Aquaculture 291 (2009) 230–236

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Short communication

Detection and persistence of Lymphocystis disease virus (LCDV) in *Artemia* sp

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ARTICLE INFO

Article history:
Received 30 July 2008
Received in revised form 6 February 2009
Accepted 12 March 2009

Keywords:
Artemia
Lymphocystis disease virus
Viral transmission
PCR-hybridisation
In situ hybridisation
Immunofluorescence assay
Immunohistochemistry

ABSTRACT

Lymphocystis disease virus (LCDV) was detected by PCR-hybridisation in *Artemia* cysts and metanauplii, and the infectivity of these viral particles was confirmed by inoculation on SAF-1 cells. Viral genome and antigens have been detected by whole-mount *in situ* hybridisation (ISH) and immunofluorescence assay (IFA) in the digestive tract of nauplii hatched from LCDV-contaminated cysts, but not at the umbrella and instar I stages. Instar II nauplii also became LCDV-contaminated after bath challenge. LCDV was detected by ISH and immunohistochemistry (IHC) in the digestive tract and in some cells in the ovisac of adults reared from LCDV-positive instar II nauplii. Reproductive cysts arising from LCDV-contaminated *Artemia* breeders, as well as their offspring nauplii, were also LCDV-positive. No viral genome and antigens were detected in the eggs, which may indicate an external cyst contamination during spawning. Moreover, viral DNA on cysts disappears by the decapsulation treatment, which may be applied to prevent the transmission of LCDV in *Artemia*. Infective LCDV particles persist along crustacean life cycle, as demonstrated by cell culture. These findings suggest that *Artemia* might act as a reservoir of LCDV, although further studies are necessary to establish its role as a vector of this virus to cultured fish.

1. Introduction

The brine shrimp *Artemia* sp. (Crustacea, Branchiopoda, Anostraca) is of great importance in aquaculture, being essential in the dietary regimen of larval stages of fish and crustacean species (Lavens and Sorgeloos, 1996; Naessens et al., 1997). *Artemia* nauplii have been considered as possible vectors for the introduction of different microbial pathogens into fish and shrimp rearing systems, including infectious pancreatic necrosis virus (IPNV) and nodavirus (Austin and Allen, 1982; Mortensen et al., 1993; Skliris and Richards, 1998), shrimp viruses (Sahul Hameed et al., 2002; Sudhakaran et al., 2006), several bacterial pathogens (Muroga et al., 1987; Nicolas et al., 1989), and protozoa (Mendez-Hermida et al., 2006). In most studies, a mechanical carrier state has been proposed (Overstreet et al., 1988; Mortensen et al., 1993; Chang et al., 2002).

Lymphocystis disease virus (LCDV), a member of the *Iridoviridae* family, is the causal agent of a common chronic and usually benign infection, named lymphocystis disease (LCD), which is characterised by the hypertrophy of fibroblasts in the fish connective tissue, proliferating sometimes as true epithelial tumours (Samalecos, 1986). Fish showing these symptoms cannot be commercialised, causing important economic losses (Masoero et al., 1986). Several factors may provoke the appearance of the disease symptoms, such as water contamination (Overstreet, 1988), and stress caused by nutritional deficiencies, oxygen depletion, or human manipulation and overcrowding (Paperna et al., 1982; Mellergaard and Nielsen, 1995; Sindermann, 1996). Direct contact among fish specimens is the main via for LCDV transmission (Wolf, 1988), although the oral route has also been recently proposed (Cano et al., 2005, 2009; Sheng et al., 2007).

Lymphocystis disease (LCD) affects over 125 different fish species, belonging to 42 families (Anders, 1989). It is the main viral infection reported to affect gilthead seabream (*Sparus aurata*, L.) (Borrego et al., 2001), which is one of the most important species in the Mediterranean aquaculture. Although this infection is rarely associated with massive mortalities of fish, they can occur under determined culture conditions, possibly due to the gill impairment and the presence of intrusive lesions that may cause alteration in the swimming and feeding behaviour.

During the course of virological analyses carried out in a gilthead seabream farm affected by LCD, LCDV was detected by PCR-hybridisation in the *Artemia* metanauplii used in the hatchery. The objective of the present study was to elucidate the presence of LCDV in *Artemia* from several seabream farms (both affected and free of LCD), and, in addition, to establish the source of LCDV contamination in the brine shrimp.

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doi:10.1016/j.aquaculture.2009.03.018

2. Materials and methods

2.1. Virological analysis

Artemia cysts (50 mg) or animals (nauplii to adults) (100 mg) were homogenised in 1 ml of Leibovitz medium (L-15) (Gibco) supplemented with 10% penicillin–streptomycin (Gibco). Homogenates were centrifuged at 5000 ×*g* for 10 min at 4 °C, filtered (0.45 μm pore-size filter), and used for the inoculation of SAF-1 cells. Cell cultures were maintained at 20 °C until the development of cytopathic effects (CPE) (up to 14 days post-inoculation) (Garcia-Rosado et al., 1999).

2.2. Polymerase chain reaction and dot-blot hybridisation

DNA was extracted from cysts (50 mg) and nauplii (or other developmental stages of brine shrimp) (100 mg) using DNazol (Invitrogen), according to the manufacturer's instructions.

A specific PCR combined with dot-blot hybridisation was used to detect LCDV genome as described by Cano et al. (2007). Briefly, a 270-bp fragment of the LCDV major capsid protein (MCP) gene was amplified by PCR. PCR products (10 μl) were denatured and blotted onto a Hybond-N nylon membrane (GE Healthcare). Hybridisation assay was carried out using an LCDV-specific digoxigenin (DIG)-labelled probe obtained by PCR (Cano et al., 2007), and the signal was detected by chemiluminescence using CSPD solution (Roche Applied Science).

2.3. Whole-mount in situ hybridisation (ISH) and immunofluorescence assay (IFA)

Animals were fixed with neutral buffered formalin (NBF, Merck) at 4 °C overnight, washed twice with phosphate-buffered saline pH 7.2 (PBS) supplemented with 0.1% (v/v) Tween-80 (PBT) for 5 min, and maintained in 100% methanol at −20 °C until use.

For ISH, brine shrimp nauplii were rehydrated using decreasing concentrations of methanol, and finally washed in PBS. Permeabilisation was carried out by sonication (Manzanares et al., 1993), followed by a treatment with proteinase K (20 μg μl^{-1}) in Tris buffer saline (TBS) (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl) for 5 min at 37 °C. The DIG-labelled probe above mentioned was denatured at 95 °C for 5 min prior to hybridisation overnight at 42 °C. After hybridisation, animals were washed in saline sodium citrate buffer (SSC) with 6 M urea and 0.2% (w/v) bovine serum albumin for 10 min at 42 °C, blocked with 6% (w/v) skimmed milk in TBS, and incubated with an anti-DIG monoclonal antibody conjugated to alkaline phosphatase (Roche Applied Science) for 1 h. The endogenous phosphatase activity was removed by a treatment with 1 mM levamisole, and the hybridisation signal was detected using NBT/BCIP (Roche Applied Science). Finally, animals were post-fixed in NBF for 2 h at room temperature and, after two washes with PBT, kept at 4 °C in PBS supplemented with 0.1% (w/v) sodium azide. Samples were placed on slides and mounted with a coverslip by using 80% (v/v) glycerol in PBS.

For IFA, fixed, rehydrated and washed brine shrimp nauplii were permeabilised with 0.1% (w/v) Triton X-100 (Sigma) in PBS for 5 min at 4 °C, washed twice with 30% (w/v) skimmed milk in PBS during 30 min, and incubated for 2 h with an anti-LCDV serum (Garcia-Rosado et al., 2002). Anti-rabbit IgG conjugated with FITC (Sigma) was used as secondary antibody. Samples were mounted as described above.

2.4. LCDV detection in histological sections

Adult *Artemia* specimens were fixed as described, dehydrated, and embedded into paraffin following standard histological protocols. Sections (5–7 μm) were mounted on silane-treated slides (Sigma).

ISH assays were carried out as described by Cano et al. (2009). Briefly, deparaffinised and rehydrated sections were permeabilised with 0.3% (w/v) Triton X-100 (Sigma) in TBS for 30 min, and proteinase K (100 μg ml^{-1} in TBS) for 30 min at 37 °C. The hybridisation protocol was that described in Section 2.3. Tissue sections were dehydrated and mounted with a coverslip by using a permanent mounting medium (Entellan, Merck).

For immunohistochemistry (IHC), deparaffinised and rehydrated sections were incubated in 3% H_2O_2 (Merck) for 10 min for endogenous peroxidase blocking. Tissue sections were permeabilised with Triton X-100 (0.3% in TBS), washed with blocking solution, and incubated with the first antibody as described for IFA. HRP-conjugated anti-rabbit IgG (Sigma) was used as secondary antibody. Peroxidase activity was developed with a solution of diaminobenzidine (Sigma) and H_2O_2. Finally, tissue sections were mounted with a coverslip using Aquatex (Merck).

For histological studies, animal sections were stained with haematoxylin–eosin (HE).

2.5. LCDV transmission experiments

For horizontal transmission studies, three groups of animals were considered: a negative control group (LCDV-free cysts), a positive control group (naturally LCDV-contaminated cysts), and a challenged group (LCDV-free cysts to be used for challenging). Naturally LCDV-contaminated and LCDV-free cysts were tested by dot-blot hybridisation of the PCR products as described in Section 2.2.

Cysts from each group (50–100 mg l^{-1}) were hatched in sterile seawater (30 ppt salinity) at 20 °C (Lavens and Sorgeloos, 1996). After 35 h incubation, hatched instar II nauplii were separated from the unhatched and empty cysts and transferred to aquaria with fresh sterile seawater. At this stage, the nauplii from the challenged group were introduced in 500 ml of seawater containing a gilthead seabream LCDV isolate at 2×10^2 $TCID_{50}$ ml^{-1}. After 24 h, the animals were filtered through a synthetic net, washed and transferred to a tank with fresh sterile seawater. The three groups of animals were then reared to the adult stage at 20 °C, with continuous aeration and a 24 h photoperiod. Nauplii were fed on commercial baker's yeast. From the 10th day post hatching on, the alga *Dunaliella salina* (10^6 cells ml^{-1}) was also administered weekly. The algal cultures used were LCDV-negative (tested by PCR-hybridisation).

The challenged group was sampled at 1, 2, 3, 6, 10 and 30 days post-infection (p.i.) to be analysed by PCR-hybridisation. Infectivity studies based on cell culture inoculation were carried out at 1 and 10 days p.i.

In parallel, brine shrimps from the negative and positive control groups were sampled at the umbrella and instar I stages for whole-mount ISH. The three groups of animals were sampled at 3 and 10 days after hatching, and analysed by whole-mount ISH and IFA as specified in Section 2.3.

For vertical transmission studies, adult brine shrimps from the same three groups described above were used. Seawater salinity was increased up to 150‰ to achieve the optimal conditions for oviparous reproduction (Lavens and Sorgeloos, 1996). After spawning, cysts and their respective offspring nauplii were collected for LCDV detection using PCR-hybridisation and whole-mount ISH analyses. The presence of infective viral particles was tested by inoculation on SAF-1 cells. In addition, reproductive adults were selected for histological, ISH and IHC assays.

2.6. Decapsulation of Artemia cysts

Hydrated *Artemia* cysts from the positive control group were decapsulated using a mixture of sodium hypochlorite and sodium hydroxide, following the procedure specified by the FAO (Moretti et al., 1999). The presence of LCDV on cysts after their decapsulation was studied by PCR-hybridisation. After hatching, samples from the