Journal of Virological Methods 225 (2015) 9–15

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Short communication

Broad-range detection of arboviruses belonging to Simbu serogroup lineage 1 and specific detection of Akabane, Aino and Peaton viruses by newly developed multiple TaqMan assays

Hiroaki Shirafuji[a,*], Ryu Yazaki[b], Yozo Shuto[c], Tohru Yanase[a], Tomoko Kato[a], Youji Ishikura[d], Zenjiro Sakaguchi[e], Moemi Suzuki[f], Makoto Yamakawa[g]

[a] Kyushu Research Station, National Institute of Animal Health (NIAH), National Agriculture and Food Research Organization (NARO), Japan
[b] Kusu Livestock Hygiene Service Center, Oita Prefectural Government, Japan
[c] Oita Livestock Hygiene Service Center, Oita Prefectural Government, Japan
[d] Domestic Livestock Disease Identification Office, Food Safety Promotion Division, Shimane Prefectural Government, Japan
[e] Kagoshima Central Livestock Hygiene Service Center, Kagoshima Prefectural Government, Japan
[f] Okinawa Prefectural Institute of Animal Health, Okinawa Prefectural Government, Japan
[g] Viral Disease and Epidemiology Research Division, NIAH, NARO, Japan

Article history:
Received 23 January 2015
Received in revised form 30 August 2015
Accepted 30 August 2015
Available online 2 September 2015

Keywords:
Arbovirus
Akabane virus
Diagnosis
Orthobunyavirus
TaqMan assay

A B S T R A C T

TaqMan assays were developed for the broad-range detection of arboviruses belonging to Simbu serogroup lineage 1 in the genus *Orthobunyavirus* and also for the specific detection of three viruses in the lineage, Akabane, Aino and Peaton viruses (AKAV, AINOV and PEAV, respectively). A primer and probe set was designed for the broad-range detection of Simbu serogroup lineage 1 (Pan-Simbu1 set) mainly targeting AKAV, AINOV, PEAV, Sathuperi and Shamonda viruses (SATV and SHAV), and the forward and reverse primers of the Pan-Simbu1 set were also used for the specific detection of AKAV with another probe (AKAV-specific set). In addition, two more primer and probe sets were designed for AINOV- and PEAV-specific detection, respectively (AINOV- and PEAV-specific sets). All of the four primer and probe sets successfully detected targeted viruses, and thus broad-range and specific detection of all the targeted viruses can be achieved by using two multiplex assays and a single assay in a dual (two-color) assay format when another primer and probe set for a bovine β-actin control is also used. The assays had an analytical sensitivity of 10 copies/tube for AKAV, at least 100 copies/tube for AINOV, 100 copies/tube for PEAV, one copy/tube for SATV and at least 10 copies/tube for SHAV, respectively. Diagnostic sensitivity of the assays was tested with field-collected bovine samples, and the results suggested that the sensitivity was higher than that of a conventional RT-PCR. These data indicate that the newly developed TaqMan assays will be useful tools for the diagnosis and screening of field-collected samples for infections of AKAV and several other arboviruses belonging to the Simbu serogroup lineage 1.

The genus *Orthobunyavirus* is a group in the family *Bunyaviridae*, and the viruses of the family have three-segmented, negative single-stranded RNA as the viral genome (Elliott and Blakqori, 2011; Plyusnin et al., 2012). Akabane, Aino (Shuni virus serogroup), Peaton (Shamonda virus serogroup), Sathuperi and Shamonda viruses (AKAV, AINOV, PEAV, SATV and SHAV, respectively) belong to Simbu serogroup lineage 1 in the genus *Orthobunyavirus* (Elliott and Blakqori, 2011; Saeed et al., 2001; Plyusnin et al., 2012), and all of these viruses are arthropod-borne viruses (arboviruses) that are transmitted principally by *Culicoides* biting midges (Schmaljohn and Nichol, 2007; Tsuda, 2000). AKAV and AINOV are causative agents of Akabane disease and Aino virus infection, respectively, and the features of these diseases in cattle are abortion, stillbirth, premature birth and congenital abnormalities (Coverdale et al., 1978; Tsuda, 2000). PEAV caused congenital malformations in sheep *via* an experimental infection (Parsonson and McPhee, 1985). The pathogenesis of SATV and SHAV is not clear, but the closely related Schmallenberg virus (SBV) has been causing congenital abnormalities in ruminants in Europe (Beer et al., 2013; Doceul et al., 2013; Goller et al., 2012; Yanase et al., 2012).

Large outbreaks of Akabane disease occurred widely in Japan in the 1970s, and epidemics of this disease have also been reported frequently in Japan and South Korea in recent years (Forman et al., 2008; Tsuda, 2000). Besides the typical bovine cases of Akabane

* Corresponding author.
E-mail address: shirah@affrc.go.jp (H. Shirafuji).

http://dx.doi.org/10.1016/j.jviromet.2015.08.021
0166-0934/

disease with the clinical manifestations mentioned above, bovine cases of Akabane viral encephalomyelitis occurred in 2006 and 2010 (Kono et al., 2008; Oem et al., 2012), and swine cases of encephalomyelitis occurred in 2011 were suspected to have been caused by Akabane virus (Honda et al., 2013). Field cases of Akabane disease were also found in China and Israel (Brenner et al., 2004; Qiao et al., 2013). Akabane disease has thus been one of the major obstacles in the livestock industry, similar to the large impact that the SBV infections of ruminants have had on the livestock industry in Europe (Beer et al., 2013).

Several arboviruses belonging to the Simbu serogroup lineage 1 have been isolated in Japan over the past decade. PEAV, which was originally isolated in Australia in 1976 (St George et al., 1980), was first isolated in Japan in 1999 (Matsumori et al., 2002). Another virus in the genus, SATV, which was originally isolated in India in 1957 (Dandawate et al., 1969), was first isolated in Japan in 1999 (Yanase et al., 2004). SHAV, which was first isolated in Japan in 2002 (Yanase et al., 2005), was originally isolated in Nigeria in 1965 (Causey et al., 1972). Since their first isolation in Japan, the viruses described above have been repeatedly isolated from bovine blood or *Culicoides* biting midges (Yanase et al., 2010, 2012).

To date, quite many TaqMan assays have been developed for the detection of arboviruses (Barros et al., 2013; Dyer et al., 2007; Garcia et al., 2001; Pang et al., 2014; Rodriguez-Sanchez et al., 2008), including TaqMan assays for arboviruses belonging to the genus *Orthobunyavirus* (Fischer et al., 2013; Rodrigues Hoffmann et al., 2013; Van Eeden et al., 2014). However, there have been no TaqMan assays that are optimized for use in Japan and other Asian countries to detect arboviruses belonging to the genus *Orthobunyavirus*. Since it is often the case with TaqMan assays for arboviruses that some topotypes (distinct geographical variants) (Mertens et al., 2009; Samuel and Knowles, 2001) of an arbovirus can be detected with a set of primers and a TaqMan probe, other topotypes cannot be detected with it (Shaw et al., 2007; Wilson et al., 2009), TaqMan assays targeting arboviruses need to suit the topotype in each area where the targeted arboviruses are distributed. Based on this idea, the aim of this study was to develop TaqMan assays for the detection mainly of AKAV, AINOV and PEAV – which are of veterinary importance in Japan – as well as SATV and SHAV, with the use of Japanese isolates of the Simbu serogroup lineage 1.

Forty four isolates of arboviruses were used in the present study. Japanese isolates of the Simbu serogroup lineage 1 were 19 isolates of AKAV, seven isolates of AINOV, six isolates of PEAV, four isolates of SATV and three isolates of SHAV in the 44 isolates. The other species of arboviruses were four isolates of the genus *Orbivirus* including epizootic hemorrhagic disease virus (EHDV) No. 2 isolate, bluetongue virus (BTV) TO2-1 isolate (serotype 21) (Shirafuji et al., 2012), Chuzan virus (CHUV) 31 isolate, D'Aguilar virus (DAGV) B8112 isolate, and an isolate of the genus *Ephemerovirus*, bovine ephemeral fever virus (BEFV) ON-1/B/04 isolate. All 44 of the isolates were propagated in BHK-21 or HmLu-1 cell cultures until approx. 90% of the monolayer showed cytopathic effects. Viral RNA was extracted from the supernatant of the virus-infected cell cultures using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, USA).

The sequences of the primers and probes for the TaqMan assays are shown in Table 1. Based on conserved regions of S segment of AKAV, AINOV, PEAV, SATV and SHAV, a set of primers (Simbu1_F and Simbu1_R) and a TaqMan probe (Simbu1_Probe) were designed for the broad-range detection of Simbu serogroup lineage 1 mainly targeting the five arboviruses listed above (Pan-Simbu1 set). Then, another TaqMan probe (AKAV_S2_Probe) was designed based on a conserved sequence of AKAV in between the binding sites of Simbu1_F and Simbu1_R primers. The AKAV_S2_Probe was used for specific detection of AKAV together with the Simbu1_F and Simbu1_R primers (AKAV-specific set). A set of primers (AINOV1-2F and AINOV1-2R) and a probe (AINOV1-3_Probe) was designed for specific detection of AINOV based on conserved region of M segment of AINOV (AINOV-specific set), and another set of primers (PEAV6F and PEAV6R-2) and a probe (PEAV6_Probe) was designed for specific detection of PEAV based on conserved region of M segment of PEAV (PEAV-specific set). Signatures of all the primers and probes were prescreened with a free web-based tool for analyzing primers, Net Primer (Premier Biosoft International, Palo Alto, USA), for the TaqMan assays to work properly, and the sequences of the primers and probes were also checked by the Basic Local Alignment Search Tool (BLAST) to prevent non-specific reactions.

The conditions for the assays were optimized with the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen/Life Technologies, Carlsbad, USA) and a real-time PCR system, MyiQ$_2$ (BIO-RAD, Hercules, USA). For each RNA sample, 2 μl was mixed with 23 μl of reaction mix containing 12.5 μl of 2× Reaction Mix (included in the kit), 10 pmol of each primer, 3 pmol of each probe, 0.5 μl of SuperScript III RT/Platinum *Taq* Mix (kit), 0.5 μl of RNase OUT Recombinant Ribonuclease Inhibitor (Invitrogen) and nuclease-free water. Since the MyiQ$_2$ can detect two excited fluorophores, the assays were designed to be multiplex assays to detect 6-carboxyfluorescein (FAM) and hexachloro-6-carboxyfluorescein (HEX) fluorophores in each tube or a single assay to detect the HEX fluorophore. The probes of Pan-Simbu1 and PEAV-specific sets ("Simbu1_Probe" and "PEAV6_Probe") were both labeled with FAM at the 5′ end, and the probes of AKAV- and AINOV-specific sets ("AKAV-S2_Probe" and "AINOV1-3_Probe") were both labeled with HEX at the 5′ end, respectively. A primer and probe set for bovine β-actin gene (β-actin set) was also used for bovine samples (Wernike et al., 2015), and 5 pmol of each primer and 2.5 pmol of the probe were used per reaction. Both FAM-labeled and HEX-labeled probes for the bovine β-actin gene were prepared, and one of them was selected depending on fluorophore of the other probe used in the multiplex assays. All of the probes were labeled with the dark quencher, BHQ-1, at the 3′ end. The conditions for the assays were as follows: 50 °C for 15 min for reverse transcription (RT), 95 °C for 2 min for inactivation of the RT enzyme and initial denaturation, 40 cycles of 95 °C for 15 s (denaturation), and 60 °C for 30 s (annealing and extension).

Analytical specificity of the assays was determined by using RNA samples extracted from the 44 virus isolates described above. Each of the virus isolates was tested in the same titer in all of the assays for the analytical specificity. The samples were tested in duplicate in multiplex assays with a combination of Pan-Simbu1 and AKAV-specific sets and another combination of AINOV- and PEAV-specific sets, respectively.

In order to produce standard RNA for the TaqMan assays, artificial RNA templates were synthesized containing target sequences by molecular cloning and following *in vitro* transcription as previously described (Niwa et al., 2015). The AKAV KM-1/Br/06 isolate, the AINOV KS-1/E/02 isolate, the PEAV KSB-1/P/06 isolate, the SATV KSB-1/P/08 isolate and the SHAV ON-3/P/07 isolate were selected for the synthesis, and cDNA containing target sequences was amplified by RT-PCR with the QIAGEN OneStep RT-PCR Kit (Qiagen). The primers used for the RT-PCR are shown in Table 1. The products of the *in vitro* transcription were purified with an RNeasy Mini Kit (Qiagen) and then the concentration and purity of the purified RNA were measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). After the measurement, 10-fold dilutions were prepared as 10^7 to 10^{-1} copies/tube and used as external standards to determine RNA copy numbers in virus samples (cell culture supernatant) of the five virus isolates described above. Then, 10-fold dilution series of the virus samples were prepared by using Simbu serogroup virus-free bovine plasma, and RNA was extracted from the diluted samples and then used as standards to determine analytical sensitivity of the assays. The analytical sensitivity was determined with the standards in