from 18 to 39 years old (average age 21 years, SD 3.01) (Table 1).

According to the ELISA results, active HBV infections were present in 0.15% of the population, resolved infections in 0.61% and isolated anti-HBc in 1.1% of the population. In total, 1.85% (n = 24) of university students have had previous contact with HBV. In addition, 30.2% (n = 392) of the participants presented anti-HB antibodies titers resulting from vaccinations which were greater than or equal to 10 IU/L while 67.9% (n = 882) had no serological markers identifiable with the serological tests employed and were defined as susceptible (Table 2). In conclusion, we observed a low prevalence of HBV infection and antibodies from vaccination with a high proportion of students susceptible to acquiring the virus.

Molecular testing by nested PCR allowed confirmation of results of active infection in the 2 HBsAg(+)/ anti-HBc(+) serum determined by ELISA. However, no HBV genome presence was detected in the 14 samples that were positive for isolated anti-HBc nor was any detected in the 8 resolved infections nor in the 10% (n = 88) of the three negative serological markers (Table 2). In conclusion, we found 0% prevalence of OHB in the samples analyzed.

Table 1. Distribution of students participating in the study according to university in the city and metropolitan area of Bucaramanga.

Institution	Participants n(%)	Average age (range)	Female n(%)	Male n(%)
Universidad Santo Tomás de Aquino –USTA–	268 (20,7)	21 (18-38)	164 (61,2)	104 (38,8)
Universidad Industrial de Santander –UIS–	263 (20,3)	22 (18-36)	84 (31,9)	179 (68,1)
Universidad Autónoma de Bucaramanga –UNAB–	259 (19,9)	21 (18-33)	134 (51,7)	125 (48,3)
Universidad de Santander –UDES–	256 (19,7)	22 (18-34)	157 (61,3)	99 (38,7)
Unidades Tecnológicas de Santander –UTS–	252 (19,4)	22 (18-39)	74 (29,4)	178 (70,6)
Total	1298 (100)		613 (47,3)	685 (52,7)

Table 2. Frequency of HBV infection stages in the analyzed sample.

Stage	Frequency	
	n (%)	IC95 ^a
Active Infection	2 (0,15)	0,019-0,55
Resolved Infection	8 (0,61)	0,26-1,20
Isolated Anti-HBc	14 (1,1)	0,59-1,80
Vaccinated	392 (30,2)	27,71-32,78
Susceptible	882 (67,9)	65,33-70,48
OBH	0 (0%)	
Total	1298 (100)	

^aIC95: 95% confidence interval.

The numbers of participants who tested positive for active infections and isolated anti-HBc stages were similar for both men and women except for resolved infections (37.5 and 62.5% respectively). Overall, the average age range for men was 21 to 26 years, while for women it was 19 to 25 (Table 3).

Table 3. Demographic characteristics of students with HBV infection markers.

Type of Infection	Male		Female		n total
	n (%)	Average age in years	n (%)	Average age in years	
Active	1 (50)	26	1 (50)	19	2
Resolved	3 (37,5)	21	5 (62,5)	25	8
Isolated Anti-core	7 (50)	21	7 (50)	23	14
Total	11 (45,8)		13 (54,2)		24

Out of the total study population, 882 (67.9%) were positive for evaluated serological markers (susceptible stage) while 392 (30.2%) were confirmed to be vaccinated against HBV by ELISA testing. The average ages and sexual ratios were similar for both groups (Table 4).

Table 4. Características demográficas de estudiantes vacunados y susceptibles.

Gender	Susceptible			Vaccinated		
	n (%)	IC95 ^a	Average age (SD) ^b	n (%)	IC95 ^a	Average age (SD) ^b
Male	487 (55,2)	51,86- 58,53	22 (3,0)	187 (47,7)	42,66-52,77	20,9 (2,49)
Female	395 (44,8)	41,47- 48,14	21 (3,0)	205 (52,3)	47,22-57,33	20 (2,0)
Total	882 (100)			392 (100)		

^a IC95: 95% Confidence Interval.

^b SD: Standard Deviation.

DISCUSSION

This study investigated the prevalence of serological infection markers in 1,298 students from five universities in the city of Bucaramanga and its metropolitan area during the period between July and November 2010. Demographic variables such as gender and age were analyzed as well as positive tests for three HBV serological markers, anti-HBc, anti-HBs and HBsAg as determined by ELISA. In addition, two nested PCR protocols were implemented to investigate OHB frequency in cases that were positive for isolated anti-HBc and in the 10% of cases that were negative for all three markers.

To our knowledge this is the first epidemiological study to determine the prevalence of hepatitis B among university students in our region. Overall, the 1.1% anti-HBc and 0.15% HBsAg prevalence were lower than those previously described in the department of Santander by others (12, 16, 33). However, data collected in those studies is mainly related to the blood donor population which consists of symptomatic cases reported to the public health surveillance system (SIVIGILA) and the general population.

It is likely that the level of education and socioeconomic status which have raised awareness of safe sex practices have roles in protection against HBV infection. In a cohort of adolescent students in the Central African Republic there was a 15.5% prevalence of HBV infection associated with sex without the use of condoms, poverty and low levels of education (40). Age and sex were associated with infection or immunity to the virus by vaccination as observed in the present study.

Our results contrast with the findings of a recent epidemiological study in four Colombian departments. That study evaluated HBsAg and anti-HBc serological markers to determine the prevalence of hepatitis B in the general population. The findings showed anti-HBc prevalence of 54.55 in Amazonas, 18.38 in Chocó, 3.95 in San Andrés and 31.69% in Magdalena. HBsAg prevalences reported were 7.95, 3, 70, 1.97 and 8.39% in the same order. The

average frequency of anti-HBc and HBsAg for these four departments was 28.43% and 5.66% respectively which confirms that our country is a buffer zone for HBV prevalence as defined by WHO and others (5-7).

The low prevalence of HBV infection in the university population is an interesting fact that demonstrates the importance of screening for HBV in different risk groups to define local public health policies. This screening strategy for risk groups in Australia has been implemented in order to channel resources to populations with higher HBV infection prevalences and to reduce the community health care burden in the country (41).

In other studies of the prevalence of hepatitis B among university students, the HBsAg serological marker has perhaps been evaluated more than any other marker. Data reported for this marker among students from Peru, Japan, Albania, China, Korea and Taiwan have been in the range of 2.5% to 25% (42-45). Nakamura, in a sample of 162 students from Tohoku University in Japan, and Ramirez et al. in a study of 240 students from three universities in Peru (42,44), both found prevalences of HBsAg of 2.5%. The prevalences in those two countries and periods of time were higher than those determined in this study in which only 0.15% of students presented active infections.

Carvalho and colleagues recently reported a 0.5% prevalence of hepatitis B determined by reactivity to anti-HBs and anti-HBc in a sample of 652 students aged 17 to 48 years in the health area at the Federal University of Bahia, Brazil (46). These results do not differ greatly from those found in this study where the prevalence of anti-HBc was 1.1% and the sample included participants from all the major areas of study offered by the institutions of the city of Bucaramanga.

A survey of 198 medical students (ages 18 to 30 years) in Peru established that 35.4% had complete vaccination (47). This percentage was similar to that reported in this study (30.2%). However, in our case the finding is supported by the presence of anti-HBs protective antibodies by ELISA in the absence of a vaccination card which was not required as a condition to participate in our study.

In 2008, de la Hoz and colleagues published a paper on the impact of recombinant vaccines against HBV in Colombian children during eight years since its inclusion in the PAI (Plan Ampliado de Inmunización – Broad Immunization Plan) (48). As anticipated, the study showed a 60% to 75% overall reduction in the prevalence of HBV infections and a vaccination coverage of 91% in a representative sample of 2,145 children aged 1 to 12 years of age obtained from 4 regions of the Amazon basin which is an area considered to have high HB prevalence. In contrast to this, there is lower HBV vaccination coverage among Colombian adults. Recently, the work of Alvarado-Mora et al. (2011) showed

a frequency of anti-HBs antibodies from vaccination of between 15.3% and 32.88% among adults. These numbers are similar to those observed in the present study in which 30.2% of university students had levels of protective antibodies against HBV as the result of vaccinations of 10 IU/L or higher.

Currently there is a consensus on the protection against infection conferred by recombinant HBV vaccine even in the absence of anti-HBs protective antibodies (49). Similarly, it is known that the vaccine protects against acute and chronic liver disease even in individuals with anti-HBs titers lower than 10 IU/L. The contrasting results of low prevalence of HBV infection in our study and the previously reported intermediate prevalence by Alvarado-Mora et al. (2011) combined with the similar rates of vaccination coverage in both studies confirms that other factors besides vaccinations may have an important role in protection against HBV infection (17). For university students in the area of health care, vaccination against hepatitis B is a prerequisite for advancing their practice due to the risk from contact with patients' bodily fluids. For this reason it is common to find that these people present immunity to the virus (50, 51).

Occult HB is an unusual form of HBV infection that causes a dissimilar pattern of serological markers. Experts have defined it as the presence of HBV DNA in liver tissue in the absence of HBsAg in peripheral blood independently of the presence or absence of serum viral genome (30). In healthy subjects occult HB represents an elevated risk of chronic disease and further transmission of the virus mainly during voluntary blood donation (52,53).

A study of blood donors showed that 22 out of 55 individuals who received blood products from 5 cases of OHB acquired the infection. Of these, three recipients in a state of immunosuppression developed sudden hepatic failure (54). A longitudinal study of cirrhotic Japanese patients recently demonstrated that OHB increases the risk of developing HCC 8.25 times (29).

Considering that a significant number of units of blood in Colombia come from donation campaigns in universities, we decided to investigate occult HB in this population. Our results of 0% prevalence were expected considering the low frequency of positive cases of active HBV infections among students. In Venezuela, a study with 2,075 blood donors with isolated anti-HBc showed the same result (34). Also in Colombia, Beltran and colleagues did not detect the HBV genome in 129 serum samples from blood banks with the same serological profile (55). However, in both cases pooled sera was used to detect the viral genome, which eventually could reduce the sensitivity of molecular biology tests considering that occult B presents very low viral loads (56).

This research used an extraction kit and two nested high sensitivity PCR assays. However, one of the limitations for identifying occult HB was the low number of individuals who had isolated anti-HBc and that were analyzed with these tests.

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Conflict of interest statement

The authors declare that they do not have any direct or indirect conflict of interest in any academic, scientific, financial or personal matter related to the publication of this manuscript.

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