Author's response to reviews

Title: In vitro evaluation of antibiotics' combinations for empirical therapy of suspected methicillin resistant Staphylococcus aureus severe respiratory infections

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Author's response to reviews: see over
Dear Editor,

We send you the revised version of our manuscript entitled “*In vitro* evaluation of antibiotics’ combinations for empirical therapy of suspected methicillin resistant *Staphylococcus aureus* severe respiratory infections” which has been modified in accordance with comments of the referees. Moreover it has been formatted following the Manuscript formatting checklist of the Journal and revised for written English.

Here below you will find our answers for the referees.

Hoping that in this new version it could be suitable for acceptance,

Sincerely Yours

Lorenzo Drago

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**Answers to referee 1 (Prof. Stefani)**

**General**

In the general comment on our paper, you defined methods used in the present study as “popular” methodologies. We would like to point out that they are still considered as the gold standard for evaluation of synergy. In recent years some authors have evaluated the use of E-test for studying antibiotics combination, but no definitive methods to perform the test have been recognized (i.e. how put the strips on agar -at cross or one upon the other?-), and so on). We know that checkerboard assay and time kill curves are based on different approaches since the first considers MIC values and the latter bactericidal activity, thus they can lead to different conclusions.

1. we have changed title according to your and the other referee’s comments
2. The aim of the study was not the comparison between checkerboard and time kill curves. This issues has been addressed by several authors. If our aim had been comparison between methods, we should have evaluated the same number of strains. Instead we perform a first evaluation on a larger number of strains by using
checkerboard, while time kill curves were performed in order to better define the interaction observed in the checkerboard assay.

3. we have evaluated antibiotics used for empiric therapy of severe pneumonia, when among aethiological agents one may consider both gram-negative bacilli and MRSA. Rifampin is generally used once the MRSA aethiology has been confirmed. As far as the use of linezolid is concerned, we were more interested in comparing teicoplanin with vancomycin, which, at the time of the study, was the usual anti-MRSA agent used in empirical therapy of pneumonia.

4. We believe that evaluation of frequency of mutation (not induction) are an important feature of our study. At our best knowledge, most of the studies on antimicrobial combinations determine only synergy; but it is well known that this may not be enough for a fully evaluation of an antimicrobial combination (for instance see Nature 2007, 446:668-71). Moreover, we did not induce resistance in our strains, but calculate the frequency of spontaneous mutations allowing growth of a strain in presence of antibiotics in single or in combination. In literature you can easily find several studies by different authors dealing with frequency of mutation also on antibiotics which don’t target DNA, such as cephalosporins, carbapenems and macrolides (see for instance Antimicrob. Agents Chemother. 2007;51:826-30; J. Antibiot. 2006;59:220-8; Antimicrob. Chemother. 2000;46:909–915).

Major compulsory Revisions

1. We have now changed the title according to your and the other referee’s comments
2. We have not deleted tables 4 and 5 for the reasons stated above (see point 4).
3. We have added number of strains in the method section
4. We have not used the same number of strains for the two assays, because, as stated above, our aim was not the comparison between them. Strains for time kill study were randomly chosen among those tested in the checkerboard assay. Time kill experiments were performed to better characterize antibiotics’ interaction. Moreover, as you know, they are quite labour intensive and expensive. This is an approach, that other authors already followed for their synergy studies (J Chemother. 2005;17:614-21. J. Antimicrob. Chemother. 2003;51:1203-11. Antimicrob. Agents Chemother. 1998;42:2002-5. Antimicrob Agents Chemother. 1997;41:1475-81.)
5. See comments above
6. As stated above, the aim of the study was not the comparison between checkerboard and time kill assays. This issue has been already investigated by other authors, which
have evidenced advantages and drawbacks of each assay. The actual aim was the in vitro comparison of different antimicrobial combinations, usually used for the starting empiric therapy of severe pneumonia when MRSA is considered. Such therapies, as you know, involve both anti-gram negative agents and anti staphylococcal antibiotics.
Answers to referee 2 (Prof. Solorzano)

General

1. The choice of antimicrobial combinations was based, as you noted, on the ATS guidelines for the empirical therapy of pneumonia with suspected involvement of MRSA. In these cases, you may not exclude involvement of gram-negative bacilli. In the new version we have modified the text, making it more clear than the previous version.

2. we have added references for the methods used in the study. However, Checkerboard technique is not limited to only two antibiotics. (See Eliopoulos, G. M., and R. C. Moellering, 1991. Antimicrobial combinations, p. 432-492. In V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore, Md.) In case of three molecules combinations, FIC index is calculated by sum of FIC$_A$, FIC$_B$ and FIC$_C$ where FIC of each antibiotic is calculated by ratio between MIC in combination and MIC alone. Now, calculation of FIC index has been added to the text. As far as time kill curves are concerned, strains were randomly chosen. Time kill curves were performed with the aim to better characterize activity of antimicrobial combinations. Since this technique is rather labour intensive, time consuming and expensive, we decided to use a limited number of strains, as already performed by other authors (J Chemother. 2005;17:614-21. J. Antimicrob. Chemother. 2003;51:1203-11. Antimicrob. Agents Chemother. 1998;42:2002-5. Antimicrob Agents Chemother. 1997;41:1475-81). Time kill assays were performed in duplicate. Beside synergy, also indifference and antagonism were considered for interpretation of results. Now we have added interpretation criteria in methods and in results section.

3. For MIC determination we used a quality control (S. aureus ATCC 29213), but there are no quality controls for synergy testing. We have modified tables 2,3,4 to improve information.

4. Sorry, but what do you mean with “FIC index values should be more specific”? In the checkerboard assay we tested 50 strains with double combinations and 25 with the triple ones. Report of each single FIC value for each tested strain would be quite difficult and we do not believe that the mean of all values would give the same information, since for instance if you have FIC of 0.25, 0.25, and 1.5, with a mean of 0.67, you could classify the combination as no interaction, while for two strains you have synergy. Perhaps, the median or the modal value could be more informative.
than the mean. However, in these cases, information will be the same, we gave by FIC index interpretation.
5. We have modified discussion, according to your comment.
6. In the title and in the abstract we underlined the fact that we tested antibiotics used for empirical therapy of infections with a probable MRSA ethiology. Now we have changed it, in accordance with your and the other referee’s comments.

**Major Compulsory Revisions**

In the revised version we have included description of the methods used.

**Minor Essential Revisions**

We have corrected ref 24.