Dr. P.M. Zunino

Recommender,

PCI Microbiology

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Montpellier, 25 March 2023

Dear Recommender,

Herewith a revised version of our manuscript that has been prepared in response to your comments and to your reviewer's comments. The manner in which these comments have been incorporated is outlined below.

Sincerely yours,

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Answers to recommender:

The correct links to the data used in the study and to supplementary Fig1 are provided in the "Data, scripts, code, and supplementary information availability" section line 372.

The surname of the first author was changed to Totté line 4.

Four additional references were added to the list.

Answers to anonymous reviewer, 17 Feb 2023 13:51:

Methods

Line 74: the method for CFU counting should be presented in detail.

- *details on the method for CFU counting were added lines 77-79.*

Line 78: the detailed information about the cattle form which the sera were collected should be added (the origin, breeding conditions, epizootic status especially for mycoplasma infections)

- *detailed information about the cattle from which the sera were collected was added lines 84-85.*

Line 80: the range for room temperature should be added

- *the range for room temperature was added line 88.*

Line 82: the authors should explain such concentration range

- *the reason for using such concentration range is given lines 92-93.*

Line 85: the authors should add more information about the animals from which the sera were collected (the origin, medical history, the methods used for the status confirmation)

- *more information about the animals from which the sera were collected was added lines 93-97.*

Line 91: the information about the Ethics Committee agreement should be transferred to sub-section of 'bovine complement and antiserum')

- *the information about the Ethics Committee agreement was transferred to lines 85-87 in the 'bovine complement and antiserum' section.*

Line 108: there should be added the detailed conditions for culturing

- *the sentence 'CFU Mmm titers were determined…' line 108 was replaced by the sentence 'Mmm CFU titers were determined as described above' line 117.*

Line 140: the detailed information about the ELISA reader used here should be added

- *detailed information about the ELISA reader was added line 149*

Results

Lines 157, 175: a statements 'results not shown' or 'data not shown' - the authors should give the reason for such explanation, it is not possible to reliably evaluate such presented statement

- *the sentence ' The killing effect was due to complement since it was neutralized by heating sera at 56°C for 30 min (results not shown). ' line 157 was replaced by the sentence 'The killing effect was due to complement since it was neutralized by heating sera at 56°C for 30 min a method known to inactivate complement (Volanakis, 1998).' Lines 166-168. Furthermore, results obtained with bovine serum pre-treated at 56°C for 30 min were included in Figure 1 and the legend was modified accordingly (line 174).*

- *the sentence 'Similar trends were observed at MOIs of 1 and 100 whereas only survival of Mmm without growth was observed in the absence of macrophages (data not shown)' lines 173-175 was replace by the sentence '*Similar trends were observed at MOIs of 1 and 100 whereas only survival of *Mmm* without growth (i.e., CFU titers remained constant) was observed in the absence of macrophages*).' Lines 183-185.*

Line 184: 'Mmm' in 'Anti-Mmm' should be in italics

- 'Anti Mmm' was replaced by 'Anti-*Mmm*' in line 194

Discussion

Lines 310, 335: 'not shown' should be avoided; the authors should describe it in another way or based on the published data

- *the sentence 'It should be noted that pre-incubation of Mmm with bovine complement for 30 min at 37°C before infecting macrophages yielded similar results (not shown).' Lines 309-310 was replaced by the sentence 'It should be noted that pre-incubation of Mmm with bovine complement for 30 min at 37°C before infecting macrophages rather than concomitantly did not change the results ' in lines 319-321.*

- *the sentence 'Also, it should be noted that bovine complement did not improve Mmm capacity to bind to the surface of macrophages, as measured by PI-dependent extracellular fluorescence (not shown).' Lines 334-335 was removed.*

Answers to anonymous reviewer, 14 Feb 2023 07:13:

The authors used macrophages purified from the blood instead of alveolar macrophages obtained directly from the bovine lungs. Will the results be the same if the authors used alveolar macrophages instead of circulating macrophages? May be this point should be discussed in the discussion section.

- *it is written in paragraph two that our results are in agreement to what has been observed previously for other mycoplasmas (Marshall et al., 1995; Howard et al., 1976). These previous studies used alveolar macrophages.*

Did the authors quantify the opsinization of Mmm when using non bactericidal concentration of non decomplemented bovine serum? May be the difference between non decomplemented sera and antiserum come from a low binding of the complement proteins onto the Mmm cells?

-*affinity binding experiments were not performed.*

Regarding TNF production experiments: Is the effect of bovine complement on TNF production specific or not? The production of TNF by macrophages seems to be dependent of the titer of viable Mmm, reducing the MOI or killing them by adding bovine complement lead to similar results. Do you think that similar result will be obtain with heat-killed Mmm?

- *this is a good question which warrants further analysis using heat-killed Mmm.*

Finally, did the authors observe that Mmm is able to reduce the viability of macrophages?

- *no obvious signs of cytotoxicity were observed at any MOI used as stated lines 181-182. This means that detachment or even rounding of cells were not detected.*

Minor comments

Line 1: "subsp." between mycoides mycoides.

-*"subsp." has been added.*

Line 78: maybe it would be worth to mention that the animals are CBPP-free.

This information has been added in lines 84-85.

Line 84: remove the dot after CO2.

- *done.*

Line 157: The effect of bovine serum decomplementation (30 mn at 56°C) has to be shown, it is an important result as innate defenses are present in serum other than complement (Bacterial self-defence: how Escherichia coli evades serum killing by Helen Miajlovic & Stephen G. Smith).

- *results obtained with bovine serum pre-treated at 56°C for 30min were included in Figure 1 and the legend was modified accordingly (line 174).*