# Bactericidal Activity of Serum By *Brucella Abortus* RB51 Outer Membrane Protein's Combined By *Brucella Abortus* S99 Lipopolysaccharide Induction

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**Research Article**

**Keywords:** Brucella abortus RB51, Bactericidal activity, Brucella abortus S99, Outer Membrane Protein's

**Posted Date:** February 19th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-231416/v1

# Abstract

Brucellosis vaccines are designed to induce the cellular immunity. An effective brucellosis vaccine is one that could induce both cellular and humoral immunity. Serum Bactericidal Assay(SBA) is an important method for the determination of vaccine humoral immunity. This study is the first to observe humoral immunity in brucellosis by SBA. Extracted *B.abortus* LPS and OMP's were injected to rabbits. Group1 injected by 25µg of LPS, Group2 injected by 50µg of OMP 's and Group3 injected by 1ml of combined vaccine, 3 times every 2 weeks. The Groups were challenged with *B.abortus 544* in the second injection. Sera were separated 2 weeks after the last injection. SBA was performed, each well was streak cultured into a plate of Brucella Agar. Colony count was done for each plate. Results have shown, the third injection of the combined vaccine, had the highest titer of 1/64, and the efficacy of the vaccine was %87.71.


# Introduction

The first effective vaccine against brucellosis was the live attenuated vaccine *Brucella abortus S19* strain. Although this vaccine protects cattle against *Brucella abortus*, but its side effects has led it to be used with caution [2]. Despite these complications, the *Brucella melitensis* vaccine strain, Rev-1 leads to the control of brucellosis in sheep and goats, only in endemic areas [3]. Many researchers have been performed live attenuated vaccine strains. One of the first strains of *Brucella abortus* 45/20, which could transform back into the pathogenic form. *Brucella abortus* strain RB51 vaccine protects cattle against *Brucella abortus* and has fewer side effects [4]. The vaccine is a preventive tool, which is used to eradicate brucellosis. In the classical serological tests, the antibody or antigen against S19 and Rev-1 vaccine, is the LPS [5]. Therefore, no specific serological tests can not distinguish between animals vaccinated with S19 and Rev-1, or a naturally infection of a brucella strain, to detect the disease. Recently, mutant strains of *Brucella abortus* RB51 were proposed to be used in the immunization of cattle. Thus there are no effective vaccines against brucellosis in human or live stocks [6].

The major outer membrane proteins (OMPs) of *Brucella* spp. were first introduced in the 1980s [7]. They were soon characterized as potential immunogenic and protective antigens. They were classified according to their apparent molecular mass as group 1, 36–38 KDa OMPs, group 2 porin proteins and 31–34 and 25–27 KDa OMPs which belong to the group 3 proteins [7]. The use of recombinant protein technology and monoclonal antibodies (MAbs) has shown that the major OMPs appear to be of little relevance as antigens in smooth (S) *B. abortus* or *B. melitensis* infections i.e. low or no protective activity in the mouse model of infection and low or no immunogenicity during host infection. However, group 3 proteins in particular Omp31, appear as immunodominant antigen in the course of rough (R) *B.ovis* infection in rams and as important protective antigen in the *B.ovis* mouse model of infection. The major OMP genes display diversity and specific markers have been identified for Brucella species, biovars, and strains, including the recent marine mammal Brucella isolates for which new species names have been proposed.

The *Brucella abortus* LPS, compared to the *Enterobacteriacae*, has an unusual structure that is significant in making T-cell mediated responses [8]. It can induce IgM and $IgG_3$. In addition, according to new studies, the *Brucella abortus LPS* can show adjuvancity [9]. Moreover, it is also a Hapten. The LPS has a crucial role in the pathogenesis of *Brucella* ssp., therefore, by the characteristics described above, it is likely to consider it as a qualified vaccine candidate [10].

Susceptibility of microorganisms to antimicrobial agent is often measured in vitro by measuring the inhibiting activity of growth. These measurements often do not provide sufficient information to determine the appropriate treatment of certain infections, such as endocarditis [11]. In addition, a specific β- Lactam antibiotic, which have bactericidal activity do not have bactericidal activity for all organisms [12]. Therefore, there is a requirement for new experimental methods which have the ability to observe the bactericidal activity in optimal conditions. However, Schlichter and Mac Lean are considered as the founders of the SBA methods. They discovered the inhibitory effects of blood serum. Although, they were unable to define a criteria for characterization of bactericidal effects. In fact, Fisher completed Schlichter's path discovery. He cultured the tubes which had no visible bacterial growth [13]. However, fisher disclaimed the urge to determine the concentration levels of bactericidal effect [14]. However, there are no accepted methods for Microdilution and Macrodilution of SBA in the world. This test can be performed at any places. Although, the implementation of this technique, due to a variety of variables will be different at any laboratory. Our aim in this study was to determine the bactericidal activity of the serum induced by the potential *Brucella abortus* LPS and OMP's combined subunit vaccine.

## Materials And Methods

# Strains used in this study

*Brucella abortus* S99 was provided from Bacterial Cell Bank of Pasteur Institute of Iran (CSBPI) as the strain for smooth LPS extraction.

*Brucella abortus* RB51 was provided as a live attenuated vaccine, which was used after resuscitation, as the source of the OMP's extraction.

*Brucella abortus 544 was* provided by the Department of Bacteriology, Tarbiat Modares University, and used as the challenge strain for vaccine immunization in mice and rabbits.

# LPS extraction

The LPS of *Brucella abortus* S99 was extracted and purified by a modification of the modified hot phenol method of the Westphal (1986). Phenol with 68°C was added to a batch cultured *Brucella abortus* S99, in fermentor (Novo-Paljas Netherland) and centrifugated at 2900 RPM. 4 phases had been obtained. In order to precipitate the proteins and nucleic acids, cold methanol was added to the phenolic phase, after separation. Trichloroacetic acid was then added, 0.5 grams for each liters of solution and stirred at 4°C for 30 minutes. After centrifugation, the supernatant was dialyzed (cut-off = 20 KDa) 3 times against

distilled water for 24 hours. Then, the white precipitate accumulated inside the dialysis sack was packaged in 100 cc vials and lyophilized [15].

# LPS Detoxification

The LPS obtained was detoxified by the addition of 0.2 N NaOH to the purified LPS, placed in 100°C for 2 hours. After it cooled down, the pH was to set to 7 by 0.1 N HCL. The fatty acids released by performing dialysis against distilled water for 4 days, were cleared. The dialysate (consisted of D-LPS) was then lyophilized. In order to assure LPS detoxification, quality assurance tests, such as LAL, Bradford test, and bioassay were also done [16].

# OMP extraction

its weight in moisture was suspended in Tris buffer, 10 mM (Sigma) containing ethylene diamine, tetrachloroacetic acid, 10 mM (EDTA) W/V. The 100% power for 4 minutes sonication was done within half a minute at 3500 rpm at 4°C and was then centrifuged. Then the cell free fluid was centrifuged for 60 minutes at 42000 RPM and ultracentrifugation was done at 4°C. the precipitate was suspended in 20ml sodium N-Lauryl sarcosinate %2 added to 0.1M Tris buffer containing 10 mM EDTA and kept in room temperature for 1 hour. It was ultracentrifuged again at 42000 RPM for 1 hour at 4°C. The OPM's precipitated in the tube were solubilized in 15 ml water, then dialysed against 0.2M NaCl, with three replacements every 8 hours, for 24 hours at 4°C. the OMP's deposits in 15 ml distilled water and against NaCl 0.2 M. The Dialysed OMP's were devided in sterile 20 and 50 ml glass vials, after passing through 0.22 $\mu m$ filters and kept in -20°C. quality control tests were also done.

**Combination of the** Brucella abortus **LPS and OMP's**

The combined vaccine was made containing 25 mg/ml LPS and 50 µg/ml OMP's. 10 ml of the vaccine mentioned was provided [17].

# Colony count of Mice spleens

First, the mice were sacrificed and the spleens removed were crushed in plates containing 1 ml saline. The original spleen concentration was diluted $\frac{1}{10}$, $\frac{1}{100}$ and $\frac{1}{1000}$ and then streak cultured on brucella agar medium and incubated at 37°C. The plates were controlled for up to 1 week. The colonies formed on the agar plate, were counted next [18].

# Immunization of mice and rabbits

Five BALB/c mice were considered, and 0.2 ml of the combined vaccine was injected to each of them, contemporarily and with the same injection program for the rabbits.

Four groups consisted of two white New Zealand rabbits were chosen. Group 1 was injected by 25 µl LPS, Group 2 was injected with 50 µl of the OMP's, the next group was injected with 1 ml of the combined vaccine. The injections were done twice, with a 14 days interval. In order to simulate brucellosis in all



*ella abortus* 544, two weeks after the first

injection. Two weeks after the last injection, the total blood for all groups was drawn and their sera were separated, in order to perform Serum Bactericidal Assay [19].

# Serum Bactericidal Assay

Serum Bactericidal Assay was performed by, serial two-fold dilution of the group's sera in DPBS; by placing 50 µl DPBS in $\frac{1}{2}$to $\frac{1}{128}$ wells (the second well in each row to the last well). The first well of each group of serum was placed by 100 µl of undiluted serum.50 µl of the first well content was placed in the $\frac{1}{2}$well and serially diluted by the same sampler tip to the $\frac{1}{128}$. Then, 50 µl of $10^5$ *Brucella abortus* 544 concentrations was placed in each well as challenge [20].

The following controls were also considered:

**Negative serum**

50 µl Negative serum + 50 µl $10^5$ Bacterial concentration (*Brucella abortus* 544) + 50 µl DPBS

**Cellular Control**

50 µl inactivated Baby Rabbit Serum + 50 µl $10^5$ Bacterial concentration + 50 µl DPBS

**Positive Control**

50 µl Antigen (LPS) Positive Serum + 50 µl $10^5$ Bacterial concentration + 50 µl DPBS

**Complement Control**

50 µl Active serum + 50 µl $10^5$ Bacterial concentration + 50 µl DPBS

**Bacterial Control**

only 50 µl $10^3$ Bacterial concentration

After all steps above were done, the microplate was incubated for 30 minutes in 37°C. Then, 1 µl of each well was transferred to Brucella Agar plates and streak cultured at once. Then, all plates were kept in the incubator for 72 hours at 37°C. the colonies grown on all plates were then counted. Referring to the control plates, any plate count, less or equal to %50 of the colonies were considered as positive.

# Results

# Results for the Mice Spleen Bacterial Count

In order to evaluate the immunogenicity of the groups vaccinated, *Brucella abortus* strain 544 was injected into the mice groups. Results of bacterial counts were determined four weeks after

challenge.

# Results for the Bactericidal Activity of sera

Since, evaluation of the serum bactericidal activity is one of the most important vaccine immunological tests, Serum Bacterial Assay was performed on immunized animal sera, challenged by of Brucella abortus 544 challenge strain, and the result have shown, the antibody against the combined vaccine has arisen at day 45, which is two weeks after the last vaccine injection. The positive bactericidal titre of this group was $\frac{1}{64}$. Stimulation of antibody against the combine vaccine was significant, compared to all groups at this time. Chart – 2 shows the overall efficacy of the combined vaccine vs. the RB51 live vaccine.

**Table 1** Bactericidal antibody OMP+LPS vaccines combine the first and second injection combine first injection first injection of vaccine, **T2:** Combine vaccine injection, **L:** Suicide live vaccine strain RB51 title.

| | $\frac{1}{2}$ | $\frac{1}{4}$ | $\frac{1}{8}$ | $\frac{1}{16}$ | $\frac{1}{32}$ | $\frac{1}{64}$ | $\frac{1}{128}$ |
|---|---|---|---|---|---|---|---|
| T1 | + | + | + | + | - | - | - |
| T2 | + | + | + | + | + | + | - |
| L | + | + | + | + | - | - | - |

# Discussion

Although, the S19 and RB51 live vaccines used against brucellosis are safe, even in a few cases could cause disease in animals or humans (and personal associated with the cultivation of bacteria). In addition, by the inoculation of these vaccines, the ability to distinguish vaccinated from infected animals to microbes in natural ways is very difficult. Therefore, efforts to achieve a subunit vaccine, due to the problems of using conventional vaccines not in humans has risen; by this reason, brucellosis subunit vaccine development is one of the important research areas.

Since, a variety of protein and non-protein antigens of brucella, or a combination of them have been proposed as vaccine candidates. Induction of immunity against brucellosis; both immune responses are required, particularly cellular immunity. The characteristic of pure brucella antigen are not sufficient to induce these responses. Therefore, an effective subunit vaccine against brucellosis seems likely to be a combination of different brucella antigen [17, 21, 22].

In general, since the injection of pure LPS can induce a few antibodies, although it does not stimulate the immune system with high protection. This is due to the role of LPS as a thymus-independent antigen. The LPS was extracted from *Brucella abortus* S99, a smooth strain, which is used in the Pasteur Institute of Iran to produce antigens, and this component of the vaccine, was considered as the Hapten, due to the

stimulation of the immune system. In order to correct the flaws of this vaccine, the OMP's of *Brucella abortus* RB51 were used. Since, the candidates do not cause long-lasting immunity.

Attempts to develop vaccines for brucellosis have been done through the world. Despite all these efforts, no one has been able to develop an effective vaccine for the use in humans and animals with minimal side effects.

In order to develop an effective vaccine against brucellosis, now Smooth Brucella Strains:

*B.abortus* S19, *B. melitensis* Rev-1 and Rough Brucella strains: *Brucella abortus* RB51 are used in many countries [21, 23, 24].

Although S19 and Rev-1 have disadvantages of causing abortions in pregnant animals and are pathogenic in humans. However, more significant problems are the production of specific antibodies against O-LPS, and cross immunity seen in the conventional serological tests using S-LPS, 9, 10 and 23. Therefore, the best strain using to develop a brucellosis vaccine is *Brucella abortus* RB51 which, is a rough strain and does not have the disadvantages of the S19 and Rev-1 vaccine. In addition, studies have shown that the OMP's of *Brucella abortus* RB51 strain are immunogenic. However, each one of them by itself is not an effective inducer of the immune system itself. However, they are all used together, they show a synergistic effect in the vaccine. In this study, all the 11 OMP's were used [17, 25, 26].

SBA is superior to the ELISA method, because ELISA measures the antibody production that can only be achieved by a vaccine. Nevertheless, SBA, antigenicity and the serum bactericidal titers against bacteria can be achieved at the sane time, as desired. SBA was the main method for testing vaccine safety against Meningitis, and could likely be used to observe the vaccine immunity for brucellosis.

Despite the fact that Brucella is an intracellular bacterium, since the new developed vaccines are killed, it will not be a problem against the intracellular bacteria. Moreover, by the application of both LPS and the OMP's of *brucella abortus*, the humoral and cellular immune responses to vaccines would be significant. The colony count of mice spleen clearly demonstrated the effectiveness of this vaccine compared to the control plate. Thus, indications are that the use of these two antigens may be useful in vaccine development.

Compared to the S19 vaccine, this vaccine did not show any sign of abortion. Bioassay in mice and rabbits did not show recurrence. Moreover, this vaccine does not have the risk of transformation of rough strains to soft strains. Since, there are risks of using cattle brucellosis vaccine in human. However, by using the combination of LPS and the OMP's of brucella, it will be cleared.

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# Conclusions

The results in this study have shown, the LPS responses in the vaccine induced the T-cell Mediated response. The OMP's in this vaccine had acted as a carrier for the LPS and boosted the immune response more for the LPS.

Due to the economic damage that brucellosis causes in livestock, the production of meat and dairy products can hopefully be raised; regardless of any concerns.

Since, conjugation of two or more molecules can lead to better immunogenicity in humans or animals, further studies in the future could be done on the evaluation of the immunity caused by the conjugation of these two antigens by Serum Bactericidal Assay.

# Declarations


### Acknowledgements

We would like to thank the Pasteur Institute of Iran,


### Compliance with Ethical Standards

### Conflict of interest

The authors have declared that no competing interests exist.

### Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AA, MK, HA.MSS The first draft of the manuscript was written by AA,MSS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.


### Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.


### Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

### Informed consent

Informed consent was obtained from all individual participants included in the study

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# Figures

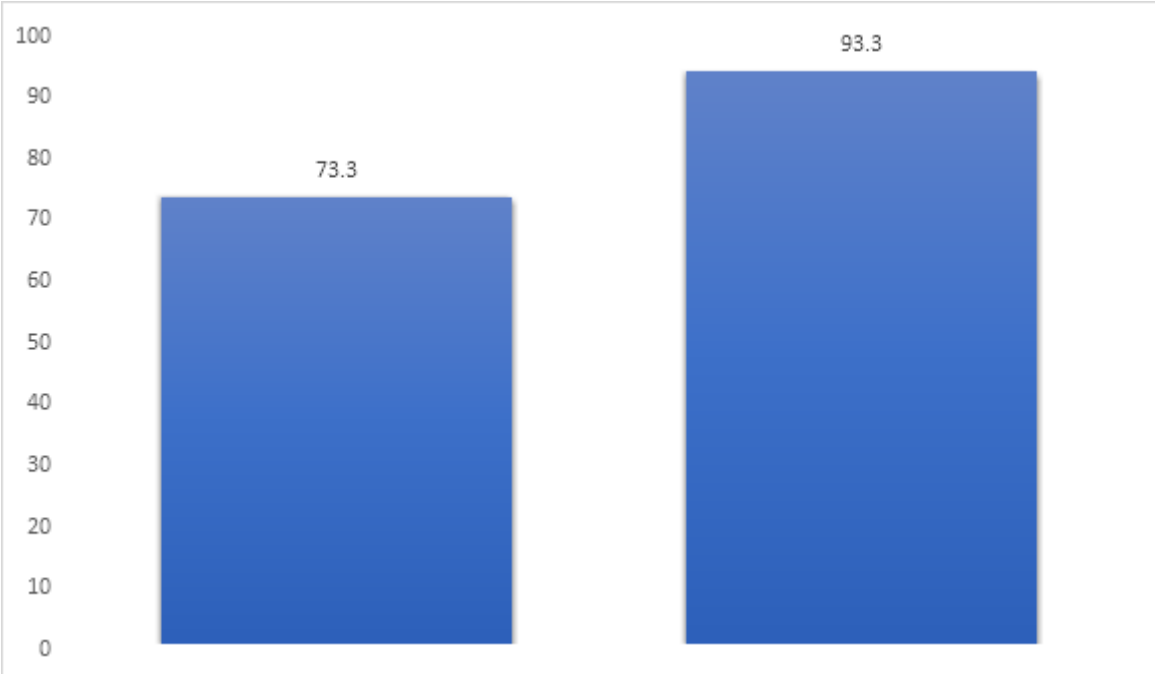


**Figure 1**

1) The mean number of colonies per spleen for each group 2) The number of colonies combined vaccine group OMP+ LPS

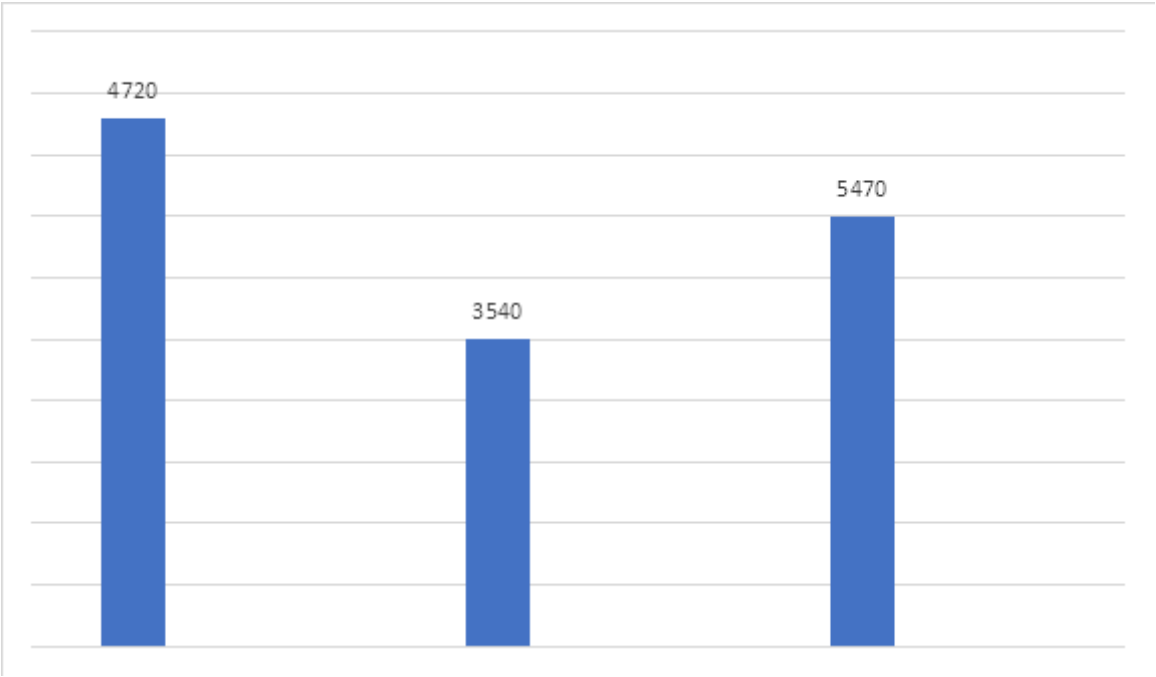


**Figure 2**

The first column on the left side indicates the spleen colony count for pure Brucella OMP's. The second column in the middle is the spleen colony count for the Brucella OMP+LPS. The column on the right is the spleen colony count done in comparison done with the current RB51 live vaccine.

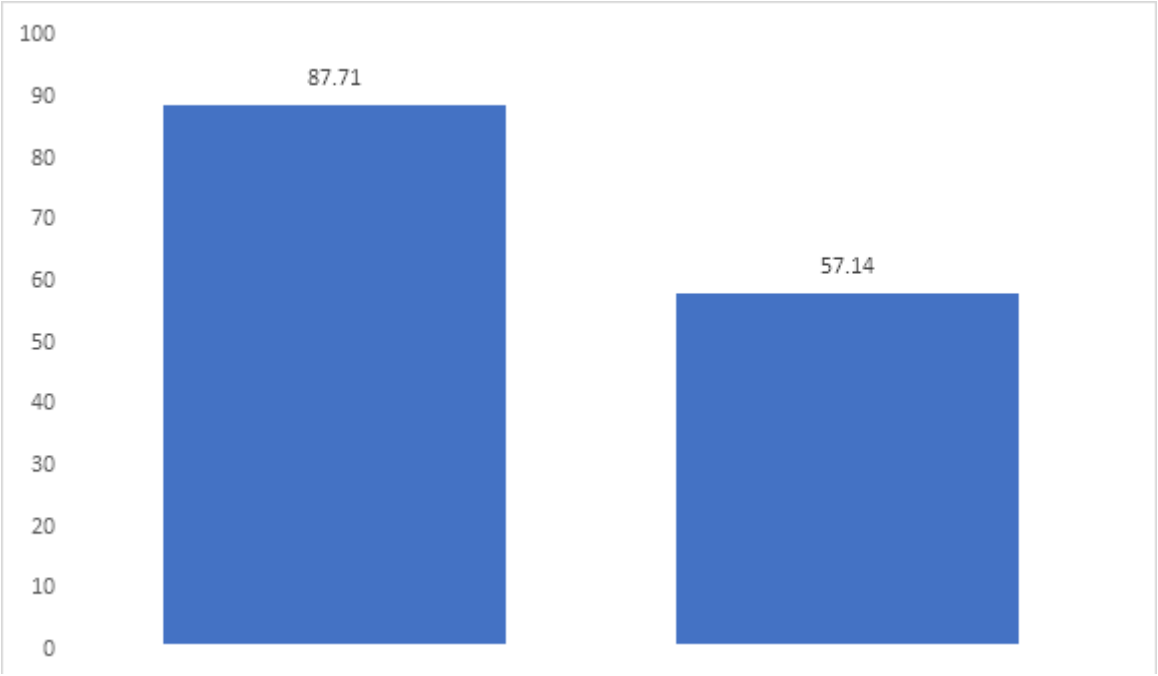


**Figure 3**

Percentage of subunit vaccine protects against OMP+LPS vaccine RB51: 1) The immunogenicity of combine vaccine: OMP+LPS (%87.71) 2) The percentage of live vaccine immunogenicity RB51 (% 57.14)