IMMUNITY TO *RHODOCOCUS EQUI* INFECTION IN HORSES

Ljiljana Suvajdžić[1*], Aleksandar Potkonjak[2], Dubravka Milanov[3],
Maja Bekut[4], Anika Trudić[5], Milenko Lazić[6], Zoran Suvajdžić[7]

[1] Department of Pharmacy, Faculty of Medicine,
University of Novi Sad, Novi Sad, Serbia

[2] Department of Veterinary Medicine, Faculty of Agriculture,
University of Novi Sad, Novi Sad, Serbia

[3] Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia

[4] Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia

[5] Center for Microbiology, Institute for Pulmonary Diseases
of Vojvodina, Sremska Kamenica, Serbia

[6] Hemofarm Stada, Vršac

[7] Veterinary practice STRIKS, Čerević, Serbia

Abstract

Rhodococcus equi is an opportunistic bacterium that commonly infects foals and immunocompromised patients. Due to the large economic losses that it can cause in the field of horse breeding, the microorganism has been studied in details, including its immunological aspect. Within the humoral immunity, the most important immunoglobulins are those of the class G (IgG), produced as a response to the surface antigen associated with virulence (virulence associated protein A, VapA). IgG antibodies provide resistance to pneumonia in foals and have a dose dependent protective effect. In addition to them, the protective role of plasma is achieved through various cytokines. Cellular mechanisms are important for killing bacteria within the macrophage. Virulent strains which carry a plasmid with the gene for VapA stimulate the production of interferon gamma (IFN-γ), a key cytokine to kill these bacteria. The presence of IFN-γ is crucial for the removal of microorganisms from the lungs and prevention of formation of pulmonary granulomas. For the complete removal of bacteria cooperation of the humoral and cellular immunity is necessary. Particularly significant is opsonization, which increases phagolysosomal fusion. Vaccination and

[1*] corresponding author: Ljiljana Suvajdžić; email adress: giba57@eunet.rs

application of hyperimmune plasma play a vital role in the treatment of disease. Only alive and virulent bacterium is capable of producing protective immunity in horses. The use of hyperimmune plasma in foals results in a lower percentage of sick animals and less severe clinical progression of the disease. Further research is needed in order to create a safe and effective vaccine.

Key words: *Rhodococcus equi*, immunity, horses, vaccine

IMUNITET KOD KONJA NA
RHODOCOCCUS EQUI INFEKCIJU

Ljiljana Suvajdžić[1*], Aleksandar Potkonjak[2], Dubravka Milanov[3], Maja Bekut[4], Anika Trudić[5], Milenko Lazić[6], Zoran Suvajdžić[7]

[1] Departman za farmaciju, Medicinski fakultet, Univerzitet u Novom Sadu, Novi Sad, Srbija

[2] Department za veterinarsku medicinu, Poljoprivredni fakultet, Univerzitet u Novom Sadu, Novi Sad, Srbija

[3] Naučni institut za veterinarstvo "Novi Sad", Novi Sad, Srbija

[4] Medicinski fakultet, Univerzitet u Novom Sadu, Novi Sad, Srbija

[5] Centar za mikrobiologiju, Institut za plućne bolesti Vojvodine, Sremska Kamenica, Srbija

[6] Hemofarm Stada, Vršac

[7] Veterinarska ambulanta STRIKS, Čerević, Srbija

Kratki sadržaj

Rhodococcus equi je uslovno patogena bakterija koja najčešće inficira ždrebad i imunokompromitovane pacijente. Zbog velikih ekonomskih gubitaka koje može da izazove u uzgoju konja, mikroorganizam je detaljno proučen, između ostalog sa imunološkog aspekta. U okviru humoralnog imuniteta, najznačajniji su imunoglobulini klase G (IgG), nastali na površinski antigen udružen sa virulencijom (virulence associated protein A, VapA). IgG povećavaju otpornost ždrebadi na pneumonije, a imaju dozno zavisno zaštitno delovanje. Pored antitela, zaštitna uloga plazme ostvaruje se preko različitih citokina. Celularni mehanizmi ubijanja su značajni zbog ubijanja bakterije unutar makrofaga. Virulentni sojevi koji su nosioci plazmida sa genom za VapA stimulišu produkciju gama interferona (IFN-γ),

ključnog citokina za ubijanje bakterija. Prisustvo IFN-γ je presudno za ukla-
njanje mikroorganizma iz pluća i sprečavanje nastanka plućnih granuloma.
Za potpuno odstranjenje bakterija, neophodna je kooperacija humoralnog
i celularnog imuniteta. Posebno je značajna opsonizacija, koja povećava fa-
golizozomalnu fuziju. Vakcinacija i primena hiperimune plazme zauzimaju
značajno mesto u terapiji bolesti. Samo živa i virulentna bakterija je sposob-
na da izazove zaštitan imunitet kod konja. Primena hiperimune plazme kod
ždrebadi rezultuje manjim procentom obolelih jedinki i manje ozbiljnim
kliničkim tokom bolesti. Neophodna su dalja istraživanja u cilju kreiranja
bezbedne i efikasne vakcine.

Ključne reči: *Rhodococcus equi*, imunitet, konji, vakcine

INTRODUCTION

Rhodococcus equi (*R. equi*) is an intracellular bacterium that survives
mechanisms of phagolysosomal fusion, which compromises the effectiveness
of antibiotic therapy (von Bargen and Haas, 2009). The microorganism is pre-
sent in the soil, on all continents except Antarctica (Prescott, 1991).

It causes diseases in various animal species (Takai *et al*., 1996; Suvajdžić,
2000; Suvajdžić *et al*., 2001; Flynn *et al*., 2001; Takai *et al*., 2003; von Bargen
and Haas, 2009) but predominantly in horses (Prescott, 1991; Muscatello,
2012a; Suvajdžić *et al*., 2015), which is why this bacterium is called "equino-
centric" (Vázquez-Boland *et al*., 2013).

It can be found in the soil of 50-95% households which breed horses, as
R. equi is present in horses' feces in very high concentrations. In the feces of
mares, *R. equi* is present in concentration from 10^2 to 10^3 colony forming units
(CFU) per gram. It is isolated from the feces of foals, starting from the first
week of life. In the feces of four weeks old foals it is present in the amount
from 10^4 to 10^5 CFU. Such high concentration stays up to 8 and 10 weeks of
age, when it starts to decrease and, as foals are maturing, settles to the con-
centration characteristic for mares (Takai, 1997). This is in accordance with
the research of optimal doses for the foal experimental challenge. The dose of
10^2 CFU gave mild clinical presentation, mimicking natural infection (Sanz *et
al*., 2013). The highest excretion by feces, 10^6 to 10^8 CFU per gram, appears in
the period until 8 weeks of life, precisely at the time when the foals are most
receptive to infections (Takai, 1997).

In human population, infections by *R. equi* often affect immunocompromi-
sed patients (Weinstock and Brown, 2002; Suvajdžić and Považan, 2006; Tuon
et al., 2007). Ten percent of infected patients were already being treated with

immunosuppressive drugs, which are an integral part of the therapy during organ transplantation and in autoimmune diseases (La Rocca *et al.*, 1998). It is believed that about two-thirds of patients infected with *R. equi* suffer from HIV (Harvey and Sunstrum, 1991). However, it can also cause diseases in immuno-competent persons (Kedlaya *et al.*, 2001; Suvajdžić, 2004; Suvajdžić *et al.*, 2015).

Managing control and prevention of this disease in veterinary medicine represents an economic category for the single reason that losses which may occur in the case of an epidemic in stables, especially of thoroughbred horses, would be enormous. In addition to general hygiene and sanitation, specific active and passive prophylaxis should occupy the place they deserve in the system of overseeing animal health (Muscatello, 2012a).

IMMUNITY IN *RHODOCOCCUS EQUI* INFECTIONS

Rhodococcus equi usually infects foals and very rarely adult horses (Vázquez-Boland *et al.*, 2013). Infections occur sporadically, except in foals, where they can have enzootic character. The immune system has a key role in development of infection. In foals, the infection is associated with the period of vanishing of maternal antibodies present in colostrum. In adult animals, accommodation, food and environmental conditions have a significant role in the course of infection, mainly due to their effect on the general immunological conditions (Muscatello, 2012b).

Infection of foals occurs within the first week of life. The onset of clinical signs occurs approximately in 50 days. This also indicates that the incubation period is longer in natural infections, when compared to extremely high inocula sizes used in experiments. (Horowitz *et al.*, 2001).

There are implications that some mares give birth to foals that are particularly sensitive to *R. equi* infections. Such sensitivity can be explained by low levels of colostrum antibodies, functional immaturity of neutrophils in some foals, or genetic predisposition to infections (Wilks *et al.*, 1982).

Due to the big impact on the health of horses, studies of immune response to infection with *R. equi* were mostly conducted on horses, in addition to murine models. Many aspects of immunological reactivity of horses were investigated, including both cellular and humoral mechanisms.

HUMORAL IMMUNITY IN HORSES

The digestive tract is probably the main source of antigenic stimulation of *R. equi* in horses. In foals, the level of maternal antibodies decline to the

lowest level in the period of eight weeks of age, after which foals are actively producing antibodies. Foals with low levels of colostral antibodies, detected by an ELISA test, are particularly vulnerable to rhodococcal pneumonia (Hietala and Ardans, 1987). Those with higher levels of antibodies show a milder form of the disease (Martens *et al.*, 1989). Accordingly, the maximum sensitivity of foals occurs in the period between 2 and 6 months of age (Hines *et al.*, 1997).

The protective role of antibodies that are produced as the response to the surface antigen virulence-associated protein A (VapA) was shown. The G class of immunoglobulins (IgG), created against this antigen was present in bigger concentration when compared to the concentration obtained by immunization with intact but killed *R. equi*. Also, the increase in the mare serum opsonizing activity is more pronounced in the group that received the VapA antigen compared to the group that received the whole bacteria (Cauchard *et al.*, 2004).

Immunoglobulins created to VapA can be considered a diagnostic parameter in horses' infection, (Sanz *et al.*, 2016), bearing in mind that this antigen is unique to equin *R. equi* - human or other animal's isolated strains did not posses this antigen (Occampo- Sosa *et al.*, 2007)

In the study of IgG to VapA and IgG subclasses, it is shown that VapA- specific IgG(T) is the first antibody with the increase of titar. Also, this immunoglobulin is potentially revised as a prognostic parameter in detection of naturally occurring rhodococcal pneumonia (Sanz *et al.*, 2015).

After infection, subclasses of detected antibodies include IgGa, IgGb, IgGc and IgM, with significantly higher levels of IgGa and IgGb subclasses in foals than in horses. (Jacks *et al.*, 2007a) Isotype IgGa is the most important in the prevention of pneumonia in foals (Hooper-McGrevy *et al.*, 2003).

Antibodies against the VapA antigen administered to mice showed a dose-dependent protective effect. After exposure to *R. equi,* the control group without applied antibodies died. The group that received a lower dose of the antibodies was partially protected, although the pathohistological examination showed bacteria in the spleen or lungs. In the mice that received the full dose, there were no deaths or signs of the disease, nor was the microorganism found in their tissues (Fernandez *et al.*, 1997).

However, in a study that examined the passive immunization, in a colt with very low titer of antibodies, there have been no clinical manifestations of the disease, while another colt with a high titer developed serious illness with *infausto* prognosis. Levels of antibodies are not always in correlation with the opsonizing activity. Therefore, it is assumed that non-specific plasma factors, such as lymphokines and interferons, can also impact the protective effect of the immune plasma (Martens *et al.*, 1989).

CELLULAR IMMUNITY IN HORSES

Bearing in mind the intracellular nature of *R. equi*, it is believed that cell-mediated mechanisms are the most effective against this disease (Vázquez-Boland *et al*., 2013). Aside from the disappearance of maternal antibodies, the decreased killing capacity of macrophages in foals contributes to their susceptibility in age of 1-5 months. (Berghaus *et al*., 2014)

The study which compared bronchoalveolar and monocyte- derived macrophages, revealed that the replication of *R. equi* is greater in bronchoalveolar macrophages, with 3- 100 fold increase during 48 hours (Berghaus *et al*., 2014). This is logical, having in mind that predominant route of infection is aerosol inhalation of *R. equi* (Giguère *et al*., 2011a).

Rhodococcus is able to survive intracellularly and to replicate in macrophages, both in murine peritoneal, as well as equine alveolar. The number of bacteria increases with time after the initial phase lag period of the first 6-12 hours of infection, during which the number of intracellular bacteria remains unchanged. During the subsequent 36 hours, the number increases five fold or more, to the inability of quantification, while preserving cell viability. A similar phenomenon was observed with mycobacteria, with the fact that *R. equi* more rapidly divides. Replication within macrophages is characteristic of *R. equi* strains that possess VapA (Hondalus and Mosser, 1994).

The first step in intracellular killing of pathogens is macrophage activation by interferon gamma. The CD4+ and CD8+ T lymphocytes from the lungs are able to produce interferon gamma (INF-γ) (Hines *et al*., 2003). Pulmonary clearance of the pathogen depends precisely of the increase in the number of pulmonary T cells and IFN-γ production (Kanaly *et al*., 1995; Hines *et al*., 2001; Hines *et al*., 2003).

In studies conducted on mice, it has been shown that there are two phenotypes of CD4+ T lymphocytes, which are referred to as Th1 and Th2. The classification was based on the cytokines they produce: IFN-γ is characteristic of the Th1 phenotype, IL-4 of the Th2. The transfer of adequate cell lines, demonstrated that Th1 phenotype led to the complete removal from the lungs (zero CFU), while in the Th2 lines led to the development of pulmonary granuloma. Natural killer (NK) cells can also produce INF-γ, but transferring them alone does not lead to clearance of bacteria from the lungs (Kanaly *et al*., 1996). The production of IFN-γ in horses is triggered by VapA. It causes the same later response in horses as well as in mice (Hines *et al*., 2001; Lopez *et al*., 2002). Since the protein VapA is encoded by the *vap*A gene located on the plasmid (Tan *et al*., 1995), only the virulent strains, carriers of this plasmid, may be removed from the lungs (Hines *et al*., 2003).

Infection of macrophages by *R. equi* leads to a rapid translocation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) from cytoplasm into nucleus, resulting in production of large amounts of inflammatory cytokines. Some of the most important are TNF, interleukin IL-12 and nitric oxide, NO. There is an assumption that these paths of non specific immunity are the main reason why the microorganism rarely causes diseases in immunocompetent adult animals (Darrah *et al.*, 2004).

In a study on normal human peripheral blood mononuclear cells it has been demonstrated that they secrete TNF-α, IL-6 and IL-8 when they are stimulated with killed *R. equi*. Levels of TNF-α were increasing during the first 48 hours, wherein the 90% of secreted cytokines were detected after 36 hours. For IL-6, this level was reached after only 12 hours. Production of IL-8 was at its maximum at the beginning, and leveled off between 12 and 48 hours (Pece *et al.*, 1997).

The effect of foal's age on cytokine production is also revised. The expression of production signals in bronchoalveolar macrophages for IL-1β, IL-10, IL-12 p40 and IL-8, as well as IL-1β and IL-12 p40 production signals in bronchoalveolar lavage macrophages in 1-3 days old foals is higher than in foals of other age. This also indicates exposure to infection with *R. equi* in very early period of life (Berghaus *et al.*, 2014).

It has been shown that the lack of galectin-3 receptor increases resistance to *R. equi* infections. Mice with and without this receptor were compared. Macrophages lacking this receptor are less susceptible to *R. equi*, its reproduction and survival. This receptor normally provides a balanced response to nonspecific immunity, affecting the production of IL-1β by macrophages (Ferraz *et al.*, 2008).

COOPERATION OF HUMORAL AND CELLULAR IMMUNITY

The best descriptions of important interactions between cell-mediated immunity and humoral immunity come from *in vitro* studies that have investigated the mechanisms of killing *R. equi* by macrophages (Hietala and Ardans, 1987).

Alveolar macrophages in foals experimentally exposed to *R. equi* phagocyte and kill both non opsonized and opsonized microorganisms more efficiently than alveolar macrophages in foals that were not exposed to the bacteria. Macrophage activity in foals which were already in contact with *R. equi*, was similar to the macrophage activity in adult horses. However, the rate of killing of opsonized *R. equi* was slightly lower in foals than in horses. In

all tested alveolar macrophages, opsonization of *R. equi* significantly increases phagolysosomal fusion when compared to nonopsonized bacteria (Hietala and Ardans, 1987; Giguère *et al*., 2011).

The use of plasma with antibodies also decreased bacterial viability in the extracellular matrix, but not in the macrophages (Dawson *et al*., 2011).

IMMUNOPROPHYLAXIS AND IMMUNOTHERAPY IN HORSES

Conventional therapy with antibiotics, with some being more effective than others (Giguère *et al*., 2015), is practically impossible without immunological support, with nature taking its course (Berghaus *et al*., 2011). Scientific attempts, with various approaches to increasing antibiotics in target sites included streptolysin O (Horohov *et al*., 2011; Gurel *et al*., 2013) and liposomal forms of antibiotic (Burton *et al*., 2015). In this way, we can "help nature" with immunomodulating support.

Field research conducted by Magnusson in 1923 and 1924 failed to justify vaccination with an inactivated culture of *R. equi*. Vaccination of foals with isolates of *R. equi,* which were inactivated with formalin, failed to protect animals from a strong intratracheal exposure to the bacteria (Magnusson, 1923).

Lopez *et al*. (2008) tested a live attenuated strain of *R. equi* which was a riboflavin auxotroph on a mouse and a colt. This strain has kept the virulence plasmid. However, in a vaccine it is important to balance immunogenicity and attenuation. It is shown that such a vaccine must increase immunogenicity to demonstrate its success in neonatal foals. In order to make disease prevention more effective on endemic farms, a vaccine should be able to induce an immune response very early in life (Lopez *et al*., 2008). Currently, there is no vaccine, but new candidates are being tested (Cauchard *et al*., 2013).

Other attempts with immunostimulans containing *Propionibacterium acnes* showed no effect as imunomodulators in therapy in foals (Sturgill *et al*., 2011).

Pneumonia in foals can be prevented by vaccination of pregnant mares. These vaccines contained VapA and exoenzim. The result of this research was the decline of mortality rates in cases of rhodococcal pneumonia on farms that have vaccinated mares from 3% to 1.2% (Becú *et al*., 1997).

Studies on a few endemic farms in Japan, United States and Argentina have shown that administration of hyperimmune plasma in foals led to protection in the earliest period of life. Some of the recommendations for prevention of pneumonia are: separation of foals in clean and ventilated paddocks, serological testing on 30th and 45th days after birth and immunization with plasma

immediately after birth (Higuchi *et al.*, 1999), no later than second day of life (Giguère et al., 2011a; Vázquez-Boland *et al.*, 2013)

Unspecific measures, such as feces removal from paddocks, avoiding spreading manure on pastures, irrigation of holding pens, foaling at pastures- gave unsatisfying results (Giguère *et al.*, 2011b)

CONCLUSION

Only alive and virulent *R. equi* bacteria can cause immunity in mice. Avirulent live strains and the strains that were killed by heat did not show this activity (Takai *et al.*, 1999). Effective immunization against intracellular bacterial pathogens requires the use of living microorganisms, rather than inactivated, in order to initiate a cellular immune response (Collins, 1988).

In passive immunization, the quality of donor's plasma is crucial: immunized donor's plasma protects significantly from severe illnesses when compared to non immunized donor's plasma (Martens *et al.*, 1989). *In vitro* reaction of passively immunized foals was similar to actively immunized mares (Becú *et al.*, 1997).

In our country there is no immunoprophylaxis or immunotherapy for *R. equi*. Would it be reasonable to take into consideration collecting indigenous isolates, determining antigens and creating a vaccine that covers the antigenic determinants (including VapA) present in our area? Serum production on the same isolates could be professionally, scientifically and economically justified.

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