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# Development of Specific Nano-Antibody for Application in Selective and Rapid Environmental Diagnoses of *Salmonella arizonae*

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**Abstract:** Treatment of human and animals for protection from pathogens infection has significant economic value, especially with the harmful *Salmonella arizonae*. Beneficial cameloid heavy-chain antibodies as single-domain antigen-binding fragments known as VHHs or nano-bodies may be the acceptable option for producing the treatment and/or diagnostic agents. In the current study, we developed a sandwich ELISA based nano-body towards *S. arizonae* as the first report for the treatment of *S. arizonae*. using the cDNA synthesized from immunized camels RNA to isolate 700 bp DNA fragment, which contains all VH domains of IgG2 and IgG3 isotypes followed by the second amplification VHH PCR with amplified fragments at 450 bp. The final PCR products were cloned into the phagemid vector pMECS then via phage display technique. Nano-antibodies protein was purified and separated under non-denaturing conditions by SDS-PAGE. The reactivity of each VHH of the selected clones was analyzed by Western blot assay. The isolated nano-antibodies showed binding not only to *S. arizonae*, but also to other bacterial strains, indicating that these nano-antibodies can be used in treatment but cannot use in diagnostic.

**Keywords:** *S. arizonae;* nano-antibodies; cameloid VHH; environmental treatment.



## 1. Introduction

Microbial group of *Salmonellae* is a Gram-negative, facultative, non-spore-forming, bacilli bacteria belonging to *Enterobacteriaceae* family. It usually causes food-borne diseases. Also, it is a part of bacterial flora found in humans and animals, though the occurrence regularity is variable, reflecting the general level of *Salmonella* in water, food, and environment [1]. They are widely distributed into environments that have disturbed by human activities. *Salmonella arizonae* is considered one of the less common *Salmonellae* members [2]. It was reported firstly by Caldwell and Ryerson in 1939 and named *Salmonella* dar-es-salaam [3]. Subsequently, it also named *S. arizonae, Arizona hinshawii, S. cholerasuis* subsp. *arizonae* and finally re-classified as *S. enterica* subsp. *arizonae* in 1983 [4]. *S. arizonae* was reported to cause the human disease spectrum, including bacteremia, gastroenteritis, vascular infection, joint, and bone infection [5, 6–8]. Cold-blooded animals are the typical *S. arizonae* habitats and other animals like poultry, dogs, and rats [9, 10], as well as it has been involved in human infection. Around 20 pediatric patients below 18 years old sorrow from *S. arizonae* infection have been noted in the papers [11, 12].

Hamers-Casterman and his colleagues in 1993 established the antibodies' existence devoid of light chains that are selected as heavy-chain antibodies [13]. The binding sites of antigen include amino acids from only a single variable domain of heavy-chain antibodies (VHHs) [14]. The encoding VHH of DNA (~400 bp) can be manipulated for cloning, expression, and mutation. For building the phage display library, the VHH coding sequence was bonded with the minor capsid protein p3 of non-lytic filamentous phage. The nano-body would show on the filamentous phage tip after liberation by the helper phage that provides replication factors for the modified display phage [15]. Compared with conservative monoclonal and polyclonal antibodies, the generation of phage display antibody fragments is cost-effective and time-saving. It is possible to design or modify the DNA sequence of coding the phage-displayed antibody fragments. The recombinant antibodies could be easily expressed in various protein expression hosts. Newly, nano-bodies, which specifically bind to the LM invasion, were isolated by the selection of the non-immunized or immunized VHH library [16].

To find treatment technology for *S. arizonae* infection, our aim in this study was to develop specific nano-bodies for *S. arizonae* with lower cost and higher stability compared with traditional antibodies. The immune library of phage-displayed nano-body was constructed after immunization of *S. arizonae* camel. *S. arizonae*-specific VHH antibodies were achieved after biopanning. Furthermore, we have demonstrated the construction of a nanobody-based sandwich ELISA which could be applied in environmental sample analysis.

## 2. Materials and Methods

### *2.1. Preparation of Salmonella arizonae vaccine.*

*Salmonella arizonae,* as pathogenic bacterium used in this study to produce the nano-body for the camel immunization, was kindly obtained from the Egyptian Microbial Culture Collections Network (EMCCN) at National Research Centre (NRC), Cairo, Egypt.

Firstly, the formalin killed vaccine was prepared from activated bacterium culture. A single colony from freshly streaked *Salmonella arizonae* on nutrient agar plates was inoculated into one flask contained 20 ml tryptic soy broth for immunization at an early stage. The inoculated flask was incubated at 37 $^{o}$C overnight at shaking conditions. After incubation, the broth culture was centrifuged at 6000 xg at 4 $^{o}$C for 15 min [17]. The supernatant was decanted, and the precipitated cells were washed twice with acetone followed by twice diethyl ether. The washed cell pellet was re-suspended into 1% formalin saline, and Tween 80 was added to the suspension with the final concentration of 3-4 %. The suspension was washed three times with Phosphate buffer saline (PBS)/Tween and centrifuged at 6000 xg at 4 $^{o}$C for 15 min, and the pellet was dissolved in 500 µl PBS as an inactivated bacterial cells for injection into camel [18].

### *2.2. Camel Immunization.*

One camel was immunized by intramuscular injection with 6 doses from the prepared *Salmonella arizonae* vaccine. At zero time, the camel was given the first dose of vaccines emulsified into complete adjuvant (1:1 vaccine to adjuvant). In the other five doses, the vaccines were formulated in incomplete adjuvant (1:1 vaccine to adjuvant) and injected into the camel. The injection was done every week. At the end of immunization, the blood sample was collected in a sterile blood collection tube contained 200 µl of 1M EDTA and then centrifuged at 4500 xg for 10 min at 4$^{o}$C. Plasma was transferred to new 1.5 ml tubes and

diluted by PBS to $10^{-2}$,$10^{-3}$ and $10^{-4}$ to check the immune-response against *S. arizonae* by ELISA technique [19] with few modifications using ELISA reader (BIO TEK ELX800) at 450 nm. The control was used, PBS as a sample against *S. arizonae* coated well.

*2.3. Bactrian camel immunization and nano-body library construction.*

The VHH libraries were constructed as previously presented [20]. Principally, mRNA was isolated from 100 ml blood of vaccinated camel using E.Z.N.A.® Blood RNA Maxi Kit (OMEGA Bio-TEK, USA). All materials were treated by RNase Away (RNase Away, MOLECULAR BIO PRODUCTS, USA). Consequently, the first-strand cDNA was synthesized using SuperScript kit (INVITROGEN, GERMANY) with oligo-dT primers. All VH domains, including the VHH domains from Heavy-chain antibody IgG2 and IgG3 isotypes, were amplified with the primer CALL001 (5' GTCCTGGCTCTCTTCTACAAG 3') and CALL002 (5' GGTACGTGCTGTTGAACTGTT 3') primer [18] in VERITY 96 well, AB, Singapore thermocycler using Fast Start DNA Taq Polymerase (ROCHE, USA). The reaction was performed as follows, a 2 µl of cDNA were mixed with 5 µl of 10 x Fast Start DNA Taq Polymerase buffer with $MgCl_2$, 1 µl dNTP's, 10 pmol of each primer (Call 001 and Call 002), and adjusted to a final volume of 50 µl by nuclease-free water. PCR amplification was accomplished by denaturation at 94°C for 5 min followed by 35 Cycles of 94°C for 30 s, 55 °C for 1 min, 72 °C for 1 min and terminated with a final extension at 72 °C for 7 min. The VHH gene fragments (coding for the Nbs) were purified from agarose gel and re-amplified using nested primers containing the restriction sites for PstI and NotI restriction VHH (5' GATGTGCAGCTGCAGGAGTCTGGRGG 3') and pMECS (5' CTAGTGCGGCCGCTGAGGAGACGGTG 3') primers using Fast Start DNA Taq polymerase (Roche, USA). 8 µl of purified PCR were mixed with 5 µl of 10 x Fast Start DNA Taq polymerase buffer with $MgCl_2$, 1 µl dNTP's, 1 µl of VHH primer, 1 µl of pMECS primer and adjusted to final volume of 50 µl by nuclease-free water. The cycling conditions were 94°C for 5 min followed by 20 Cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 7 min [21]. The final PCR products were cloned into the phagemid vector pMECS. Ligated material was transformed into Escherichia coli TG1 cells by electroporation (BIO-RAD). Colonies were harvested by scraping in the culture medium, washed, and the VHH library stored at -80°C in LB medium supplemented with glycerol (30% final concentration). The transformation of VHH/pMECS into TG1 competent cells was checked through colony PCR for 20 random colonies by using G III (5' CCACAGACAGCCCTCATAG) and MP57 (5' TTATGCTTCCGGCTCGTATG) primers using the following cycling parameters: denaturation at 94°C for 5 min and 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min with a final extension at 72°C for 10 min.

*2.4. Phage Display Enrichment for Specific Binders.*

Production of rescue phage of VHH library (Pan 0) was prepared according to Xue et al. [22] with some modifications. In brief, 2 ml of the library representing cells was diluted in 2 l 2xty media with ampicillin (Amp) at an optical density ($OD_{600}$) 0.143 and incubated at 37ºC with shaking until the OD reached to 0.576, then 2 ml of KM13 (M13KO7) helper phage at a concentration of $2x10^{12}$ was added and incubated for 2 h at 37ºC without shacking. Finally, 2 ml of kanamycin (Kan) (200 mg) was added and incubated at 37ºC overnight with shaking. The overnight culture was centrifuged at 6000 rpm for 6 min, and the supernatant was mixed

with 20 % $PEG_{6000}$/ 2.5 M NaCl at dilution 4:1, followed by incubation on ice and centrifuged at 3200 xg for 30 min at 4 $^{o}$C. The pellet was dried and re-suspended in 12 ml of PBS and centrifuged at 8500 xg for 2 min. The supernatant was mixed with PEG/NaCl with a dilution factor of 6:1 and centrifuged at 3200 xg for 30 min at 4$^{o}$C. The pallet was re-suspend in 1 ml of PBS and stored at 4$^{o}$C.

*2.5. Panning against S. arizonae.*

Construction of Pan1 for *S. arizonae* strain was prepared in two wells of ELISA plate (Maxisorb 96-well microtiter plate, NUNC) that was coated by *S. arizonae* cells and blocked by skimmed milk. A 100 µl of Pan0 rescue phage was added to the coated wells and incubated for at least 1 h at room temperature. The phage solution was removed and discarded in bleaching water. The wells were rinsed 10× with PBS/Tween. To elute bound phages, 100 µl of freshly prepared 100 mM triethylamine (pH ~11.5) was added and incubated for 10 min at room temperature. The eluted phages were transferred to a fresh tube containing an equal volume (100 µl) of 1 M Tris HCl, pH 7.4 to neutralize the solution. After that, 190 µl from each tube contains neutralized solution of phage particles was mixed with 2 ml of TG1 cells with $OD_{600}$ 0.6 at 37$^{o}$C for 1 h without shacking, and then 8 ml of 2xty-Amp were added and incubated at 37$^{o}$C for 1h with shacking, 100 µl M13k07 helper phage as superinfection were incubated at 37$^{o}$C for 1h and diluted in 250 ml 2xty-Amp (100 mg/ml)-Kan (100 mg/ml) and incubated at 37$^{o}$C overnight with shacking. One well was coated with *S. arizonae* cells, and the other was coated with PBS as control. A second panning round of enrichment for phages with antigen-specific nano-bodies was started at the next day, where the overnight culture was used instead of the VHH original library as described in Pan1 and the PBS as a control sample. After serial dilutions, the remaining 190 µl of phages was discarded in bleaching water, the colony quant in LB agar media with Amp and glucose. Rescue phage was titrated by taking 10 µl of the neutralized solution of phage particles and prepared 8-fold serial dilutions of eluted phage followed by mixing 10 µl from each dilution with 90 µl of Tg1 cells with $OD_{600}$ 0.6 and incubated at 37$^{o}$C for 1 h then 10 µl from each dilution was plated onto the 2xty-Amp-glucose-agar medium. These steps were repeated from Pan1 to Pan4.

*2.6. Testing of rescue phage against S. arizonae by phage ELISA.*

Rescue phage of *S. arizonae* was screened by coating 4 wells of ELISA plate with 100 µl of *S. arizonae*, then blocked using skimmed milk. The OD of each rescue phage was measured at 260 nm then diluted to lowest OD value, and 2 µl from each dilution was re-diluted to 200 µl, and 100 µl from this solution was added to the coated and control wells and incubated at RT for 1 h. The phage solution was removed and discarded in bleaching water. The wells were rinsed 3× with PBS/Tween using an antibody (anti M13, 1:1000) followed by TMB, the ELISA plate was read on ELISA reader at 450 nm.

*2.7. Screening for S. arizonae specific nano-antibody by ELISA.*

One ELISA plate was coated by *S. arizonae* cells or carbonate buffer as the control, and all wells were blocked by skimmed milk. 48 colons contain VHH fragment against *S. arizonae* from each were selected, inoculated on 1000 µl TB medium, and incubated for 5 h at 37$^{o}$C. After the incubation period, 10 µl of IPTG was added for each culture and incubated at 37$^{o}$C overnight. The incubated culture was centrifuged at 2054 xg for 15 min, and the pellet was re-

suspended in 200 µl TES buffer and incubated on ice for 45 min with shaking. And then, 300 µl 1:4 TES buffer was added to the suspension and incubated for 1 h on ice with shaking. The suspension was centrifuged at 2054 xg for 15 min, and 200 µl from each sample was added to the ELISA plate and incubated at room temperature for 1 h. First antibody (anti HA 1:2000), a second antibody (Goat anti-Mouse HRP 1:2000), and the substrate (TMB) were used, ELISA reader was used to reading the ELISA plate at 450 nm.

*2.8. Preparation and Purification of Plasmid.*

The bacterial clones were inoculated into 5 ml LB medium containing 5 µl Amp (100 mg/ml) for isolation of plasmid containing VHH gene. The mixture was incubated with vigorous shaking at 37ºC for 14-16 h. Then the bacteria were harvested by spinning at 11000 xg for 1 min, and the pellet (contain the plasmid) was purified according to the protocol of Qiagen company (Qiagen Kit, Germany).

*2.9. Amplification of VHH (Nanobodies) using Polymerase Chain Reaction.*

Amplification of VHH (Nanobodies) was performed by the PCR technique using G111 (5’ CCACAGACAGCCCTCATAG ’3) and PM57 (5’ TTATGCTTCCGGCTCGTATG ’3) primers. The reaction was performed in a final volume of 25 µl as following, 1 µl of purified plasmid was mixed with 12.5 µl of 2x master mix Promega and 1 µl of each primer at a concentration of 10 pmol/µl". Parameters of PCR amplification were started by initial denaturation at 94°C for 5 min followed by 20 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min and ended with Final extension at 72°C for 7 min in a thermocycler (Veriti 96 well, AB, Singapore). The success of amplification was tested by loading the amplified samples on 1% agarose gel and running on the gel electrophoresis system and investigated on gel documentation system (UVP, USA).

*2.10. Expression and purification of nano-bodies.*

Nano-bodies were expressed in *E. coli* WK6 cells and then purified according to protocols described by Vasylieva et al. [23] using a Ni-NTA Spin Kit (Qiagen, Germany) according to the manufacturer’s protocol. Nanobodies purification was confirmed by loading 20 µl of each nano-body on 12% mini SDS-PAGE separating gel and 4% Stacking Gel stained with coomassie blue dye and the samples were run under the running condition of 200 volts for approximately 45 min [24]. The reactivity of nano-bodies was analyzed by Western blot using the standard transfer protocol from the Bio-Rad preprogrammed protocols or the user-defined protocol and binding with monoclonal anti-poly-histidine antibody (Sigma-Aldrich, Germany) for the detection.

*2.11. Nanobody-based sandwich-ELISA.*

ELISA reaction was performed to test the selected specific nano-bodies against *S. arizonae*. A microplate was coated with 0.25 µg $ml^{-1}$ of rabbit polyclonal antibody (100 µl/well) overnight at 4 °C. The unbound sites in the microplate were blocked with 3% (w/v) skimmed milk (300 µl/well) in PBS. After rinsing each well with PBST, serial concentrations of *S. arizonae* was added to the wells (100 µl/well) and incubated for 1 h. Then, 100 µl of purified nano-body (4 µg $ml^{-1}$) was added and incubated at 37 °C for 1 h. After washing, the microplate was incubated with an anti-HA tag antibody conjugated with HRP for another 1 h and

subsequently washed. Freshly prepared TMB substrate (100 µl/well) was added to each well. Incubation was carried out at 37 °C for 10 min, and the reaction was stopped by 50 µl of 2 M $H_2SO_4$. Then the absorbance value of each well at 450 nm was detected using a microplate reader.

## 3. Results and Discussion

### *3.1. Camel immunization.*

*S. arizonae* is the common environmental pathogenic bacteria in various vital fields, and it can cause serious illnesses for humans and animals [25]. Thus, the current research manuscript was conducted to produce nano-bodies for the fasting diagnostic of *S. arizonae*. To achieve this aim, the construction of VHH library was based on immunization of one male camel with formalin killed vaccine of *S. arizonae*. The detection of camel immune response against *S. arizonae* was determined by isolation of plasma antibody (Ab) that measured by ELISA, as described in Fig. (1). Immunized camel with *S. arizonae* produced a high ELISA Ab titer, so the sample diluted $10^3$. The $OD_{450}$ value of the diluted sample was 1.353, and the $OD_{450}$ value of PBS as a sample control was 1.252.

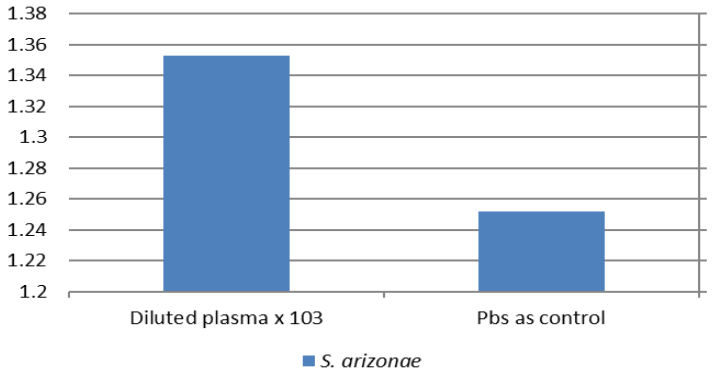


**Figure 1.** The response of immunized camel antibodies against *S. arizonae* in plasma measured by ELISA, where the plasma dilutions $10^{-3}$ $OD_{450}$ value was 1.353, while PBS control was 1.252.

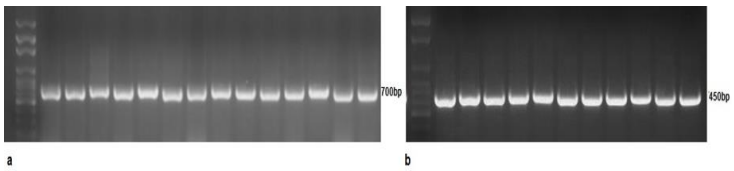


**Figure 2.** Amplification of VHH through two successive PCR CAL and nested VHH were performed; a, showed success of CAL PCR amplification using Cal001 and Cal002 primers on 1% agarose gel electrophoresis, the isolated fragment was at 700 bp; b, showed amplification and success of nested PCR for VHH domains at 450 bp.

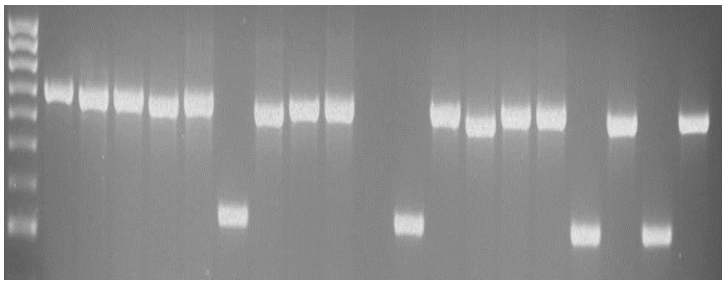

**Figure 3.** The camel VHH library construction where the success of a positive colony reached to 74% by investigation on 1% agarose gel electrophoresis.

*3.2. Production of immunized VHH library (IVHHL).*

To produce the immunized VHH library, the total RNA was isolated from the collected vaccinated camel blood and reversely transcribed into cDNA in order to amplify VHH fragments through two successive PCR amplification. The first amplification was Cal PCR that produced fragment length at 700 bp, which contains all VH domains of IgG2 and IgG3 isotypes followed by the second amplification VHH PCR with amplified fragments at 450 bp (Fig. 2a and 2b). The nested PCR and pMECS vector were digested with PstI and NotI restriction enzymes and then ligated together. The ligated vector and VHH were prepared for cloning step by electro-transformation into *Escherichia coli* TG1 cells (the preferred and suitable *E coli* cells for producing the high yield of VHH by phage), the success of transformation was tested by colony PCR producing 85% success by testing on 1% agarose gel (Fig. 3).

*3.3. Phage display selection of nano-antibody to S. arizonae.*

To select specific phages displaying an immunized library (IVHHL) against *S. arizonae*, IVHHL was added to helper phage and bio-panning Pan 0 was prepared for isolating rescue phage. Besides, four rounds of bio-panning Pan 1, Pan 2, Pan 3, and Pan 4 were performed using *S. arizonae* as antigen such that used in camel vaccination. All the pans showed complete fusion between nano-bodies and antigen. Furthermore, the concentration of rescue phage was determined for four pans where Pan 3 and Pan 4 exhibited the highest specificity of nano-antibodies against the antigen, in corresponding to the control rather than Pan 1 and Pan 2. All Pans were diluted to the lowest one in order to isolate the rescue phage. The success of phage display analysis and the maturation of camel nano-antibodies were checked by colonies counting and phage ELISA.

Colonies counting was detected as an increase in colony quant from Pan 1 to Pan 3 then become stable in Pan 4 where, Pan 1 expressed $3*10^4$, $2.3*10^4$ in Pan 2 corresponding to $1*10^2$ control and $1.1*10^5$ in Pan 3 and $7*10^5$ in pan4, the control was zero in pan 3 and 4 control (Table 1).

The Phage ELISA was proved the success of phage display and the maturation of nano-bodies, wherever the specify of nano-bodies was increased from P1 to Pan 3, while Pan 4 possessed the same specifications as in Pan 3 (Fig. 4).

**Table 1.** Colony quant in LB agar media with AMP and Glu for each Pan to detect the specify of camel nano-bodies against *S. arizonae*.

| | Pan1 Antigen | Pan2 antigen | Control | Pan3 antigen | Control | Pan4 antigen | Control |
|---|---|---|---|---|---|---|---|
| *S. arizonae* phage | $3.0*10^4$ | $2.3*10^4$ | $1*10^2$ | $1.1*10^5$ | 0 | $7*10^5$ | 0 |

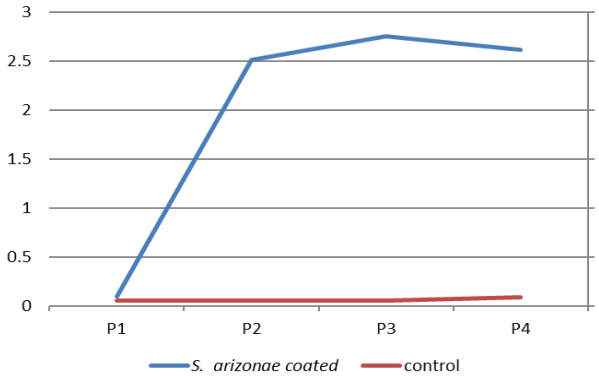


**Figure 4.** Screening for *S. arizonae* specific nano-bodies by Phage ELISA.

*3.4. Screening for S. arizonae specific nano-antibody by ELISA.*

ELISA measures were performed for screening specificity of VHH fragments for *S. arizonae* using 48 different colonies of Pan 4 wherever Elisa OD/450nm value of nano-bodies for each clone was measured referring to the control, therefore, 5 out of 48 nano-bodies (Nb-S. a-15: 0.954), (Nb-S. a-20: 0.977), (Nb-S.a-29 : 1.003), ( Nb-S.a-30 : 0.971), and (Nb-S.a-43 : 1.06) that possessed the highest OD/450nm were chosen (Fig. 5). Nano-bodies were purified cloned and isolated under non-denaturing conditions for preserving their functional protein configuration, and the reactivity of each VHH of the selected nano-bodies was analyzed by Western blot assay.

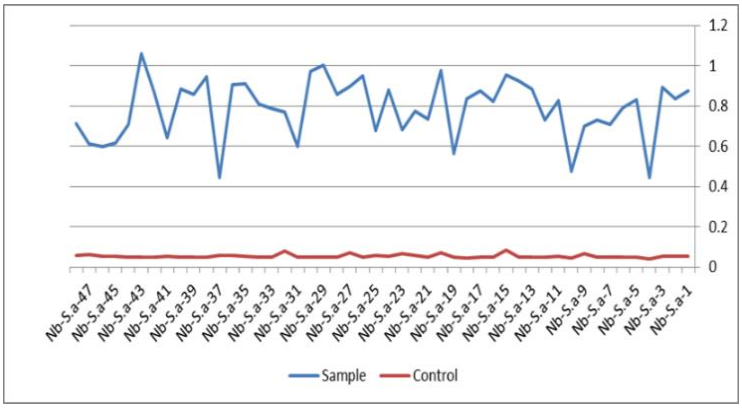


**Figure 5.** ELISA OD/450nm value of different 48 colonies marked with blue columns and its control with red columns, 5 Nano-antibodies (Nb-S.a-15 : 0.954), (Nb-S.a-20 : 0.977), , (Nb-S.a-29 : 1.003), ( Nb-S.a-30 : 0.971), and (Nb-S.a-43 : 1.06) according to highest ELISA OD value were selected against *S. arizonae*.

Nano-bodies protein was purified and separated under non-denaturing conditions by SDS-PAGE and visualized by Coomassie staining [26]. The reactivity of each VHH of the selected clones was analyzed by Western blot assay, where nano-bodies were probed with monoclonal anti-poly-histidine antibody and GAMAP and stained with NBT/BCIP substrate (Fig. 6).

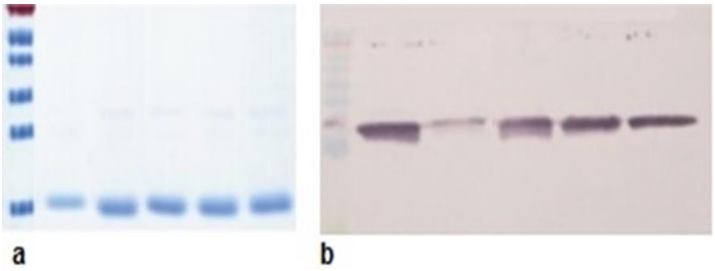


**Figure 6.** The selected nano-bodies were separated by SDS-PAGE and visualized by Coomassie staining (Fig. 6a). Western blot assay was performed for detecting the binding specificity of nano-bodies Fig. 6b) where VHH was probed with monoclonal anti-poly-histidine antibody and GAMAP and stained with NBT/BCIP substrate.

*3.5. ELISA Competition.*

The ability of nano-bodies binding to *S. arizonae* was evaluated by competitive binding assays of nano-bodies with *Salmonella typhimurium*. Fig. (7) illustrated the competitive assays of previously selected five clones of *S. arizonae*, Nb-S.a-15, Nb-S.a-20, Nb-S.a-29, Nb-S.a-30, and Nb-S.a-43. Nano-bodies showed the highest immune response against *S. arizonae* regarding the *Salmonella typhimurium*. In the clone Nb-S.a-43, OD/450nm value of nano-bodies showed the highest value (1.044) against *S. arizonae* in comparison with OD/450nm value 0.556 against *S. typhimurium*, in the clone Nb-S.a-29, it has high OD/450nm value

possessed the highest value 1.025 against *S. arizonae* compared against *S. typhimurium* OD/450nm value was 0.67, in the clone Nb-S.a-20 possessed the highest OD/450nm value 1.023 against *S. arizonae*, whereas OD/450nm value demonstrated 1.009 against *S. typhimurium*, in the clone Nb-S.a-30 possessed the highest OD/450nm value 0.871 against *S. arizonae*, whereas OD/450nm value demonstrated 0.867 against *S. typhimurium*, while in the clone Nb-S.a-15 determined the higher value of OD/450nm 0.834 against *S. typhimurium* than, OD/450nm value was 0.795 against *S. arizonae* [27-29].

The proposed assay didn't show much superiority in terms of sensitivity compared with other reported immunoassays. The reason might due to the traditional colorimetric reaction using TMB as a substrate. In order to improve its sensitivity in the future, this method could be combined with other HRP-mediated assays, including chemiluminescent [30,31], fluorescence [32], and plasmonic ELISA [33,34]. Nanobodies, with advantages of high stability and easy genetic manipulation, make it possible to generate recombinant chimeric or tagged fragments that enable improvements in immunoassays [35-40].

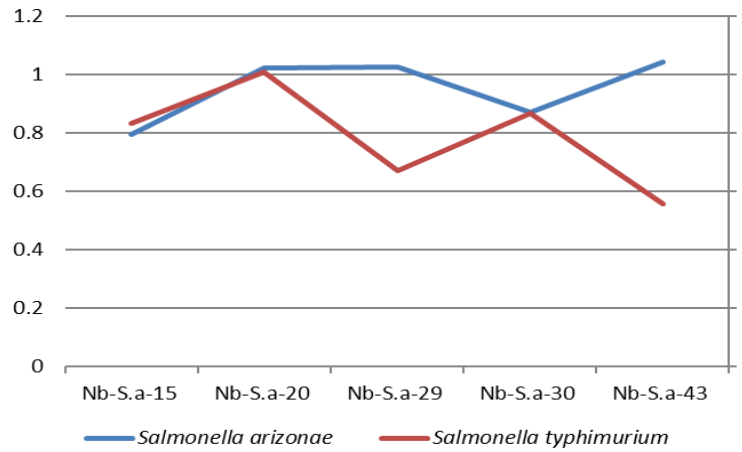


**Figure 7.** The competitive ELISA assays of 4 selected nano-bodies against S. arizonae, Nb-S.a-20, Nb-S.a-29, Nb-S.a-30, and Nb-S.a-43, showed the highest immune response regarding the *S. typhimurium*. In contrast, the nano-body Nb-S.a-15 showed OD/450 nm value agents *S. arizonae* lower than *S. typhimurium*.

## 4. Conclusions

In the current study, we developed a sandwich ELISA based on a nano-body towards *S. arizonae* as the first step for the detection of these pathogens. This is considered the first report of the development of nano-bodies for the treatment of *S. arizonae*. The isolated nano-bodies showed binding not only to *S. arizonae*, but also to another *Salmonella* strains, indicating that these nano-bodies can be used for treatment but can't use in diagnostic. So, many trials should be done to improve its sensitivity in the future.

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## Conflicts of Interest

The authors declare no conflict of interest.

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