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RESEARCH ARTICLE

Molecular identification and *in vitro* assessment of zoonotic-potential of a novel *Orthobunyavirus* isolated from broiler chicken in Malaysia

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ABSTRACT

We have previously isolated a novel avian *Orthobunyavirus*, Kedah Fatal Kidney Syndrome (KFKS) virus from a broiler farm in Kedah, Malaysia in 2020 with a severe kidney lesion in chickens. The virus was designated as KFKS2_CS virus. Sequence analysis of partial nucleocapsid (N) and nonstructural (NSs) sequence of this virus showed the highest sequence identity with previous KFKS1 from Malaysia (100%) and 97% with a zoonotic Umbre (UMB) virus, which was reported to cause encephalitis in immunocompromised humans in India. Phylogenetic analysis revealed that this virus was clustered together with previous KFKS1 virus from Malaysia, UMB and Cristoli viruses. This study aimed to assess the zoonotic potential of this KFKS2_CS virus *in vitro* by determining its ability to inhibit the production of interferon (IFN) in human glioblastoma multiforme (GBM) brain cells using reverse-transcriptase polymerase reaction (RT-PCR). This virus blocked the production of interferon-α in this human brain cells. In conclusion, this KFKS2_CS virus may have a zoonotic potential and become a public health concern in the future.

Keywords: Zoonotic-potential; Novel *Orthobunyavirus*; broiler; Malaysia; interferon-α.

INTRODUCTION

Orthobunyavirus genus comprises over 170 named mosquito- and midge-borne viruses, several of which cause severe disease in animals or humans (Elliott, 2014). A novel *Orthobunyavirus*, Kedah Fatal Kidney Syndrome (KFKS1) virus was first isolated in 2014 from a broiler farm in Kedah, Malaysia. The virus caused severe kidney lesions in chickens (Palya & Banyai 2018). The virus sequences were closely related to a zoonotic UMB virus with nucleotide sequence identity of 90% of complete nucleocapsid (N) gene (Palya & Banyai 2018). UMB virus has been reported to cause encephalitis in immunocompromised people in France in 2013 and 2018 (Perot *et al*., 2021). KFKS virus and UMB viruses are classified into the same *Orthobunyavirus* genus, which is transmitted by mosquitoes (Palya & Banyai 2018; Rodriguez *et al*., 2020). Some orthobunyaviruses are zoonotic including UMB and Shuni viruses (Oymans *et al.,* 2020; Perot *et al*., 2021). Thus, this KFKS virus may be also pathogenic to other animal species including humans.

Interferons (IFNs) are an antiviral factor that interferes with viral replication in mammalian cells (Isaacs & Lindenmann, 1957). They are secreted from infected cells and involved in activating of innate immune response which involves in promoting cytokine production and natural killer cell functions (Ivashkiv & Donlin, 2014). Currently, three distinct classes of IFNs have been described (Type I, II, and III). The type-I IFN family includes numerous IFN-α variants and a single IFN-β (Kopitar-Jerala, 2017). Type I IFNs are important for host defense against viruses (McNab *et al*., 2015).

To know more about the zoonotic potential of this virus, we have undertaken this study to determine the zoonotic potential of this KFKS virus by *in-vitro* evaluating its ability to inhibit IFN -α and -β in human glioblastoma GBM brain cells. This study is important to create public health awareness for future disease control.

MATERIALS AND METHODS

Source of the virus

Chicken kidneys with severe lesion from one of the poultry farms in Kedah, Malaysia was submitted to the virology laboratory, Universiti Malaysia Kelantan in year 2020. The samples were confirmed containing the KFKS virus by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using designed primers targeting partial N and NSs genes of KFKS1 (MK047474) and UMB (MK330174) viruses from the National Center for Biotechnology Information GenBank (NCBI-GenBank). To isolate the virus, the samples were ground, filtered using 0.22 µM syringe filter (Merck Millipore, USA) and inoculated into the 25 cm^3 tissue culture flask containing the confluent Vero E6 cells. The cytopathic effect (CPE) was observed after the third passages in the Vero E6 cells (Figure 1) which were purchased from American Type Culture Collection (ATCC). The virus was further propagated in Vero cells and determined its titer as 10^5 of 50% Tissue Culture Infectious Dose (10^5 TCID50)/mL using Spearman-Karber method (Lei *et al.,* 2021). The virus was titrated in 10-fold serial dilutions and 100 µl of each dilution was seeded in 10 wells of the 96-well tissue culture plate. The wells were incubated

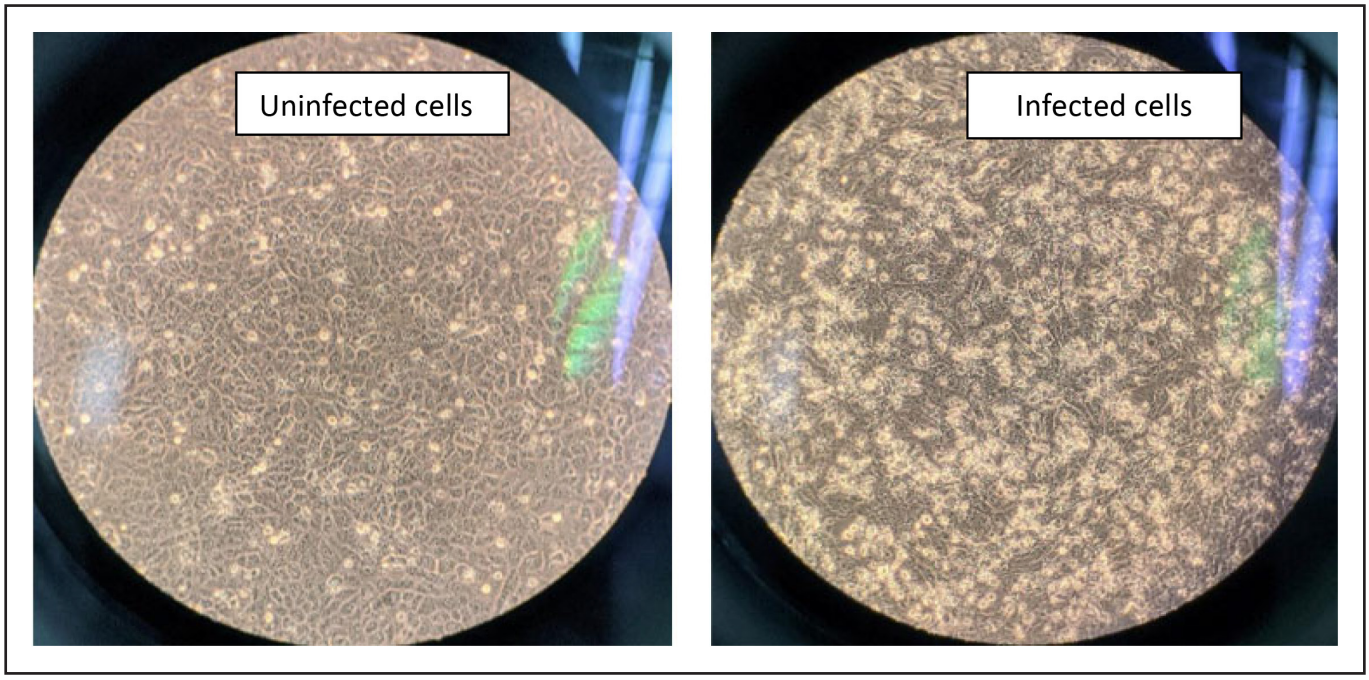

Figure 1. Cytopathic effect caused by KFKV2_CS virus in Vero cells.

in 37°C incubator with 5% CO_2 and examined for the presence of CPE for 7 days. The 10^5 TCID50/mL of the virus was determine using this calculation;

Log ID_{50}= log (Highest dilution giving 100% CPE) +0.5 -Total number of tests unit showing CPE/Number of test units per dilution.

Preparation of Vero and Human glioblastoma multiforme (GBM) cells

Vero E6 and GBM (ATCC CRL-1690) cells were obtained from ATCC. The cell was grown and maintained in Dulbecco's minimum essential medium (DMEM) (Gibco, USA) with 10% and 2% fetal bovine serum (FBS) (Gibco, USA), respectively, with the addition of 1% penicillin-streptomycin (5000 U/ml) (Nacalai Tesque, Japan). The cells were incubated for seven days at 37°C with 5% CO_2 with 95% humidity.

Inoculation of KFKS2_CS virus in Vero E6 and Human glioblastoma multiforme (GBM) cells

Vero E6 and GBM cells were seeded at 10^5 cells/mL in a 6-well tissue culture plate and incubated in a 37°C incubator with 5% CO_2 and 95% humidity for 24 hours. Then, the media was removed and washed twice with 1 X phosphate buffer saline (PBS), pH7.5 and each of the wells was filled with two ml of DMEM with 2% FBS. The GBM cells were chosen for IFN study because KFKS virus was closely related to UMB virus that causes encephalitis to the human brain. Vero E6 cells infected with KFKS2_CS virus were used as negative controls in this study. Two plate wells were infected with 10^2 $TCID_{50}$ KFKS2_CS virus and uninfected Vero cells were served as negative controls. The plate was incubated for 24 hours at 37°C with 5% CO_2 and 95% humidity. After 24 hours of incubation, the medium was removed and 1 mL of GENEzol reagent (Geneaid Biotech Ltd, Taiwan) was resuspended onto the monolayer cells. The cells were scraped with a cell scraper (Corning, USA) and the supernatant was transferred to a 1.5 mL microcentrifuge tube.

Ribonucleic acid (RNA) extraction of the cells

Infected Vero, GBM cells and uninfected cells (negative controls) suspended in GENEzol reagent were extracted following the manufacturer's protocol (Geneaid Biotech Ltd, Taiwan). The purity and concentration of the RNA were checked using NanoPhotometer (Implen, USA).

Transcription-Polymerase Chain Reaction (RT-PCR) reaction

The reaction was carried out in a 25µl reaction mixture using AccessQuick RT-PCR reagent kit (Promega, USA) on the extracted RNA from GBM infected and uninfected cells with the later served as a negative control. The RT-PCR reaction was conducted following the manufacturer's recommendation with the addition of 0.2 uM of each forward and reverse primers, nuclease-free water (Promega, USA), and template RNA (0.1 to 1 mg total RNA). The primers used were as shown in Table 1. All the primers were designed in this study except for GAPDH_F and GAPDH_R house keeping gene (Xie *et al*., 2017). The OBV, IFN-α and IFN-β primers were designed manually based on sequences alignment of reference KFKS1 and Umbre viruses, human IFN-α and IFN-β sequences retrieved from NCBI-GenBank using ClustalX 2.1 program (Thompson *et al*., 1997). The specificity of the primers was checked using NCBI-BLAST software. RT-PCR was carried out for one cycle at 42°C for 60 min, inactivation of the reverse-transcriptase enzyme at 94°C for 2 min, followed by 35 cycles of PCR amplification at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec and one final extension cycle at 72°C for 7 min. The reaction was conducted in a thermocycler (Biorad T100, USA). The products were run at 100 Volt for 50 minutes in 2% Midori green (Nippon Genetics, Europe) stained agarose gel and visualised using Gel DocTM EZ Gel Imager (Bio-Rad, USA). The RT-PCR product was sent to Apical Scientific Sdn. Bhd. Malaysia for sequencing in both forward and reverse directions. The sequences were edited using Bioedit 7.2 software (Informer Technologies, Inc).

BLAST and Phylogenetic analysis of KFKS2_CS virus

The sequences were compared with reference orthobunyaviruses sequences in the GenBank using NCBI-Basic Local Alignment Search Tool (NCBI-BLAST) software to determine the sequence identity of this virus. Both obtained forward and reverse sequences of RT-PCR products were analysed using ChromasPro software (Technelysium Pty Ltd, Australia). Multiple alignment of the sequences was prepared using the ClustalX 2.1 program (Thompson *et al*., 1997) and phylogenetic analysis was conducted by neighbor-joining methods

Table 1. Primer sequences for RT-PCR

Primer	Sequence (5'…3')	Gene	Expected Size (bp)	Reference
IFN-α_ F IFN-α_ R	GACTCCATCTTGGCTGGCTGTGA TGATTTCTGCTCTGACAACCT	IFN- α	140	This study
IFN-β_ F IFN-β_ R	CAACTTGCTTGGATTCCTACAAA TATTCAAGCCTCCCATTCAATT	IFN- β	101	This study
OBV_F OBV_R	CACTAGCATTTATCCAGGAT GACACCCACAAACGTATCGT	Partial N and NSs	110	This study
GAPDH_F GAPDH_R	GAGTCAACGGATTTGGTCGT GACAAGCTTCCCGTTCTCAG	GAPDH	195	Xie *et al*., 2017

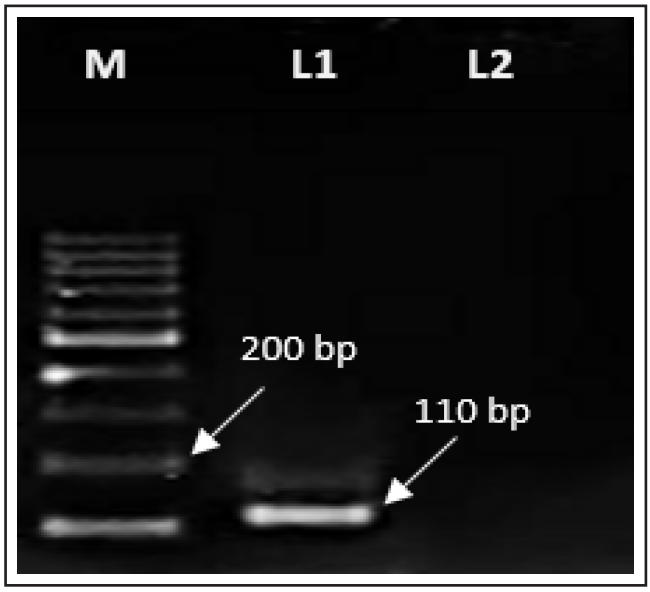

Figure 2. RT-PCR product of KFKS2_CS based on partial N and NSs genes. M-100 bp DNA marker, L1-KFKS2_CS virus. L2-Uninfected cells.

using NJ plot software (Perriere & Gouy, 1996) based on partial N and NSs sequences of 11 reference sequences of orthobunyaviruses from the NCBI-GenBank (Figure 3). Anopheles A virus (KY793539) was used as an outgroup virus. All the accesion number of the sequences used to contruct the tree were as shown in Figure 3.

RESULTS

RNA purity and concentration.

Concentration and purity of extracted RNA from GBM, Vero cells inoculated with KFKS virus and uninfected cells as a negative control were almost with the same RNA concentration (0.1-0.2 mg) and purity ratio (260/280 nm ratio of >2), which mean that they are valid for use in the RT-PCR reaction.

RT-PCR and Sequence analysis

A band of an expected size of 180 base-pairs (bp) of partial N and NSs was obtained from Vero infected cells as shown in Figure 2. No band was observed in uninfected cells (negative control). NCBI-BLAST result of the virus sequence has showed that it has 100% sequence identity with previously published sequence of KFKS1 virus from Malaysia (MK047403) and 97% identity with UMB virus (NC_043035). The virus is designed as KFKS2_CS and the sequence was deposited in the NCBI GenBank (Accession number of 2688521).

Phylogenetic analysis of KFKS2_CS virus

Phylogenetic analysis based on the partial N and NSs gene segments showed that KFKS virus of the current study (KFKS2_CS) is in the same clade as previous KFKS1 virus from Malaysia (Palya & Banyai, 2018) as shown in Figure 3. These two viruses were clustered together with UMB (MK30174) and Cristoli (MN488996) virus sequences.

Inhibition of interferon α and β in human GBM brain cells

A band of the expected size of IFN-α was only observed in uninfected GBM cells but not in the GBM cells infected with KFKS2_CS virus as shown in Figure 4. IFN-β band was not observed in both infected and non-infected GBM cells. No IFN-α and -β band was expressed in Vero cells. RT-PCR for the GAPDH house-keeping gene gave a

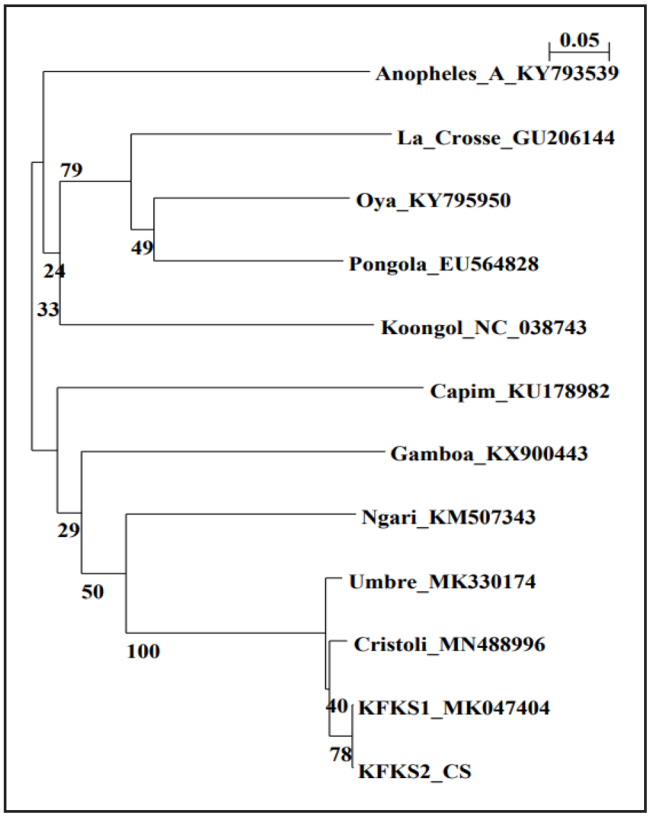

Figure 3. Phylogenetic trees of KFKS virus and selected reference orthobunyaviruses based on partial N and NSs gene. Scale bars indicate nucleotide substitutions per site. KFKS1 is previously isolated virus (Palya *et al*., 2019) and KFKS2_CS is the virus identified in this study.

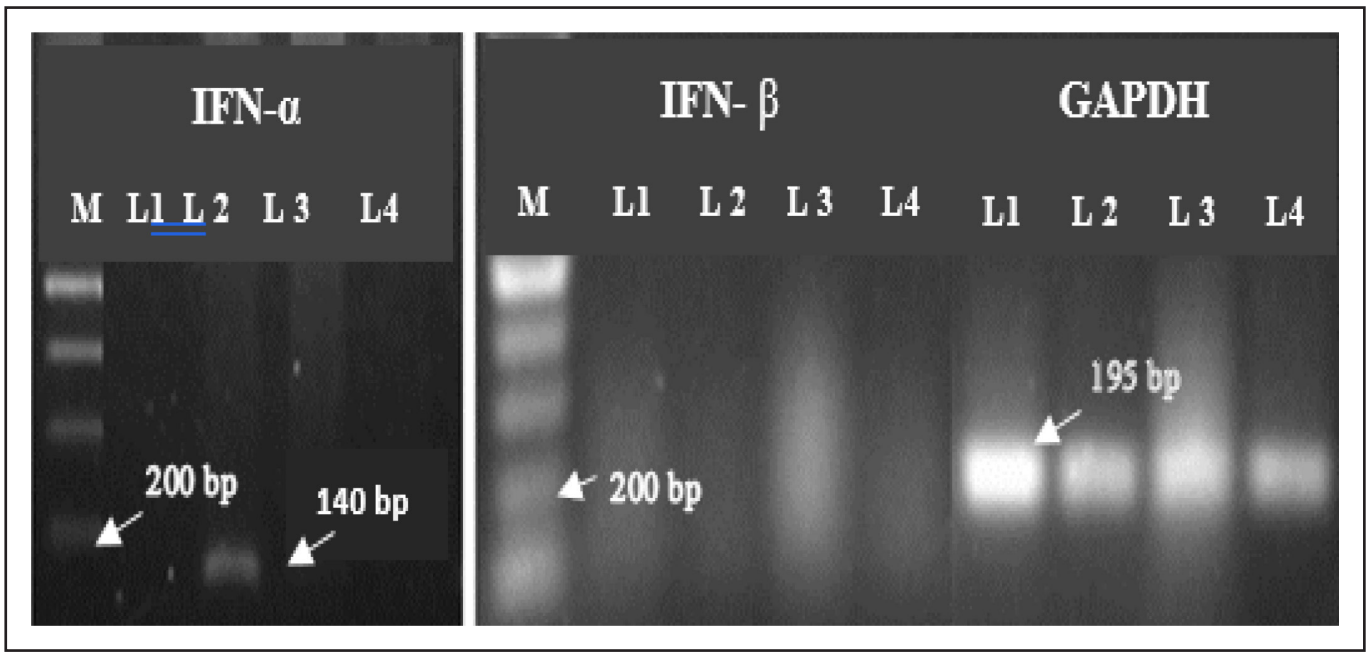

Figure 4. RT-PCR of IFN α, β and GADPH. M-100 bp DNA marker, L1-Uninfected GBM cell; L2-GBM cell infected with KFKS2_CS; L3- Uninfected Vero cell; L4-Vero cell infected with KFKS2_CS.

strong band signal in all the infected and uninfected cells (negative control) wells, indicating that the RNAs were prepared in the same concentrations and qualities in all the cells.

DISCUSSION

BLAST result of the partial N and NSs sequences obtained in this study confirmed the virus as KFKS virus with 100% nucleotide sequence similarity with previous KSKV1 virus detected in Malaysia (Palya & Banyai, 2018). Phylogenetic analysis in Figure 3 supported this finding which showed that it was in the same clade as the previous KFKS1 virus and clustered together with the UMB virus from India and Cristoli virus from France. These UMB and Cristoli viruses were shown to have the ability to infect humans and cause encephalitis in immunocompromised people (Rodriguez *et al*., 2020; Perot *et al*., 2021), which indicates that they are able to inhibit human IFN in human brain cells. This KFKS2_CS virus might also share the same ability as UMB and Cristoli viruses to act as an interferon antagonist and causes disease in human. The current result supported this hypothesis which showed that the KFKS2_CS virus was able to inhibit the synthesis of IFN-α in human glioblastoma GBM brain cells *in vitro* (Figure 4). IFN-β was not expressed in infected and non-infected GBM cells, which indicated that IFN-β is not functional in these cells. IFN-α and -β were not expressed in treated and untreated Vero cells, which were used as negative controls in this study. This is expected results since Vero cells are deficient in IFN production, causing the cells not able to secrete interferon during viral infection (Chew *et al*., 2009). Since the KFKS2_CS virus able to counteract the IFN α production in GBM cells, this indicates that it might have a zoonotic potential to humans. The ability of this KFKS2_CS virus to inhibit the IFN- α is in agreement with previous studies where members of orthobunyaviruses; Tacaiuma, Lac Crosse and Bunyamwera viruses were also able to inhibit the production of IFN- I (IFN-α/β) in the mammalian cells (Weber *et al*., 2002; Hart *et al*., 2009; Mohamed *et al*., 2009). Previous studies have shown that NSs protein of orthobunyaviruses play a vital role in the shut-off of the host cell protein synthesis of mammalian cells and induced the zoonotic potential of this virus (Hart *et al*., 2009; Mohamed *et al*., 2009; Leventhal *et al*., 2021) by targeting the components of RNA polymerase II (Thomas *et al*., 2004). Hence, further study is needed to determine the role of the NSs protein of the KFKS2_CS virus in shutting off protein synthesis of mammalian cells to get a clearer picture of the zoonotic potential of this novel virus.

CONCLUSION

KFKS2_CS virus was identified using RT-PCR and sequencing. It was closely related to previous KFKS virus from Malaysia (KFKS_MK047404), UMB (MK330174) and Cristoli (MN488996) viruses. This KFKS2_CS virus was able to inhibit the production of interferon-α in human glioblastoma brain cells, which indicates that it might possess a zoonotic potential similar to its closely related UMB virus that causes encephalitis in humans. However, further research such as a reverse genetic study on the NSs deleted mutant is needed to further confirm this statement.

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Conflict of interest

There is no conflict of interest for this manuscript and no informed consent is needed for this manuscript.

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