Author's response to reviews

Title: Molecular evidence of Ureaplasma urealyticum and Ureaplasma parvum colonization in preterm infants during respiratory distress syndrome.

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Author's response to reviews: see over
To The Chief Editor of 

*BMC Infectious Diseases*

Dear Editor,

We would like to submit the revised manuscript entitled “**Molecular evidence of *Ureaplasma urealyticum* and *Ureaplasma parvum* colonization in preterm infants during respiratory distress syndrome**”, by R. Cultrera et al., for publication in *BMC Infectious Diseases*. Authors considered the general comments indicated by BioMed Central Editorial board and the comments of the Reviewers.

You will find enclosed the figures and tables. This paper is original and has not been submitted in equivalent form for publication elsewhere. All Authors have read and approved this revised version and they have contributed significantly to the work. We declare there are no competing financial or non-financial interests (political, personal, religious, ideological, academic, intellectual, commercial or any other) in relation to this manuscript.

With best regards,

Rosario Cultrera

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Molecular evidence of *Ureaplasma urealyticum* and *Ureaplasma parvum* colonization in preterm infants during respiratory distress syndrome.

**Reviewer 1 (Prof. Robert Schelonka)**

**ANSWERS**

1. **Lack of a true “gold standard”**.

   *Ureaplasma* spp., such as other *Mollicutes*, are fastidious microorganisms to identify by culture methods. *Ureaplasma* detection is usually based on urea hydrolysis tests and culture techniques which are often complex and inadequate for routine application in clinical laboratories. The International Committee on Taxonomy of *Mollicutes* issued recommended tests for mycoplasma identification including those required to define new isolates, as well as tests for genus and species determination. The detection of mycoplasmas and ureaplasmas by culture techniques could not be considered a true “gold standard” because of the lower sensitivity than in PCR methods. Although the culture technique may be considered the reference standard for the detection of *M. hominis* and *Ureaplasma* spp., it is expensive and requires specialized media and expertise (Waites, 2005). Moreover, a possible detection of *Mycoplasma* spp. and *Ureaplasma* spp. by cultures needs additional tests, including molecular techniques to identify the species. Nucleic acid amplification has become the method of choice for routine detection, differentiation and classification of some *Mollicutes* (e.g. phytoplasmas) (Razin S., 2002). For the detection of *Mycoplasma* genomes, PCR methods may be considered a true “gold standard” technique although the results should be
confirmed by other molecular techniques, such as Southern blot or sequence analysis of the amplicons, in order to decrease the rate of false positivity or false negativity. We employed a culture identification assay to identify \textit{M. hominis} and \textit{Ureaplasma} genus as a reference technique in comparison with PCR amplification. Our findings suggested that the PCR methods described in our work could be considered as a possible true “gold standard”, because of the selection of primers with wide specificity which react with the DNA of \textit{Ureaplasma} or \textit{Mycoplasma} derived from target sequences in the highly conserved regions of the genes.

2. Possibility of DNA contamination.

DNA contamination is an effective problem of the PCR amplification. In addition to the employment of specific primers for the PCR, as mentioned above, we employed laboratory measures to minimize the probability of DNA contamination. Four different and separate working spaces for each step (sample lysis, DNA extraction, amplification mixture constitution and addition of DNA samples) together with aliquoting autoclaved reagents, UV-irradiation treatment for surface laboratory benches, filter-tips, adding DNA last were used in order to avoid the risk of contamination by DNA or PCR product carryover. In addition, each PCR assay for \textit{Ureaplasma} and \textit{Mycoplasma} included a preliminary PCR tests to rule out the presence of inhibitory substances of amplification assay (Cultrera R. et al., 1998).

The specificity of the PCR technique was confirmed by sequence analysis of the amplification product. Although PCR detection of mycoplasmas is still too expensive and complex for the wide species to be carried out routinely in clinical microbiology laboratories, PCR assay for \textit{Ureaplasma} spp. could become a major method for diagnosis of ureaplasmal infections of newborns with respiratory failure.

3. Calculation of sensitivity of the PCR technique.
The sensitivity of PCR assays is usually in the order of magnitude of a few femtograms of mycoplasmal DNA, considering that 1 fg of mycoplasmal DNA is approximately equivalent to the genomic DNA of a single mycoplasma cell (S. Razin, 2002). In a previous study, we demonstrated that our PCR technique was able to detect 5-15 microorganisms in clinical specimens (Cultrera R. et al., 1998). Considering that a positive mycoplasma culture requires an inoculum of about 100 to 1,000 cells, PCR technique may be considered the most sensitive detection method available.

4. Treatment with clarithromycin.

We stated that the administration of clarithromycin led to clinical and microbiological eradication as demonstrated by the improvement of clinical outcome and culture and PCR amplification negativity in both NF and TA specimens, before the removal of tracheal intubation. In the “Discussion” section (page 17) we added this sentence: “Moreover, as shown by culture and PCR DNA detection, this treatment led to eradication of *Ureaplasma* spp. in TAs as well as in NF specimens, before the removal of tracheal intubation.”

All studied babies, which resulted either positive or negative for *Ureaplasma* spp. in respiratory secretions, were tested a second time by PCR and/or culture techniques after treatment or clinical improvement. In the “Results” section (page 9) we added the sentence “Clinical specimens of all patients with or without clarithromycin treatment were tested before the tracheal intubation was removed or after the clinical improvement, respectively.”

5. Concordance between TA and NF samples.

The Authors have not matched TA and NF samples for DNA detection. The Reviewer has made a correct and well-observed comment. The study did not foresee this analysis, due to the difficulty of obtaining TA and NF specimens from the same newborn at the same time, especially from those newborns that did not need the tracheal intubation. Moreover, we were not authorized by the Ethics Committee to obtain TA specimens from infants without tracheal intubation. Nevertheless, we
observed that there was a correspondence among TA specimens, obtained from the infants in group 1 (with RDS), and evidence of *Ureaplasma* spp. by PCR in contrast with the negativity of ureaplasma detection by PCR in NF specimens, obtained from the newborns in group 2 (without RDS).

6. **Statistical analysis**

We attempted to match birth weight and gestational age with group 1 and group 2 infants to analyse the association between Ureaplasma-RDS and PDA. A logistic regression was also made correlating as independent variables PCR results, PDA, gestational age and birth weight with significant statistical differences between two groups (p <0.0004).

7. **Spelling and grammatical errors.**

The manuscript has been completely reviewed by an English translator.
Reviewer 2 (Prof. Dimitris A. Kafetzis)

ANSWERS

1. In the “Patient and Methods” section (page 9) we added this sentence “Newborns that resulted positive for \textit{U. urealyticum} or \textit{U. parvum} by microbiological or PCR analyses, received intravenous (i.v.) clarithromycin at a dosage of 10 mg kg\textsuperscript{-1} twice a day.” The sentence “Patients that had a positive PCR assay for \textit{U. urealyticum} and \textit{U. parvum} were treated with intravenous clarithromycin at a dosage of 10 mg kg\textsuperscript{-1} twice a day for ten days or until clinical improvement” was moved to the “Results” section (page 15). The sentence “Although in our study antibiotic therapy was restricted to newborns that resulted PCR positive for \textit{Ureaplasma} spp. without a placebo-treated control group, the improvement of the clinical outcome observed in patients after clarithromycin treatment may be a further suggestion that the colonization of the respiratory tract by \textit{U. urealyticum} and \textit{U. parvum} could be considered an important factor that contributes to acute lung injury” was moved to the “Discussion” section (page 17) in order to discuss the possible effect of clarithromycin on clinical improvement.