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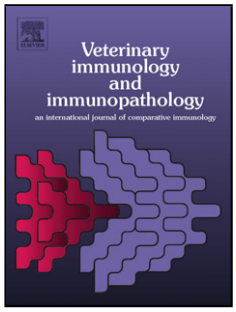

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Polymorphonuclear cells and reactive oxygen species in contagious bovine pleuropneumonia: new insight from in vitro investigations

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Highlights

- *Mmm* produces small amounts of reactive oxygen species *via* the glycerol metabolism
- *Mmm* is capable of adhering to the membrane of bovine polymorphonuclear cells
- *Mmm* enhances the respiratory burst of bovine neutrophils

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Abstract

Reactive oxygen species (ROS) are suggested to play a role in the pathogenesis of contagious bovine pleuropneumonia, a severe respiratory disorder caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). The present study investigated the generation of ROS by different strains of *Mmm*, as well as their effect on the oxidative response of bovine neutrophils. The production of ROS was indirectly measured using a luminol-based chemiluminescence assay. Our results confirm that *Mmm* can produce ROS *via* the metabolism of glycerol, significant differences existing between African and European strains. *Mmm* was capable of adhering to the external surface of neutrophils. Interestingly, *Mmm* enhanced the respiratory burst of bovine neutrophils. This activity was particularly pronounced with the African field strain and in presence of glycerol. Taken together, our data argue in favour of a major role for neutrophils as the main source of ROS in contagious bovine pleuropneumonia.

Abbreviations: CBPP = contagious bovine pleuropneumonia; *Mmm* = *Mycoplasma mycoides* subsp. *mycoides*; ROS = reactive oxygen species; GtsABC = glycerol transport system ABC; PMNs = polymorphonuclear cells; CL = chemiluminescence; AUC = area under curve; TLR = toll-like receptor.

Keywords: contagious bovine pleuropneumonia, *Mycoplasma mycoides* subsp. *mycoides*, polymorphonuclear cells, reactive oxygen species, chemiluminescence

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Introduction

Contagious bovine pleuropneumonia (CBPP) is a severe infectious disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). To date, most continents are free from CBPP, which was eradicated through onerous policies of stamping out and biosecurity practices. In contrast, CBPP is still endemic in sub-Saharan Africa, where it negatively impacts the profitability of livestock production (Anonymous, 2014a).

Although *Mmm* is a small, "simple" and widely investigated microorganism, its virulence factors (e.g. adhesins, toxins, spreading factors etc.) have not been fully identified and the pathogenesis of CBPP remains largely unknown (Pilo *et al*., 2007).

Reactive oxygen species (ROS), produced *via* the metabolism of glycerol, are suggested to play a role in the pathogenicity of *Mmm* (Pilo *et al*., 2005). It is known that *Mmm* African strains – which produce larger amount of ROS and are considered more virulent – take up glycerol through a specific import system (glycerol transport ABC, GtsABC). Afterwards, glycerol is phosphorylated and metabolized to dihydroxyacetonephosphate. The latter conversion is catalysed by L-alpha-glycerophosphateoxidase and results in the production of hydrogen peroxide (H_2O_2), a highly toxic compound included in the ROS group. Conversely, European strains are considered less virulent and produce much less H_2O_2, due to lack of a complete and functional GtsABC (Vilei *et al*., 2000). However, mycoplasmas could also induce the production of ROS by phagocytic cells, which could further contribute to the tissue damage (Köppel *et al*., 1984; Pilo *et al*., 2007).

Considering that, the present study investigated the generation of ROS by African and European strains of *Mmm*, as well as their effect on the respiratory burst of bovine neutrophils (polymorphonuclear cells, PMNs). The production of ROS was indirectly quantified *in vitro*, using a suitable luminol-based chemiluminescence (CL) assay (Rinaldi *et al*., 2007). Data could provide further insights into the CBPP pathogenesis representing, at the same time, a useful model to investigate other pathogens and respiratory disorders.

Materials and methods

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***Mycoplasma mycoides* subsp. *mycoides* strains**

Three *Mmm* strains were tested in the present study: i) "Caprivi", a highly virulent African strain, isolated in Namibia in 2003 (Scacchia *et al*., 2011); ii) "57/13", a European strain isolated in Italy in 1992 (Orsini *et al*., 2015); iii) "T1/44", an attenuated African strain commonly used as a vaccine (Anonymous, 2014a). All *Mmm* strains were available at the OIE Reference Laboratory for CBPP in Teramo (Italy) and were cultured in modified pleuropneumonia-like organism (PPLO) broth (Difco™ PPLO broth, Becton Dickinson) at 37 °C with 5% CO_2 for three days (Anonymous, 2014b). Bacterial cells were harvested by centrifugation at 9,000 × *g* at 4 °C for 40 min and washed three times with isotonic phosphate-buffered saline (PBS, pH 7.2). Afterwards, *Mmm* pellets were re-suspended in PBS and titrated by serial 10-fold dilutions; the titer was expressed as colony-forming units (CFU) *per* ml.

An aliquot of the 57/13 strain was inactivated by heating at 100°C × 10 min; inactivation was confirmed by failure to culture in PPLO modified media.

Isolation and purification of bovine PMNs

Blood samples were obtained after jugulation from clinically healthy and regularly slaughtered cattle, which were *Mmm*-negative, homogeneous in terms of breed (Jersey) and age (24-36 months). PMNs were purified by standard procedures, as previously published by Grinberg *et al*. (2008). Briefly, the blood was collected in EDTA containing tubes and centrifuged at 1,000 × *g* for 20 min. Then, plasma, buffy coat and about 50% of red cell layer were discarded. The remaining packed cell volume was incubated at 37 °C for 20 min in lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.12 mM EDTA), in order to completely eliminate the erythrocytes. After an additional centrifugation at 400 × *g* for 10 min, a pellet mainly containing PMNs was obtained, washed three times and finally re-suspended in PBS supplemented with 0.9 mM $CaCl_2$, 0.2 mM $MgCl_2$ and 5 mM glucose (pH 7.2).

Purification of PMNs was microscopically assessed by modified Giemsa stain (Hemacolor® rapid stain kit, Merck), while the viability and the concentration of the cell suspensions were checked by

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means of an automated cell analyser (Vi-Cell, Beckman Coulter). Only suspensions with ≥ 90% of purified and viable PMNs were used and then adjusted to 5×10^6 cells/ml.

From each cattle, an additional blood sample was also collected in a serum-separating tube. All sera proved to be negative at the complement fixation test (Anonymous, 2014a).

Evaluation of ROS production by different *Mmm* strains

Mmm strains (200 µl, 5×10^8 CFU/ml) were dispensed in a 96-wells microplate, both in absence and presence of glycerol (100 µM). Then, luminol (30 µl, 1 mM, Sigma Aldrich) was added to each well and CL was monitored for 2 h at 37 °C by a multi-mode plate reader (Sinergy H1, Bio-Tek), with 1 min time interval between consecutive readings of the same well. Each test was performed in triplicate in four independent experiments. The intensity of the CL was expressed as the area under curve (AUC). Wells containing either *Mmm* or luminol alone were used to determine reference values of luminescence. Data were aggregated and reported as the mean ± standard deviation (SD).

Analysis of the CL response of PMNs in presence of *Mmm*

PMNs (200 µl, 5×10^6 cells/ml) were dispensed in a 96-wells microplate with luminol (30 µl, 1 mM, Sigma Aldrich) and incubated at 37 °C for 10 min. Both live and heat-killed *Mmm* cultures were first opsonised by incubation at 37 °C for 30 min with the "autologous" serum (i.e. serum derived from the same animal from which PMNs were also purified), washed and re-suspended with PBS (pH 7.2), in absence or presence of glycerol (100 µM). Then, each *Mmm* strain (30 µl; 5×10^8 CFU/ml) was added to PMNs and CL monitored, calculated and expressed as above described. Overall, blood samples from 12 cattle were analysed and each test was carried out in triplicate. Wells with PMNs or luminol alone provided reference values of CL. The PMNs respiratory burst induced by heat-killed, opsonized *Saccharomyces cerevisiae* (Baker's yeasts, Sigma Aldrich; 30 µl, 1.5 x 10^9/ml) acted as positive control.

Immunocytochemistry for *Mmm*

The interaction between PMNs and *Mmm* was investigated by immunocytochemistry (ICC). PMNs (200 µl, 5×10^6 cells/ml) and opsonised, live or heat-killed *Mmm* (30 µl; 5×10^8 CFU/ml) were mixed

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and incubated at 37 °C for 1 h, smeared on a glass slide and fixed with methanol. An anti-*Mmm* murine monoclonal antibody was used as primary antibody (Luciani *et al*., 2017). Immune reactions were revealed by a biotin-streptavidin amplification method and using 3-3′-diaminobenzidine as chromogen (Dako, Real detection system).

Statistical analysis

The sample size was calculated by G*Power software. One-way analysis of variance and *Tukey-Kramer* multiple comparisons analysis were applied to verify significant variations of CL. *Student*-t test was applied in paired groups to compare the response in presence and absence of glycerol. Only p values (p) <0.01 were accepted as statistically significant.

Results

ROS production by different *Mmm* strains

The generation of ROS by *Mmm* was negligible in absence of glycerol, no significant difference being demonstrated among different strains ($p \geq 0.01$). On the other hand, ROS production significantly increased after adding glycerol to all live *Mmm* strains ($p < 0.001$) and reached the highest level with the Caprivi one (Figure 1).

Effect of *Mmm* on the PMNs oxidative response

The incubation of PMNs with *Mmm* strains significantly increased the CL response when compared with the reference level (i.e. PMNs or luminol alone), both in presence and absence of glycerol.

No significant difference was observed among the PMNs incubated with the three *Mmm* strains ($p \geq 0.01$) in absence of glycerol. The addition of glycerol did not significantly modify the production of ROS with the only exception of Caprivi strain, which induced the highest CL response (Figure 2).

Immunocytochemistry for *Mmm*

ICC consistently demonstrated the presence of *Mmm* adhering to the membrane of PMNs (Figure 3). No difference was observed among *Mmm* strains or between live and inactivated bacteria (data not shown).

Discussion

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PMNs play a pivotal role in the immune response, representing one of the earliest lines of innate defence against bacterial and mycotic pathogens. The bactericidal power of PMNs depends on a number of well-coordinated activities: the ability to respond to chemotactic stimuli, to recognize and engulf bacteria, to kill the infectious agents by producing and/or releasing microbicidal molecules. Among these molecules are ROS, which are generated within lysosomes *via* the NADH-oxidase and the myeloperoxidase enzymes (Paape *et al*., 2003; Hodgson *et al*., 2006). Despite their protective function, the excessive and/or prolonged activation of PMNs can result in an overabundant generation of ROS, which contribute to the cell and tissue damage creating a detrimental, vicious circle (Ackermann, 2007).

The interaction between mycoplasmas and phagocytic cells has been long investigated, *in vivo* and *in vitro* (Marshall *et al*., 1995). In the bovine species some data are currently available about the effect of *Mycoplasma bovis* (*M. bovis*) on PMNs. It has been shown that *M. bovis* does not elicit a CL response in bovine neutrophils (Thomas *et al*., 1991; Gondaira *et al*., 2017), but does suppress their capacity to form extracellular traps (Gondaira *et al*., 2017) and to mount an oxidative response towards other stimuli (Thomas *et al*., 1991). More recently, it has been reported that *M. bovis* can have different and partially opposite effects on PMNs, aiming to evade the host innate immunity (Alabdullah *et al*., 2015; Jimbo *et al*., 2017).

Alveolar macrophages and PMNs are abundantly present in CBPP-affected lungs, mostly during the acute-to-subacute stages of the disease, thus likely participating both in the host defence and in the typical pathological changes (Thiaucourt *et al*., 2004; Nicholas *et al*., 2007). Few mixed data exist about the interaction between *Mmm* and macrophages; *Mmm* was shown to induce tumor necrosis factor-alpha – a relevant proinflammatory cytokine – in alveolar macrophages (Jungi *et al*., 1996), while its free exopolysaccharides demonstrated anti-inflammatory activity by inducing the production of interleukin-10 (Totté *et al*., 2015). Though, to the best of our knowledge, the effect of *Mmm* on PMNs has never been investigated at all.

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Therefore, the present study aimed to evaluate the production of ROS by different *Mmm* strains, on one side, and the PMNs ability to mount an oxidative response towards those mycoplasmas, on the other. Our results confirm that live *Mmm* can generate ROS in presence of glycerol, significant differences existing among strains (Rice *et al*., 2001; Bischof *et al*., 2008). However, we remark that the CL response generated by *Mmm* was overall weak, this finding suggests that the cytotoxic effect of ROS, if any, may result only from the close contact between *Mmm* and the hosts' cells (Marshall *et al*., 1995; Pilo *et al*., 2005; Bischof *et al*., 2008). In this respect, we point out that the highly virulent Caprivi strain was not able to induce any obvious pathological change in bovine airways *in vitro* (Di Teodoro *et al*., in press); such finding supports the hypothesis that cell damage is mainly due to the immune and/or inflammatory response, rather than to a direct effect of the *Mmm* infection.

As shown for *M. bovis*, both live and inactivated *Mmm* were capable of adhering to the external surface of PMNs. However, in contrast to what has been reported for *M. bovis*, *Mmm* stimulated the PMNs respiratory burst, regardless of the *Mmm* strain, viability and metabolism of glycerol. The CL response of neutrophils incubated with *Mmm* was dozens-to-hundreds times greater when compared with that of *Mmm* alone. Reasonably, the induction of the PMNs respiratory burst could result from the interaction of *Mmm* with PMNs toll-like receptors (TLRs) (El-Benna *et al*., 2016); in particular, *Mmm* lipid-associated membrane proteins could act as TLR2 agonist (Wang *et al*., 2017). Further studies are needed to reinforce what still remains a speculation and to better understand the link between *Mmm*, on one side, and TLRs and ROS production by PMNs, on the other (e.g. using TLR antagonists or NADPH-oxidase inhibitors).

It is interesting to note that the highest CL response was achieved after the incubation of PMNs with the Caprivi strain in presence of glycerol. This suggests that the generation of ROS by *Mmm* could further elicit the PMNs activation. In this respect, it is known that H_2O_2 can modulate the respiratory burst of human PMNs in a dose-dependent manner. In particular, micromolar concentrations of H_2O_2 (such as those physiologically generated by PMNs or *Mmm*) were demonstrated to enhance the CL response, probably by damaging the membrane integrity and by inducing the degranulation of PMNs

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(Winn *et al*., 1991; Marshall *et al*., 1995). Considering that, we could speculatively advance the following scenario: 1) *Mmm* colonizes the lower airways; 2) PMNs are recruited within bronchioles and alveoli by *Mmm* chemotactic factors; 3) *Mmm* adheres to the surface of PMNs and induce their respiratory burst *via* TLRs; 4) virulent *Mmm* strains produce and translocate higher amounts of ROS inside the host phagocytic cell, thus damaging the host cell membranes, triggering degranulation and boosting several times the amount of ROS released at tissue level ; 5) as a result, the inflammatory reaction is further enhanced and contributes to the impairment of the lung morphology and function.

Conclusions

Taken together, data provided herein indicate that bovine PMNs are able to mount an oxidative response against *Mmm*. Such response is particularly intense in presence of the virulent strain, which is able to generate higher amounts of ROS. Assuming that ROS are relevant for CBPP pathogenesis, the present study indicates that PMNs should be regarded as the main source of such toxic metabolites. According with literature (Rosengarten *et al*., 2000), our data support the hypothesis that host cell damage could mainly result from the inflammatory response rather than from a direct effect of ROS generated by *Mmm*.

Authors contribution

GDT, FM, ADP: carried out the laboratory investigations and acquired the data. GDT, GM: designed the study, drafted the manuscript. GDT, GM, FS: analysed and interpreted the data. FS, PGT, MS: critically revised the article for important intellectual content. All authors read and approved the final manuscript.

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Declarations of interest

None

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Figures' legends

Figure 1. Production of ROS by *Mmm* strains. In presence of glycerol, the CL response significantly increased in all live *Mmm* strains (*p<0.001), while no significant difference was demonstrated in the inactivated 57/13 strain (p≥0.01). Moreover, CL was significantly higher in the Caprivi strain when compared with the 57/13 and T1/44 strains (°p<0.001).

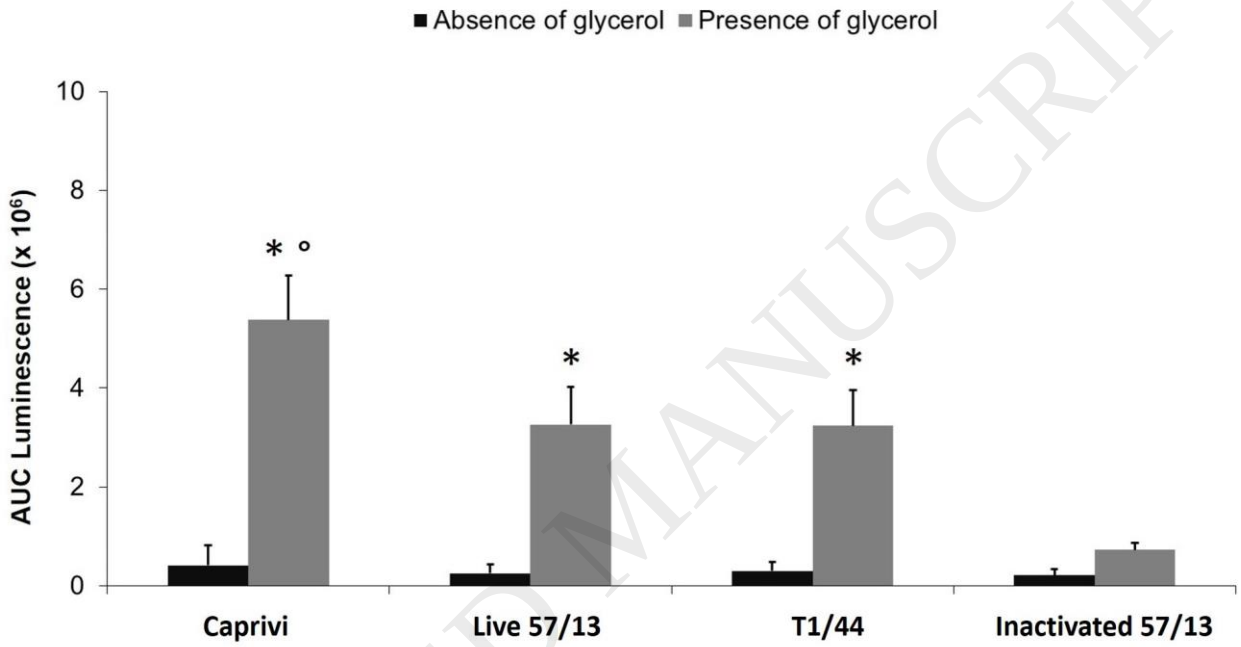

Figure 2. Oxidative response of PMNs towards *Mmm* strains. Live and inactivated *Mmm* strains proved to stimulate the PMNs respiratory burst, the CL response being significantly higher when compared with the reference value (PMNs alone), both with and without glycerol (*p<0.001). No significant difference was demonstrated among *Mmm* strains aside from the Caprivi one, which remarkably increased the CL response only in presence of glycerol (°p<0.001). The CL response of PMNs stimulated with yeasts was particularly intense and not significantly modified by glycerol; such data indicate that the assay conditions did not bias the PMNs respiratory burst.

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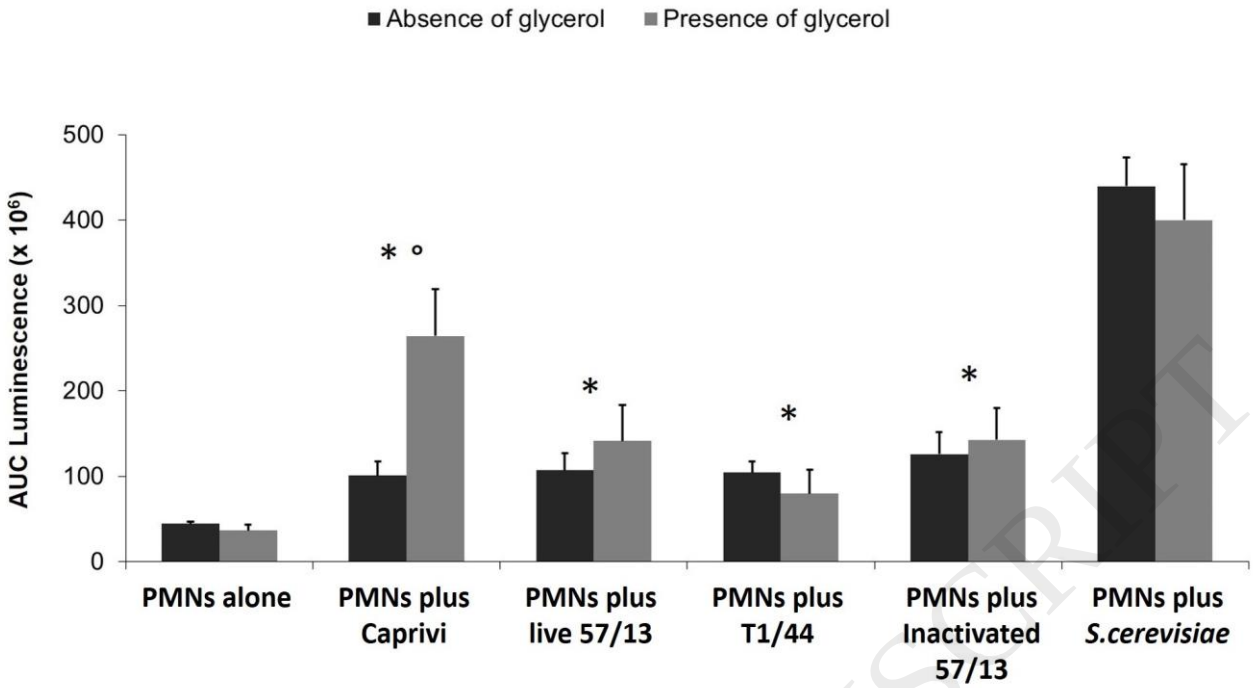
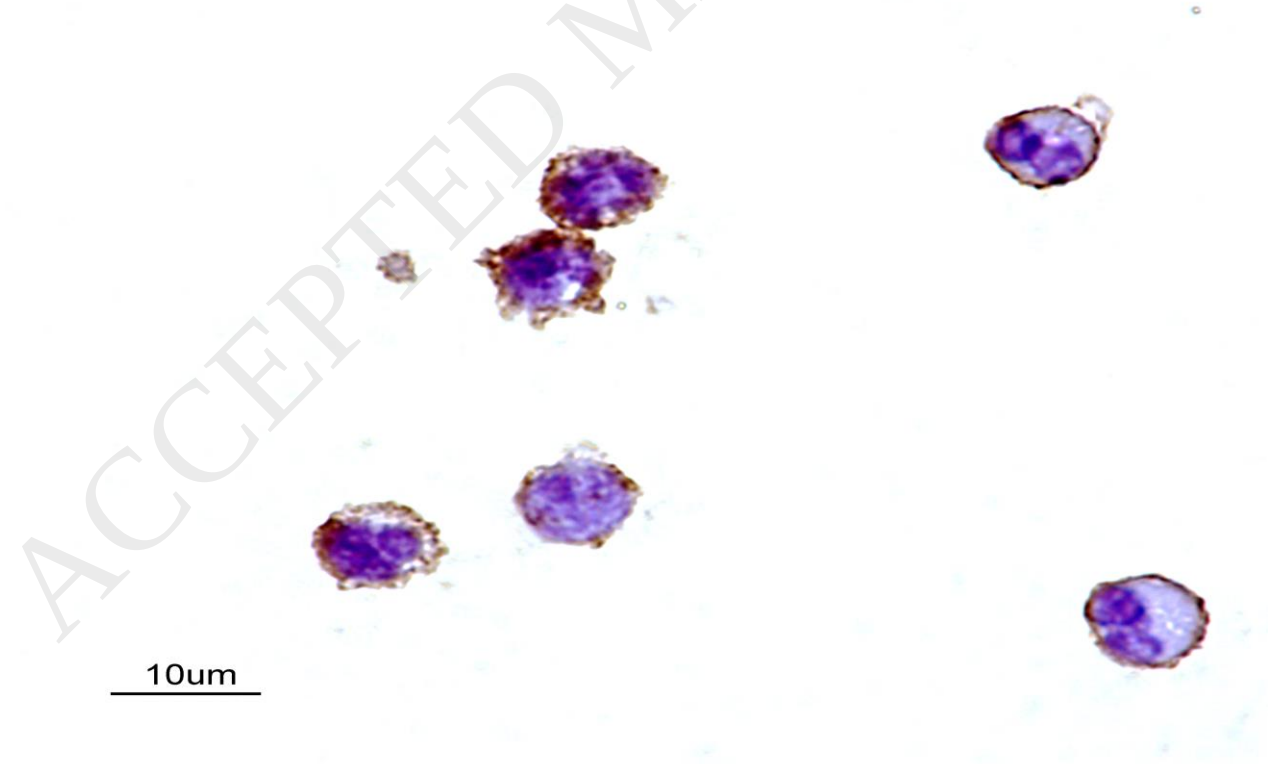


Figure 3. Immunocytochemistry for *Mmm*. *Mmm* antigens (Caprivi strain; brown colour) are abundantly distributed on the cellular membrane of PMNs. Mayer's hematoxylin counterstain. Scale bar = 10 µm.



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