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Evaluation of Flinders Technology Associates Cards as a Non-lethal Sampling Device for Molecular Diagnosis of Betanodavirus in Asian Seabass, *Lates calcarifer* (Bloch, 1790)

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Abstract

This study aimed to evaluate the utility and efficiency of the Flinders Technology Associates (FTA®) Elute Card (Whatman®) as a sampling device and storage platform for RNA from Betanodavirus infected biological sample (*viz*., cell culture supernatant). The retrieval of target RNA from FTA Elute Card discs with a diameter ranging from 1.2 - 2 mm was found to be satisfactory for detection of Betanodavirus by reverse transcription-nested polymerase chain reaction (RT-nPCR). The viral RNA on the cards could be detected by RT-nPCR for a minimum period of 30 days of storage at 4°C, though at lower efficiency after 21 days of storage. In conclusion, FTA cards protocol provides a supplementary method for quick and easy collection of samples, preservation of RNA on a dry storage basis and detection of Betanodavirus infected fish.

Keywords: Viral nervous necrosis; Viral encephalopathy and retinopathy; Betanodavirus; Diagnosis; RT-nPCR; FTA elute card

Introduction

Viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) affects more than 120 fish species is one of the major disease constraints in the finfish aquaculture, mostly marine [1-5]. The disease is caused by Betanodavirus (family: *Nodaviridae*) which is small, icosahedral (25 nm) RNA virus. The virus causes acute as well as latent infections [2] and exhibits different clinical signs depending on age and fish species. Latent infection may develop an acute phase with biological and environmental stress factors [6] or facilitate vertical/horizontal transmission of the virus [1,7].

Recently, the Betanodavirus has been reported as the main cause of significant mortality in many species of hatchery/farm grown marine fish species [8-13]. The most common method of detection is by PCR, based on the gene sequences of RNA1 and RNA2 and is now accepted as the gold standard for its confirmatory diagnosis [14,4]. Sampling of the most suitable material is required to facilitate the confirmatory diagnosis of Betanodavirus in marine fish and to develop a suitable management strategy for finfish breeding programme. Non-lethal sampling and transport of suitable biological sample from broodstock fish in hatchery condition is a challenging task, especially when sample sizes are small and when operating away from diagnostic laboratories. FTA Elute Card (Whatman®) is a paper-based matrix designed to fix and store nucleic acids directly from fresh tissues, hence has the potential for sampling, retrieval and subsequent downstream applications in molecular research. This study investigates the potential of using FTA Elute Cards as a sampling device for molecular detection of Betanodavirus from biological samples.

Materials and Methods

Test samples

Betanodavirus infected Asian seabass (*Lates calcarifer*) larvae (9 days post-hatch) collected and preserved at -80°C from an acute disease outbreak of viral nervous necrosis were used as a source of virus sample for cell culture propagation. A homogenate of larval samples in Hank's balanced salt solution (HBSS) at 1:10 dilution was prepared, filtered using 0.22 µm syringe filter and inoculated in a monolayer of SISS cell line grown in L-15 medium containing 10% foetal bovine serum [13] for viral propagation. Following the observation of cytopathic effect (CPE) in the cell culture, the supernatant fluids containing virus were harvested after freeze-thawing for three times and clarified by centrifugation at 3000 g for 15 min at 4°C and stored at -80°C until use. Virus titration was conducted on a monolayer of cells in 96 well plates using 10-fold serial dilutions in triplicate. The 50% of the tissue culture infective dose ($TCID_{50}$ ml^{-1}) was calculated as described by [15]. The titer of the culture supernatant was 10^3 $TCID_{50}$ ml^{-1} at 4 days post-infection (dpi) and 10^8 $TCID_{50}$ ml^{-1} at 7 dpi. The culture supernatant on 7 dpi was used for application on FTA Elute Card and to maintain homogeneity of the sample on the card.

Control samples (positive and negative)

Betanodavirus infected Asian seabass (*Lates calcarifer*) larvae after 9 days post hatch (dph) collected and preserved at -80°C from an acute disease outbreak of viral nervous necrosis were used as a source of positive control in this study. A VNN-free stock of Asian seabass larvae (28 dph) collected earlier from a hatchery and archived at -80°C was used as virus-free negative control. These samples were used for conventional RNA extraction using TRIzol Reagent (Invitrogen) as per the manufacturer's instructions for comparison.

Sample application and extraction of RNA from FTA elute card

A fresh homogenous suspension of each sample (30 µl) was spotted on FTA® Elute Card (Whatman®) starting in the center, in an outward spiral motion as per manufacturer's recommendations (Figure1a), labeled for identification and dried for 2 hours at room temperature. These cards were stored at 4°C in a multi-barrier pouch until used for weekly tests. Two discs of different diameters (0.5 mm, 1.2 mm and 2 mm) were cut out on day 1, 7, 14, 21 and 30 after application of samples using a coring device, Harris uni-core punch (Figure 1b) or an office paper punch (for 5 mm diameter discs), and one disc was placed directly in a PCR amplification tube and another one into a 1.5 ml tube for RNA extraction as recommended by the manufacturer. Essentially, two different protocols were used for the preparation of RNA template from FTA card. In the first protocol, RNA extraction was carried out using an elution buffer ('disc elution') as per the Whatman protocol for RNA isolation. The extracted RNA was quantified, and its purity was checked using a Nano Photometer (Implen, Germany). In the second protocol, a single disc of different diameter was treated with FTA purification reagent (Whatman) as per the manufacturer's protocol prior to use as 'RNA template' for cDNA synthesis ('disc-in-PCR') in reverse transcription polymerase chain reaction (RT-PCR) as described earlier [16].

Figure1: FTA Elute Card for sample application (a) and the Harris Micro Punch (b) for cutting disc dimension of 0.5, 1.2 and 2 mm diameter (note the encircled area).

cDNA synthesis

The RNA samples prepared from FTA Elute Cards by RNA processing buffer and the dried discs were used separately for each type of samples for cDNA synthesis and subsequent PCR protocol using the same cycling conditions. First-strand synthesis was done using an iScript cDNA synthesis kit (Bio-Rad, USA) in 10 µl reaction as per manufacturer's instructions. The RNA from control (positive and negative) sample was also processed for cDNA synthesis. The cDNA was quantified using a NanoPhotometer (Implen, Germany) at 260 nm.

Polymerase chain reaction

The nested PCR method used in this study has been previously described [13]. For first step PCR, the primers BARL-F1 (5'-GTACGCAAAGGTGAGAAGAAA-3') and BARL-R1 (5'-GTCCCAGATGCCCCA-3') were used, while the nested PCR primers include BARL-F2 (5'-AACTGACAACGACCACACCTT-3') and BARL-R2 (5'-TGTGGAAAGGGAATCGTTG-3'). For first-step PCR, 2 µl of the cDNA product was used as the template, whereas in the nested PCR, 2 µl of the primary PCR product was used, with same thermal cycling conditions.

Duration of storage (days) at 4°C	PCR	PCR results using a single disc of different diameter (by direct 'disc in PCR' and 'disc elute PCR')			
		0.5mm	1.2 mm	2.0 mm	5.0 mm
1	First step	-	+	+	+
	Nested	-	+	+	+
7	First step	NA	NA	-	+
	Nested	NA	NA	-	+

14	First step	NA	+	+	+
	Nested	NA	+	+	+
21	First step	NA	+	+	+
	Nested	NA	+	+	+
30	First step	NA	+	+	-
	Nested	NA	+	+	+

Table 1: PCR analysis on FTA elute card discs after application of cell culture suspension of Betanodavirus sample (note: positive (+), negative (-), not tested (NA).

PCR was carried out in 25 µl reaction mixture containing 12.5 µl ready-to-use PCR master mix (Ampliqon Taq 2x Master Mix Red), 9.5 µl nuclease-free water and, 10 picomol of forward and reverse primers (1 µl each) by following cycling conditions: 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR amplicons were analysed by electrophoresis in 1.5% agarose gel containing 0.5 µg ml^{-1} XR^{+} Imaging system (Bio-Rad, USA).

Results

In the present study, we evaluated the FTA Elute Card (Whatman®) as a sampling device and retrieval of Betanodavirus nucleic acid for diagnostic applications in fish. The recovery of RNA concentration from punched discs measuring 1.2, 2.0 and 5.0 mm diameter was found to be relatively satisfactory in terms of quality for diagnostic PCR. However, the stability and concentration of total RNA on the FTA disc were found to decrease during storage at 4°C throughout three weeks.

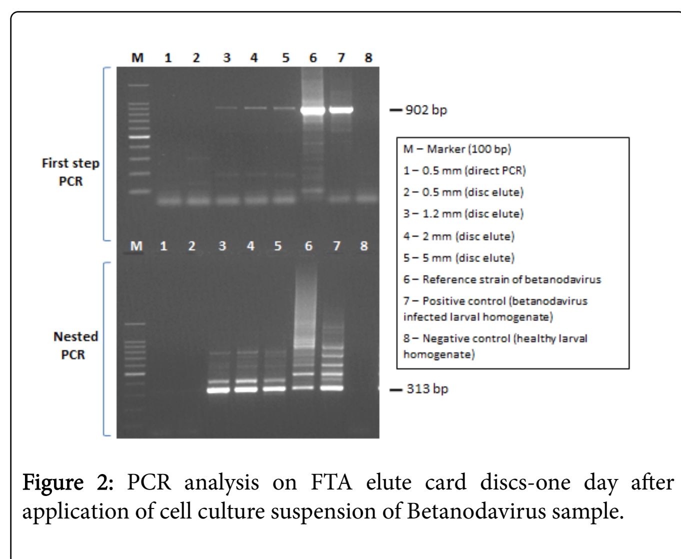

Figure 2: PCR analysis on FTA elute card discs-one day after application of cell culture suspension of Betanodavirus sample.

The RNA concentration from 0.5 mm diameter disc was poor from day 1 onwards and hence eliminated from further testing by PCR. The mean concentration of RNA extracted (day 1 post application of sample) from a single disc of 1.2, 2 and 5 mm was 861, 1011 and 2563 ng µl^{-1}, respectively. A positive control (reference strain of Betanodavirus in cell culture suspension or VNN infected Asian seabass larval homogenate) and negative control (healthy seabass larval homogenate) were also used for conventional RNA extraction followed by RT-nPCR analysis. The conventional RNA extraction protocol of larval homogenate of positive and negative control revealed mean RNA concentration of 1089 and 825 ng µl^{-1}, respectively. Of the two RNA template preparation protocols from FTA Elute Card discs, both the 'disc elution' and 'disc-in-PCR' techniques employed in this study gave almost similar results by RT-PCR with amplification of predicted sizes (902 bp and 313 bp by first step and nested PCR, respectively) from all sample discs at weekly intervals (Table 1).

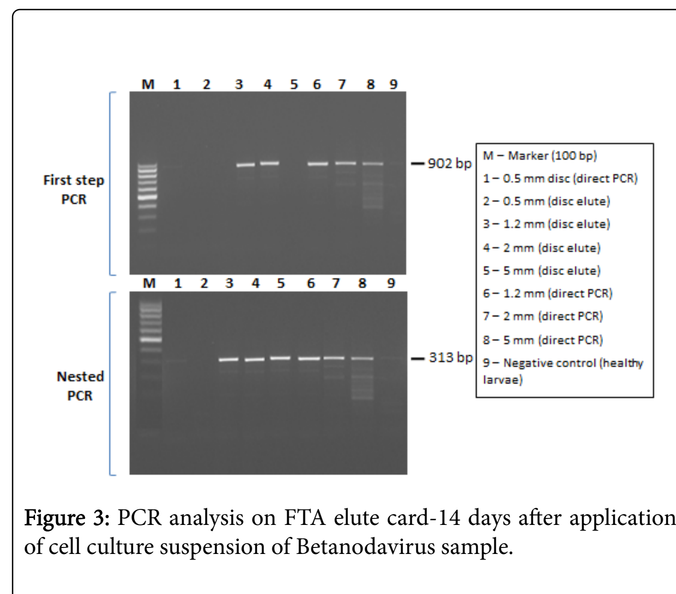

Figure 3: PCR analysis on FTA elute card-14 days after application of cell culture suspension of Betanodavirus sample.

No amplification was observed from the healthy larval samples used as a negative control at each test point. The PCR analysis using FTA Elute Card on day 1, 14 and 30 days after application of cell culture suspension of Betanodavirus sample is shown in Figure 2-4. On test day 1, most of the Betanodavirus-positive samples showed multiple bands in nested reaction possibly due to carryover PCR products from the first step reaction due to excess template of RNA, which was resolved by dilution (1: 5) of primary PCR product in subsequent nested reactions.

The disc diameter of 1.2 and 2 mm gave better results consistently at different test points, while 5 mm discs often manifested inhibition, probably due to excess template concentration. PCR products of predicted sizes were obtained from frozen samples of all virus-positive samples processed by traditional method and gave more prominent band by visual assessment (Figures 2 and 4).Thus effective retrieval of

viral nucleic acid from the cell culture supernatants applied on FTA Elute Card has been demonstrated in this study for diagnostic assays up to 30 days. This protocol eliminates the traditional multi-step extraction and purification of RNA using hazardous chemicals.

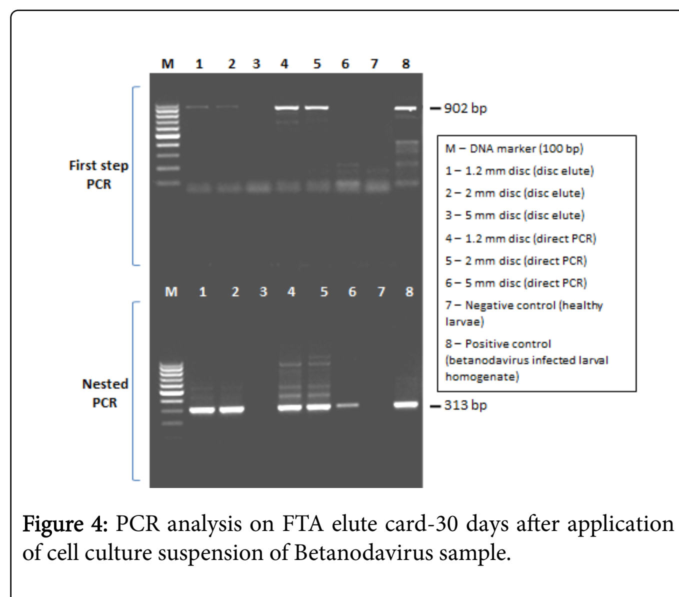

Figure 4: PCR analysis on FTA elute card-30 days after application of cell culture suspension of Betanodavirus sample.

Discussion

The Whatman FTA Elute Card is a paper-based matrix impregnated with proprietary reagents that lyse cells, degrade proteins and stabilize nucleic acids directly from fresh tissues applied on to the treated paper and hence has the potential for sampling and retrieval of nucleic acid from biological samples. Infectious pathogens in samples are rendered inactive on contact and can be shipped safely, making them useful for dry transportation of clinical samples and subsequent recovery of nucleic acid for molecular detection. This technology has been in use for diagnostic and epidemiological studies on pathogens of human, animals and plants [17-22]. The use of FTA cards for diagnostic and epidemiological investigation has been very limited except a few reports on molecular diagnostics of aquatic animal diseases such as white spot syndrome virus in shrimp [23] and viral nervous necrosis in finfish [16]. However, for FTA technology to be effectively used as a routine tool for PCR-based diagnostics, it must allow the stability and retention of viral nucleic acids originated from biological samples (target tissues, reproductive fluids, cell culture supernatants etc.) in a manner equivalent to that offered by traditional isolation methods. In this study, Betanodavirus detection on stored FTA elute card at 4°C was possible up to 30 days, although a decrease in sensitivity was observed after 21 days of storage. Similar results were also observed in the case of Betanodavirus infected seabass using different biological samples [16]. This could be due to degradation of RNA quality and yield at ambient temperature as reported earlier [16,17].

Further, the stability of RNA on the FTA card depends on the storage temperature, duration and the type of biological samples to some extent (unpublished data). Hence, storage of FTA impregnated samples at a lower temperature, and the use of a more number of punched discs per reaction might allow detection of the pathogen beyond this period and enhance sensitivity. A single disc with a diameter of 1.2 to 2 mm was found to be satisfactory for adequate RNA for reverse transcription reaction in 'disc-in-PCR' format. The discs with 0.5 and 5 mm diameter did not yield desired results due to poor recovery of nucleic acid in the former and possibly the non-homogenous distribution of nucleic acids and excessive leaching of nucleic acid from the latter. In spite of these disadvantages, easy handling and transport of a large number of samples at ambient temperature in non-infectious form are clear advantages for field application, especially when samples cannot be transferred to the lab immediately. The specificity of the RT-PCR detection from the FTA Elute Cards proved to be the same as that obtained by RT-PCR from frozen tissues. However, the sensitivity was found to be less optimal beyond 30 days of storage at 4°C but could be improved by storage at -20°C or even at -80°C.

Finfish breeding programmer involves routine examination of spawners by collecting samples from the ovary of female fish and sperms from male fish using cannulation. We see the feasibility of using FTA cards for a non-lethal sampling of reproductive fluids containing ova/milt or blood from broodstock for ascertaining the carrier status of Betanodavirus infection before spawning. Samples drawn from the reproductive tract of broodstock fish during routine maturity check-up may be directly blotted on FTA Elute card for selection of nodavirus-free broodstock and prevent vertical transmission of Betanodavirus to the progeny. In conclusion, the use of FTA Elute Card as a storage and transport device for handling a large number of samples from fish hatcheries to disease diagnostic laboratory needs to be explored for managing Tran's boundary diseases in aquaculture.

Competing Interests

The authors declare that they have no competing interests.

Statement on Ethical Approval

The authors certify that we have followed and complied the animal ethics and protocol as per the institutional, national, or international guidelines for using *Lates calcarifer* in the study.

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