Molecular detection of ephemeral fever virus among large ruminants in the Philippines

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Abstract In the Philippines, bovine ephemeral fever (BEF) is currently undetected and considered as an exotic disease of both cattle and water buffaloes. The Philippines until now has no official data regarding the occurrence of BEF. There were no existing control programs or vaccine used for the prevention of the disease. However, there are claims of BEF existence in different water buffalo and cattle farms based on the clinical signs but never confirmed using laboratory test yet. Detection of BEF virus in cattle and water buffalo blood samples was conducted using reverse-transcription PCR targeting the glycoprotein (*G*) gene, a conserved region in the BEF virus genome. The samples were collected from 22 cattle and 50 water buffaloes with clinical signs suggesting of BEF infection. All water buffalo blood samples were negative while four cattle blood samples turned positive for BEF virus. The *G* gene partial sequence analysis from two BEF virus positive samples showed close relationship to Australian isolates.

Keywords Bovine ephemeral fever · RT-PCR · Phylogenetic tree analysis · Philippines

Bovine ephemeral fever (BEF) is an arthropod-borne viral disease caused by infection with BEF virus, which belongs to genus Ephemerovirus within the family Rhabdoviridae [10]. The BEF is an economically important vector-borne viral disease of cattle and water buffaloes [1]. The infected animal usually recovers 3 days later so, the disease is also known as 3-day fever or sickness [7]. Economic losses are attributed to mortality of stock and draught animals, abortion, decreased milk production, reduced weight gain and lowered fertility of bulls. The costs of supportive care and treatment, and live cattle trade restrictions [5] also adds to the loss due to BEF. The virus is enzootic in ruminant populations in tropical and subtropical regions of Africa, the Middle-East, Asia, and Australia [2, 11]. Vaccination is currently the only useful preventive measure against ephemeral fever. To date, live attenuated and killed vaccines against BEF virus were already available in some countries like Australia, South Africa and Japan.

In the Philippines, livestock is firmly integrated into the economic structure of farm and village life and is being recognized as a key element which contributes to rural income and to the efficient use of the available resources in the rural sector. The raising of farm animals is still on a small scale basis since it is intimately tied with farmers' activities and way of life. Cattle and water buffaloes are mainly raised for draught purposes and as source of income. Also, these animals offer a means whereby crop products and farm residues as well as native vegetation in uncultivated areas are converted into meat, milk, hides and other by-products (<http://www.bas.gov.ph/?ids=cattlesituation>). The total water buffalo inventory in the country, as of

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July 1, 2014, was 2.86 million heads while 2.52 million for cattle (<http://www.bas.gov.ph/?ids=cattlesituation>).

To date, BEF is undetected in the Philippines. Livestock agencies in the country have no official data, no control programs or vaccine used for the prevention of the disease. However, field reports based on clinical signs suggests BEF existence in the country but has never confirmed by any available laboratory test yet. Thus, this present study was conducted to confirm presence of BEF using molecular test and construct a phylogenetic tree to compare the local isolates with the existing BEF submission in the GenBank.

Blood samples were collected from 50 water buffaloes and 22 cattle that showed similar clinical signs of BEF. Approximately 5 mL of blood was aseptically collected from the jugular vein of the animals using a disposable 5 mL syringe with a 23G $\times$ 1" needle. The collected blood was transferred to a 5 mL tubes coated with EDTA. After collection, blood samples were stored in an ice box and transported to the laboratory for examination.

About 500 L of the buffy coat from the whole blood was transferred in a 1.5 mL microcentrifuge tube and a 2–3 volumes of 0.14 M NH_4Cl were added and then mixed by vortexing. The mixture was subjected to centrifugation for 5 min at 3000 rpm. The supernatant was poured out to gather the pellet then dissolved by adding 1000 μL of Trizol[®] (Invitrogen, USA) reagent mixed by pipetting and incubated at room temperature for 5 min. 200 μL chloroform was added then mixed by vortexing. The mixture was centrifuged at 14,000 rpm for 10–15 min at 4 °C or room temperature. The aqueous phase was transferred to a new tube with 500 μL isopropanol and mixed by vortexing. The mixture was incubated at room temperature for 10 min and centrifuged at 14,000 rpm at 4 °C for 10–15 min. The pellet was collected and washed with 1 mL 70% ethanol. The solution was centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was air-dried at room temperature until no droplets or traces of alcohol were visible. DEPC water or RNase-free water was used to rehydrate the pellet. The cDNA was synthesized using the TaKaRa 1st Prime script cDNA synthesis Kit (TaKaRa, Japan). The cDNA was stored at -20 °C if not amplified immediately.

A semi-nested PCR primers used in the study were designed from the conserved region of the glycoprotein (G) gene of BEF virus using National Center for Biotechnology Information (NCBI) Primer BLAST software (forward primer 1: 5'AGGATGGAGAATGGTGGAGT3'; forward primer 2: 5'AGGATGCACACAGATAGACAA3'; and reverse primer 5'GTCTCGTGGGAGCCAAGTA3'). These primers were designed to amplify 145 bp region of the BEF virus G gene.

The PCR was carried out in total volume of 10 μL reaction mixture was composed of 1 μL of synthesized

cDNA, 6.15 μL of double-distilled water, 2 μL of 5 $\times$ PCR buffer, 0.2 μL of 25 mM MgCl_2 , 0.2 μL of deoxynucleoside triphosphatase (dNTP), 0.2 μL of each forward and reverse primers and 0.5 μL of Taq polymerase (5U/ μL). The PCR reaction mixture was subjected to initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, primer extension at 72 °C for 30 s and then final extension at 72 °C for 10 min. The same PCR mixture volumes and thermal conditions were used in the 2nd PCR of BEF virus amplification. A 3 μL of PCR product was electrophoresed on 2% agarose gel stained with GelRed. Since there is no reported case or isolated BEF virus or even vaccine available in the Philippines, a synthetic plasmid of G gene was used as positive control. The use of positive control terminates the possibility of a false-negative due to contamination or other related reasons.

A 10-fold dilution series were used for cDNA synthesis to determine the sensitivity of RT-PCR. For specificity, the RT-PCR was performed on extracted RNA from a rabies vaccine and a bluetongue virus positive control in order to evaluate if the designed primers and optimized protocol was specific only for BEF virus.

RT-PCR products were submitted for purification and sequencing at the Fist BASE Laboratories, Malaysia. The generated sequence of the BEF virus was aligned and compared with the sequences of BEF viruses in other countries using Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Center (NCBI). The aligned DNA sequences of the isolated BEF virus and its subsequent reference BEF virus sequences from GenBank were used as a reference for the construction of the phylogenetic tree using MEGA version 6 software [9].

Results showed that all of the animals screened, four cattle blood samples were found positive of BEF virus (Fig. 1a). The specificity of the optimized RT-PCR protocol was checked using RNA templates extracted from BEF virus and other virus including the rabies virus (extracted from vaccine) and bluetongue virus (synthetic plasmid). Specificity refers to the ability of an assay to measure one particular organism or substance, rather than others [8]. In this study, the BEF virus produced a single compact band on the expected target amplicon size of 145 bp while band was not observed from rabies virus and bluetongue virus (Fig. 1b). Thus, the optimized RT-PCR protocol was specific only for the detection BEF virus. Sensitivity represents the smallest amount of substance in a sample that can accurately be measured by an assay. The smaller the detectable change, the greater is the sensitivity [8]. In the present study, the lowest detection limit by the tenfold dilution was at 1×10^{-9} using the starting concentration of 4 ng/ μL of the stock sample (Fig. 1c). Bonds of the

Fig. 1 **a** Gel electrophoresis analysis of RT-PCR results targeting *G* gene of BEFV in cattle blood samples. M—100 bp molecular ladder; lanes 1–9, cDNA from blood samples of cattle; lane 10, positive control; lane 11, negative control (RNase free water). **b** Specificity of detection was checked with, lane 2: rabies virus, lane 3: bluetongue virus. **c** Using synthetic RNA of the partial *G* gene, detection limit was assessed by tenfold dilutions (10^{-1} to 10^{-10} copies/tube), lanes 1–10: tenfold serial dilutions containing cDNA templates of BEFV; lane 11: negative control

dilutions 1×10^{-7} to 1×10^{-9} were faintly seen in the gel plate.

The partial sequence of the *G* gene from the positive samples was composed of 145 nucleotides on Isolate 1 (LC315929.1) and 144 nucleotides on Isolate 2 (LC315930.1). The result of DNA alignment and nucleotide search for reference homologous DNA sequences in the GenBank confirmed the BEF virus in the Philippines. Isolate 1 and 2 showed homology to various isolates of BEF virus from other countries. Both isolates in this study were 99% homologous to isolate from Australia with an accession number of KF679473.1 and 98% homologous to another isolate from Australia (Accession Number: KF679486.1). Isolates 1 and 2 were compared to Australian BEF virus isolates, since Australian isolates were the ones that gave the highest sequence homology compared to other BEF virus isolates from GenBank database. One nucleotide polymorphism of both isolates in this study was observed in locus 78 of the nucleotide sequence that differ them from the Australian isolates. Moreover, one nucleotide deletion was observed in Isolate 2 in locus 74 of the nucleotide sequence.

Seventeen (17) BEF virus isolates were grouped into three distinct lineages (Fig. 2). Cluster I contained the strains isolated from Iran, Israel, Japan and Turkey. The Australian and Philippine isolates were grouped into Cluster II, and other East Asia BEF virus isolates from China and Taiwan were placed in the independent Cluster III. The phylogenetic findings of the BEF virus isolates from Philippines (Isolates 1 and 2) were closely related to Australian isolates with a bootstrap value of 99%. However, BEF virus isolates from China and Taiwan have a distant relationship with the Japan, Philippine and Australian BEF virus isolates and the BEF virus isolates from Middle East (Iran, Israel and Turkey).

The results of this study verified the existence of BEF virus in the Philippines. With the presence of this disease, water buffaloes and cattle in the country are at risk of being infected by BEF virus. The BEF was classified as a potential hazard since the disease is most severe in food producing animals.

The RT-PCR technique which was also applied in this study had been developed with many advantages. Previous researches reported that it is possible to detect as little as 2 fragments of viral RNA from infected tissue [13] using this technique. There is no need for virus replication, moreover, RT-PCR is not time consuming since all procedures involved take about 6 h to be completed [3]. The RT-PCR was also used by Kasemet al. [6] in identifying BEFV in Delta Provinces in Egypt. In their study they found out that RT-PCR was sensitive, specific and of value for rapid diagnosis of viral diseases as it confirmed and diagnosed the BEF outbreak in Egypt. The optimized RT-PCR protocol can be used as a routine diagnostic tool for imported animals, which are possibly carriers of the BEF virus.

The surface glycoprotein is considered as the major neutralizing and protective antigen of BEF virus [4]. The *G* gene is a useful genotyping marker as it displays reliable alignment with adequate sequence variation to obtain precision and resolution, and the absence of genetic recombination suggests it is likely to be representative of the entire genome [12]. In the present study, Australian lineage is a complete distinct lineage together with Philippine isolates; Iranian, Israeli, Turkish and Japan isolates were grouped into a separate lineage; and other East Asian isolates from China and Taiwan construct the third lineage (Fig. 2). From the results of this study, it was speculated that the same BEF virus from Australia might be responsible for BEF cases in the Philippines since Australia is one of the countries where Philippines imports live cattle. The BEF virus was believed to exist as a single serotype however, phylogenetic studies using *G* gene sequences have shown that BEF virus isolates cluster geographically into 3 lineages: East Asia, Australia and the Middle East [11, 12].

Fig. 2 Phylogenetic profiles of the BEFV isolates and reference GenBank sequences in 1000 bootstrap based on the comparison of the partial *G* gene sequences. A total of 17 isolates were used to construct the phylogenetic tree using MEGA 6 program. Philippine Isolate 1 (LC315929.1) and Isolate 2 (LC315930.1)

To date, the livestock agencies in the Philippines still considers BEF as an exotic disease, although there were some reports in the past years suggesting that BEF virus was present in the country. Moreover, these BEF suspected cases were diagnosed only based on clinical signs and no confirmatory tests to prove its presence in the country. In this study, RT-PCR was a useful tool in detecting BEF virus in cattle and buffaloes, and clearly showed existence of BEF virus in the Philippines. The isolated Philippine BEF virus isolates were related to the Australian isolates and can be possibly introduced locally through live cattle importation. However, for surveillance purposes, increasing the number of animals is highly recommended. It is also recommended that the use of other diagnostic method like Reverse-Transcription Loop Mediated Isothermal Amplification (RT-LAMP) developed by Zheng et al. [14] can be attempted. Quarantine of newly imported live animals from different countries should be done in order to minimize the spread of diseases from infected areas to non-infected areas. Additionally, molecular testing of different samples from imported live animals is also an important tool to detect if such animals do carry infectious pathogens. In addition, further studying of the whole *G* gene of the BEFV is recommended for the future development of vaccines against BEF.

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