**Author’s response to reviews**

**Title:** Co-infection of human parvovirus B19 with Plasmodium falciparum contributes to malaria disease severity in Gabonese patients

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**Author’s response to reviews:** see over
Dear Editor,

please find enclosed our revised manuscript (MS: 1663982090876671) “Co-infection of human parvovirus B19 with Plasmodium falciparum contributes to malaria disease severity in Gabonese patients” by N. L. Toan et al.

which we would like to have considered for publication in the BMC Infectious Diseases.

Thank you very much for reviewing our manuscript and for the comments and suggestions of the five reviewers which were very helpful. We have addressed and carefully considered the comments from all reviewers point by point and made amendments to the manuscript accordingly. Specific replies to each reviewer comment are detailed in the “Answers to the editor and reviewer comments” letter.

We hope that the reviewers find the changes agreeable. We believe that our data are of interest for the general readership of BMC Infectious Diseases and thus trust that our findings will be now considered suitable for publication in the BMC Infectious Diseases.

Thank you very much for your time and consideration

Sincerely yours,

Prof. Dr. C.-Thomas Bock and Prof. Dr. P. Kremsner
Replies to the Editor and reviewer comments.

Thank you for your time spent on this manuscript and your constructive and valuable criticisms.

Editor’s comment:

Please remind overdue reviewer!

Reviewer #1: Svetoslav N Slavov

Human parvovirus B19 (B19V) is an ubiquitous virus throughout the human population and its clinical impact depends on the clinical and immunological status on the infected hosts. In this relation, studies examining the co-infection between B19V and the malarial plasmodium are of particular importance, where both virus and parasite could influence the outcome of the severe anemic state. In this aspect the manuscript brings significant positive value, examining the DNA prevalence and the outcome of the co-infection Plasmodium spp./B19V in children from Gabon. It also brings very interesting genotyping data, demonstrating the B19V genotype 1B in Gabon, which is considered an “Asian” genotype because of its characterization in Vietnam. The characterization of B19V genotype 2 is also of significant value, due to recent discussions that it is disappearing from circulation in the Northern hemisphere and is being substituted by the genotype 1B, which has very extensive worldwide distribution. I was surprised that genotype 3 was not detected among children in Gabon, once it is with typical African endemicity. However, regional circulations of B19V in the African continent with its extensive territory could have influenced the spread of B19V genotype 3 in Gabon. However, the manuscript suffers from some shortcomings which I suggest to be reviewed by the authors.

1. The scientific English should be revised by a native speaker. I strongly advise a correction due to the extensive use of words, the incorrect use of articles, the inadequate conjunction of verbs, where conjunction is necessary etc. This only will bring a positive value to the manuscript and clarity to the reader.

Reply: A native English speaker has carefully checked the scientific English of our manuscript. We have made changes accordingly.

2. Background:

2.1. The word “poverty” in the beginning is not used rightly. I suppose that the authors meant “poor”, but nevertheless, this is a very strong word that cannot be used in a scientific context. Malaria was a widely spread disease, even until recently, when large areas of the Southern Europe, South America, Central and Southeast Asia, were devastated by continuous epidemics and foci of the disease. I suggest to use only the words “undeveloped countries”. Moreover, the initial phrase is not very clear, and I suggest to clarified or split into two phrases.

Reply: Thank you for this valuable criticism. We have rewritten the first paragraph of the background omitting the critical phrases “poverty” and “underdeveloped countries” as suggested by the reviewer. We also split the initial paragraph into two. (page 3, 1st paragraph)
2.2. The genus of B19V is Erythrovirus and not Erythroviruses.

Reply: We have corrected the term “Erythroviruses” to “Erythrovirus” as suggested by the reviewer. (page 3, 3rd paragraph, first sentence).

2.3. It could be mentioned that B19V causes a wide spectrum of clinical diseases, but that they depend on the hematological and the immunological status of the host. In this relation the authors should clarify which are the conditions that B19V induces in healthy subjects and which are induced in patients with altered immunity and hematopoiesis.

Reply: We agree with the reviewer that the wide spectrum of clinical manifestations of B19V depend on the hematological and immunological status of the host. We have specified the clinical outcome and associated diseases according to the suggestion of the reviewer. (page 3, 3rd paragraph, last sentence)

2.4. I did not understand is there any relationship between B19V and malaria in the study from Niger. If, yes please specify in the sentence. For example “in severe anemia induced by Plasmodium spp.” etc.

Reply: The study by Jones et al. (J Trop Med Hyg 1990, 93(1):67-70) revealed that B19V-infection elevated severe anemia caused by P. falciparum among young children in the Republic of Nigeria. A direct association between malaria and B19V was not reported. However, the study was performed in a high malaria endemic region. We included a sentence according the reviewers comment that “B19V-infection has been demonstrated to elevate severe anemia caused by P. falciparum among young children in the Republic of Nigeria”. (page 3, 4th paragraph, first sentence)

2.5. ...in a cohort of P. falciparum infected patients. Please specify in the sentence

Reply: We have specified the patient cohort in more detail now. “In the present case-control study we utilized 282 healthy and P. falciparum infected Gabonese children from sub-Sahara Africa.” (page 4, 2nd paragraph, first sentence)

3. Methods

3.1. In what type of hospital was performed this study? Was it a field study or not, what type of villages it included: tribal or not. This should be clearly mentioned in the Methods section. Moreover, a brief geographic characteristic of the examined region should be provided as the majority of the readers probably do not know where is Lambarene.

Reply: The study took place at the Albert Schweitzer Hospital, in Lambaréné, Gabon, and the Centre Hospitalier de Libreville, Gabon. We have specified the type of hospitals in the Methods, Study subjects: “Patients were recruited at the Albert Schweitzer Hospital, Lambaréné, Gabon, and the Centre Hospitalier de Libreville, Libreville, Gabon. The investigated cohort is from a matched pair, case-control study, to compare severe and mild malaria in Gabon. Details of the study cohort are as

The study was designed as a case-control study. Please, see also in “Study subjects” described above and “Background” section.

Lambaréné is the capital of the political district Moyen-Ogooué in Gabon, west central Africa. The city is located 75 kilometers south of the equator. Libreville is the capital and largest city of Gabon. The city is near the Gulf of Guinea.

3.2. Substitute “pupils” by children or infants.

Reply: “pupils” has been replaced by “children”.

3.3. Substitute the “matching partners” by control individuals.

Reply: “matching partners” has been replaced by “Individuals with mild malaria were chosen from patients of the same sex, age, and locality, and admitted as soon as the severe cases were enrolled for this study”.

3.4. There is no information in the materials section how the B19V serology was performed. Please, include section named “B19V serology evaluation” or similar. In this section, please include the kits, and the methodologies, which were used for the evaluation of the titer of anti-B19V IgM and IgG.

Reply: We thank the reviewer for this comment. We have added a section “B19V serology evaluation” to the Methods as recommended.

3.5. What was the sensitivity of the real-time PCR, when the international standard for B19V was applied (in IU) and how it was compared and extrapolated to genome equivalents. If a formula was used, should be provided. Give a detailed explanation in the further correspondence.

Reply: The sensitivity and lower detection limit of our in-house B19V-specific real-time PCR was found to be $5 \times 10^2$ copies/ml which has been calculated to be $2 \times 10^2$ IU/ml in our test system using the B19V-DNA WHO-standard NIBSC 99/800. We have replaced B19V copies/ml by IU/ml in the Methods, Figures, and Results section.

3.6. Were the obtained sequences submitted to GenBank? Please provide the numbers of the submissions, because they will be used for further analyses, especially for subgenotype 1B.
Reply: The B19V genotype 1A and 1B sequence accession numbers has been denoted in the Methods section, B19V-genotype analysis (B19V genotype 1A: AB030694, AF113323, AF162273, and M13178; B19V genotype 1B: DQ357064 and DQ357065). (page 7, Methods, last paragraph, last sentence)

We have also deposited the sequences of the B19V isolates of this study to the GenBank under the following GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of B19V:

KF309501 (B19V-isolate No: Af24s), KF309502 (Af65m), KF309503 (Af87m), KF309504 (Af77m), KF309505 (Af89m), KF309506 (Af94m), KF309507 (Af81m), KF309508 (Af22m), KF309509 (Af6s), KF309510 (Af20s), KF309511 (21s), KF309512 (28s), KF309513 (Af13s), KF309514 (Af33s), KF309515 (Af53s), KF309516 (Af41s), KF309517 (Af17s), KF309518 (Af10s), KF309519 (Af2m), KF309520 (Af38s), KF309521 (Af37s), KF309522 (Af39s), KF309523 (Af91s), KF309524 (Af34m), KF309525 (Af97m), KF309526 (Af40s), KF309527 (Af35s), KF309528 (Af31m). (page 8, Methods, 1st paragraph)

3.7. What the authors mean by the “Obtained sequences were matched with the NCBI GenBank……” and cite the access number of the B19V strain Au once the authors had genotype 2 and subgenotype 1B. The Au strain is not a reference for the above mentioned genotypes. Please, make sure the adequacy of the prease.

Reply: We have rephrased this chapter and added the correct B19V sequences of genotypes 1A, 1B, 2, and 3 using the GenBank accession number. (page 7, Methods, last paragraph, first sentence)

3.8. For the sequencing analysis, was used very short fragment although amplifying one relatively variable region and Big Dye is a registered mark of the Life Technologies, USA and not Perkin Elmer. The primers for sequencing although already designed must be given in the manuscript and cited.

Reply: Recent studies have shown that the highly variable NS1/VP1u-region is sufficient to determine the B19V-genotype (Toan et al J Gen Virol 2006; Molenaar-de Backer et al PlosOne 2012). We further have replaced “Perkin Elmer” by “Life Technology Corporation”. We have also added the primer for sequencing to the Methods section. The primers used were sense (n-PS5) 5’-CGTGAACGTAGTGGGTTGTA-3’ and antisense (n-PSR) 5’-AATTGCTGATACACAGCTTTAG-3’ (page 6, Methods, 3rd paragraph and page 7, 1st paragraph, first sentence)

3.9. For the phylogenetic analysis it was used very limited number of sequences. Why the authors align the sequences using BLAST? The algorithm for the alignment which they used belongs to what software-BioEdit? How the authors confirm the reliability of the constructed alignment by BioEdit?? What is the test from the BioEdit for the reliability test? The alignment must be checked for significance using the permutation probability test and this significance must be outlined in the next version of the manuscript with the value of P. In the pylogenetic analysis must be performed with more reference strains and the bootstrap probabilities must be compulsory shown on the final cladogram? How we could evaluate the significance of the tree without the bootstrap significance on its branches?? The isolates on the tree must be represented by their original names
and not by their GenBank accession numbers. The subdivision of genotype 3 is not well demonstrated on the performed analysis.

Reply: We thank the reviewer for the important criticisms. We have re-performed with the help of Prof. Thanh Hao Le, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam, who is an expert in this field (page 16, Acknowledgements), the alignment using CLUSTALW and GENEDOC2.5 software. Genetic distances were calculated using the Kimura two-parameter model incorporated into the MEGA5.0 software. Phylogenetic trees were reconstructed by MEGA5.0 using the Neighbour-Joining method. (page 7, Methods, 3rd paragraph, first sentence)

We now show the “Genetic distance calculation (by the Kimura two-parameter model) among B19V genotypes using data from the detected and reference strains” in Figure 6B. (page 26, Figure legend 6B and Figure 6)

Additionally, we re-designed Figure 6 “Phylogenetic analysis of the B19V-NS1/VP1u region” which was reconstructed using MEGA5.0 with the Neighbour-Joining method according to the reviewers criticism. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The isolates on the tree are represented by their original names and also by their GenBank accession numbers. (page 26, Figure legend 6A and Figure 6)

4. The first paragraph of the results section repeats the materials section, where these subdivisions of the groups are described. Please, exclude the unnecessary information because it is very repetitive.

Reply: We agree with the reviewer that the first paragraph of the Results section is repetitive to the Methods section, Study Subjects. We have deleted this paragraph in the Results section and added the link to the data of Table 1 to the Methods, Study subjects section. (page 4 and 5, Methods, Study subjects section)

4.1. It is not prevalence of B19V genomes but prevalence of B19V DNA or B19V viremia. Please correct in the text. Why the authors examine the seroprevalence only in the positive patients. I think that for the next version of the manuscript should be presented the overall seroprevalence of anti-B19V IgG in the entire group and not only in the positive patients.

Reply: We have replaced “genomes” by “B19V DNA” throughout the text where appropriate as recommended. (page 8, Results, Sub-title and 1st paragraph and page 9, 1st paragraph)

We have tried to examine the seroprevalence of anti B19 IgG/IgM antibodies in the serum samples of the entire group. Unfortunately, the material left of some stored serum samples were not enough for testing seroprevalence of anti-B19V IgG and -IgM antibodies in this study. Since these anemic mild/severe malaria and healthy control individuals are young children of approx. 44 month of age,
we will not take too much blood. As a consequence we have not enough material left which are also needed for other purposes.

4.2. How the authors confirm that the detected viral load is from acute infection and not from a persistent one? B19V establishes frequent persistency and could persist at the detected values for very long periods of time. This B19V DNA coincided with the production of IgM?

Reply: The age of the study children of approx. 44 month is very young. It is therefore unlikely that the children lived through a previous B19V epidemic. It has been reported that in children of an age of 1 to 5 years only 2% to 15% showed B19V IgG antibodies, whereas children of 6 to 19 years revealed 15% to 60% B19V IgG antibodies (Heegard & Brown, Clin Microbiol Rev 2002). This is indicative that B19V-infection occurs usually in the ages up to 2 years. Moreover, in our study B19V-DNA and simultaneously B19V IgM antibodies were detectable in 71,4% (20/28) of the serum samples. Therefore the detection of B19V DNA in the sera of the study children was rather due to an acute or very recent infection than persistence of B19V DNA.

Accordingly, we have rephrased the sentence that “corresponding to an acute or recently acute B19V infection” with “The simultaneous detection of B19V-DNA and B19V IgM antibodies in 71,4% (20/28) of the serum samples is indicative for an acute or recently acute B19V-infection rather than a persistence of B19V-DNA.” (page 9, Results, 3rd paragraph)

4.3. B19V/Plasmodium spp. is not a parasitemia. It is a co-infection between B19V and the plasmodium spp. Please correct in the text.

Reply: We have now rephrased the sub-title and the text to avoid the misunderstanding that B19V/P. falciparum is a parasitemia. (page 9, Results, Sub-title and page 10, 2nd paragraph)

4.4. Please calculate the genetic distances among the detected genotypes and put them into the text in the section of genotyping.

Reply: We thank the reviewer for this comment. We have now calculated the genetic distances between the B19V-sequences using the Kimura two-parameter model incorporated in the MEGA_5.0 software. The result is shown in Figure 6B “Genetic distance calculation”. The data of genetic distances among the detected genotypes was added into the Result section “Distribution of B19V-genotypes in Gabonese children” as recommended. (page 11 and 12, Results, last and 1st paragraph, respectively, and page 26, Figure legend 6B and Fig. 6).

5. The authors should have in consideration some facts during the interpretation of their results. B19V infects the progenitor cells in bone marrow and this is not a target place for the Plasmodium spp. Please make a detailed revision of the obtained results in comparison with other findings in the field. Take care when involving genotype 2 in pathogenesis of malaria, because it can reflect simply B19V epidemiology in the region and not at disease outcome of the malaria
Reply: We have revised the statement of the B19V and P. falciparum target cells and discuss in the Discussion section that “target cells of B19V are erythroid cells and erythroid precursors which are shared by Plasmodium spp. (P. falciparum)”. Shared target cells of both B19V and P. falciparum have been described in previous publications, e.g., Rios & Bianco 2000, Wildig et al. 2006, Jones et al. 1990. (page 13, Discussion, 1st paragraph, first sentence).

Also we revised the role of B19V-genotype 2 and stated that “a geographical distribution of B19V genotype 2 could not be completely excluded as it has been shown for genotype 3” and that “The detected association of B19V-genotype 2 with severe malaria needs further analysis, although, one can postulate that variability in the host immune response to distinct B19V-genotypes may correspond with the severity of anemia in B19V/P. falciparum patients”. (page 14, Discussion, 2nd paragraph, first sentence and page 15, 1st paragraph, first sentence).

5.1 What is HI-virus? Did the authors mean HIV? Please do not use “our patients” and the patients with malaria etc. The patients are not yours.

Reply: We have replaced HI-virus by “human immunodeficiency virus (HIV)”. (page 14, Discussion, 2nd paragraph, first sentence). We have replaced “our” patients with “the” patients. (page 10, Results, last paragraph, third sentence).

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published.

Reply: the English spelling and grammar as well as the scientific style has been carefully checked by a native English speaker

Statistical review: No, the manuscript does not need to be seen by a statistician.

Reviewer #2: Quique Bassat

This is a well written relevant report on the potential impact of Parvovirus co-infection on the outcome and severity of malarial co-infections, particularly severe anemia. I believe this manuscript deserves to be published, but I do have a few minor comments to mention that would need to be addressed before accepting the manuscript for publication.

Methods: It is not 100% clear whether the study was designed as a "classic" case-control study, or whether it wasn't. Moreover, there seems to be two comparisons, those severe vs mild cases, and
those infected with P. falciparum vs non infected. I believe a better description in the methods section of the study design and the comparison groups is needed.

Reply: Our study was designed as a retrospective case-control study where we can have a look on B19V coinfection to mild and severe malaria according to its definition of an observational (epidemiological) study of persons with the disease of interest (P. falciparum and B19V-infection) and a suitable control group of persons without the disease. We have specified this in the text. (page 4, Background, 2nd paragraph, first sentence).

Nowhere in the methods section it is explained that IgM and IgG against Parvovirus were included as part of the study. This needs to be explained.

Reply: We have added a new section “B19V serology evaluation” to the Methods. (page 7, Methods, 2nd paragraph)

Results: There are some sections (definitions of severe or mild malaria) that would better be only included in the methods (not results) section.

Reply: The first paragraph of the result section was indeed repetitive to the Methods section, Study Subjects. Therefore, we have deleted this paragraph in the Results section. The information is now available in the Methods, Study subject section. Additionally, a link to the data of Table 1 has been added in the Methods, Study subjects section. (page 4, Methods, Study subjects section)

It would be good to clarify whether any of the healthy controls were parasitemic. I believe this is a critical piece of information that is not mentioned, and all HC should have malaria parasites ruled out.

Reply: The healthy controls were not parasitemic. We have added a sentence in the Methods, Study subjects to clarify this: “The control individuals were chosen of the same sex, age, and locality and the exclusion criteria “were asymptomatic P. falciparum infection and indications for concurrent diseases and malnutrition”. (page 4, Methods, Study subjects, 1st paragraph, fifth sentence)

Page 9, "...hematocrit levels were slightly significant lower...": Please rephrase this sentence which is not very clear.

Reply: We have rephrased the sentence for better reading according the suggestion of the reviewer. (page 10, Results, 3rd paragraph, first sentence)

Laman M, Rosanas-Urgell A, Michon P, Aipit S, Bona C, Siba P, Mueller I, Davis TM.) that found a critical role of Parvovirus B19 in anemia in a highly endemic area for malaria.

**Reply:** We thank the reviewer for this comment and discuss these articles in the discussion section. (page 12, Discussion, 1st paragraph)

Tables: Please include more details about what you are showing in your tables (Mean/Median, geometric mean/% etc...)

**Reply:** We have added more information to the Table 1 legend, e.g., that the values are given as median. (page 23, Table 1, also Figure legends)

Table 2 seems unnecessary, as main results are provided in figure 2, and the remaining could easily be presented as text.

**Reply:** According the reviewers suggestion we agree that Table 2 is dispensable. Therefore we have removed Table 2 from the manuscript.

Page 3: last sentence: Erase "Been" from have "been" shown

**Reply:** We have rephrased this sentence. (page 4, Background, 1st paragraph)

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable.

**Reply:** A native English speaker has carefully checked the English

**Statistical review:** No, the manuscript does not need to be seen by a statistician.
Reviewer #3: Kwabena Duedu

Major Compulsory Revisions

The authors indicate that they matched severe malaria cases with mild malaria, however, the total number of severe malaria cases are more than the mild anaemia cases. The authors, thus will have to indicate how the extra severe cases were matched and what implications they will have on the statistics.

Reply: The statistical analysis using Whitney-Mann U test allows comparing different numbers of cases in two groups. We also deleted the word “matched” and wrote instead: “The control individuals were chosen from patients of the same sex, age, and locality, but with mild malaria, and admitted as soon as the severe cases were enrolled for this study.” Additionally we removed the first paragraph of the Results section but included exact numbers of patients of each group in the Methods section, Study Subjects. (page 4, Methods, 1st paragraph)

In the discussion, the authors make reference to a recent study (page 11, paragraph 2, line 1) which was reported in 1990 and compares with “two older studies” which were rather reported in 1999 and 1997. The isn’t clear. In the context of their discussions, there have been a number of current key related studies which their review and discussions don’t cover (e.g. Wildig et al 2010 BMC Infect Dis 10:88, Wildig et al 2006 J Infect Dis 194(2):146-15, Duedu et al 2013, Asian Pac J Trop Biomed. 3(2): 129–139).

Reply: We thank for the comment of the reviewer to include current articles into the discussion section. However, the article of Duedu et al was released during the time we already had submitted our manuscript to this journal. To keep our paper up-dated we referenced the suggested articles in the discussion section and discussed the major findings therein. (page 12 and 13, Discussion, 1st and 2nd paragraph)

The authors base their key findings on B19V infection interfering with the clinical course of P. falciparum malaria. This has been demonstrated by a couple of studies already, hence, there should be a clear justification on what makes their study and approach original and different as well as what additional knowledge it adds.

Reply: Recent reports of B19V/Plasmodium spp. co-infection focused on the type and degree of anemia in highly malaria endemic regions not on the clinical course of malaria. Therefore, to our knowledge this is the first study which reports and focuses on the possible influence of B19V/P. falciparum co-infection on the course of mild and severe malaria and not (but including) type and degree of anemia. We have made a statement that this study focused on the clinical course of malaria. (page 15, Discussion, Conclusion section)

Both B19V and malaria have seasonal prevalence which may not coincide (difficult for me to tell with respect to their study area). The authors refer descriptions of study subjects to older reports
(1995 & 1998). It is uncertain whether the current study was part of the original study that was reported earlier or the same study subjects were recruited in this current study. This has both ethical and methodological concerns. In the case of the later (which is unlikely) the subjects may have had persistent B19V infection. Persistent B19V infections are potential confounders and need to be dealt with.

It is well reported that most children would have gotten B19V infection by age 2 years. Table 1 indicates the age (mean I presume) of 44.9 and 44.2 months. Children of this age group would have had an exposure (possibility) and B19V infection may be that of persistent infection rather than active infection. Were children who may have lived through a previous B19V infection immune?

Reply: The analyzed serum samples were obtained from previous studies. The samples were cryo-freezeed and stored as different aliquots at -80°C. That the serum samples were completely intact has been demonstrated also by a very recent study (Velavan et al. 29012). Additionally, other studies using this valuable material revealed no limitations of the integrity of the, e.g., DNA (Kalmbach et al. 2006, Kun et al. 1998). An ethical and methodical concern is not estimated. The study was approved by the ethics committee of the Albert Schweitzer Hospital. Finally, all controls have been undertaken to exclude false negative or positive results.

As mentioned above: “the age of the study children of approx. 44 month is very young. It is therefore unlikely that the children lived through a previous B19V epidemic. It has been reported that in children of an age of 1 to 5 years only 2% to 15% showed B19V IgG antibodies, whereas children of 6 to 19 years revealed 15% to 60% B19V IgG antibodies (Heegard & Brown, Clin Microbiol Rev 2002). This is indicative that B19V-infection occurs usually in the ages up to 2 years. Moreover, in our study B19V-DNA and simultaneously detection of B19V IgM antibodies was detectable in 71,4% (20/28) serum samples. Therefore the detection of B19V DNA in the sera of the study children should be rather due an acute or very recent infection than persistence of B19V DNA.”

In our opinion and experience the B19V-positive children must be immune against a repeated B19V-infection.

B19V viral loads are high and indicative of active infection, hence, information on other patients who might be immune will be more enlightening. In addition HIV leads to persistent B19V infection and could be a significant co-founder. Were the samples screened for HIV? This information would help to better understand the clinical course of infection better.

Reply: Unfortunately, we did not have any information or results on the course of malaria of B19V-immune patients and P. falciparum infection. This interesting topic can be analyzed in further studies.

The patients had no indications for concurrent diseases; however, HIV co-infection could not be explicitly excluded. (page 4, Methods, Study subjects section, 1st paragraph, second last sentence)

Severe malaria was defined as severe anaemia with other markers. It will be necessary to provide the reader with some data on the clinical outcomes of the patients. E.g. transfused or not, survived
or died. Another limitation of the study is lack of information on the type of anaemia. Since B19V is particularly associated with aplastic anaemia, it will be more informative to provide data on which type of anaemia was present. The references should be updated to include key similar studies conducted in similar environments.

**Reply:** The clinical data, e.g., treatment, follow up, course of disease, cases of death etc. of the patients has been described in detail in previous studies and publications. (Kun et al. 1998).

We have also updated the references to key similar studies as recommended and included and discussed these reports in the Background and Discussion section. (page 4, Background, 1st paragraph, first sentence and page 12, Discussion, 1st and 2nd paragraph)

The title and abstract are a fair representation of the study. The writing is clear and concise.

**Reply:** Thanks to the reviewer for this kind comments.

**Minor corrections**

Final editing should deal with references placed outside the full stop as well as other minor editorial work.

**Reply:** We have carefully checked the manuscript to place all in-text citation outside the full stop and have undertaken careful editorial work.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Reply:** A native English speaker has carefully checked the English (see also reviewer 1)

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Reviewer #4: Kevin K Brown**

The manuscript describes a study to investing the possible role of parvovirus B19 infection in patient with malaria. However it is not clear what hypothesis the authors are investigating. Is it that parvovirus B19 infection in the past is associated with increased severity of anemia in patients with malaria. Or is it that recent infection/co-infection with parvovirus B19 is associated with more severe anemia. It is the latter that has been hypothesized in the past and appears to be the current area of interest. Depending on the hypothesis to be tested, then the study should be set up accordingly.
Reply: Our aim was to investigate the influence and effect, and the frequency and genotype distribution of B19V in Gabonese children co-infected with P. falciparum in order to determine the impact of B19V-infection on the clinical course of malaria (mild or severe malaria) which is still elusive. Topic of recent reports was the interaction of B19V and Plasmodium spp. co-infection with regard to type and degree of anemia. Per definition anemia is one main factor of the degree of malaria, but not the only one. Accordingly, to our knowledge, our study is the first to describe the influence of B19V/P. falciparum co-infection on the course of malaria (mild and severe). For that matter, we hypothesized that an acute or recent infection/co-infection with B19V can be associated with the development of more severe malaria than B19V-negative individuals. In this regard we believe that the young patients suffered from an recent B19V-infection which is supported by the fact that the age of the study children of approx. 44 month is very young. It is therefore unlikely that the children lived through a previous B19V epidemic. As the reviewer describe in his excellent review that in children of an age of 1 to 5 years only 2% to 15% showed B19V IgG antibodies, whereas children of 6 to 19 years revealed 15% to 60% B19V IgG antibodies (Heegard & Brown, Clin Microbiol Rev 2002). This is indicative that B19V-infection occurs usually in the ages up to 2 years. Moreover, in our study B19V-DNA and simultaneously B19V IgM antibodies were detectable in 71,4% (20/28) serum samples. Therefore, the B19V-infection of the study children should be rather due an acute or very recent than a persistence B19V-infection. (page 9, Results, 3rd paragraph)

The second major concern is that the authors appear to assume that the detection of parvovirus B19 DNA is equivalent to active infection with B19. There are a number of papers in the literature that show that B19V DNA can be detected, albeit at low levels, probably for the rest of a persons life. Therefore it is not sufficient to simply detect B19V and say that this means something. Often a viral load of >10^4 IU/ml combined with IgM can be used to distinguish recent infection from past infection.

Reply: The reviewer is totally right that B19V DNA detection with B19V DNA loads up to 10^4 IU/ml are not necessarily indicative for an acute B19V-infection. However, as described above, we find in >71% of B19V-cases anti-B19V-IgM antibodies and B19V DNA. For that we believe that out B19V-positive patients are suffering from acute or very recent B19V-infection. (page 9, Results, 3rd paragraph)

Thirdly here is insufficient information to describe where and when these patient samples were collected. Are these the same samples collected in 1995-1996 and previously described. If so, then it needs to be recorded how the samples were processed and stored. If not the information on when the samples were collected, how patients were identified (ie consecutive patients attending the hospital with severe Pl falciparum infection etc). Also how the control were identified, and where they age, sex, locality and time matched.

Reply: The analyzed serum samples were obtained from previous studies and collected in 1995-1996. The serum samples were obtained from young children recruited at the Albert Schweitzer Hospital, Lambaréné, Gabon, and the Centre Hospitalier de Libreville, Libreville, Gabon. The investigated cohort is from a matched pair, case-control study, to compare severe and mild malaria in Gabon. The samples were carefully cryo freezeed and stored as diferent aliquots at -80°C. Other studies using this
valuable material revealed no limitations of the integrity of the, e.g., isolated nucleic acids (Velavan et al. 2012, Kalmbach et al. 2006, Kun et al. 1998). (page 4, Methods, 1st paragraph)

Insufficient information is provided on the statistical analysis. It appears than the mean of viral load is provided, and yet this is inappropriate for a non-Gaussian distribution. It is not stated what the confidence limits are.

Reply: We now have added missing information of the statistical analysis. Whitney Mann U test was used for determining viral loads which were presented as median. (see Figure legend 5). The confidence limits were provided in Figure legends 2 and 5. (page 8, Methods, Statistical analysis section, and Figure legends)

Minor essential revisions

No information provided on how the samples were extracted.

Reply: We have added the information on how we had extracted the samples. (page 5, Methods, last paragraph, first sentence)

No information provided on the serology

Reply: Thank you for this valuable comment. We have added a section “B19V serology evaluation” to the Methods section. (page 7, Methods, B19V serology evaluation section)

Why was serology only done on such as small subset? And why only on the PCR positive/malaria samples?

Reply: We have tried to examine the seroprevalence of anti B19 IgG/IgM antibodies in the serum samples of the entire group. Unfortunately, the material left of stored serum samples were in cases not enough for testing seroprevalence of anti-B19V IgG and -IgM antibodies in this study. Since these anemic mild/severe malaria and healthy control individuals are young children of approx. 44 month of age, we will not take too much blood. As a consequence we have not enough material left which are also needed for other purposes.

Please use standard SI units for all measurements (give Hb level not hematocrit), and IU/ml for viral load.

Reply: According to the reviewers comment we now indicate all measurements in SI units and IU/ml for viral loads. (page 23, Table 1, and page 9 and 10, Results, last and 1st paragraph)
Sequences should be submitted to GenBank or equivocal.

Reply: We have deposited the sequences of our B19V isolates to the GenBank. The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of B19 samples determined in this study are:

KF309501 (B19V-isolate No: Af24s), KF309502 (Af65m), KF309503 (Af87m), KF309504 (Af77m), KF309505 (Af89m), KF309506 (Af94m), KF309507 (Af81m), KF309508 (Af22m), KF309509 (Af6s), KF309510 (Af20s), KF309511 (21s), KF309512 (28s), KF309513 (Af13s), KF309514 (Af33s), KF309515 (Af53s), KF309516 (Af41s), KF309517 (Af17s), KF309518 (Af10s), KF309519 (Af2m), KF309520 (Af38s), KF309521 (Af37s), KF309522 (Af39s), KF309523 (Af91s), KF309524 (Af34m), KF309525 (Af97m), KF309526 (Af40s), KF309527 (Af35s), KF309528 (Af31m). (page 8, Methods, 1st sentence)

Insufficient information provided to understand what statistical tests have been done, and what confidence limits. Inadequate information provided on the figures to interpret the box graphs.

Reply: We have added the missing information of the statistical analysis (please see above) and also added confidence limits in figure legend 2 and 5. (page 8, Methods, 2nd paragraph and Figure legends)

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Reply: A native English speaker has carefully checked the English.

Statistical review: Yes, and I have assessed the statistics in my report.

Reply: We have added the missing information of the statistical analysis (please see above).

Reviewer #5: Carlos Ros

In their manuscript entitled “Co-infection of human parvovirus B19 with Plasmodium falciparum contributes to malaria disease severity in Gabonese patients”, Toan and co-authors report on the relationship of malaria and B19V infection in Gabon, a malaria endemic region. By studying a group of 282 children with (197) or without (85) malaria, the authors report an increased prevalence of B19V in the malaria infected group, an increased severity of malaria disease when B19V was also present and a higher prevalence of B19V genotype 2 in severe malaria. That B19V co-infection represents a risk and that can eventually contribute to the severity of malaria is not new. However, the present studies provide evidence that malaria and B19V co-infection is not a rare event in malaria endemic regions and that B19V co-infection should be considered in severe cases or when hemoglobin levels do not recover following effective antimalarial treatments. However, the higher
prevalence of genotype 2 of B19V in severe cases is most probably due to a particular temporal or geographical distribution.

**Major Compulsory Revisions**

Since the results presented do not clearly show an influence of the B19V genotype in the course of malaria, this observation should not appear in the conclusion section of the abstract as a fact but as a possibility.

*Reply:* Thank you for this critical comment. We have changed the sentence in the Abstract and Conclusion section accordingly and stated that our results only “signifies a possible contribution of B19V on the clinical course of malaria in a genotype-dependent manner”. (page 2, Abstract, Conclusion section)

**Minor Essential Revisions**

Clarify whether the duplicates in the PCR assay included or not the DNA extraction step.

*Reply:* We have added the information of repeated DNA extraction to the Methods. “Samples were analyzed in duplicates inclusive extraction of nucleic acids. (page 6, Methods, 2nd paragraph, last sentence)

**Minor issues not for publication**

Background, third paragraph: Erythrovirus (not cursive) instead of Erythroviruses

*Reply:* We have replaced “Erythroviruses” with “Erythrovirus” and wrote the term not cursive. (page 3, Background, 2nd paragraph, first sentence)

Background, fourth paragraph: …have shown… instead of …have been shown…

*Reply:* We have rewritten this sentence. (page 4, Background, 1st paragraph)

Methods, Polymerase chain reaction: Use a comma after the sentence: In order to prevent assay contamination.

*Reply:* We have placed a comma as recommended by the reviewer. (page 5, Methods, Polymerase chain reaction paragraph section)

Methods, DNA sequence analysis: …using primers as described previously [25].
Reply: We have added “previously” to the sentence as recommended by the reviewer. (page 6, Methods, DNA sequence analysis, last sentence)

Methods, Ethical approvals: approved instead of approval

Reply: “approval” has been replaced by “approved”. (page 8, Methods, last paragraph, first sentence)

Results, first paragraph: Change the position of the dot ... (Table 1) [21-23].

Reply: This paragraph and the respective sentence with the error of the position of the dot of the Results section has been deleted according the suggestions of the reviewers.

Results, under “B19V-loads, parasitemia, and anemia in patients with P. falciparum malaria”, third paragraph: Remove the word significant.

Reply: We replaced “significant” by “higher”. (page 10, Results, 2nd paragraph, last sentence)

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Reply: A native English speaker has carefully checked the English (see also reviewer 1)

Statistical review: No, the manuscript does not need to be seen by a statistician.

We hope that the reviewers find the changes agreeable and that you can now consider our manuscript suitable for publication in BMC Infectious Diseases.

Thank you very much.

Sincerely yours,

C.-Thomas Bock and Peter G. Kremsner