**Author’s response to reviews**

**Title:** Nearly half of Ultrio Plus NAT non-discriminated reactive blood donors were identified as occult HBV infection in south China

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**Author’s response to reviews:**

Dear Dr. Antoinette C Van Der Kuyl:

Ref: manuscript INFD-D-19-00121 entitled “Nearly half of Ultrio Plus NAT non-discriminated reactive blood donors were identified as occult HBV infection in south China”.

We would like to re-submit the new version of manuscript within one month as the journal required. According to reviewers’ and your comments and questions, the manuscript was carefully revised in the new version with changes highlighted. The explanation or answer has been addressed as indicated below under each question point by point.

If you have any more question regarding to this manuscript, please let me know.

Yours sincerely,
Response to reviewers:

Jean-Pierre Allain (Reviewer 1):
The manuscript by Ye et al reports on the difficulties introduced by a NAT triplex manufacturer with a two-step assay first with a ubiquitous signal followed by a specific discriminatory assay less sensitive than the triplex. This topic has been examined by several previous publications including two from China. It is therefore not novel and the data presented essentially confirm prior results.

Answer: we agree with reviewer comments. The relative papers has been published previously. However, during past years we accumulated more new data and analyzed them through a new direction for interpreting the NDRs we met in blood donation screening. The assay used is less sensitive than NAT triplex, but still detected a part of HBV DNA by chance, which indicated that NDRs were partly OBI and the data were helpful for dealing with the results of blood screening with NAT.

The main problem with this manuscript is that although the data appears solid, the presentation is not logically constructed and the order of text and tables makes the data unclear and difficult to follow.

Answer: the manuscript has been carefully revised in the new version.

1. In Materials and Methods, it should be made clear that the third HBsAg assay used to qualify NDRs is different from the ELISA used in screening.

Answer: We are thankful for the reviewer’s suggestion. Section “2.2. Supplemental serological testing” (Line 119) was added to make clear about the assays used to retest for HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc of suspicious or non-discriminators samples.

The authors should include a section regarding 'confirmation' of initial reactive NAT. Is it Ultrio Plus and either qPCR or NPCR positive? This reviewer considers it is confirmatory but, the importance of sensitivity of both confirmatory assays should be clearly established by indicating what amount of the 2.5ml extract is used in each assay relative to the 0.5ml of Ultrio+ and what is the limit of detection of these 2 confirmatory assays.

Answer: A section of confirmation of initial reactive NAT was added as “2.3. HBV DNA confirmation of NDRs” (Line 126). HBV DNA was extracted from 2.5mL of plasma with all NDRs by HighPure Viral Nucleic Acid Large Volume Kits (Roche Diagnostics GmbH), and were confirmed by a combination of qPCR and nested PCR amplifying the BCP/PC and S regions. Samples reactive for any test were confirmed as HBV DNA positive. The LOD of the qPCR assay and Nested PCR were 5 and 10 IU/ml (in combination with 2.5mL extraction of plasma, LOD were 2 and 4IU/per sample individually).

In the DNA sequencing section, the size of each amplicon should be given. It should also be mentioned that for genotyping only S sequences are informative.

Answer: The size of the amplicons BCP/PC (295bp) and S regions (495bp) was added in section 2.4 (Line 139). Genotype determination was performed according to S sequences by phylogenetic analysis using the MEGA5.1 program, which was informative for genotyping.

There is no section regarding ethical approval.
2. In the result section, Table 1 should be presentation of serologic data currently as Table 3. The data currently in Table 4 regarding titration of anti-HBs should equally be part of that section as Table 2. Table 1 should present a complete picture of the serological markers versus NAT confirmatory including all 421 samples, stratified into confirmed HBV DNA positive and negative (not confirmed).

Answer: Thanks for your suggestion in detail. According to your guidance, we adjusted the presentation order of these tables. The original Table 3 was changed to Table 1 to present a complete picture of the serological markers. Table 4 was renamed as Table 3 regarding titration of anti-HBs and be part of section 3.4 as Table 1. The primary Table 1 was improved according to your next suggestion and rename as table 4. Since the total number of NDRs was 259, not including HBV NAT yielded 162 samples, we put the data of 121 NDRs with confirmed HBV DNA and the rest 138 unclassified in Table 1.

The section 3.3 should be divided into 2 subsection: 1) confirmatory with Table 2 (currently Table 3) and 2) overall data related to sequencing. The latter should present in Table 4 (currently Table 1) samples with both S and BCP/PC sequences, BCP/PC only and S only. It might be then useful to add in this table the number of sequences in each group that presented 1727 mutations, 1762-1764 mutations and most importantly 1896 mutations and combinations of mutations in those 3 regions. In that respect, it would be useful to indicate which 1896 mutated samples were anti-HBe positive.

Answer: The authors thank you very much for the kind guidance.

1) We revised section 3.3 and the related new Table 2.

2) According to your suggestion, the new Table 4 was made to give the number of BCP/PC only, S only, and both S and BCP/PC positive samples obtained from different serological groups, in addition presenting the number of BCP/PC mutations T/G1727A/C, A1762T/G1764A, and G1896A in each group. The mutation analysis with serological patterns was added to section “3.6. Mutation analysis on the BCP/PC in HBV DNA+ donations”.

Section currently 3.5 should also be divided into 2 subsections 1) genotyping and 2) MHR or outside of MHR mutations. In this section, the authors take a group of mutations as 'vaccine related' without justifying their choice by references. To my knowledge, only the G145 aa is clearly related to vaccination and appears in this data as the most frequent substitution. A second group of substitutions should be those demonstrated as interfering with HBsAg assays (supported by adequate references). In that respect, the authors did not mention that contrary to many reports on OBI, their sequences did not include mutations of P120 or any C 121, 124, 137, 139, 147, 149 which are shown to have a major impact on HBsAg detection. They did not either find substitutions at aa 75, 100 or 178, shown responsible in OBIs for lack of excretion of HBsAg.

Answer: Thank you. We rearranged the structure of the results. The genotyping of NDRs according to S genes was renamed as section 3.5. The analysis of MHR or outside of MHR mutations related to vaccination, HBsAg detection and excretion was renamed as section 3.7. According to our data obtained from Shenzhen and Guangzhou, the OBI with genotype B and C were mostly wild-type and less frequency of critical mutations were found in S region of strains. Adequate references supporting these meaningful substitutions were provided.

The discussion should be structured around key questions raised by this and previously published data on the same topic:

1. Critical importance of design and sensitivity of screening assays. Increased sensitivity with the 2-stage assay carries increasing difficulties in confirmation (13% with Ultro and 57% of NDR with Ultro Plus in New Zealand). Note that this problem has been eliminated by the latest Roche assay
directly identifying viruses.

Answer: Thank you very much for sorting out the discussion in detail. We emphasized the critical importance and the problems arising from the NDR results. We add “Being dependent on the same configuration and the same reagents as the screening assay, the NAT assay cannot qualify for confirmation [25]. By this strategy, lots of non-discriminators appeared.” in the paragraph 1 of Discussion. And “For the increased sensitivity with the 2-stage NAT assay carried increasing difficulties in confirmation, even further evaluation by repeat testing or with an alternative NAT assay didn’t resolve all cases.” in the paragraph 7 of Discussion.

Algorithm for confirmation. There are several publications advocating repeat testing with the screening assay from the plasma bag (not addressed). Alternative assay as done here is another approach highly dependent on respective sensitivity of assays and choice of regions amplified. Pros and cons should be discussed.

Answer: Yes, we totally accepted. The confirmation of low viral load HBV DNA such as NDRs relies on HBV-NAT assays as well as the amount of samples modified to improve sensitivity. Repeat testing with the screening assay from the plasma bag was used in Europe and South Africa in an attempt to discriminate between false and true screening results on the primary test tube and increase the chances of the presence of HBV DNA, but cannot be used for confirmation of HBV infection [25]. This study adopted standard nested PCR method targeting different regions of the genome and qPCR with the relatively short amplified region for NDRs’ HBV DNA confirmations. However, these amplification methods may lack the sensitivity required for appropriate confirmation of the screening results with the current triplex NAT blood screening assays. Ultracentrifugation of large volumes of plasma was the first choice in low HBV endemic area [13, 25]. Considering the relatively high background of extremely low level HBV in the anti-HBc+ qualified blood donors in China [28], increasing the volume of plasma extracted from 0.5ml of the screening method input volume to 2.5ml was proved to be the best choice, highly provided good sensitivity and succeeded in identifying nearly half of the non-discriminators as OBIs by nested PCR or alternative qPCR in this study. This part was added in the paragraph 2 of Discussion.

Usefulness of serology. Here anti-HBc is not helpful contrary to other publications from China and from other parts of the world. This 'anomaly' requires discussion.

Answer: Thank you. Complementary serological testing of index and follow-up donations might not be sufficient to confirm the infection of non-discriminated donations due to anti-HBc background prevalence especially in high-endemic areas and when there were discrepancies between serological and molecular markers [3]. Additionally, in China where the prevalence of anti-HBc exceeds 40% of the donor population, a positive anti-HBc assay does not provide a reliable adjunct in deciding between a true and false positive HBV DNA result [25]. This was added in the middle part of paragraph 4 of Discussion.

What to do with and tell the donors? The authors do not consider in their analysis that serology negative may well be 'primary OBI' rather than window period.

Answer: Thanks. We add “Deferred donors can be informed to the effect that WP has 95% chance of resolving spontaneously and that this needs to be verified by a physician.” in the end of paragraph 3 of Discussion.

Implications for transfusion risk. It was clearly shown (ref 21) that Ultrio Plus negative donations can be infectious. Therefore NDR not confirmed but anti-HBc positive might be a safety risk. Only detection of anti-HBs >100IU/L appears protective.

Answer: Thank you for your revision. “Because it was clearly shown that Ultrio Plus negative
In conclusion, this manuscript needs to be entirely re-written to make good data palatable to the readers.

Answer: We benefit the reviewer’s guidance a lot. The manuscript has been entirely re-written. Thank you very much!

Angelita Silva de Miranda Corrêa (Reviewer 2):
The manuscript is relevant for public health, since it addresses the issue of transfusion safety, being a current problem and of worldwide interest. However you need some adjustments, listed below:

1. Line 188. Replace "Fig.2" by "Figure 2".
   Answer: Thank you. We revised the format of the whole manuscript.

2. Line 250 "In this study, the percent of anti-HBc reactivity among non-discriminated donations (91.1%) was significantly higher than that was found in a random group of donors in a previous study (P<0.001) [19]." Include the percent of anti-HBc reactivity of previous study (of ref. number 19)
   Answer: The previous study [now 28] detected 47.5% anti-HBc reactivity in a random group of donors, which indicated the background anti-HBc reactivity, so 91.1% anti-HBc reactivity among non-discriminated donations at this study suggested high rate of HBV infection in the non-discriminated donations. We included the number in the manuscript (Line 274-276).

3. Line 262 "Multiple studies estimated OBI transmission rate for all components varied between 3% and 48% [4,20]." The author uses the term "multiple studies" and cites only two references. Add more references or remove "Multiple".
   Answer: Thanks. We removed the word “Multiple” (Line 307).

4. Tables
   Tables 1 and 2: The symbol "%" on top of the column "HBV DNA +" should be removed.
   Table 4. Remove the IU/L from the first column and place on top of the following columns.
   Answer: Thank you. The symbol "%" on top of the column "HBV DNA +" actually indicated the number in the table were the percentage of 259 NDR donations. To make it clear, we made a revision in the new Table 2 and place it on top of the following columns. In the new Table 3, which was previously Table 4, we made the same revision as you suggested.

5. The author should describe the limitations of this study. It is possibility of underestimating.
   Answer: Thanks for your suggestion. We add the limitations of this study in the paragraph 7 of Discussion.

6. Which HBV genotype was dominant in China?
   Answer: In South China, genotype B is more prevalent, while genotype C is mainly distributed in North China. In Hongkong, a city in close vicinity to Shenzhen, China, genotyping studies of HBV revealed that genotype C was the commonest (62.6%) and was followed by genotype B (32.5%) in chronic hepatitis B patients. Shenzhen is one of the pilot cities, which has a population migrated from most provinces of China, making it representative of the whole situation in China in a certain extent.

7. The author mentions in the text that China has high prevalence of HBV but does not cite values.
Include in the text this information. In addition, the author describes that in China the presence of the anti-HBc marker is not a reason for bag discarding. However, Brazil, also considered high country endemicity, the anti-HBc marker is used for the discard of the bag and permanent refusal of the donor. The author could discuss this issue as well as describe and compare it with other countries of high endemicity.

Answer: Thanks. We add “HBV has been highly epidemic in China, where epidemiological studies showed about 10% prevalence of HBsAg in general population in 1992.” in the second paragraph of introduction.

Screening for anti-HBc would have identified and excluded almost all the cases of infectious OBI donors reported. In Brazil, which was also considered high country endemicity, the anti-HBc marker was used for the discard of the bag and permanent refusal of the donor, while the prevalence of anti-HBc was 2.05%-6.12% in Brazil blood donors [29]. However, in Shenzhen, China, screening for this marker would exclude more than 40% of blood donors and impair blood supply [28]. This part was added in the start of the paragraph 3 of discussion (Line 298-303).

Syria Laperche, MD PhD (Reviewer 3):

This study presents the results of investigations performed in Shenzhen blood setting in HBV/HCV/HIV multiplex NAT initially reactive blood donations, seronegative for markers included in the routine screening (HBsAg, HIVAb and HCV Ab) which resulted negative when further tested with discriminatory NAT assays (n=259).

Authors show that 91% (236/259) of these NDR donations were anti-HBc positive and 47% (121/259) had HBV DNA when tested with alternative amplification methods (classified as OBI). On remaining 53% (n=138), 89% (123) had anti-HBc alone or associated with HBsAb

This study is certainly helpful to adopt an adapted strategy to avoid HBV transmission by transfusion from OBI donations since anti-HBc cannot be systematically tested in China due to the high HBV infection prevalence and helps to define a reliable algorithm to permit non-infected donors to come back to donate.

General comments:
- The paper is difficult to read especially because results are presented several times in different ways ex: anti HBc positive are detailed in section 3.2 then 3.3 ; anti HBs in section 3.2 and 3.4... The presentation according the flow showed in figure1 should clarify the text and lead to a better understanding.

Answer: Thanks for your very nice suggestion. We adjusted the presentation of the results and Table 1-4 as in the revised manuscript. It could be given a better understanding.

- 53% of NDR were inconclusive as HBV DNA was not detected. Although nearly 90% of these samples were anti HBc pos, suggesting that HBV infection could be the cause of them, HIV or HCV early infections with low viral loads cannot be totally ruled out. Donor follow up (not performed) would have helped to conclude.

Answer: Yes, thanks. Before 2014, we screened nearly 400,000 donations, and only 1 WP of HCV, no HIV WPs was found by follow up study [ref 10]. Since 2014 until now, we have screened nearly 500,000 donations, no HCV WPs, only two WPs of HIV were found. We added the limitations of the study with no follow up in the paragraph 7 of Discussion.

- Except the 121 NDR donors who have been confirmed HBV DNA positive by alternative PCRs, no confirmation of NDR samples has been presented: were Ultrio Plus initially positive repeated?

Answer: No, we just studied NDR donors with the Ultrio Plus initially reactive, dHBV-. Repeat testing with the screening assay from the plasma bag is used in Europe and South Africa in an attempt
to discriminate between false and true screening results on the primary test tube and increase the chances of the presence of HBV DNA, but cannot be used for confirmation of HBV infection [25]. We discussed about it in the paragraph 2 of Discussion.

- Were anti-HBc positive results confirmed?
  Answer: Yes, thanks. Section “2.2. Supplemental serological testing” was added to make clear about the assays used to retest for HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc of suspicious or non-discriminators samples. Anti-HBc reactive samples were re-tested by a domestic EIA kits (Wantai Diagnostics, Beijing, China).

- Were presence of mutations confirmed with another sequencing?
  Answer: Yes, some important mutations were cloned for further proving.

- Authors showed that 91% of NDR donations were antiHBc positive, is this rate the same in blood donors negative for all viral markers?
  Answer: No, our previous study [ref 28] detected 47.5% anti-HBc reactivity in a random group of donors, which can be regarded as the background rate (Line 274-276).

- 121 donors have been classified as OBI: were the VL determined?
  Answer: Yes, they were determined. Of 82 (31.7%) qPCR+ donations, the maximum and median viral loads were 65.2IU/ml and 7.9IU/ml, respectively. 24 (29.3%) presented VL<LOD. The data were added in line 191-193.

- Authors did not clearly claim whether observed mutations are responsible for OBI.
  Answer: Thank you for your suggestion. We indicated the OBI related mutations in Table 5 with *.

- Follow up results to confirm OBI should have been useful
  Answer: Yes, we agree. Since no follow up was performed, we added the limitations of the study in the paragraph 7 of Discussion.

Specific comments
- Introduction: refer to Candotti et al GUT 2009 to show that OBI are potentially at risk in transfused patients
  Answer: Thanks. We refered to it in Line 54 of introduction.

- Line 107: please mention if IR samples with multiplex NAT were repeated
  Answer: Thanks. We indicated in Line 111 “The initial reactive samples with multiplex NAT were repeated testing with discriminatory Procleix Ultrio plus test to identify the virus responsible for NAT reactivity (HBV, HCV or HIV-1)”. In China, IR samples were further tested with discriminatory Procleix Ultrio plus test, while in Europe it will be repeated again.

- Line 114: HBeAg and HBeAb results were not provided
  Answer: No HBeAg+ only samples was found. And the distribution of anti-HBe+ samples was
indicated in Table 1’s footnote.

- line 118 : give the LoD of qPCR . Why VLs were not provided? Clearly clarify which samples have been tested with qPCR?
  Answer: Thank you very much for your suggestions. We revised the presentation more clearly. The LOD of the qPCR assay and Nested PCR were 5 and 10 IU/ml (in combination with 2.5mL extraction of plasma, LOD were 2 and 4IU/per sample individually) (Line 131-133). The VLs were provided in Line 191-193. All NDR samples were tested with qPCR (Line 127-130).

- Line 138 : 215 samples were HBsAg pos only. This is a surprisingly high number, knowing (i) the expected high rate of chronic carriers who should have been NAT pos and/or HBcAb pos and (ii) the high sensitivity of NAT. Have these samples been confirmed HBsAg pos?
  Answer: Yes, it’s a high number due to dual EIA kits screening in SZBC, thanks for attentions. We have been detected about 157 HBsAg EIA pos only samples, 76 were confirmed HBsAg+. Sequencing was undergoing. In Europe, HBsAg pos only was rare, which might be related with viral mutations, because frequency of HBV mutations was Asia>Africa>America>Europe. Reference: Gencay M, et al., Ultra-deep sequencing reveals high prevalence and broad structural diversity of hepatitis B surface antigen mutations in a global population, PLoS One 12 (5) (2017) e0172101.

- Lines 161-169 : difficult to understand "68 donations of 82 qPCR pos" 82 sequences : I assume that some samples have been amplified in 2 regions? Please clarify
  Answer: Thank you. We made a revision: BCP/PC and S genes were amplified from 259 NDR samples by nested PCRs, 68 donor samples (OBIs) generated 82 HBV sequences, in which 46 S sequences and 36 basic core promoter (BCP)/precore (PC) sequences were included. (Section 3.5, Line 219)

- Line 172 : Have the 2 donors classified as WP been followed up be to attest that they were early infections?
  Answer: Although the two WPs had been detected repeatedly and got concordant results excluding contamination, we failed in following up the two donors. We discussed this in the 7th paragraph of Discussion (Line 400). 1 WP had sequence, another one we tested it with MPX2.0 ID HBV-NAT, and 1/3 times showed positive.

- Lines 179-190 : were anti-HBs pos donors vaccinated?
  Answer: Most of donors born after 1992 were neonatally vaccinated if they had anti-HBs.

- Lines 211-232 : were strains with S mutations affecting HBs detection controlled with an alternative HBsAg assay ? if positive they must be excluded from OBI classification
  Answer: Yes, two cases were alternative HBsAg assay positive, which were excluded from OBIs.

- Tables 1-2 to be merged
  Answer: Thanks. We merged Table 1 and 2 to be the new Table 2.