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Vitellogenin is an acute phase protein with bacterial-binding and inhibiting activities

Zhou Tong [1], Lei Li [1], Ravindra Pawar, Shicui Zhang *

Department of Marine Biology and Laboratory of Evolution and Development, Ocean University of China, Qingdao 266003, PR China

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ABSTRACT

Previous studies have shown that vitellogenin (Vg) is an immune-relevant molecule, but its potential immunological role *in vivo* remains obscure. We demonstrated here that injection of lipopolysaccharide (LPS) and lipoteichoic acid (LTA) into male *Danio rerio* rapidly induced a significant up-regulation of Vg at both transcriptional and translational levels, and that serum Vg produced was able to bind to both *Escherichia coli* and *Staphylococcus aureus* and to inhibit their growth in a dose-dependent manner. All these data suggest that serum Vg in zebrafish *D. rerio* is an acute phase protein with bacterial-binding and inhibiting activities. It also bolsters the notion that factors normally involved in control of female reproduction are linked with immunity in organisms that rely on Vg for oocyte development.

Introduction

Vitellogenin (Vg) constitutes the precursor of major yolk proteins in all egg-laying organisms. In non-mammalian vertebrates, Vg is usually synthesized in the liver of female animals, released into the bloodstream and transported via circulation to the ovary, where it is internalized by the maturing oocytes and proteolytically cleaved to form the yolk proteins, providing the energy reserves for later developing embryos and larvae (Arukwe and Goksøyr 2003; Finn and Kristoffersen 2007; Matozzo et al. 2008). However, the roles of Vg appear to extend beyond this nutritional function. For example, in the advanced eusocial honeybee, Vg has been shown to be involved in the social organization, temporal division of labor and foraging specialization, regulation of hormonal dynamics and change in gustatory responsiveness (Amdam et al. 2003; Amdam et al. 2006; Guidugli et al. 2005; Nelson et al. 2007). Besides, the honeybee Vg has been demonstrated to be able to reduce oxidative stress by scavenging free radicals, thereby prolonging the lifespan in the facultatively sterile worker castes and reproductive queen castes (Seehuus et al. 2006). Similar antioxidant activity has also been observed for the nematode (*Caenorhabditis elegans*) Vg (Nakamura et al. 1999).

Another novel function of Vg is linked with the host immune defense. Vg has been demonstrated to possess both hemagglutinating and antibacterial activities in the protochordate *Branchiostoma belcheri* as well as in the bony fish rosy barb *Puntius conchonius* (Shi et al. 2006; Zhang et al. 2005). More recently, piscine Vg has been revealed to be a multivalent pattern recognition molecule capable of identifying non-self components including lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA) and glucan, and to act as an opsonin that can enhance macrophage phagocytosis (Li et al. 2008; Liu et al. 2009). However, the potential immunological role of Vg *in vivo* remains open. Interestingly, injection of *Escherichia coli* into the male rosy barb has been shown to be able to lead to an increase in serum Vg (Shi et al. 2006). This suggests that Vg may be related to infection-resistant immunity, but little is known regarding the dynamics of Vg synthesis as well as the significance of serum Vg in the male fish. We therefore sought to address these issues in this study.

Materials and methods

Fish

Zebrafish, *Danio rerio* aged approximately 5 months were purchased from a local fish dealer. The male fish were selected and acclimated for at least 3 days before experiments. They were fed and cared following the established protocols of Westerfield (1995).

Abbreviations: hpi, Hour post injection; i.p., Intraperitoneal; LPS, Lipopolysaccharide; LTA, Lipoteichoic acid; qRT-PCR, Quantitative real-time PCR; Vg, Vitellogenin

* Corresponding author. Tel.: +86 532 8203 2787.
E-mail addresses: sczhang@ouc.edu.cn, milyandtt@163.com (S. Zhang).

[1] These authors contributed equally to this work.

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Injection and sampling

Pilot experiments revealed that intraperitoneal (i.p.) injection of LPS and LTA (Sigma-Aldrich, USA) at a dose of 5 μg/g was able to induce a significant increase in Vg mRNA within 1 h in male *D. rerio* (data not shown). Therefore, in the following experiments, male *D. rerio* weighing about 0.32 ± 0.05 g were injected intraperitoneally with ~10 μl of 160 μg/ml LPS and LTA (yielding approximately a dose of 5 μg/g body weight), individually. Control fish were similarly injected with saline. Each experimental group consisted of about 100 male *D. rerio*, which were observed daily for mortality and abnormal swimming and feeding behavior, if any. Of each group, 5 fish were sampled at 0, 0.5, 1, 2, 3, 5, 9 and 12 h post injection (hpi), respectively, for RNA preparation, and 5 individuals sampled at the same intervals for blood collection. The control and experimental fish were separately maintained in 55 L glass aquarium tanks and cared following the protocols of Westerfield (1995).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to investigate the response of *Vg1* gene to LPS and LTA at mRNA level. Two PCR primer sets specific for *Vg1* (GenBank accession no: CAK03614) and β-actin genes (Keegan et al. 2002) were designed using primer 5 program. The primer sequences and amplicon lengths are listed in Table 1.

Total RNAs were prepared with Trizol (Invitrogen) from the whole *D. rerio* sampled (Tong et al. 2004). After digestion with RQ1 RNase-free DNase (Promega) to eliminate the genomic contamination, cDNAs were synthesized with reverse transcription system using oligo d(T) primer, and used as templates. After qualification of the cDNA templates, qRT-PCR was performed on ABI 7500 real-time PCR system. SYBR® *Premix Ex Taq*™ (Takara) was used for real-time PCR reaction, with a primer concentration of 200 nM. Reaction conditions consisted of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 34 s. Reaction of each sample was performed in triplicates. Zebrafish *β-actin* was used as control to normalize the starting quantity of RNAs. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with ABI 7500 SDS software (Applied Biosystems), and quantified using the comparative C_T method ($2_T^{-\Delta\Delta C}$ method) based on C_T values for both *Vg1* and *β-actin* in order to calculate the fold increase (Livak & Schmittgen 2001). All data were given in terms of relative mRNA expressed as mean SD. Statistical analysis was performed using SPSS 13.0 for *Windows*. The data obtained from real-time PCR analysis were subjected to One-way Analysis of Variance (ANOVA) followed by Dunnett's 2-sided *post-hoc* test to determine the differences in the mean values among the treatments. Significance was set at $p < 0.05$.

Table 1
Primers used for real-time PCR analysis.

Genes	Primers (5′–3′)	Amplicon (bp)	GeneBank accession no.
Vg1	cttctggatgctcttcctgctgt tctgaatgaactcgggagtggta	100	CAK03614
β-actin[a]	cgagcaggagatgggaacc caacggaaacgctcattgc	102	

[a] The sequence of β-actin primers was according to Keegan et al. (2002).

Enzyme-linked immunosorbent assay (ELISA)

ELISA technique was used to investigate the response of Vg to LPS and LTA at protein level as described by Wang et al. (2009). Male *D. rerio* were bled by cardiac puncture, and the blood samples were collected, and mixed with 1 volume of 20 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl, 0.5 mM PMSF, and 600 IU/ml sodium heparin. After centrifugation at 3000*g* at 4 °C for 20 min, the supernatant (sera) were pooled and stored at −80 °C until used.

For ELISA, the sera were thawed on ice and an aliquot of 20 μl sera was mixed with 80 μl of the coating buffer (50 mM sodium carbonate buffer, pH 9.6). The wells of 96-well microtiter (Costar) were coated with 100 μl of the coating buffer and incubated overnight at 4 °C. After washing 5 times with PBS containing 0.1% Tween-20, the wells were each blocked with 100 μl of 5% BSA (1 g of BSA in 20 ml of PBS, pH 7.4) at 4 °C for 12 h, followed by addition of 100 μl of rabbit anti-zebrafish Vg antibody (DR-264; Biosense Laboratories AS, NORWAY) diluted at 1:500 with 5% BSA, and incubated at 37 °C for 4 h. Subsequently, an aliquot of 75 μl of 0.4 mg/ml o-phenylenediamine (OPD) in 51.4 mM Na_2HPO_4, 24.3 mM citric acid and 0.045‰ H_2O_2 (pH 5.0) was added to each well, and incubated at 37 °C for 20 min in dark. To terminate the reactions, 25 μl of 2 M H_2SO_4 was added into each well, and absorbance at 492 nm was monitored by a microplate reader (GENios Plus, Tecan).

Bacterial binding assays

Since the serum Vg peaked at 30 min after LPS injection, the blood samples collected at 0.5 hpi were used for the bacterial binding assays conducted according to Fan et al. (2008). In brief, the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *Staphylococcus aureus* were each washed with 0.9% NaCl, and re-suspended in 100 μl of 20 mM Tris–HCl buffer containing 150 mM NaCl and 10 mM $CaCl_2$ (pH 7.5). A total of 100 μl sera from LPS-injected male fish was mixed separately with the same volume of *E. coli* and *S. aureus* suspensions (2×10^9 cells/ml) and incubated at 26 °C for 50 min with gentle agitation. For control, 100 μl of 0.9% NaCl was mixed with 100 μl of *E. coli* and *S. aureus* suspensions (2×10^9 cells/ml) and incubated under the same conditions. After incubation, the bacteria were washed three times with 0.9% NaCl, and harvested by centrifugation at 3000*g* at room temperature for 5 min. The bacterial pellets were again washed three times with 0.9% NaCl, re-suspended in 40 μl of 4 M urea in 10 mM Tris–HCl (pH 8.0) and subjected to mild agitation for 15 min followed by centrifugation at 3000*g* for 5 min. The supernatant was then collected and run on a 7.5% SDS-PAGE under reducing condition. The gel was washed for 15 min in 20 mM PBS containing 0.1% Tween-20, and proteins on the gels were blotted on PVDF membrane (Amersham). Blotted membranes were incubated in 20 mM PBS containing 3% defatted milk powder at 30 °C for 2 h, and then in the rabbit anti-zebrafish Vg antibody diluted 1:500 with 20 mM PBS containing 0.1% Tween-20 for 2 h. After washing in 20 mM PBS, the membranes were further incubated in horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Zhongshan, China) diluted 1:1000 in the similar manner as that of Vg antibodies, at 30 °C for 2 h. Bands were visualized using DAB and 0.03% H_2O_2.

Antibacterial activity assay

As in the bacterial binding assays, the sera collected from LPS-injected male fish at 0 and 0.5 hpi were used for the antibacterial activity assays. The sera from saline-injected male fish were used

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for control. To inactivate the complement activity, the sera were heated at 45 °C for 30 min (Wang et al. 2009). Aliquots of 20 μl of the heated sera were mixed with 5 μl of anti-zebrafish Vg antibody diluted with 0.9% saline at different ratios, or with 5 μl of anti-ß actin antibody (AA128-1; Beyotime, Nan Tong, China) or with 5 μl of 0.9% saline. Subsequently, the mixtures were mixed with 1 μl of *E. coli* and *S. aureus* suspensions (10^6 cells/ml) and incubated at 26 °C for 60 min, diluted 1000-folds with 0.9% saline and plated onto 3 LB agar plates at 30 μl/plate. After incubation at 37 °C for 16 h, the resulting bacterial colonies in each plate were counted. The control was processed similarly except that the serum was replaced by 0.9% saline. The percent inhibition was calculated as follows: [number of colonies (control − test)=number of colonies (control)] × 100 (n=3 experiments).

Results

Up-regulation of Vg1 expression by LPS and LTA

qRT-PCR assays for determination of *Vg1* expression and its response to injection with LPS and LTA in *D. rerio* are presented. The expression levels of the gene were given in fold increase compared to the same gene expression level in control fish. The dissociation curve of amplification products showed only a single peak, indicating that the amplification was specific (data not shown).

Injection of LPS soon resulted in a significant up-regulation of *Vg1* expression (Fig. 1A). The expression level of *Vg1* reached about 100-fold at 0.5 hpi, peaked (about 200-fold) at 2 hpi and then decreased gradually. However, it remained significantly higher than control till the end of experiment (12 hpi). Similarly, injection of LTA induced a marked increase in *Vg1* expression, rapidly reaching the maximum (50-fold) at 0.5 hpi, followed by a sharp decrease (Fig. 1B). These data indicated that both LPS and LTA were able to induce a rapid increase in *Vg1* expression in male *D. rerio*.

Increase in Vg following LPS/LTA injection

ELISA was performed to test if injection of LPA and LTA could cause an increase in serum Vg in male *D. rerio*. As with *Vg1* expression, injection of LPS induced a rapid and significant increase (~128%) in serum Vg at 0.5 hpi (Fig. 2A). At 1 and 2 hpi following LPS injection, serum Vg in male *D. rerio* decreased to the control level but it increased significantly again at 3 and 5 hpi (~110 and ~112%). In contrast, injection of LTA caused little increase in serum Vg in male *D. rerio* at the initial 3 h, but it induced a significant increase in serum Vg at 5 hpi (~104%), peaking (~116%) at 9 hpi (Fig. 2B). These results showed that LPS and LTA were both able to induce a significant increase in serum Vg in male *D. rerio*, agreeing with our observations above that they both triggered a rapid increase in *Vg1* expression.

Binding of serum Vg to bacteria

Next we detected if serum Vg is able to bind the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *S. aureus*. Western blotting showed two bands of Vg with molecular masses of approximately 170 and 140 kDa that were monitored in the elution derived from both bacteria incubated with the sera collected at 0.5 hpi from LPS-injected fish, whereas no bands were found in the bacteria incubated with the saline (Fig. 3). These results demonstrated that serum Vg was capable of binding both *E. coli* and *S. aureus*.

Antibacterial activity of serum Vg

We then sought to examine if serum Vg was able to inhibit the growth of *E. coli* and *S. aureus*. The sera from control and LPS-injected male *D. rerio* all exhibited conspicuous antibacterial

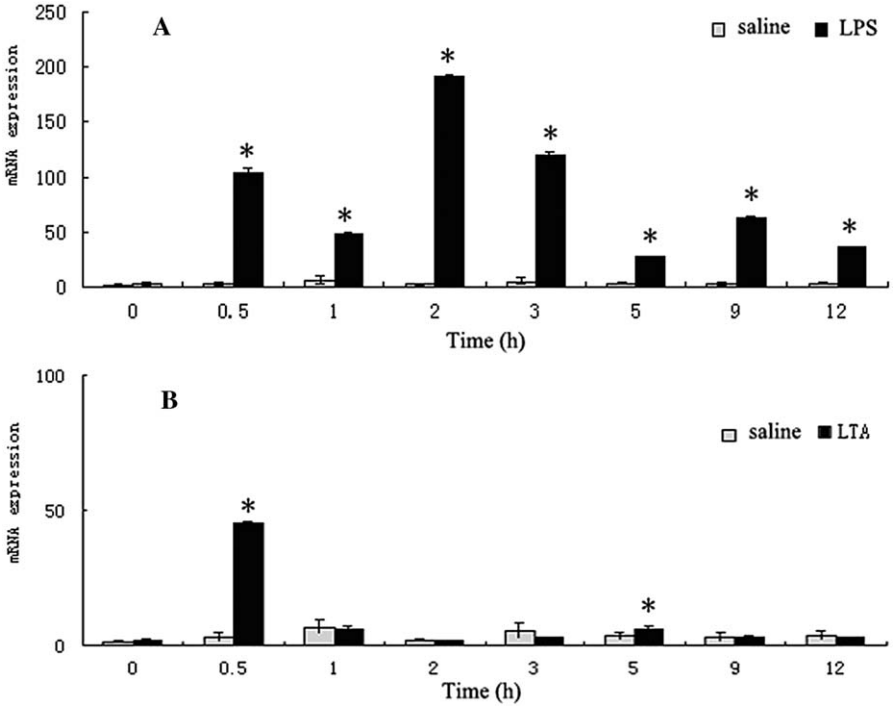

Fig. 1. Expression of *Vg1* in response to LPS (A) and LTA (B). Statistically significant differences ($p < 0.05$) between the treated fish and controls at each time-point are indicated by (*).

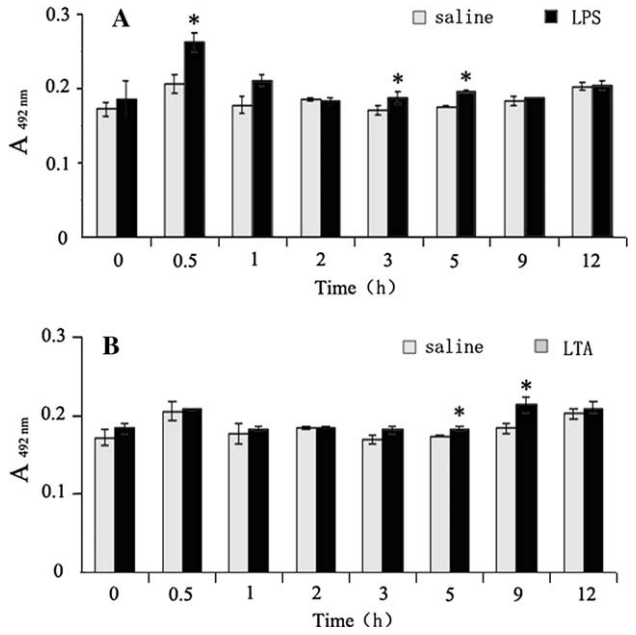

Fig. 2. Changes in serum Vg levels in response to LPS (A) and LTA (B). Statistically significant differences ($p < 0.05$) between the treated fish and controls at each time-point are indicated by (∗).

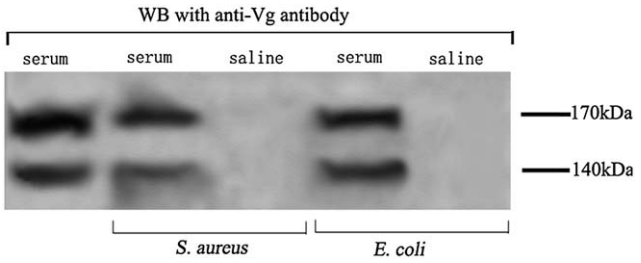

Fig. 3. Binding of Vg to bacteria. *In vitro* binding assays were performed using *E. coli* and *S. aureus*. The bacteria were incubated with the sera and saline (negative control). After stringent washing, the proteins bound to the bacteria were eluted by 4 M urea and subjected to SDS-PAGE and Western blot analysis using anti-Vg antibody.

activities against *E. coli* and *S. aureus*, with the antibacterial activities of the sera collected at 0.5 hpi from LPS-injected fish being significantly higher than those from saline-injected fish (Fig. 4A and B). When the complement activities of the sera were inactivated by heating, their antibacterial activities against *E. coli* and *S. aureus* were remarkably reduced. However, some antibacterial activities remained in all the heated sera. In particular, after heating, the antibacterial activities of the sera collected at 0.5 hpi from LPS-injected fish were still significantly higher than control, suggesting the presence of additional antibacterial molecules in these sera. Notably, the antibacterial activities of the heated sera against *E. coli* and *S. aureus* were significantly inhibited by the pre-incubation of the sera with anti-zebrafish Vg antibody, but not by the pre-incubation with anti-actin antibody; and this inhibitory effect showed a clear dose-dependence (Fig. 4A and B). Moreover, consistent with higher Vg levels in the sera collected at 0.5 hpi, the antibacterial activities in these sera, after heating, were also greater than control (Fig. 4A and B). These data together implicated that in addition to complement, Vg was an important factor responsible for the antibacterial activities in the sera.

Discussion

Acute-phase response comprises a complex series of reactions initiated in response to infection, physical trauma, or malignancy, which forms part of the host's first line of systemic defense and begins to act well in advance of any antibody-mediated immune responses. It involves a large number of acute phase proteins functioning in a variety of defense-related activities such as preventing ongoing tissue damage, isolating and destroying the infective agents and activating the repair processes necessary to restore the host's normal function (Steel and Whitehead 1993). In this study, we demonstrate that Vg expression is inducible, and injection of LPS and LTA into male *D. rerio* rapidly causes a marked up-regulation of Vg expression at both transcriptional and translational levels. Vg has repeatedly been reported to be a female-specific protein involved in reproduction (Chang et al. 1994;Hara et al. 1980;Wallace 1985) and the physiological significance of the production of Vg in male and juvenile fish is uncertain (Harries et al. 1997;Purdom et al. 1994). Our results suggest that Vg is a novel candidate of immune-relevant molecules involved in acute phase response, opening an avenue for exploring the physiological role of Vg.

An important part of innate immunity is that a group of proteins have bactericidal activity in addition to their immune recognition function. Our previous studies have shown that in addition to being involved in yolk formation, piscine Vg *in vitro* is a multivalent pattern recognition receptor capable of identifying Gram-negative and positive bacteria as well as fungus and an opsonin capable of enhancing macrophage phagocytosis (Li et al. 2008; Liu et al. 2009). However, the potential immunological role of Vg *in vivo* remains to be determined. We show here that serum Vg can bind to both *E. coli* and *S. aureus* and inhibit their growth, implying that Vg is not only a pattern recognition receptor capable of identifying these bacteria but also an effector molecule capable of directly damaging these bacterial cells. As with purified Vg *in vitro*, serum Vg *in vivo* is able to kill the Gram-negative bacteria like *E. coli* and the Gram-positive bacteria like *S. aureus* via interacting with and destroying the bacterial cell walls (Li et al. 2008; Liu et al. 2009), suggesting that Vg physiologically performs pleiotropic functions including a wide spectrum of antibacterial activities capable of identifying and killing invading bacteria *in vivo*.

LPS is a large molecule consisting of lipid and a polysaccharide joined by a covalent bond, found in the outer membrane of Gram-negative bacteria, while LTA is a surface-associated adhesion amphiphile from Gram-positive bacteria. It is of note that compared to LPS, LTA induces a slower and weaker response in Vg production at the protein level. The mechanism for this is not clear at present, but it is apparently in line with the consensus that LPS acts as an endotoxin and elicits strong immune responses in animals (Swain et al. 2008).

In summary, the present study highlights that serum Vg in *D. rerio* is an acute phase protein capable of binding to both *E. coli* and *S. aureus* and inhibiting their growth. It also supports the notion that some molecules like Vg normally involved in control of female reproduction are linked with immunity in oviparous organisms.

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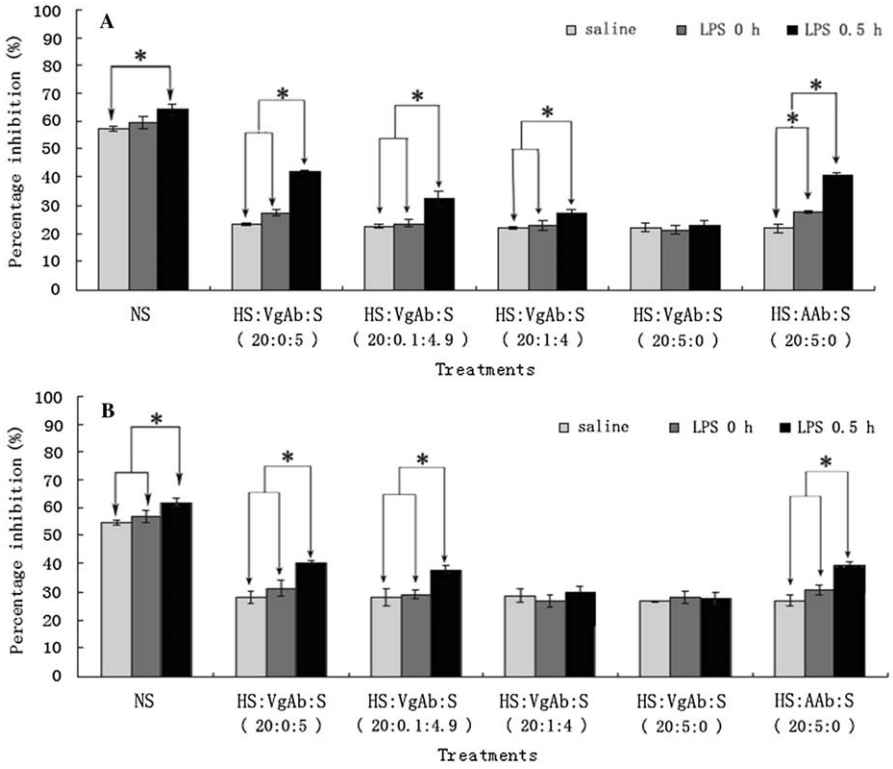

Fig. 4. Percent inhibition (%) of the sera against the growth of *E. coli* (A) and *S. aureus* (B). NS, normal serum; HS, heated serum; VgAb, anti-zebrafish Vg antibody; AAb, anti-β actin antibody; S, saline. The numbers in brackets indicate the ratios (volumes) of different parts.

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