Vol. 160: 7–12, 2024
https://doi.org/10.3354/dao03817

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published October 10

NOTE

PirA- or PirB-binding nanobodies can protect whiteleg shrimp from the acute hepatopancreatic necrosis disease toxin

Sylvia Cheung[1], Slade A. Loutet[1], Sarah Zaytsoff[1], Filip Van Petegem[2], Loc H. Tran[3], Hamlet Abnousi[1,*]

[1]NovoBind Livestock Therapeutics, Vancouver, BC V6E 0C3, Canada
[2]University of British Columbia, Department of Biochemistry and Molecular Biology, Vancouver, BC V6T 1Z3, Canada
[3]ShrimpVet Laboratory, Nong Lam University, Ho Chi Minh City 720371, Vietnam

ABSTRACT: Acute hepatopancreatic necrosis disease (AHPND) is a devastating shrimp disease caused by a binary toxin, PirAB, produced by *Vibrio parahaemolyticus* and other closely related bacteria. To address AHPND, over 300 unique single-domain antibodies (also known as nanobodies) derived from the V_HH domains of *Lama glama* heavy-chain-only antibodies were raised against either PirA or PirB and characterized. Nanobodies were shortlisted based on their affinities for either PirA or PirB, their relative stability in intestinal fluids, and their ability to reduce PirAB-induced death in brine shrimp *Artemia salina*. From these data, a subset of nanobodies was tested for their ability to reduce AHPND in whiteleg shrimp *Penaeus vannamei*, and nanobodies targeting either PirA or PirB provided significant disease protection to whiteleg shrimp. These results show that nanobodies can be a new option for shrimp farmers to reduce or eliminate the impact of AHPND on their operations.

KEY WORDS: *Vibrio parahaemolyticus* · *Penaeus vannamei* · Heavy-chain-only antibodies · Early mortality syndrome

1. INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND) is an infectious disease of penaeid shrimp characterized by atrophying hepatopancreas, empty guts, and high mortality (Tran et al. 2013). Cumulative global shrimp production losses caused by AHPND exceed $43 billion USD, with production reduced to approximately 60% in affected areas (Kumar et al. 2021).

A *Vibrio parahaemolyticus* plasmid-expressed binary toxin, PirAB, is necessary for the disease (Han et al. 2015, Lee et al. 2015). PirA and PirB share similar 3-dimensional topology to the insecticidal Cry toxin of *Bacillus thuringiensis* (Lee et al. 2015). Based on structural similarities, PirB is hypothesized to bind receptors and form pores, while PirA assists with receptor binding through interactions with sugar ligands (Lin et al. 2017). PirA and PirB interact with each other *in vitro* (Lin et al. 2019). New therapeutics could inhibit PirA and PirB by blocking receptor binding sites, disrupting the PirA–PirB interface, or inhibiting pore formation.

Typical pond management strategies such as aeration control, feed quality assessment, disinfection of input materials, and antibiotic applications are often insufficient, with AHPND-causing bacteria frequently resistant to several antibiotics (Kumar et al. 2021).

*Corresponding author: hamlet@novobind.com

Publisher: Inter-Research · www.int-res.com

Other mitigation strategies include probiotic bacteria, lytic bacteriophages, and phytochemicals, but none target PirAB. A new therapeutic option targeting PirAB, suitable for inclusion in shrimp feed, is needed. Nanobodies, derived from the V_HH domain of llama heavy-chain-only antibodies (Muyldermans 2013), offer one such option. Since anti-PirA chicken IgY antibodies can reduce AHPND mortality in experimental infections (Nakamura et al. 2019), antibody treatments for AHPND are viable. Here, we show that nanobodies targeting either PirA or PirB protect whiteleg shrimp from the AHPND toxin, supporting the use of nanobodies for AHPND control in shrimp aquaculture.

2. MATERIALS AND METHODS

2.1. Protein purification and nanobody discovery

Vibrio parahaemolyticus PirA and PirB were recombinantly expressed with C-terminal 6X-histidine tags similarly to previously described methods (Lee et al. 2015). Llamas were immunized with PirA and PirB, either alone or in combination, by CedarLane Laboratories (Burlington, Canada) under CedarLane's approved animal use protocol PL148AB. Nanobodies to PirA and PirB were discovered using well-described protocols (Baral et al. 2013) and expressed with N-terminal TEV-cleavable 6X-histidine and thioredoxin (TRX) tags as previously described (Loutet et al. 2024). Further details on nanobody discovery and purification protocols can be found in the Supplement (www.int-res.com/articles/suppl/d160p007_supp.pdf).

2.2. Nanobody ELISA

A 96-well plate was coated overnight at 4°C with 100 µl of either PirA (10 µg ml^{-1}) or PirB (20 µg ml^{-1}) in phosphate-buffered saline (PBS). Wells were washed 3 times with 300 µl of PBS and blocked with 200 µl of 5% skim milk powder in PBS + 0.05% Tween-20 (PBST) for 2 h at room temperature (RT). Wells were then washed once with 300 µl of PBST. Nanobodies were diluted in blocking solution to final concentrations of 4 pM to 400 nM; 100 µl were then added to the wells, and plates were incubated for 1 h at RT. Wells were washed 3 times with 300 µl of PBST, and 100 µl of HRP-conjugated rabbit IgG anti-V_HH cocktail (GenScript) diluted 1:8000 in blocking solution were added to wells and incubated for 1 h at RT. Wells were washed 3 times with 300 µl of PBST, and 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Abcam) were added to wells and incubated for 30 min at RT. Reactions were stopped with the addition of 50 µl of 1 M HCl, and absorbance at 450 nm was measured.

2.3. Gastrointestinal tract extract stability assay

Gastrointestinal (GI) tract stability assays were conducted as previously described (Loutet et al. 2024) using either shrimp GI tract or chicken jejunal extracts. Chicken intestinal tracts were removed at slaughter by a local poultry processor under NovoBind's approved animal use protocol GUT001. See the Supplement for further details.

2.4. *Artemia in vivo* tests

Brine shrimp *Artemia salina* cysts were purchased from Aquarium Direct and rehydrated in 1.7 l of artificial seawater (pH 8.4–8.6; alkalinity 3.2–3.8 mEq l^{-1}) prepared following the manufacturer's instructions (Salinity™; Aquavitro®) in an Artemia hatchery blender (ZH-2000, Ziss Artemia Blender 2.0 l; 18 cm × 14 cm × 13 cm). After 48 h of incubation between 26 and 28°C with constant aeration and light, the growth stage of *Artemia* was confirmed under a microscope.

Stage II *Artemia* (n = 10–12) were allocated into 0.5 ml artificial seawater per well in 24-well plates and acclimatized for 1 h at 26–28°C. Diluted protein treatment solutions (0.5 ml) were added to each well to achieve final concentrations of 3 µM PirA, 3 µM PirB, and 15 µM nanobody in 10% PBS. The 24-well plates were shaken at 90 rpm for 5 min to mix the treatment solutions, then incubated at 26–28°C with constant light and no shaking. Mortality was measured at 42 h post challenge. Each treatment group had 12 replicates. All experiments included a negative control group with 10% PBS and a positive control group receiving PirA and PirB without nanobody. *A. salina* infection experiments were conducted at NovoBind under approved animal use protocol ART001.

2.5. Whiteleg shrimp *in vivo* tests

Ten-day post-larval (PL10) whiteleg shrimp *Penaeus vannamei*, 9–11 mm in length, were used for whiteleg shrimp challenges. One PL10 was put into 0.5 ml seawater (salinity 20 ppt, pH 7.8–8.2, alkalinity 120–160 ppm) per well in 24-well plates and allowed to acclimatize for at least 1 h. PirA, PirB, nanobodies, and seawater were mixed as a 2× treatment solution

and allowed to equilibrate for 30 min. After PL10 acclimatization, 0.5 ml of 2× treatment solutions were added to each well. Final nanobody and toxin concentrations were 8 µM and 125 nM, respectively. Each treatment group consisted of 12 PL10s. Shrimp mortality was monitored every 2 h for 24 h. Additional groups included a negative control with seawater and a positive control receiving PirA and PirB without nanobody. Whiteleg shrimp infection experiments were conducted at ShrimpVet under approved animal use protocols IT-23005C, IT-23005D, IT-23005E, and IT-23005F.

2.6. Statistical analysis

All descriptive statistics (means and standard deviations), nonlinear regressions, and unpaired Student's *t*-tests were calculated using GraphPad version 10.1.2.

2.7. Animal use

All experiments involving the use of animals were approved by NovoBind's internal animal use committee.

3. RESULTS

3.1. Identification of unique PirA- and PirB-binding nanobodies

Nanobodies to PirA and PirB were obtained from several immunized llamas and, in total, 127 and 205 unique nanobodies were raised to PirA and PirB, respectively. Of these, more than 90% could be successfully expressed in and purified from *E. coli* and were tested *in vitro*.

3.2. Nanobody binding screen

The binding of all purifiable nanobodies to either PirA or PirB was measured by ELISA. To exemplify this, the binding of nanobody NBX-PA1 to PirA and nanobody NBX-PB1 to PirB are shown in Fig. 1. For both, binding is detected in the sub-nM range, with midpoints of saturation below 5 nM. The mean ± SE midpoints from 5 independent experiments were 2.1 ± 0.5 and 0.6 ± 0.1 nM for NBX-PA1 and NBX-PB1, respectively. Although these are not equilibrium assays, this result suggests a high inherent affinity.

3.3. Nanobody proteolytic stability screen

All nanobodies were screened for proteolytic stability in a GI tract extract. In early experiments, a GI extract derived from shrimp was available; however, such samples were difficult to obtain in Canada due to import restrictions to prevent the spread of infectious hypodermal and hematopoietic necrosis (IHHN) disease. Later experiments were conducted with a chicken jejunal extract that could be more easily obtained. Some nanobodies were tested in both samples and although survival times in different extracts varied, the relative ranking of nanobody stability was consistent across extract types. Fig. 2 shows the results for NBX-PA1 and NBX-PB1 in a chicken jejunal sample. NBX-PA1 was visible on the SDS-PAGE gel until the last measured time point (24 h), while NBX-PB1 was visible until 6 h. In total, 167 unique PirA- or PirB-binding nanobodies were tested for stability in

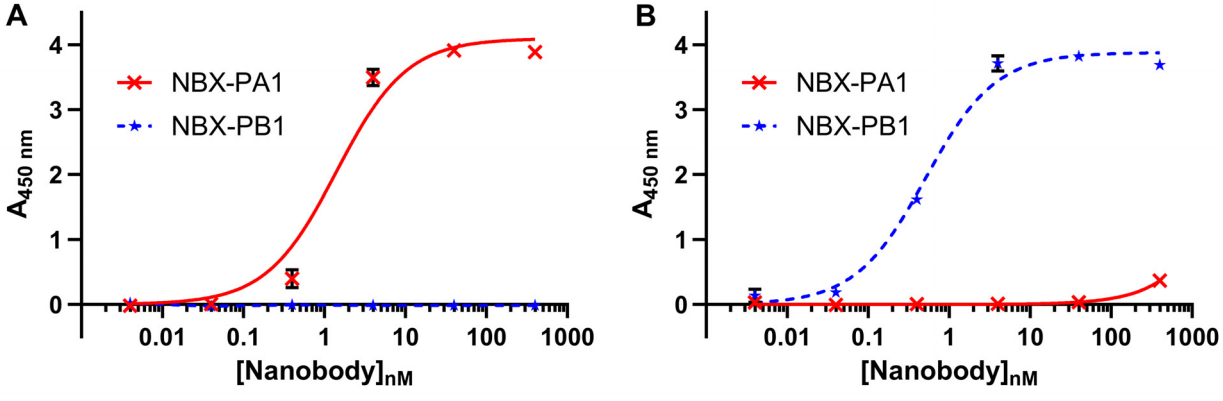

Fig. 1. Nanobodies NBX-PA1 and NBX-PB1 bind to target antigens in an ELISA-based assay at low nM concentration. (A) NBX-PA1, but not NBX-PB1, is able to bind to PirA. (B) NBX-PB1, but not NBX-PA1, is able to bind to PirB. Data shown are from a representative experiment for each nanobody. Data are means ± SD from triplicate wells

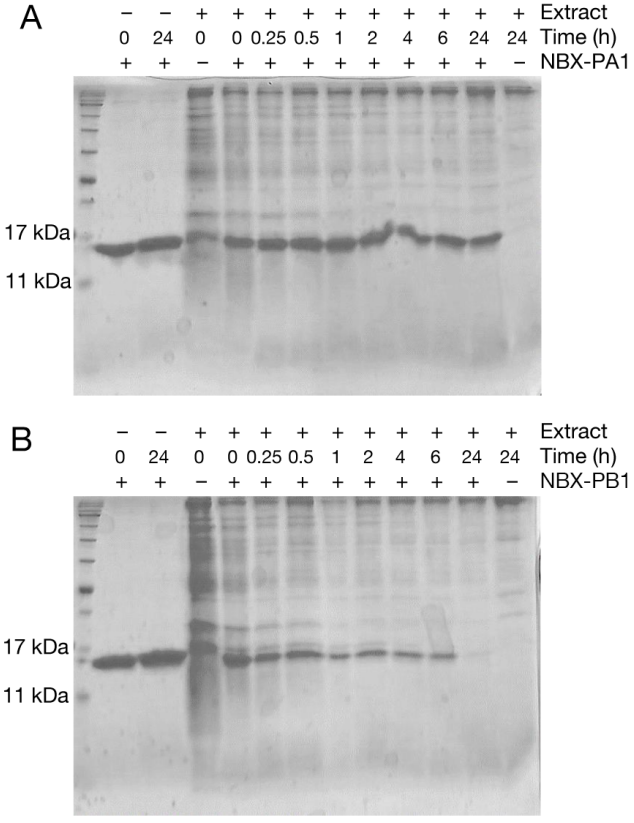

Fig. 2. (A) NBX-PA1 and (B) NBX-PB1 (both have predicted molecular weights of 12.5 kDa) survive for 24 and 6 h, respectively, in the presence of a chicken jejunal extract. For each nanobody, the SDS-PAGE gel shows the following from left to right: 11 and 17 kDa molecular weight markers; the nanobody, incubated at 42°C in the absence of extract at the start (0 h) and endpoints (24 h) of the experiment to show that any observed degradation is extract dependent; the extract without nanobody at the start of the experiment to aid in the identification of the nanobody on the gel; a time course (0–24 h) of the nanobody incubated at 42°C in the presence of the extract; and the extract without nanobody at the end of the experiment to aid in the identification of the nanobody on the gel

this batch of GI tract fluid. Only NBX-PA1 and 1 other nanobody were visible in the fluid after 24 h, meaning that NBX-PA1 is in the top 1.2% of the most stable nanobodies. A total of 20 nanobodies, including NBX-PB1, are visible at 6 h, indicating that NBX-PB1 is in the top 12% of the most stable nanobodies. A control nanobody, known to degrade between 6 and 24 h, was included in all assays to ensure expected behavior.

3.4. Nanobody protection of brine shrimp

Based on the combined results of nanobody binding and proteolytic stability studies, a collection of 50 nanobodies was selected for PirAB neutralization studies in brine shrimp *A. salina*. NBX-PA1 and NBX-PB1 were both able to reduce the mortality induced by PirAB by approximately 50% (Fig. 3). Using a cut-off of 30% mortality reduction or better during nanobody treatment, the nanobody collection was reduced to 20 for testing in whiteleg shrimp *P. vannamei* PL10s.

3.5. Nanobody protection of whiteleg shrimp

Nanobodies were tested for the ability to protect *P. vannamei* PL10s from treatment with PirAB in 24 h immersion experiments. Of those tested, 5 were able to reduce PL10 mortality by 50% or more after 24 h. Both NBX-PA1 and NBX-PB1 reduced PL10 mortality induced by PirAB to levels comparable to the untreated control PL10s (Fig. 4).

It was evident that PL10s were more susceptible to PirAB than brine shrimp. PL10s were killed more quickly (average of ~80% mortality at 24 h) than brine shrimp (average of ~60% mortality at 42 h), despite the fact that PL10s were treated with less PirAB (125 nM) than brine shrimp were (3 µM). Similar nanobody concentrations were used for each species, and lower PirAB levels in PL10 experiments may have improved protection by neutralizing nanobodies.

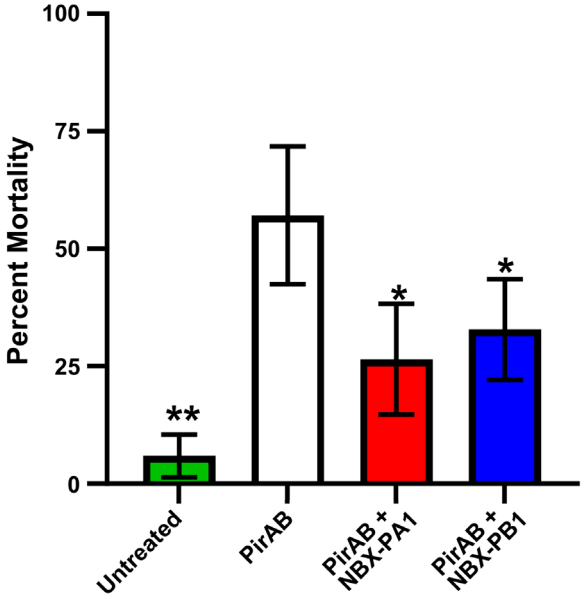

Fig. 3. NBX-PA1 and NBX-PB1 reduce PirAB-induced mortality in brine shrimp *Artemia salina*. Data are means ± SD from 3 to 7 independent experiments. Nanobody and toxin concentrations were 15 and 3 µM, respectively. All other groups are statistically different from the PirAB-treated group by unpaired Student's *t*-test (*$p < 0.05$, **$p < 0.0001$)

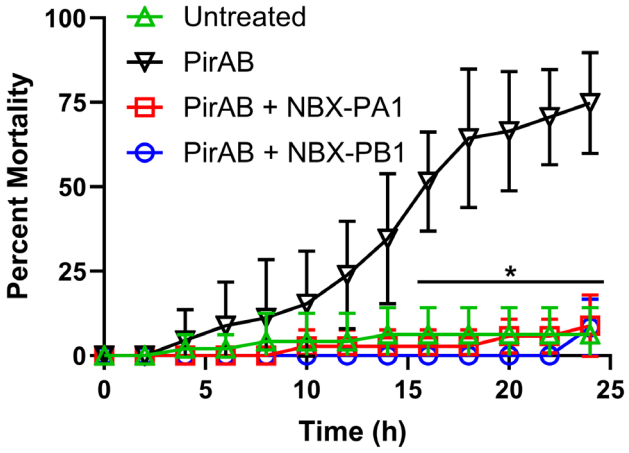

Fig. 4. NBX-PA1 and NBX-PB1 protect whiteleg shrimp *Penaeus vannamei* from PirAB. Data means ± SD from 3 or 4 independent experiments for untreated, PirAB treated, PirAB + NBX-PA1 treated, and PirAB + NBX-PB1 treated whiteleg shrimp. Nanobody and toxin concentrations were 8 µM and 125 nM, respectively. From 16 h onwards, the difference in percent mortality between PirAB treated and either PirAB + NBX-PA1 treated or PirAB + NBX-PB1 treated is statistically different (*$p < 0.01$) by unpaired Student's *t*-test

4. DISCUSSION

To our knowledge, this is the first use of nanobodies for disease prevention in shrimp; however, other antibody types have been tested previously. Single-chain variable fragment antibodies or conventional antibodies can be used to reduce white spot syndrome, AHPND, or bacterial vibriosis (Lu et al. 2008, 2009, Gao et al. 2016, Kumaran et al. 2018, Nakamura et al. 2019, Cui et al. 2022). Compared to these, nanobodies are smaller and consist of a single compact domain. Nanobodies can be more easily produced through large-scale fermentation processes using organisms such as yeast, which should reduce their cost for widespread use in aquaculture.

Previous attempts to develop antitoxin nanobodies have included *in vitro* functional assays during the nanobody discovery pathway (Andersen et al. 2016, Gangaiah et al. 2022, Loutet et al. 2024). This enables the selection of nanobodies with direct antitoxin effects. When such a nanobody is applied *in vivo*, it likely acts by directly reducing the ability of the pathogen to cause disease. Without an immortalized cell line susceptible to PirAB, no such *in vitro* screen could be included here. Despite this, nanobodies were obtained that protect whiteleg shrimp from PirAB toxicity. This was accomplished by starting with several hundred nanobodies and filtering them through a series of protein–protein interactions, intestinal tract proteolytic stability, and brine shrimp *in vivo* tests to shortlist nanobodies for whiteleg shrimp protection experiments. These experiments showed that nanobodies can reduce shrimp mortality in the presence of PirAB to levels comparable to unchallenged control shrimp in immersion trials.

Although the mechanism by which the lead nanobodies protect shrimp from the toxin was not confirmed, the lead nanobodies are hypothesized to bind at functionally relevant epitopes, thus blocking toxin function. These epitopes could be important for the PirA–PirB interface, receptor binding, or cell lysis. Several non-neutralizing PirA- or PirB-binding nanobodies with binding and stability characteristics comparable to the lead nanobodies described here were obtained. These non-neutralizing nanobodies suggest that toxin binding alone is insufficient for disease protection. However, the possibility that the lead nanobodies bind the toxins in a way that recruits an antitoxin or antibacterial component of the shrimp innate immune systems cannot be excluded.

In vertebrates, nanobodies have been applied as fusion proteins with host immunoglobulin Fc regions to engage the host immune system (Hussack et al. 2018, Virdi et al. 2019) or in multivalent formats (Yang et al. 2014, Fiil et al. 2022). Fusing the lead nanobodies with a protein able to elicit antitoxin or antibacterial elements of the shrimp innate immune system or expressing them in multivalent formats are intriguing options. However, this increasing complexity may negate the benefits of the small compact structure of nanobodies, resulting in higher production costs.

Future studies will include larger trials with nanobodies supplied via feed. Nanobodies have been shown to protect other animal species from GI diseases when applied in feed (Virdi et al. 2019, Lessard et al. 2020, Loutet et al. 2024). It is anticipated that nanobodies in shrimp feed should also be effective. The results presented here demonstrate the great potential nanobodies have for the protection of shrimp from AHPND. Nanobodies offer an excellent new opportunity to the shrimp aquaculture sector for the reduction of this disease, which at this time causes billions in dollars of losses to producers every year (Kumar et al. 2021).

Conflict of interest. S.A.L, S.C., S.Z., and H.A. are all employees of NovoBind and possess NovoBind shares and/or share options. F.V.P. possesses NovoBind shares. L.H.T. is the founder of ShrimpVet Laboratory, and ShrimpVet was remunerated for their work presented here. NovoBind has filed 2

PCT patent applications (WO2020008254A1 and WO2024 092360A1) related to this work, the second of which contains the hundreds of nanobody sequences identified as part of this study.

Data availability. The present work is available in report form on the NovoBind website (https://www.novobind.com/news-updates; Report 20230923).

Acknowledgements. Funding was provided by the National Research Council of Canada Industrial Research Assistance Program. This work would not have been possible without many NovoBind employees. The authors thank the Editor, Fuhua Li, and the 3 anonymous reviewers for their constructive comments concerning this manuscript.

LITERATURE CITED

Andersen KK, Strokappe NM, Hultberg A, Truusalu K and others (2016) Neutralization of *Clostridium difficile* toxin B mediated by engineered lactobacilli that produce single-domain antibodies. Infect Immun 84:395–406

Baral TN, MacKenzie R, Ghahroudi MA (2013) Single-domain antibodies and their utility. Curr Protoc Immunol 103:2.17.1–2.17.57

Cui C, He L, Tang X, Xing J, Sheng X, Chi H, Zhan W (2022) Monoclonal antibodies (mAbs) and single chain variable fragment (scFv) antibodies targeting envelope protein VP28 of white spot syndrome virus provide protection against viral infection. Fish Shellfish Immunol 127:508–520

Fiil BK, Thrane SW, Pichler M, Kittilä T and others (2022) Orally active bivalent V_HH construct prevents proliferation of $F4^+$ enterotoxigenic *Escherichia coli* in weaned piglets. iScience 25:104003

Gangaiah D, Ryan V, Van Hoesel D, Mane SP and others (2022) Recombinant *Limosilactobacillus* (*Lactobacillus*) delivering nanobodies against *Clostridium perfringens* NetB and alpha toxin confers potential protection from necrotic enteritis. MicrobiologyOpen 11:e1270

Gao X, Zhang X, Lin L, Yao D and others (2016) Passive immune-protection of *Litopenaeus vannamei* against *Vibrio harveyi* and *Vibrio parahaemolyticus* infections with anti-*Vibrio* egg yolk (IgY)-encapsulated feed. Int J Mol Sci 17:723

Han JE, Tang KFJ, Tran LH, Lightner DV (2015) *Photorhabdus* insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Dis Aquat Org 113:33–40

Hussack G, Ryan S, van Faassen H, Rossotti M, MacKenzie C, Tanha J (2018) Neutralization of *Clostridium difficile* toxin B with V_HH-Fc fusions targeting the delivery and CROPs domains. PLOS ONE 13:e0208978

Kumar V, Roy S, Behera BK, Bossier P, Das BK (2021) Acute hepatopancreatic necrosis disease (AHPND): virulence, pathogenesis and mitigation strategies in shrimp aquaculture. Toxins 13:524

Kumaran T, Thirumalaikumar E, Lelin C, Palanikumar P, Michaelbabu M, Citarasu T (2018) Physicochemical properties of anti *Vibrio harveyi* egg yolk antibody (IgY) and its immunological influence in Indian white shrimp *Fenneropenaeus indicus.* Fish Shellfish Immunol 74:349–362

Lee CT, Chen IT, Yang YT, Ko TP and others (2015) The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proc Natl Acad Sci USA 112:10798–10803

Lessard PA, Parker M, Bougri O, Shen B and others (2020) Improved performance of *Eimeria*-infected chickens fed corn expressing a single-domain antibody against interleukin-10. Nat Food 1:119–126

Lin SJ, Hsu KC, Wang HC (2017) Structural insights into the cytotoxic mechanism of *Vibrio parahaemolyticus* $PirA^{vp}$ and $PirB^{vp}$ toxins. Mar Drugs 15:373

Lin SJ, Chen YF, Hsu KC, Chen YL and others (2019) Structural insights to the heterotetrameric interaction between the *Vibrio parahaemolyticus* $PirA^{vp}$ and $PirB^{vp}$ toxins and activation of the Cry-like pore-forming domain. Toxins 11:233

Loutet SA, Cheung S, Zaytsoff S, Hofacre C, Jones MK, Van Petegem F, Abnousi H (2024) *Clostridium perfringens* NetB and CnaA neutralizing nanobodies in feed reduce the incidence of poultry necrotic enteritis. Poult Sci 103: 103578

Lu Y, Liu J, Jin L, Li X and others (2008) Passive protection of shrimp against white spot syndrome virus (WSSV) using specific antibody from egg yolk of chickens immunized with inactivated virus or a WSSV-DNA vaccine. Fish Shellfish Immunol 25:604–610

Lu Y, Liu J, Jin L, Li X and others (2009) Passive immunization of crayfish (*Procambius clarkiaii* [sic]) with chicken egg yolk immunoglobulin (IgY) against white spot syndrome virus (WSSV). Appl Biochem Biotechnol 159:750–758

Muyldermans S (2013) Nanobodies: natural single-domain antibodies. Annu Rev Biochem 82:775–797

Nakamura R, Pedrosa-Gerasmio IR, Alenton RRR, Nozaki R, Kondo H, Hirono I (2019) Anti-PirA-like toxin immunoglobulin (IgY) in feeds passively immunizes shrimp against acute hepatopancreatic necrosis disease. J Fish Dis 42:1125–1132

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner DV (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Org 105:45–55

Virdi V, Palaci J, Laukens B, Ryckaert S and others (2019) Yeast-secreted, dried and food-admixed monomeric IgA prevents gastrointestinal infection in a piglet model. Nat Biotechnol 37:527–530

Yang Z, Schmidt D, Liu W, Li S and others (2014) A novel multivalent, single-domain antibody targeting TcdA and TcdB prevents fulminant *Clostridium difficile* infection in mice. J Infect Dis 210:964–972

Editorial responsibility: Fuhua Li, Qingdao, PR China
Reviewed by: C. Zhao and 2 anonymous referees

Submitted: February 2, 2024
Accepted: August 16, 2024
Proofs received from author(s): October 4, 2024