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Evaluation of recombinant protein superoxide dismutase of 

*Haemophilus parasuis* strain SH0165 as vaccine candidate in a mouse model

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Abstract

*Haemophilus parasuis,* can cause a severe membrane inflammation disorder. It has been documented that superoxide dismutase is a potential target to treat systemic inflammatory diseases. Therefore, we constructed an experimental *H. parasuis* subunit vaccine SOD and determined the protective efficacy of SOD using a lethal dose challenge against *H. parasuis* serovar 4 strain MD0322 and serovar 5 strain SH0165 in a mouse model. The results demonstrated that SOD could induce a strong humoral immune response in mice and provide significant immunoprotection efficacy against a lethal dose of *H. parasuis* serovar 4 strain MD0322 or serovar 5 strain SH0165 challenge. IgG subtype analysis indicated SOD protein could trigger a bias toward a Th1-type immune response and induce the proliferation of splenocytes and secretion of IL-2 and IFN-γ of splenocytes. In addition, serum in mice from SOD immunized group could inhibit the growth of strain MD0322 and strain SH0165 in the whole-blood killing bacteria assay. It was the first time reported that immunization of mice with SOD protein could provide protective effect against a lethal dose of *H. parasuis* serovar 4 and serovar 5 challenge in mice, which may provide a novel approach against heterogenous serovar infection of *H. parasuis* in future.

**Keywords:** *Haemophilus parasuis*; Glässer's disease; superoxide dismutase; vaccine
Introduction

*Haemophilus parasuis* (*H. parasuis*), is the causative agent of the Glässer's disease in swine with phenotypic presentation of polyserositis, meningitis and arthritis (Oliveira and Pijoan 2004). In recent years it has become one of the most important respiratory bacterial pathogens of livestock worldwide and can cause gross economic losses (Rapp-Gabielson et al. 1997). To date, fifteen serovars of *H. parasuis* have been identified, however, up to 20% isolates could not be sero-typed in some countries (Kielstein and Rapp-Gabrielson 1992). Serovar 4 and serovar 5 are the most frequently detected in most countries (Rúbies et al. 1999; Cai et al. 2005). Although vaccine immunization is thought to be the best method to control and prevent infectious disease (Rappuoli et al. 2002), currently no good vaccine is available which can provide protection against the challenge of all serovars of pathogenic *H. parasuis*.

The pathogenesis of *H. parasuis* infection is poorly understood - only a few number of virulence-related factors have been linked to the pathogenicity of Glässer's disease. *ClpP* plays an essential role in stress tolerance, and negatively regulates biofilm formation by *H. parasuis* (Huang et al. 2016). *cheY* plays a crucial role in growth, colonization, biofilm formation and autoagglutination of *H. parasuis* (He et al. 2016). Deletion of *rfaE* gene can attenuate resistance, adhesiom and invasion of *H. parasuis* to PUVEC and PK-15 cells (Zhang et al. 2014). *capD* gene is a novel pathogenicity-associated determinant and involves in serum-resistance ability of *H. parasuis* (Wang et al. 2013). Previous reports demonstrated that immunization against virulence-related proteins, which mostly were outer membrane proteins
(OMPs), could confer protection against the infection of microbe (Tavares et al. 2003; Vilanova et al. 2004). Importantly, some protective or immunogenic antigens have been identified that potentially can be used as vaccine candidates, for example, Omp26, VacJ, HAPS-0742 (Li et al. 2016), PfIA, Gcp, Ndk, HsdS, RnfC, HAPS-0017 (Li et al. 2015), TbpB (Frandoloso et al. 2015), rGAPDH, rOapA, rHPS-0675 (Fu et al. 2013a) and 6PGD (Fu et al. 2012a). Even though some potential vaccine candidates have been studied, the mechanism of immune protection triggered by vaccines is poorly understood.

The genomic sequencing of H. parasuis SH0165 strain has been completed (Yue et al. 2009). We have found superoxide dismutase (SOD) gene present in H. parasuis genome. SOD is a family of antioxidant enzymes that function as a frontline defense against superoxide anion radicals (O2−) which were produced in all aerobic cells (Fridovich 1998). It has been documented that SOD is very useful to treat systemic inflammatory diseases (Shafey et al. 2010). To date, using H. parasuis SOD protein as a vaccine candidate has not been reported previously.

In this study, we constructed an experimental H. parasuis SOD vaccine and determined the protective efficacy of the H. parasuis SOD using a lethal dose challenge of H. parasuis serovar 4 and serovar 5 in a mouse model.

Materials and methods

Bacteria strains and growth conditions

H. parasuis (serovar 4, strain MD0322; serovar 5, strain SH0165) (Cai et al. 2005) were grown in tryptic soy broth (TSB) (Difco, USA) or tryptic soy agar (TSA) (Difco,
USA) supplemented with 10 µg/ml NAD (Sigma, St. Louis, MO) and 10% newborn calf serum (Gibco, USA). *Escherichia coli* was grown in Luria-Bertani (LB) broth or agar (Difco, USA) at 37 °C in the presence of 25 µg/mL kanamycin (Sigma, USA) when necessary. All bacterial strains were grown at 37 °C.

**Purification of recombinant SOD protein**

*sodA* gene was amplified by PCR using the following primers: sodAF (5’-GCGGATCCATGGCATACACATTAC-3’; underlined, *Bam*HI site) and sodAR (5’-GCAAGCTTTTATGCTTGGGATTCA-3’; underlined, *Hind*III site). Then the PCR products were sub-cloned into the pET28a expression vector.

*E. coli* BL21 (DE3) (OD₆₀₀=0.6) were induced with 1mM IPTG (Sigma, USA). A pilot experiment was performed and the molecular mass of the SOD protein was assessed by SDS-PAGE. The SOD protein was purified, as previously described with some minor modifications (Fu et al. 2013a). Briefly, the cell pellet was suspended in 20 mL of buffer solution (50 mM Tris-HCl at pH 8.0, 500 mM NaCl and 1 mM PMSF) and disrupted in a French pressure cell. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant was mixed with 5 mL of Ni²⁺-NTA agarose (Qiagen, Germany) in a column. Proteins not bound to Ni-NTA agarose were removed using 150 mL binding buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 5 mM imidazole). The target protein was eluted by 5 mL elution buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 500 mM imidazole) and stored at -80 °C for further study.

**Animals and vaccination**

This study was carried out in strict accordance with the recommendations in the
China Regulations for the Administration of Affairs Concerning Experimental Animals 1988 and the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals 2005. The protocol was approved by China Hubei Province Science and Technology Department (Permit Number: SYXXK(ER) 2010-0029). The animals were euthanized at the end of the experiments or when moribund during the experiments.

Before immunization, the purified SOD protein was mixed with Marcol 52 (ESSO). 99 numbers of four to five weeks old female BALB/c mice were divided randomly into three groups. Mice were immunized subcutaneously with 50 µg of SOD protein (100 µL, 50 µg) mixed with a 1.5-fold volume of Marcol 52 adjuvant (immunized group). The other two groups were injected PBS (100 µL) emulsified in the same adjuvant as negative control (negative group) or PBS only as blank control (blank group). Mice were injected a vaccination boost of the same dose on day 14 following the first immunization. The blood samples were collected for immunologic assays by tail bleeding on day 14 after the booster immunization.

**Determination of antibody titers**

Serum for IgG titers against SOD protein was analyzed by enzyme linked immunosorbent assay (ELISA), as described previously (Fu et al. 2012b). Briefly, 96-well microtiter plates were coated with purified SOD protein and serially-diluted mouse serum was added and incubated for 45 min at 37 °C. Hence goat anti-mouse IgG-HRP (CST, USA) was added and the samples were incubated for 30 min at 37 °C. To determine IgG subclass, coated plates were incubated with dilutions of mice sera.
and added 100 µL of goat anti-mouse IgG1-HRP or IgG2a-HRP (Santa Cruz Biotechnology, USA) diluted 1:5000. The color was developed by adding activated substrate solution (sodium citrate buffer containing 1 mg/mL of 3, 3', 5, 5'-tetramethylbenzidine and 0.03% H$_2$O$_2$) and the reaction was stopped by adding 0.25% hydrofluoric acid to each well. The plates were read at an absorbance of 630 nm.

**Lymphocyte proliferation**

Lymphoproliferation assays were determined, as previously described with some minor modifications (Khan et al. 2006; Fu et al. 2013a). Three mice from immunized group, the negative control group and the blank control group, were sacrificed on the 14th day following the boost immunization, and the spleens from the mice were isolated as recorded previously (Silva and Benitez 2005). Briefly, spleens were aseptically harvested and processed by gentle disruption with sterile stainless steel sieve and glass pestle and suspension of splenocytes in RPMI incomplete medium (Gibco, USA). Cell suspensions were centrifuged for 20 min at 190 × g. Erythrocytes were lysed by treatment with 0.84% ammonium sulfate for 15 min on ice. Then the cells were gently washed three times with Hank’s Balanced Salt Solution (HBSS) (Hyclone, USA), then resuspended in complete RPMI medium (Gibco, USA). 200 µL of cells were cultured (1 × 10$^5$ cells/mL) in 96-well culture plates at 37 °C. The cells were stimulated with the SOD protein and concanavalin A (4 µg/well) (WAKO, Japan) *in vitro* and incubated for 72 h at 37 °C in a 5% CO$_2$ incubator. Lymphoproliferation assays were determined using
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent with a cell proliferation kit (Promega, USA), according to the manufacturer’s instructions. The lymphocytes were co-cultured in 96-well culture plates with MTS reagent for 4 h, and the absorbance was read at 490 nm wavelength using ELISA reader.

**Determination of cytokines by ELISA**

The supernatants from splenocytes cultures were collected after 72 h and stored at -80 ◦C. The production of IL-2, IL-4, and IFN-γ from culture supernatants were measured using ELISA kits (R&D, USA), according to the manufacturer’s instructions.

**Whole blood killing bacteria assay**

The bactericidal assay was determined as described previously (Furano and Campagnari 2003; Fu et al. 2013b), with some minor modifications. Briefly, live bacteria of *H. parasuis* strain SH0165 or strain MD0322, were washed and diluted in sterile physiological saline. Samples of diluted bacteria (20 µL; 10⁶ cells) were mixed with 180 µL inactivated test sera which were inactivated in a 56 ◦C water bath for half an hour diluted 2-fold with physiological saline, or diluted bacteria were mixed with saline alone. After incubation at 30 min under 37 ◦C and then 45 min on ice, nonimmune whole heparinized (10 U/mL) mouse blood (100 µL) was added, and the mixture was incubated at 37 ◦C with shaking for 1 h. Bacteria were then plated on TSA containing 10% newborn calf serum and 10 µg/mL NAD. Bacterial colony counts (CFU) were recorded after 36 h. Results were expressed as percent
killing according to the following formula: (CFU after 1 h of growth with control sera - CFU after 1 h of growth with immune sera)/CFU after 1 h of growth with control sera × 100. All assays were performed in triplicate and repeated three times.

**Challenge experiments in mice**

On day 14 following the booster immunization, mice immunized with SOD protein were randomly divided into groups 1 and 2 (15 mice/group) (immunized groups), mice immunized with Marcol 52 adjuvant were randomly divided into group 3 and 4 (15 mice/group) (negative control groups), mice immunized with PBS were randomly into 5 and 6 (15 mice/group) (blank control groups), respectively. Hence, the mice from groups 1, 3, and 5 were challenged intraperitoneally against a lethal dose of $6.0 \times 10^9$ CFU of *H. parasuis* SH0165 strain and the mice of groups 2, 4, and 6 were challenged intraperitoneally against a lethal dose of $6.0 \times 10^9$ CFU of *H. parasuis* MD0322 infection, respectively. All mice were monitored regularly for 7 days following the challenge, and morbidity and mortality were recorded.

**Histopathology**

Lung tissues of mice from immunized group, negative control group and blank control group were fixed by immersion in 10% neutral buffered formalin and embedded in paraffin. And then 4 μm tissue sections were cut and stained with hematoxylin and eosin (H&E) according to a standard protocol and examined under light microscopy.

**Passive immunization**

Passive immunization experiments were conducted as previously described (Sun
et al. 2011; Fu et al. 2012b). Briefly, the newly purchased 40 numbers four to five weeks old female BALB/c mice were randomly divided into four groups which were named groups 7-10. The groups 7 and 9 (ten mice per group) were intraperitoneally injected with 100 µL of serum from immunized mice and groups 8 and 10 (ten mice per group) were intraperitoneally injected with 100 µL of serum from adjuvant-immunized mice (negative group). At 24 h post-immunization, groups 7 and 8 were challenged against a lethal dose of $3.0 \times 10^9$ CFU of SH0165 strain of serovar 5, and groups 9 and 10 were challenged against a lethal dose of $3.0 \times 10^9$ CFU of MD0322 strain of serovar 4. All mice were monitored as described above and morbidity and mortality were recorded.

**Statistical analysis**

The experimental data were expressed as mean ± SD. The difference between two groups was analyzed using Student’s t-test and the difference among three groups was analyzed using the ANOVA and survival analysis was used the Logrank test. $p$ values of $<0.05$ were considered significant. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

**Results**

**Expression and purification of the recombinant SOD**

The recombinant SOD protein was successfully cloned and expressed as HIS fusion protein which corresponded to its predicted size (Fig. 1A), and the His-tagged SOD protein was purified by Ni+NTA affinity chromatography. We showed that the purified recombinant SOD migrated as a single band in SDS-PAGE (Fig. 1B).

**Antibody response to SOD recombinant protein in mice**
The results demonstrated that the mice immunized with the purified SOD protein exhibited significant antibody immune responses \( (p < 0.01) \) (Fig. 2A). However, the antibody production was not observed in mice from negative control group or blank control group (Fig. 2A).

To further determine the type of immune response, the subclass of IgG1 and IgG2a were investigated. We showed that the levels of antibody isotypes were higher in the SOD-immunized group compared to the negative control group and blank control group \( (p < 0.001) \) (Fig. 2B). Furthermore, IgG2a immune responses predominated over IgG1 immune responses \( (p < 0.01) \) (Fig. 2B).

**Determination of cytokine production**

We showed that a significant proliferative T-cell immune response was displayed in the mice from immunized with SOD protein \( (p < 0.001) \) (Fig. 3A). However, the blank control group and the negative control group could not induce a proliferative T-cell immune response (Fig. 3A). In addition, a strong T cell proliferative response was elicited by concanavalin A (Con A) \( (p < 0.001) \) (Fig. 3A).

The levels of IL-2 and IFN-\( \gamma \) from the culture supernatants of splenocytes isolated from SOD-immunized group mice were significantly higher than that from the negative control group and the blank control group \( (p < 0.01) \) (Fig. 3B, 3C). These results strongly suggested that immunization with SOD protein in mice could elicit a Th1-type immune response.

**Mouse whole-blood killing bacteria assay**

The results demonstrated that serum from the negative control group and the blank
control group could not inhibit the growth of either SH0165 strain or MD0322 strain (Fig. 4). However, the serum from SOD immunized group displayed a clearly reduction of the viable titers of SH0165 strain and MD0322 strain ($p < 0.01$) (Fig. 4), which demonstrated that the antibodies induced by the recombinant SOD protein potentially provide a certain immunoprotection against the challenge of $H. parasuis$.

**Protective efficacy against $H. parasuis$ challenge in mice**

The results of immunization and challenge experiments showed that the survival of mice from immunized with SOD protein against MD0322 strain challenge was significantly better than that group immunized with SOD protein against SH0165 strain challenge, 80% and 73.3%, respectively ($p < 0.05$) (Fig. 5A and 5B). In addition, the immunoprotective efficacy in groups immunized with SOD protein were markedly greater compared with the blank control groups and the negative control groups ($p < 0.01$) (Fig. 5A), in which no immunoprotective efficacy was observed, because all mice in the blank control group died within 2 days after challenge (Fig. 5B).

**The significance of antibody response to immunoprotection effect**

The results showed that the percentages of surviving mice immunized with the serum from SOD protein against SH0165 strain or MD0322 strain challenge were 37.5% and 50%, respectively, which were significantly higher than that from the negative control group ($p < 0.01$) (Fig. 6A, 6B).

**Histopathologic analysis**

All of the mice from the blank control groups and from the negative control groups displayed severe tissue damage in the lung. However, all survival mice from
SOD protein immunized groups did not appear obvious pathological damage. Lung tissues from blank control groups or negative control groups challenged by either *H. parasuis* MD0322 strain or SH0165 strain exhibited extensive edema with massive proliferation of fibroblasts and connective tissue formation. The bronchioles were suffused with cellular exudates composed of neutrophils (Fig. 7C, 7D, 7F, 7G). However, only minor pathological damages were detected in the tissue of lung from mice immunized with SOD protein (Fig. 7E, 7H). These results indicated that immunization with SOD protein could inhibit tissue pathological damage following *H. parasuis* challenge, which also confirm that SOD protein could confer immunoprotection efficacy against infection of *H. parasuis*.

**Discussion**

The current studies were carried out to explore the role of superoxide dismutase (SOD) of *H. parasuis* in triggering immunoprotection efficacy against a lethal challenge in mice. In this study the *sodA* gene of *H. parasuis* was cloned and expressed and its immunoprotection effect was determined. To our best knowledge, this is the first report documenting that SOD protein of *H. parasuis* has immunogenic characteristic and can trigger humoral and cell mediated immune responses in the SOD-immunized mice. In addition, immunization of mice with SOD protein could provide protective effect against the lethal dose of *H. parasuis* serovar 4 and serovar 5 challenge in mice.

Previous studies have reported that SOD can control the levels of reactive oxygen species (ROS) and reduce neutrophil recruitment to the lung and dampen
inflammatory responses during noninfectious insult (Bowler et al. 2004; Yao et al. 2010). Extracellular SOD has a high affinity in binding to heparin sulfate and collagen in the extracellular matrix (ECM) and has important effects on protecting tissues from oxidative damage and inflammation (Petersen et al. 2004; Gao et al. 2008). Extracellular SOD also plays a detrimental effect during *L. monocytogenes* infection which decreased host survival, bacterial clearance, TNF-α and peroxynitrite production, and neutrophil function (Break et al. 2012). SOD has been used in many pharmaceutical compositions for treatment of diseases. To evaluate the immune responses and immunoprotection effect against *H. parasuis* challenges, a murine model has been developed in the past (Zhou et al. 2009; Fu et al. 2012a). Again, we used this well-developed murine model and demonstrated that the SOD protein can protect against lethal dose of *H. parasuis* infection, which provides a novel approach for the development of a *H. parasuis* SOD subunit vaccine.

Previous research has been showed that the subtype of IgG and the type of Th immune responses are important for protective immunity against certain disease (Chiang et al. 2009; Fu et al. 2013b). The production of IgG1 subclass is representative of T helper 2 (Th2) immune response, whereas the IgG2a is the marker of Th1 immune response. In this study, we showed that SOD protein of *H. parasuis* can trigger the production of both subclasses of IgG1 and IgG2a. Furthermore, the higher levels of IgG2a demonstrated the dominance of Th1 immune response over Th2 immune response. To further verify the types of immune response, the productions of cytokine from the splenocytes were measured. Our results showed that
SOD protein of *H. parasuis* also induced the secretion of IL-2 and IFN-γ of splenocytes, but not the production of IL-4. Th1 immune cells mainly produce IL-2 and IFN-γ and Th2 immune cells mainly produce IL-4 (Gagliani and Huber, 2017). These results, together with the data of passive immunization assay and whole-blood killing bacteria assay, indicate that the effect of SOD protein of *H. parasuis* is likely related to cellular immune response which elicits strong immunoprotection function against *H. parasuis* infection.

It has been documented that serum resistance is thought to be an important virulence pathological mechanism in *H. parasuis* leading to pig systemic disease and virulent strain were mainly more resistant to the bactericidal effect of the serum than the non-virulent strains (Cerdà-Cuèllar and Aragon 2008). Serovars are commonly considered as a marker of virulence in *H. parasuis* (Oliveira and Pijoan 2004). In general, serovar 5 was thought to be highly virulent, whereas serovar 4 was considered to be moderately virulent (Amano et al. 1994). In this study, we elected to use the Chinese local isolates, MD0322 (serovar 4) strain and SH0165 (serovar 5) strain, as the challenge strains. These virulence strains allow us to simulate a natural situation of infection. In addition, we showed that both highly virulent SH0165 strain and moderately virulent MD0322 strain were killed by the antiserum induced by SOD protein with different degrees and serovar 4 MD0322 strain is more sensitive to bactericidal effect of the antiserum. This new finding is somehow contradictory to our previous finding (Fu et al. 2013a). To address this discrepancy, we are in the process of designed new experiments to dissect the underlying mechanism. Furthermore, the
result of blood killing bacteria assay also suggests that the serovar 5 is more virulent than serovar 4, which implies that antibody responses seems to play an important role in SOD-triggered immunoprotection.

Our results showed that SOD protein could provide immunoprotection effect in the female BALB/c mice against lethal dose challenge of serovar 4 and serovar 5 of *H. parasuis*.

**Acknowledgements**

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**REFERENCES**


Break, T.J., Jun, S., Indramohan, M., Carr, K.D., Sieve, A.N., Dory, L., and Berg, R.E. 2012. Extracellular superoxide dismutase inhibits innate immune responses and


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Vilanova, M., Teixeira, L., Caramalho, I., Torrado, E., Marques, A., Madureira, P.,


Legends to figures

Fig.1. Expression of SOD protein in E. coli BL21 (DE3). The E. coli BL21 (DE3)
cells were grown to log-phase and induced with 1mM IPTG for 3 h at 37 °C. The target protein was eluted using 500 mM of imidazole. (A) (1) (2) (3) induced with IPTG; M: molecular weight marker. (B) (1): elution; M: molecular weight marker.

Fig.2. (A) Antibody titers in mice from immunized group, the negative control group and the blank control group. Blood samples were collected on the 14 day after the booster immunization, and the antibody response was determined by ELISA; optical density readings of > 0.3 were scored as positive. (B) The levels of IgG1 and IgG2a in mice from immunized group, the negative control group and the blank control group. Results are expressed as means ± SD. **, significance at a p value of < 0.01; ***, significance at a p value of < 0.001. △ versus the negative control group and the blank control group.

Fig.3. (A) Lymphocyte proliferation assay. On the 14th day following the boost immunization, 200 µL of splenocytes from immunized group, the negative control group and the blank control group were cultured (1 × 10^5 cells/mL) were stimulated with the SOD protein (4 µg/well) and concanavalin A (4 µg/well) and incubated for 72 h at 37 °C in a 5% CO_2 incubator. (B) and (C) Levels of IL-2 and IFN-γ secretion in cultured splenocytes of mice from immunized group, the negative control group and the blank control group. Results are means ± SD; ***, significance at a p value of < 0.001. △ versus the negative control group and the blank control group.

Fig.4. The whole blood bactericidal activity of SOD immunized, negative control and blank control mice. The results are expressed as the percent killing bacteria activity according to the percentage of reduction of bacteria growth in the presence of
immune blood serum. The results were performed from three independent assays. “*” indicates significance at p < 0.05 and “**” indicates significance at p < 0.01. △ versus the negative control and the blank control.

**Fig.5.** (A) Survival of mice from SOD immunized group, negative control group and blank control group following challenge against *H. parasuis* MD0322 strain. (B) Survival of mice from SOD immunized group, negative control group and blank control group following challenge against *H. parasuis* SH0165 strain.

**Fig.6.** (A) Survival of mice immunized with serum from immunized group and negative control group following challenge against *H. parasuis* MD0322 strain. (B) Survival of mice immunized with serum from immunized group and negative control group following challenge against *H. parasuis* SH0165 strain.

**Fig.7.** Representative lung sections of mice in different treatment groups (immunized groups, the negative control groups and the blank control groups). (A) Negative control groups, blank control groups and SOD-immunized groups challenged against *H. parasuis* MD0322 strain. (B) Negative control groups, blank control groups and SOD-immunized groups challenged against *H. parasuis* SH0165 strain. Negative control group: C, F; Blank control group: D, G; SOD protein immunized group: E, H.
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Fig. 5. (A) Survival of mice from SOD immunized group, negative control group and blank control group following challenge against *H. parasuis* MD0322 strain. (B) Survival of mice from SOD immunized group, negative control group and blank control group following challenge against *H. parasuis* SH0165 strain.
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black and white
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