April 20th , 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 17056_TufmRNA_Loonen.doc)

Modified title: Tuf mRNA rather than 16S rRNA is associated with culturable *Staphylococcus aureus*

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The manuscript has been improved according to the suggestions of reviewers:

- Format has been updated.

a) Answers to comments of reviewer 00506623:

1) Define more specifically your target?
   The *Tuf* gene encodes for elongation factor Tu which promotes GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. Most prokaryotes contain the *tuf* gene in their genome. In the abstract and introduction sections we now state that *Tuf* encodes elongation factor Tu.
2) What was your rationale for choosing the tuf gene?
   Our rationale for choosing both the 16S rRNA and *tuf* gene is the fact that both genes are household genes with relatively high expression [Chaffin *et al.* Plos One, 2012]. High expression is indicative of protein expression and thus most likely bacterial viability. Furthermore, we developed the *tuf* gene PCR assay for detection of coagulase negative Staphylococci. This gene has greater discriminatory power as compared to 16S rDNA for Staphylococci. All Staphylococci poses both *tuf* and the 16S rDNA gene, therefore for this proof-of principle study we decided to compare the *tuf* gene with 16S rDNA on a DNA, mRNA and rRNA level. By using these two genes we were also able to compare mRNA and rRNA targets in relation to viability.
   In the introduction section we added our rationale for choosing both genes: "Both the 16S rRNA and *tuf* gene are household genes with relatively high expression levels and therefore indicative of protein expression and thus most likely bacterial viability {Chaffin, 2012}."
3) Scientific nomenclature. The 16S rRNA gene is not italicized, but tuf should be italicized when you discuss nucleic acids.
   We adapted this throughout the manuscript.

4) You do not mention what controls if any you ran.
In each experiment we used samples with and without flucloxacillin. For cDNA synthesis, each sample was split in two for the plus and minus reverse transcriptase reaction to check DNA degradation. Furthermore, a positive and negative control (nuclease free water) were added in each PCR run. The latter is added to the Materials and Methods section in the manuscript.
5) If you are going to have a new detection technique described, you have to do the diagnostic sensitivity and specificity parameters for your test.
The Tuf PCR has been published in 2011. In that paper we describe the sensitivity and specificity of the assay. We adapted the real-time PCR section in Materials and Methods to: "The Tuf PCR and program used were described previously [24]".
6) All of the date revolves around a single laboratory strain of *S. aureus*. To have greater meaning, you need to at minimum test this against several strains.
The reviewer is right and more *S. aureus* strains need to be tested for this work to have greater meaning (including MRSA and VRSA). We now performed a proof-of-principle study with one *S. aureus* strain and analysed several targets in relation to antibiotic treatment. Future studies should include more mRNA targets and multiple bacteria. These studies are presently ongoing. However, these are our first results which are clinically relevant.

b) Answers to comments of reviewer 01021289:

1) The data do not have any issues; however, the interpretation and the title are overstated. I would suggest the authors change the title, for instance, "Tuf mRNA expression is associated with presence of culturable *S. aureus*". Moreover, it would be more appropriate to discuss Tuf mRNA as a viability marker for *S. aureus*-induced bloodstream infection just in the discussion section without making any conclusive statement in the title.
The reviewer raises a valid point. Therefore, we modified the title to "Tuf mRNA rather than 16S rRNA is associated with culturable *Staphylococcus aureus*".
2) Please state the rationale why the Tuf mRNA was chosen in this study.
See point 2 of reviewer 00506623.
3) Please explain how the Tuf mRNA expression is regulated. Is it down-regulated by antibiotics? Is it known that it is functionally associated with bacterial survival or proliferation?
We do not exactly know how *tuf* mRNA is regulated. We found a paper by Ahmed *et al*. (Antimicrobial Agents and Chemotherapy, 2009) in which they studied the effect of multiple antibiotics on biofilm formation. 16S rDNA and *tuf* were used as housekeeping genes in that study. *Tuf* mRNA is relatively stable and at least not affected by several antibiotics. *Tuf* mRNA seems to be highly expressed most of the time and this has been shown in a paper by Chaffin *et al*. (Changes in the *Staphylococcus aureus* Transcriptome during Early Adaptation to the Lung
Plos One, 2012).

c) Answers to comments of reviewer 02520437:

1) One MSSA isolate was used for the study since it was susceptible to fluoxacillin. Testing with one MRSA isolate is mandatory for any probable commercial exploitation?
   The reviewer is correct in that we tested one MSSA isolate which was susceptible to fluoxacillin. Testing more isolates (MRSA, VRSA) is very useful but not for a first proof-of-principle study. We do not seek commercial exploitation of this method, not at this stage at least. We think, for future studies, that it would be more useful to analyse a broader spectrum of bacteria and multiple mRNA targets, which is presently ongoing.
2) One methodology problem is how the growth medium was replenished every day. It is not acceptable to keep it unaltered for 6 days?
   We indeed did not replenish the growth medium and blood for 6 days for practical reasons. (Centrifugation was not an option due to the risk of losing bacteria, interference with bacterial viability status and it would introduce stress. Addition of fresh medium would result in dilution of the bacteria, an unwanted effect.) However, because *S. aureus* bacteria kept growing for 6 days, in absence of antibiotics, we speculated that this was not an issue. Indeed, bacteria numbers kept increasing till the end of the experiments (6 days).
3) Why was not a housekeeping gene used?
   A housekeeping gene is a gene involved in basic functions needed for the sustenance of the cell. So both 16S rDNA and *tuf* are housekeeping genes, at least in Staphylococci and Streptococci. Others have used 16S rDNA and *tuf* as housekeeping genes as well in their research (i.e. Ahmed *et al*. AI-2/LuxS Is Involved in Increased Biofilm Formation by *Streptococcus intermedius* in the Presence of Antibiotics, Antimicrobial Agents and Chemotherapy, 2009).
4) The discussion is too long.
   We have shortened the discussion section (see track changes in manuscript).

Thank you for the critical review of our manuscript. We hope the enclosed revised manuscript is adapted satisfactory for publication in the *World Journal of Clinical Infectious Diseases*.

Sincerely yours,

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