January 19th, 2022

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We have revised the manuscript entitled "Quantitative alpha-defensin testing: is synovial fluid dilution important" according to the suggestions made by your referees. We hope these changes will meet with your approval.

Sincerely,

Rodrigo Abdo

Reviewer #1:

Scientific Quality: Grade D (Fair)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: This study aimed to evaluate the potential influence of dilution on the quantitative alpha-defensin ELISA test for the diagnosis of PJI in the synovial fluid of patients with total knee arthroplasty (TKA). If there is a lack of relevant international experimental operation standards, this study has certain clinical significance.

Answer: *We thank the reviewer-1 for the kind comments on our article and for raising important points to improve our work. We reviewed the manuscript and added the necessary information in this revised version accordingly.*

1. Can you describe how to use Wilson Brown method to obtain diagnostic efficiency? Because the calculation of sensitivity and specificity requires a gold standard as a reference system.

Answer: *We have used the hybrid Wilson Brown method for computing the confidence interval of a proportion considering the Sensitivity (fraction of those with disease correctly identified as positive) and Specificity (fraction of those without the disease correctly identified as negative).*

The statistical software GraphPad Prism 8 was used for analysis. In addition, the Musculoskeletal Infection Society (MSIS) criteria for periprosthetic infection is considered the gold standard criteria, and it was used in this current study to define infected and non-infected cases (as stated in page 6). Therefore, our calculation of sensitivity and specificity was based on the gold standard diagnosis criteria.

2. Make sure the experimental operation is accurate? Does the solvent not add enough in the dilution process? From Fig.1, we can see an interesting phenomenon; with the increase of dilution ratio, the greater the dispersion of the results.

Answer: *The methodology used in the assays followed rigorous standards, accurate dilution techniques and proper selectin of the sample diluent, as indicated by the manufacturer's instructions. Increasing the sample dilution led to an increase in the result dispersion and the concentration of alpha-defensin measured by ELISA. Evaluating the role of sample dilution during alpha-defensin ELISA was indeed the main purpose of this study. We could demonstrate that by increasing the dilution, a more reliable distinction between patients with and without infection was achieved. This could be explained by the potential resolution of the prozone effect after increasing the sample dilution.*

3. We can try to measure the same sample repeatedly at the dilution ratio of 1:1000 to obtain the coefficient of variation of the result, which is a supplement to whether the method is suitable for practical experimental operation.

Answer: *This measurement was performed in our samples, and the coefficient of variation was lower than 15%*

4. Why should it be divided into affected and aseptic cases? Would it be better to use alpha defensin to distinguish between affected and aseptic cases?

Answer: *using alpha-defensin ELISA is indeed the ideal instrument to differentiate between affected and non-infected cases. However, the most adequate dilution for this assay is still to be*

determined. Therefore, we used the gold standard method proposed by the Musculoskeletal Infection Society (MSIS) to define infected and non-infected cases to evaluate the influence of sample dilution during the alpha-defensin ELISA.

5. The prozone phenomenon, also known as hook effect, this may be the reason why the dilution ratio needs to be increased.

Answer: *we agreed with the revisor's comment. We have stated in the discussion section (page 9): "The prozone phenomenon, also known as hook effect, is a false negative response that occurs in immunological tests as a consequence of an excess in either antigen or antibodies. Regarding alpha-defensin test, if the amount of antigen (i.e. alpha-defensin) is greater than the amount of antibody, the secondary antibody (i.e. peroxidase) will not bind properly, and the test will present a false result. This could be the reason for some false negative values during alpha-defensin assay. In this study we did not directly assess the influence of dilution in the prozone phenomenon. However, since lower dilutions are more likely to exhibit the prozone effect, the latest dilutions may be helpful to overcome this phenomenon, potentially allowing a more precise differentiation between TKA infection and aseptic cases."*

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: In general, when it is even mentioned, the dilution is very unclear, I agree. - Deirmengian 2015 (the AD test outperforms): "This optimization included dilution optimization of the synovial fluid to eliminate the effects of varying viscosity between samples" - Deirmengian 2014 (combined measurement): "The assays were optimized specifically for performance in synovial fluid by scientists with specific training in immunoassay development. This included dilution optimization of the synovial fluid for both assays. One purpose of the dilution optimization was to attain a synovial fluid dilution that eliminated the effect of fluid viscosity on immunoassay results, even for the samples with the highest viscosity" Most other studies seem to send it to a laboratory and just get a result. If hospitals want to use the

kit themselves, and have a spectrometer, it seems the dilution indeed is necessary. In that sens, this study is useful.

1. However, do you have any comments on the two quotes by Deirmengian that the viscosity needs to be addressed per sample? Also, you should probably comment on the methods these authors used, to show you've checked what others did, and also to underline why your study is useful for others.

Answer: *in our study we strictly followed the manufacture's instruction for the ELISA, progressively diluting the samples and comparing the results of each dilution. Therefore, no additional treatment other than serial dilution was needed in our samples.*

2. Regarding the definition, could you elaborate on the infected cases: i.e., how many had a sinus tract, how many had two or more positive samples, and how many just minor criteria, and which? Usually a table is helpful for this. This gives the reader an idea of the patient population.

Answer: *we thank the reviewer for this suggestion. We have now included a Table 1 showing the population aspects.*

3. Another question about the population: the ratio of infected - non infected is relatively high, when considering that pain >3 months is an inclusion criterion; in my experience, many patients still have pain. Could you explain who made the choice to perform aspiration in those patients?

Answer: *we agree with the reviewer that the proportion of infected patients in our study is relatively high when considering "pain > 3 months" as the only inclusion criteria. However, the majority of the patients included in this study had other criteria that indicated a suspicion for infection, which increased the infected – non infected ratio.*

4. Looking at the different studies written by Deirmengian et al, their alpha defensin mean seems to be around 60-80 mg/mL, probably twice the mean you seem to have found in the 1:5000 dilution; don't you think the effect wouldn't have been better even with 1:10000 dilution?

Answer: *in this study we only evaluated until 1:5000, therefore we cannot anticipate whether a dilution of 1:10000 would be better. In addition, using the dilution of 1:5000 and the same cutoff value (5.2 mg/L) we could achieve similar results in comparison to the literature. Importantly, 1:5000 is the maximum dilution to be used according to the manufacturer's instruction.*

5. You don't offer an explanation why the more diluted samples yield a higher concentration. Is this a matter of the spectrometer being more sensitive, a matter of calculation, or something else?

Answer: *this effect may be a consequence of the prozone effect, as discussed in our manuscript (page 9): "The prozone phenomenon, also known as hook effect, is a false negative response that occurs in immunological tests as a consequence of an excess in either antigen or antibodies. Regarding alpha-defensin test, if the amount of antigen (i.e. alpha-defensin) is greater than the amount of antibody, the secondary antibody (i.e. peroxidase) will not bind properly, and the test will present a false result. This could be the reason for some false negative values during alpha-defensin assay. In this study we did not directly assess the influence of dilution in the prozone phenomenon. However, since lower dilutions are more likely to exhibit the prozone effect, the latest dilutions may be helpful to overcome this phenomenon, potentially allowing a more precise differentiation between TKA infection and aseptic cases."*

6. Do you think 1:10000 dilution would result in concentrations ranging from 25-100 mg/L (double that of 1:5000)? I'm just a simple orthopedic surgeon I guess, but it seems strange that double dilution would result in double the concentration. Please elaborate.

Answer: *According to the manufacturer's instruction, 1:5000 is the maximum dilution to be used. Therefore, we could not address the concentrations of 1:10000 dilution.*