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Development of an immunochromatography assay kit for rapid detection of ranavirus

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A B S T R A C T

Ranaviruses are large, double-stranded DNA viruses of the family *Iridoviridae* and are known to be primary pathogens in frogs, fish and other amphibians. These viruses have been shown to be highly adaptable and have the ability to cross species barriers, making them a potent threat to global biodiversity. There is therefore, a need for rapid and efficient diagnostic methods to control the spread of these viruses. To address this, monoclonal antibodies (MAbs) were developed against ranavirus strain FV-3 (standard frog virus 3) to detect the major capsid protein and FV-3gorf19R related hypothetical protein in both the FV-3 and KRV-1 (Korean ranavirus) strains. The antibodies were then applied on a colloidal gold-immunochromatographic assay (GICA) as a kit for the detection of ranaviruses. The kit was able to detect low concentrations of the virus (10^1 $TCID_{50}$/ml) and showed analytical specificity when tested against other viral pathogens, including those belonging to the same family. It was possible to detect ranavirus in experimentally infected frogs within 30 min using the kit. The kit described here is expected to be a valuable and informative tool for on-site detection of ranavirus in frog.

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1. Introduction

Iridoviridae is a family of large, icosahedral, double-stranded DNA viruses consisting of 5 genera: *Iridovirus*, *Ranavirus*, *Lymphocystivirus*, *Chloriridovirus* and *Megalocytivirus* (Fauquet et al., 2005). *Ranaviruses* in particular, have been deemed responsible for amphibian die offs in both captive and wild amphibian populations worldwide and are capable of infecting 72 amphibian species in 14 different families; they have also been known to infect fish and reptiles as a primary pathogen (Lesbarrères et al., 2012, Miller et al., 2011).

Ranaviruses can be transmitted between animals through direct or indirect routes of infection, but may vary in virulence depending on the species of virus, and the developmental stage, species and geographic origin of the host (Chinchar, 2002, Gray et al., 2009). Pathological signs of ranavirus infection include erratic swimming, lethargy, swollen limbs and systemic organ failure involving internal organs such as the liver, spleen, kidney and gut, and these typically lead to mortality, although ranaviruses have been isolated from animals with subclinical infections (Chinchar, 2002, Miller et al., 2009, Tapiovaara et al., 1998). Individuals with subclinical infections can then act as a reservoir for the virus, leading to persistent infection, making the virus more difficult to eradicate (Gray et al., 2009). The increased incidence of ranavirus infection may indicate changes in the environment or the intragenic distribution of the virus rather than changes in the adaptability of the virus. This makes them a potential threat to both naïve and highly susceptible species of amphibians, and thus a global threat to amphibian diversity (Gray et al., 2009, Hyatt et al., 2002, Lesbarrères et al., 2012). There is therefore an urgent need to develop reliable, efficient and rapid methods to detect the virus. Diagnostic methods such as polyclonal antibody-based methods (Cinkova et al., 2010), polymerase

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chain reaction (PCR) (Couparl, 1995, Galli et al., 2006), quantitative real-time PCR assays (Holopainen et al., 2011, Jaramillo et al., 2012, Pallister et al., 2007), loop-mediated isothermal amplification (Min et al., 2013), and enzyme-linked immunosorbent assay (ELISA) (Whittington et al., 1997, Zupanovic et al., 1998) for detection of ranavirus have already been introduced. Although these are very effective at detecting ranavirus in infected amphibians, they can be time-consuming and require specialized training and equipment for their reliable performance.

Colloidal gold-immunochromatographic assays (GICA) are popular diagnostic methods used in both human and veterinary medicine, not only because they are fast, easy to use, high specificity and do not require specialized equipment, but also because the results of the assay are easily visible to naked eye. A cellulose membrane is used as the carrier and a colloidal gold-labelled antigen or antibody is used as the tracer, and the assay relies on specific antigen–antibody immunoreactions to detect a particular pathogen or protein (Peng et al., 2007). The ease of using these types of kits, coupled with their low production cost makes them widely accessible tests for detecting ranaviruses in frogs in the field (Guo et al., 2009, Li et al., 2011).

The development of a GICA-based diagnostic kit using monoclonal antibodies (MAbs) that can detect specific proteins of ranavirus strains frog virus-3 (isolates VR-567) and Korean ranavirus (KRV-1) is reported here. The kit is intended for the rapid detection of ranavirus in frogs, especially for use in on-site diagnostic testing. The sensitivity of the kit and its specificity against other known viral fish pathogens was tested. The GICA kit developed was subsequently evaluated using experimentally infected frogs.

2. Materials and methods

2.1. Viruses and cell culture

Two representative strains of ranavirus isolated from frog were selected for this study. The first was FV-3 (ATCC VR-567), a ranavirus strain originally isolated by Granoff et al. (1965), acquired from the American Type Culture Collection (ATCC), and the other was KRV-1, isolated from diseased frogs collected from Gangwon Province in South Korea by Kim et al. (2009). A previous study revealed that the sequences of major capsid protein (MCP), neurofilament triplet H1-like protein and a hypothetical protein of both strains were phylogenetically closely related (Kim et al., 2011). In the case of MCP, KRV-1 exhibited 98.1–99.9% identity at the amino acid level with MCP of other viruses such as FV-3, *Rana catesbeiana* ranavirus Japan (RCV-JP), soft-shelled turtle iridovirus (STIV), tiger frog virus (TFV) and *Rana esculenta* virus 282/I02 (REV) (Kim et al., 2011).

The other viral isolates used in this study included lymphocystis disease virus (LCDV) using a tumour collected from a virus-infected fish, sampled at a fish farm in Namhae, South Korea; red sea bream iridovirus (RSIV) which was isolated using the spleen of a virus-infected fish from a fish farm in Namhae, South Korea; viral hemorrhagic septicemia virus (VHSV) was isolated from olive flounder sampled at a fish farm in Jeju island, South Korea; Koi herpes virus (KHV) was isolated from a dead koi from a farm in Japan; and infectious pancreatic necrosis virus (IPNV) and viral nervous necrosis virus (VNNV) were isolated from farmed rainbow trout and wild marine fish, respectively, collected in the coastal areas of the Korean.

The baby hamster kidney cell line (BHK-21) (KCLB 10010; Korean Cell Line Bank, Seoul, Korea) was used to propagate FV-3 and KRV-1. The BHK-21 cells were grown as a monolayer culture in Dulbecco modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and antibiotics. Ranavirus isolates, FV-3 and KRV-1 were propagated in BHK-21 cell cultured in DMEM containing 2% FBS and infected cells showing a cytopathic effect (CPE) were collected, divided into aliquots and frozen at −70 °C.

A cell line derived from striped snakehead (E11 cell) from Seoul National University, hirame natural embryo (HINAE) cells from Tokyo University of Marine Science and Technology, chinook salmon embryo cells (CHSE-214 cell line, ATCC CRL1681), and koi fin (KF-1) cells from Tokyo University of Marine Science and Technology, were used for propagation of VNNV, VHSV, IPNV and KHV, respectively. RSIV and LCDV were prepared from respective virus infected tissues as mentioned above.

Virus purification for FV-3, KRV-1, VNNV, VHSV, IPNV and KHV was performed using cells showing CPE, which were then frozen and thawed 3 times. After centrifugation at 7000 × *g* for 30 min to obtain supernatant, viruses were concentrated using Quixstand^{TM} filtration system (GE Healthcare, Uppsala, Sweden). Virus was then centrifuged at 126,000 × *g* for 1 h and resuspended in 2 ml of TNE buffer (50 mM Tris–HCl, 150 mM NaCl and 1 mM disodium EDTA [pH 7.4]), and layered onto 30–60% (w/v) sucrose discontinuous gradients. A clear band between 40 and 50% were obtained after centrifugation at 100,000 × *g* for 1 h and resuspended in 10 ml of TNE buffer to wash the remained sucrose. The viral pellet was then kept at −70 °C until use.

2.2. Production of monoclonal antibodies

Hybridomas producing mouse monoclonal antibodies to FV-3 were generated as follows. Spleen cells of BALB/c mice (female, 6 weeks old, Koatech, Pyungtaek, Korea), immunized with purified FV-3, were fused with Sp 2/0 myeloma cells (KCLB 21581). Briefly, 10 μg of FV-3 was mixed 1:1 with complete Freund's adjuvant for the first immunization and mixed with incomplete Freund's adjuvant for the second and third immunizations every 2 weeks, for 6 weeks. A week after the third injection, mice were boosted with 10 μg of the virus without adjuvant by a tail vein injection. Three days after the final booster immunization, spleen cells were harvested from immunized mice and fused with Sp 2/0 myeloma cells using polyethylene glycol. The fused hybridomas were cultivated on a feeder layer of mouse blood cells in 96-well plates. A subsequent ELISA was performed to screen which hybridomas were producing MAbs against the virus. Positive hybridomas were selected and subcloned 3 times from a single cell by limiting dilution. Western blotting was performed to confirm the specificities of the MAbs. Isotyping of the MAbs was carried out using a mouse monoclonal antibody isotyping kit (Sigma, St. Louis, MO, USA). From the antibodies produced, one MAb with an IgG1 subtype, designated as FV-3 10E2 MAb, was selected for use as an antigen detector and conjugated with the colloidal gold, as described below, while FV-3 5D6 MAb, also an IgG1 subtype, was used as the capture MAb. The proteins detected by the MAbs were identified using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) analysis as described previously (Kim et al., 2011).

Immunization of mice and production of ascites were carried out in accordance with the animal welfare regulations of Gyeongsang National University, Gyeongnam, South Korea (GNU-LA-24).

2.3. Indirect enzyme-linked immunosorbent assay

The FV-3 was added in carbonate–bicarbonate buffer and seeded on a 96-well EIA/RIA plate (Corning, NY, USA) and incubated overnight at 4 °C. After washing 3 times with low salt washing buffer (24.2 g Trisma base, 222.2 g NaCl and 5 ml tween 20 dissolved in 1 l of distilled water [pH 7.3]), the plate was blocked with 5% skim milk (w/v) in phosphate buffered saline with 5% (w/v) tween 20 (PBS-T) for 1 h at room temperature (RT). After washing 3 times

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