Fish & Shellfish Immunology 34 (2013) 159–166

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Truncated VP28 as oral vaccine candidate against WSSV infection in shrimp: An uptake and processing study in the midgut of *Penaeus monodon*

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ARTICLE INFO

Article history:
Received 10 July 2012
Received in revised form
19 September 2012
Accepted 14 October 2012
Available online 26 October 2012

Keywords:
White spot syndrome virus
Recombinant VP28
Antigen processing
Penaeus monodon
Immunohistochemistry

ABSTRACT

Several oral vaccination studies have been undertaken to evoke a better protection against white spot syndrome virus (WSSV), a major shrimp pathogen. Formalin-inactivated virus and WSSV envelope protein VP28 were suggested as candidate vaccine components, but their uptake mechanism upon oral delivery was not elucidated. In this study the fate of these components and of live WSSV, orally intubated to black tiger shrimp (*Penaeus monodon*) was investigated by immunohistochemistry, employing antibodies specific for VP28 and haemocytes. The midgut has been identified as the most prominent site of WSSV uptake and processing. The truncated recombinant VP28 (rec-VP28), formalin-inactivated virus (IVP) and live WSSV follow an identical uptake route suggested as receptor-mediated endocytosis that starts with adherence of luminal antigens at the apical layers of gut epithelium. Processing of internalized antigens is performed in endo-lysosomal compartments leading to formation of supra-nuclear vacuoles. However, the majority of WSSV-antigens escape these compartments and are transported to the inter-cellular space via transcytosis. Accumulation of the transcytosed antigens in the connective tissue initiates aggregation and degranulation of haemocytes. Finally the antigens exiting the midgut seem to reach the haemolymph. The nearly identical uptake pattern of the different WSSV-antigens suggests that receptors on the apical membrane of shrimp enterocytes recognize rec-VP28 efficiently. Hence the truncated VP28 can be considered suitable for oral vaccination, when the digestion in the foregut can be bypassed.

1. Introduction

White spot syndrome virus (WSSV), the causative agent of white spot disease in shrimps, has been described as the most calamitous virus ever since its first appearance in Northeast Asia [1]. Therefore, there is a worldwide effort to control or manage this disease.

The morphology, molecular characterization, morphogenesis and pathogenesis of WSSV have been extensively studied [2]. The virus most likely enters *per os* when healthy shrimps scavenge on diseased individuals although entry via the gills cannot be excluded. The primary target of the ingested WSSV is the stomach, and the interaction of WSSV with the epithelial cells of the gut has been demonstrated [3,4].

Based on the viral accommodation concept suggesting the existence of immunological memory it was hypothesized that shrimps could be vaccinated against viral disease [5]. Several attempts were made to immunize shrimps against WSSV, through intramuscular injections and oral feeding of formalin-inactivated virus and/or recombinant viral envelope proteins [6–9]. VP28 is a major envelope protein of WSSV that takes part in the systemic infection of the shrimp through its attachment and entry into host cells [10,11].

Most of the vaccination trials were aimed to enhance survival, to extend the duration of protection and to increase the efficacy of vaccine delivery. However, there is a dearth of information on how the animal processes and utilizes the vaccine components. The present study describes the uptake and processing of two 'vaccine' candidates – formalin-inactivated virus preparation (IVP) and recombinant VP28 (rec-VP28) – within the gut of the shrimp after oral intubation. For comparison the oral infection process of live WSSV was studied. In addition, haemocyte aggregation and

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http://dx.doi.org/10.1016/j.fsi.2012.10.028

degranulation were documented as parameters of local immune response of shrimp.

2. Materials and methods

The present study was designed to clarify the gastrointestinal uptake of 'WSSV-vaccines' and live virus by the shrimp, *Penaeus monodon*. Oral intubation procedure was adopted to ensure precise delivery of WSSV-antigens (vaccines or live WSSV) [12] and immunohistochemical techniques were employed to understand and compare the uptake and processing of the antigens in the gut of the shrimp. The live animal experiments were conducted at the National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology, India and the immunohistochemical studies were carried out at the laboratories of Aquatic Animal Health Unit of the University of Nordland (UiN), Norway.

2.1. Experimental animals

Sub-adult black tiger shrimps, *P. monodon* (body weight 18–20 g), obtained from a local farm, were transferred to the rearing facilities of NCAAH and kept in quarantine for 48 h. WSSV-free shrimps (detected by PCR [13]) were introduced into continuously aerated rectangular fibre reinforced 30 l plastic tanks supplied with 15 parts per thousand (ppt) saline water. The shrimps were maintained under optimum conditions, exchanging 50% of the rearing water every other day and the dissolved oxygen [4.5–5 parts per million (ppm)] and ammonia content (0.5–1.5 ppm) were monitored daily. The animals were fed *ad libitum* twice a day with a commercial 'grower' pelleted feed (Amalgam Nutrients and Feeds Limited, Cochin, India).

2.2. Preparation of WSSV and inactivated virus as vaccine component

A strain of WSSV (MCCV 101) from the microbial culture collection of NCAAH was used for the experiment and an aliquot of 10 μl of the virus stock was injected intramuscularly to apparently healthy shrimps in order to generate WSSV positive animals, as described earlier [8]. Tissues from the cephalothorax region of these shrimps were macerated with a minimum quantity of PBS and an equal amount of glass fibre wool. The resulting slurry was centrifuged twice at 8200×*g* for 20 min at 4 °C to separate the supernatant containing live WSSV. The viral titer of the supernatant was determined to be 1×10^5/ml live WSSV particles, by real-time PCR [14].

IVP was generated following the method described earlier [8]. Briefly, 15 g of gills and soft tissue from the cephalothorax region of WSSV infected shrimps were homogenized (in 100 ml of sterile seawater), sieved (100 μm mesh) and subjected to two successive cycles of freezing and thawing in order to release the virus into the solution. The virus was inactivated by the addition of 0.2% formalin (v/v) and subsequent incubation for 48 h at room temperature (RT) prior to determining the virus titer. A loop-full of IVP was streaked on Zo-Bell's and Sabouraud dextrose agar plates, which were incubated for 5 days. Absence of bacteria and fungi on the streaked plates ensured the sterility of IVP. Using real-time PCR, the titer was determined to be 1×10^8/ml inactivated virus particles, as mentioned earlier [14].

A control for IVP was prepared using PCR tested WSSV-negative shrimp tissues and the above-mentioned steps.

2.3. Preparation of recombinant VP28 as vaccine component

The pET28a-VP28 construct (truncated form of VP28) expressed in *Escherichia coli* BL21 cells [9] was obtained from the Virology group of Wageningen University (WUR), The Netherlands. At UiN, His6-VP28 protein was over expressed according to the protocols of the aforementioned group at WUR. Briefly, the transformed *E. coli* BL 21 cells were cultured on Luria–Bertani (LB) medium containing kanamycin (1:1000) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 5 h. The cells were resuspended in 1 ml PBS containing 100 μl lysis solution (0.2% SDS, 1% Triton X 100, 10 mM EDTA, 0.2 M NaCl) for 1 h at RT. Later on the cells were sonicated to extract the recombinant protein, which was purified by affinity chromatography employing Ni-NTA 6×His tagged recombinant protein purification system (Invitrogen, Paisley, UK). The purified protein was confirmed as VP28 by SDS-PAGE and Western-Blotting using antibodies targeting VP28 [15]. The protein content of this purified VP28 was quantified using the Qubit® protein assay kit and the Qubit® fluorometer (Invitrogen) following the suppliers instructions. Next, aliquots of the envelope protein were prepared to conduct the animal experiments at NCAAH.

2.4. Oral delivery of WSSV vaccine candidates and live virus

The different preparations mentioned in the previous sections (VP28, IVP and live WSSV) were orally delivered to shrimps of the three treatment groups. The first two groups were intubated with either rec-VP28 (100 μl, $n = 36$) or IVP (100 μl, $n = 36$) using BusterTom Cat Catheter 1.0 × 130 mm (Jorgensen Laboratories, Loveland, Co, USA) connected to a 1 ml hypodermic syringe. The third group ($n = 36$) was intubated with live WSSV (100 μl). Each shrimp in the different treatments received either 30 μg of pure protein of VP28 or 1×10^7/100 μl of inactivated virus or 1×10^4/100 μl of live WSSV particles. The shrimps intubated with WSSV-negative shrimp extract ($n = 6$) or PBS ($n = 6$) served as controls for virus (both IVP and WSSV) and VP28 intubated groups, respectively.

2.5. Sampling from the different treatments

Six shrimps ($n = 6$) from each of the VP28, IVP and WSSV intubated groups, sampled at each time point [2, 4, 8, 24, 48 and 72 h post-intubation (hpi)] were used to isolate the entire gut. However, by 48 hpi severe mortality occurred in the WSSV intubated group, and therefore samples at 72 hpi were not taken. Samples from both control groups were procured at 4 hpi. Immunohistochemistry was performed on the isolated gut, which was divided into three equal parts namely proximal midgut, median midgut and distal midgut along with hindgut.

2.6. Immunohistochemistry

Gut samples isolated at various time points were immediately fixed in Davidson's fixative [330 ml 96% ethanol, 220 ml 37% formalin, 115 ml glacial acetic acid and 335 ml Milli Q water (Millipore S.A.S. 67120 Molsheim, France)] for 40–48 h at RT [16]. The fixed tissues were transferred to 50% ethanol and used for further studies at UiN. Tissues were dehydrated through graded ethanol series and embedded in paraffin. Subsequent sections of 4 μm were cut and deparaffinized in xylene, followed by rehydration in ethanol series. Antigen retrieval was performed on the gut sections of IVP and VP28 intubated shrimps by heating the slides at 100 °C for 10 min in Tris–EDTA buffer (10 mM Trisma base, 1 mM EDTA pH 9). Gut sections of the shrimps intubated with live WSSV did not require the antigen retrieval process for the detection of antigen since the immunoreaction was identical with or without the retrieval procedure.

All the slides were incubated with methanol and 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidase. After washing the

slides in PBS-t (1 M PBS pH 7, 0.1% Triton X 100) they were treated with 10% goat serum to block the non-specific binding sites. Later on, the slides were incubated (overnight at 4 °C) with the primary antibodies consisting of a mix of VP28 polyclonal antibody produced in rabbit [1:100; specific for rec-VP28, provided by the Virology group of WUR [17]] and the haemocyte specific monoclonal antibody (MAb) produced in mouse [WSH 8; 1:100; specific for haemocyte granules, provided by the Cell Biology and Immunology group at WUR [18]]. The slides were then washed with PBS-t before the incubation with secondary antibodies – a mix of horseradish peroxidase conjugated goat-anti-rabbit Ig (GAR-HRP: 1:200) and alkaline phosphatase conjugated goat-anti-mouse Ig (GAM-AP: 1:100) – for 1 h at RT. After performing multiple washing steps the slides were incubated with 3-amino-9-ethylcarbazole (260 mg/l sodium acetate buffer pH 5) to stain VP28 red and subsequently with NBT/BCIP (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) to stain haemocytes blue. All the chemicals used in immunohistochemistry are from Sigma (St. Louis, MO, USA), unless stated otherwise.

In an attempt to standardize the antigen retrieval protocol, various retrieval buffers (sodium citrate and Tris–EDTA), different pH conditions (pH 6 and 9) and two temperature settings (25 and 100 °C) were applied on preliminary samples. Since demasking of antigenic determinants may generate artifacts leading to false-positive results [19], comprehensive control assays were carried out to avoid ambiguity.

2.7. Impression of intensities of VP28- and haemocyte-immunoreactivity in the gut of intubated shrimps

Processing of WSSV-antigens within the gut was studied by employing immunohistochemistry using anti-VP28 polyclonal antibody. Each step of antigen processing was ranked based on VP28-immunoreactivity ('–' to '++++'). Mean ranks obtained from six shrimps at each sampling point for each of the three WSSV-antigens were compared, to allow differential analysis of antigen processing and/or virus invasion. A difference of at least two mean ranks (e.g. + vs. +++) or absence of reaction (+ vs. –) within a sampling point is considered as conspicuous. In addition, the gut samples of control shrimp were also analysed and all appeared to be WSSV negative.

Local immune responses against intubated WSSV-antigens in terms of aggregation and or degranulation of haemocytes (activation) was graded. Samples were ranked from '+' for gut with few aggregating haemocytes to '++++' for gut with abundant haemocytes and/or massive degranulation.

3. Results

3.1. WSSV-antigen transit along the gut

Following oral delivery, WSSV-antigens were transported from the proximal region of the midgut towards the hindgut during the 72 h observation period. At the early time points (2–8 hpi), WSSV-antigens were detected in the lumen and uptake/processing and associated haemocytes reaction were localized to proximal midgut (Fig. 1A). As time progressed (24–72 hpi), the antigens and their processing had progressed towards the median midgut ultimately reaching the distal midgut (Fig. 1B).

3.2. Processing of WSSV-antigens at early and later time points in midgut

At the early time points (2 hpi and 4 hpi), WSSV-immunoreactivity was restricted to the proximal midgut. WSSV-antigens were initially found to adhere on microvilli borders of the epithelial cells (Figs. 2A and 3A and D). Subsequently, they were observed in inter-cellular space (Figs. 2C and 3C and E) and later as accumulations in connective tissues underlying the epithelium and basement membrane (Figs. 2E and 3C and E). Finally, WSSV-antigens exited the gut (Figs. 2F and 3B and E).

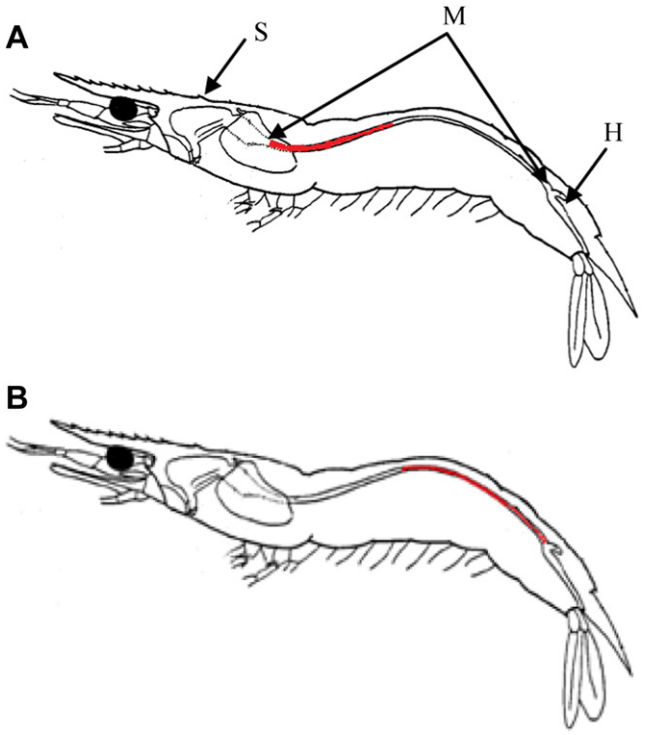

Fig. 1. Illustration of shrimp digestive tract, modified from Dall [24], showing stomach – S; midgut trunk – M; hindgut – H. Localization of WSSV-antigens in the lumen, its uptake/processing and associated haemocyte reaction are indicated by the shaded region that was observed in the proximal part of midgut from 2 hpi to 8 hpi (A); and in the median midgut along with the distal midgut from 24 hpi to 72 hpi (B). This figure is based on microscopical observations.

When WSSV-antigens were pushed to the median midgut (at 8 and 24 hpi), their initial processing and uptake were similar to that described for proximal midgut. At these time points, WSSV-antigens were seen as accumulations in supranuclear vacuoles (SNV) in the epithelium of proximal midgut (Figs. 2B and 3B). In the case of shrimps intubated with live WSSV, infected cells showing typical hypertrophied nucleus were occasionally observed in the connective tissue underlying the basement membrane at 8 hpi. Severe infection was observed in samples taken at 24 hpi onwards (not shown). Nevertheless, at all time points epithelial cells were devoid of infection.

Later at 48 and 72 hpi the processing of WSSV-antigens in the distal midgut followed the pathway that occurred at 2 and 4 hpi in the proximal midgut. However, at these later time points median midgut had several SNVs. Finally when the remaining antigens in the lumen were pushed to the hindgut, distal midgut also showed SNVs. In all control shrimps (PBS/healthy shrimp extract intubated) no immunoreactivity for anti-VP28 antibody was detected.

3.3. Haemocyte reaction to intubated WSSV-antigens

Concomitant haemocyte reactions occurred during the transit of WSSV-antigens through each segment of the gut of the shrimps. After the epithelial transport, antigens reached the connective tissues and caused aggregation of semi-granular and granular haemocytes. These haemocytes were locally activated as they were

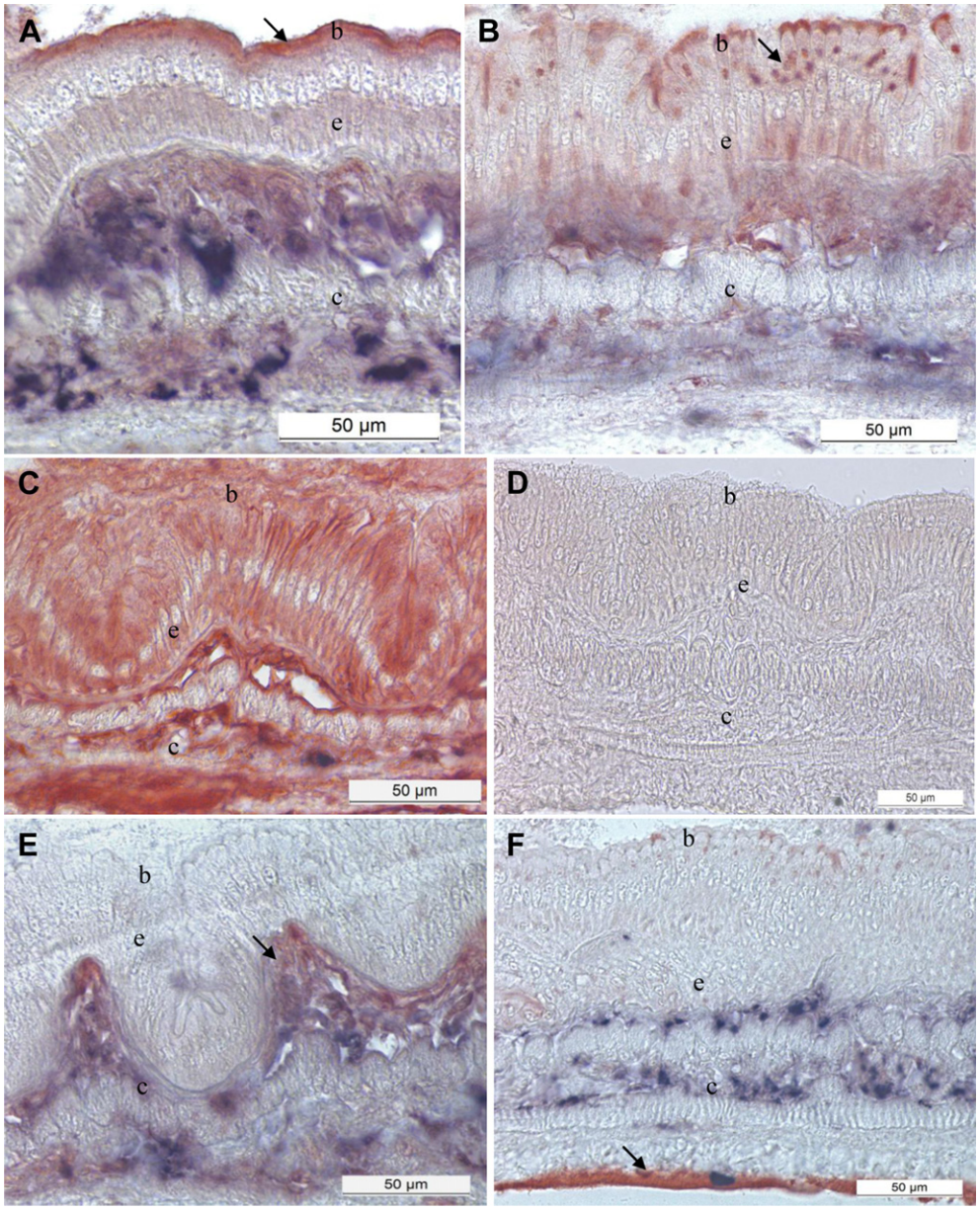

Fig. 2. Histological sections of midgut from *P. monodon* intubated with rec-VP28. VP28 antigenic determinants and haemocytes are stained with anti-VP28 antibody (red) and WSH8 antibody (blue stained haemocytes), respectively. VP28 adhering to microvilli borders of proximal midgut epithelial cells at 2 hpi (A: arrow), enclosed in SNVs at 8 hpi in median midgut (B: arrow), within the inter-cellular space at 2 hpi around proximal midgut (C: red stain), accumulated in the connective tissue at 24 hpi near median midgut (E: arrow), exiting the gut at 24 hpi around proximal midgut (F: arrow) are presented. A parallel for the gut section of shrimp presented in (C) was subjected to immunohistochemistry excluding the mix of primary antibodies (D) to confirm the absence of background staining and internal peroxidase activity. Brush borders on the apical membrane (b), entire epithelial cell (e) and connective tissues (c) underlying the epithelium are indicated in all the figures from A to F.

degranulating (Fig. 3F and G). Aggregation and degranulation of haemocytes were detected in the shrimps intubated with all the three WSSV-antigens, and to some extent in the control shrimps (PBS/healthy shrimp extract intubated).

Apart from the degranulating haemocytes, some cells with immunoreactivity for both WSH8 and anti-VP28 antibodies were observed in the connective tissues of the shrimps intubated with WSSV-vaccines and live virus (not shown).

3.4. Impression of intensities of VP28- and haemocyte-immunoreactivity in the gut of intubated shrimps

As described in Section 3.2 the processing of the antigens constituted 5 steps: initially the antigens adhered to the epithelial cells (step 1). Thereafter they were either seen as accumulations in SNVs (step 2) or in inter-cellular space (step 3). Later on, they were found in connective tissues (step 4) and finally the antigens exited the gut (step 5). Mean ranking of VP28- immunoreactivity for each of these steps, at all the sampling points, is presented in Table 1 along with the mean ranking for the associated haemocyte activity.

The adherence of VP28, IVP and WSSV to the microvilli borders of epithelial cells was almost similar (++/+++), except at 24 hpi where a conspicuous difference was observed in the adherence of IVP and WSSV (+ vs. +++). Among the three antigens, VP28 was clearly retained in SNVs (++/+++) whereas WSSV and IVP were relatively less observed in SNVs (+). The transport of vaccines and virus to the inter-cellular space did not show much variation in its

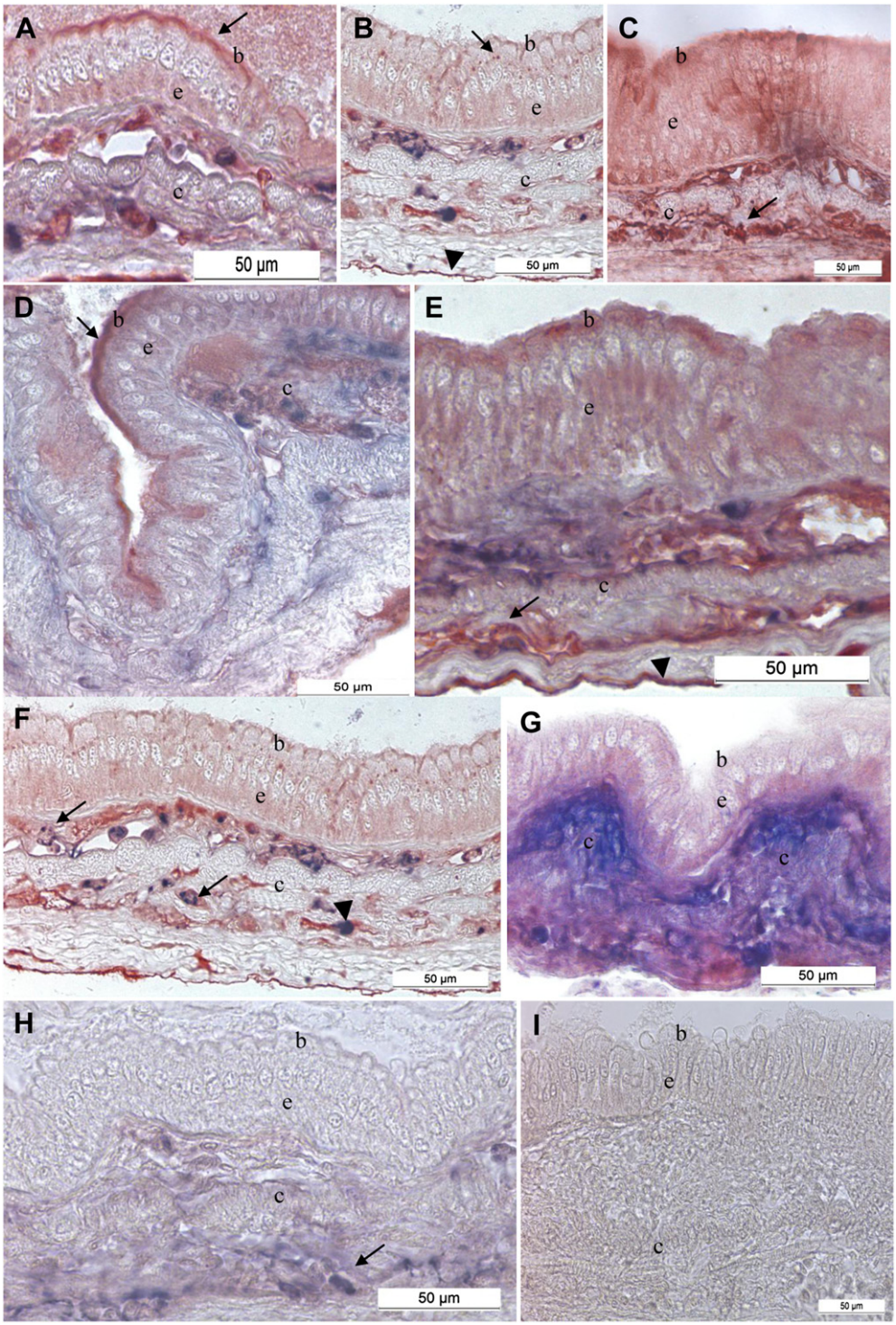

Fig. 3. Histological sections of midgut from *P. monodon* intubated with IVP and WSSV. VP28 antigenic determinants and haemocytes are stained with anti-VP28 antibody (red) and WSH8 antibody (blue stained haemocytes), respectively. Both IVP (A: arrow) and WSSV (D: arrow) adhered to microvilli borders of epithelial cells at 2 and 4 hpi around proximal midgut. The presence of IVP in SNVs (B: arrow) at 8 hpi around median midgut was observed. IVP (C: red stain) transported in the inter-cellular space at 2 hpi near proximal midgut and WSSV (E: red stain) at 8 hpi near median midgut is also presented. Later on IVP (C: arrow) and WSSV (E: arrow) accumulated in the connective tissues is also represented. The exit of IVP and WSSV from the gut is also shown in B and E (arrow heads), respectively. The non-degranulated haemocytes (F: arrow) with its granules intact and degranulating haemocytes with diffused granules at 8 hpi (F: arrow head) is shown in IVP intubated median midgut. Massive degranulation (G) at 24 hpi observed as blue stain in the connective tissues of shrimps intubated with WSSV was observed near median midgut region. PBS intubated shrimp gut section indicating positive immunoreactivity for only the WSH8 antibody (H: arrow) is presented along with a section of median midgut of shrimp intubated with IVP subjected to immunohistochemistry excluding the mix of primary antibodies (I) at 24 hpi. Brush borders on the apical membrane (b), entire epithelial cell (e) and connective tissues (c) underlying the epithelium are indicated in all the figures from A to I.

Table 1
Time-course ranking of VP28/immunoreactivity during processing of WSSV-antigens in the median midgut.

Processing steps	Antigen	Hours post-intubation (hpi)				
		2	4	8	24	48
Adherence to microvilli	VP28	++	++	++	++	++
	IVP	++	++	++	+	+
	WSSV	++	+++	+++	+++	+
Supranuclear vacuoles	VP28	+	+++	+++	++	++
	IVP	+	++	+	+	+
	WSSV	+	+	+	+	−
Inter-cellular	VP28	++	++	++	++	++
	IVP	++	+++	+++	++	++
	WSSV	++	+++	+++	+++	−
Connective tissue	VP28	+++	+++	+++	+++	++
	IVP	+++	+++	+++	++	++
	WSSV	+	++	++	+	+
External (hameolymph)	VP28	+	+++	++	++	++
	IVP	++	++	+++	+++	+++
	WSSV	+	+	+	+	+
Haemocyte activation (aggregation and degranulation)	VP28	+++	++++	++++	++++	++++
	IVP	++	+++	++++	++++	++++
	WSSV	++	+++	+++	+++	+++

The ranks are the average impression of 6 shrimps/group examined: '−' indicates no immunoreaction for anti-VP28 (polyclonal antibody) whereas '+' means slight reaction and '++++' means very strong reaction. In case of haemocyte activity the ranking '+' means slight WSH8-immunoreactivity and '++++' indicates very strong WSH8-immunoreactivity. Immunoreactivity at 72 hpi is not shown as it was not distinctly different from that at 48 hpi.

ranking (++ to +++) until 24 hpi. However, from 48 hpi the difference between live WSSV and both vaccine candidates became evident, being completely absent at 48 hpi in the case of live WSSV. Both IVP and VP28 accumulated in connective tissues at a much higher rate (mostly +++) compared to WSSV (mostly +).

Haemocyte reaction was analysed based on the number of activated haemocytes (aggregating and degranulating) in the intubated shrimps. Aggregation of haemocytes was relatively higher in the shrimps intubated with WSSV-antigens than in controls. In addition, highest activation of haemocytes was observed in the shrimps intubated with VP28 than in shrimps intubated with IVP and WSSV.

4. Discussion

4.1. WSSV-vaccines and immunohistochemistry

Oral vaccination is considered as the most practical way to immunize cultured shrimps [9]. Hence it is pivotal to understand the uptake mechanism of these oral vaccines to develop efficient delivery methods. This study demonstrates for the first time uptake and processing mechanisms of two WSSV vaccine candidates [rec-VP28 and formalin-inactivated WSSV (IVP)] and compares the mechanisms with that of live WSSV in the midgut of black tiger shrimp, *P. monodon*. Previously, live WSSV uptake was studied in the midgut of this shrimp, following bath challenge [3]. The double staining method adopted from the aforementioned study enabled us to stain rec-VP28 and haemocytes, but required antigen retrieval in case of rec-VP28 and IVP intubated shrimp and not for WSSV intubated shrimp. As the VP 28 gene sequence inserted in pET28a vector (Novagen) contained only the hydrophilic part, the protein obtained was in the soluble form. In addition, the protein might have undergone cross-linking in the presence of formalin during tissue fixation [9], necessitating antigen retrieval [20,21]. Preparation of IVP that involved addition of 0.2% formalin (v/v) might have resulted in cross-linking of the VP28 epitopes on the virus. Hence its detection by anti-VP28 antibody also demanded the antigen retrieval [8]. Nevertheless, live virus results are similar with or without antigen retrieval. This suggests that the VP28 antigenic determinant of live virus is not or hardly sensitive to the histological treatment, in contrast to those of inactivated virus and/or rec-VP28. Despite the antigen retrieval needed for rec-VP28 and IVP, the controls carried out excluded non-specific binding of the antibodies.

The envelope protein VP28 was chosen as a vaccine candidate in this study because of its credibility to provide better survival upon WSSV infection [7,9]. Moreover, VP28 is more stable in the digestive environment than VP19 or better escapes from digestion. Apart from this subunit vaccine, a conventional whole cell vaccine, IVP, was included in this study since it had provided total survival in *Fenneropenaeus indicus* after 10 days of oral vaccination against WSSV [8].

4.2. WSSV-antigen transit throughout gut

In crustaceans, the transit time of food through gut varies widely [22]. Although most of the decapod crustaceans [22,23] including non-penaeid shrimps [24] digest food within 6–12 h, it may take up to 24 h to completely evacuate the gut [22–24]. In this study, even at 72 h, VP28-immunoreactivity was observed in the lumen of distal midgut of intubated shrimps. Since the food retention time and gut motility decides the digestive efficiency and assimilation in shrimps [25], the presence of the intubated WSSV-antigens for a long period in the gut indicates that the animal gets adequate time to process the vaccine molecules.

The sequence of transport and processing of the intubated WSSV-antigens were similar in all gut segments of shrimps, during the 72 h observation period (Fig. 1). Such a sequential processing of WSSV-antigens in the gut lumen may be due to the synergistic effect of peristalsis and anti-peristalsis to promote thorough digestion in non-continuously feeding shrimps [25]. Further, feed pellets were only detected at 72 hpi, suggesting that the animals did not consume feed until two days after intubation. This may explain the rather longer transit time of WSSV-antigens.

4.3. Uptake and processing of WSSV-antigens

Enterocytes of most species take up and process luminal antigens by fluid-phase endocytosis [26,27]. Strong VP28-immunoreactivity at the microvilli borders of the gut epithelium in the first 24 hpi suggests the adherence of VP28 or VP28 derived peptides at the epithelial surface. This leads to the initiation of receptor-mediated endocytosis. It also implies that receptors present on the apical surfaces of shrimp gut epithelium efficiently recognize the rec-VP28 from the luminal WSSV-antigens. Vacuole-like supra-nuclear structures, SNVs observed in the shrimps intubated with WSSV-antigens can be considered as part of the endo-lysosomal compartment, formed by the fusion of endocytotic vesicles and lysosomes. Similar SNVs were observed in polarized epithelial cells of fish [26] and other vertebrates [27,28] that are responsible for intracellular digestion.

Antigens/macromolecules that are taken up by receptor-mediated endocytosis can also escape the degradation in endo-lysosomes and get transported to inter-cellular space [26,27]. This mechanism of transcytosis leads the antigens to enter the circulation where they encounter immune cells and components for further recognition and/or degradation as observed in common carp [29]. In this study we observed that all the WSSV-antigens are transported to the inter-cellular space, and suggest that most of these antigens successfully escape the endo-lysosomal compartment.

It is noteworthy that both rec-VP28 and IVP do not show much variation in uptake and processing within the epithelium of intubated shrimps. Although higher amount of rec-VP28 are reaching the SNVs when compared to the membrane bound VP28 (IVP), the majority of rec-VP28 appears to escape endo-lysosomal uptake in the shrimp enterocytes. However, the difference in the amount of VP28 molecules in the two preparations might have resulted in the slight variation in processing of these antigens. In mammals it has been shown that different amounts of macromolecules can lead to different uptake mechanisms [30].

live WSSV appeared to follow the same uptake and processing route as described for IVP. However, in an earlier immersion study with live WSSV showed strong accumulation of virus in SNVs of the midgut epithelium [3]. The difference in concentration of viral particles entering the gut through drinking (physiological entry) in immersion study versus the amount delivered upon oral intubation in the present study might have contributed to the discrepancy noted in the uptake pathways. Additionally, the live virus in earlier study had to undergo the digestion pressure in the foregut before reaching the midgut and hence the dysfunctional proteins might have accumulated in SNVs.

4.4. Haemocyte reaction

WSSV-antigens that accumulate in the connective tissue attract haemocytes from circulation and subsequently degranulate rapidly [31]. This degranulation of haemocytes was detected with the WSH8 antibody that stains the content of granules [18]. In several crustacean arthropods degranulated haemocytes take part in immune mechanisms by activating prophenoloxidase (proPO) system [32–34]. The limited activation of haemocytes seen in the gut connective tissues of control (PBS and healthy shrimp extract intubated) shrimps could be a response to the entry of ingested food.

Some cells with immunoreactivity for both VP28 and WSH8 were observed in connective tissues of the gut of the shrimps intubated with either VP28 or IVP, which we presume to be phagocytic cells such as hyalinocytes [33,35] or semigranular haemocytes [36,37].

It is also noteworthy that WSSV-antigens exiting the gut pass through the connective tissue, probably finding its way to the haemolymph that surrounds the external part of the gut, and subsequently may be transported to the lymphoid organ for their clearance [31,38].

5. Conclusion

This study indicates that not only rec-VP28 and IVP, but also live WSSV follow an identical uptake mechanism: receptor-mediated endocytosis. Therefore it may be acceptable to conclude that all the WSSV-antigens in this study are using VP28 to enter the polarized epithelial cells of the midgut. The subsequent processing of WSSV-antigens in enterocytes occurs partly through endo-lysosomal degradation, ultimately leading to the formation of SNVs. Majority of the antigens that escape endo-lysosomal degradation are transported to the inter-cellular space and enter the circulation through transcytosis. Minor differences were observed in the amount of SNVs present in rec-VP28 intubated shrimp. This could be due to the difference in concentration of soluble versus membrane bound VP28 in the two WSSV-preparations. Whether other viral proteins present in live WSSV or IVP play a role can presently not be excluded. Uptake of antigens attracts and activates haemocytes, thereby initiating local immune responses, a phenomenon comparable to what has been described in fish, rats and humans. Finally the present data suggest that the receptors on the shrimp enterocytes efficiently recognize VP28, leading to an uptake and processing similar to IVP or live WSSV. Therefore rec-VP28 seems to be an ideal oral vaccine candidate, when it can be protected against digestion in the foregut.

Acknowledgement

This research is part of the project “Indo-Norwegian platform on fish and shellfish vaccine development (VaccInAqua)” funded by the Research Council of Norway. We are grateful to Dr. Roy Dalmo, Senior Scientist, University of Tromsø (Norway) the project leader for his support. The authors wish to thank Professor K.M. Shankar, College of Fisheries, Mangalore, India for providing monoclonal antibodies against VP28 and MSD Animal Health (Boxmeer, The Netherlands) for their permission to use WSH8.

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