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Successful propagation of *Alkhumra* (misnamed as Alkhurma) virus in C6/36 mosquito cells

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ABSTRACT

Epidemiological data suggest that *Alkhumra* (misnamed as Alkhurma) virus (ALKV) is transmitted from livestock animals to humans by direct contact with animals or by the mosquito bites, but not by ticks. To assess the ability of the virus to replicate in mosquito cells, serum and plasma of seven acutely febrile patients with clinically suspected ALKV infection reported in Najran, Saudi Arabia in 2009 were inoculated onto *Aedes albopictus* mosquito cells (C6/36) and directly examined with ALKV-RNA-specific real time RT-PCR as well as indirect immunfluorescence assay (IFA) using ALKV-specific polyclonal antibodies. The isolated virus was titrated in the mammalian rhesus monkey kidney cells (LLC-MK2). Five of the seven specimens were RT-PCR- and culture-positive demonstrating cytopathic effects in the form of cell rounding and aggregation appearing on day 3 post inoculation with syncytia eventually appearing on day 8 post inoculation. Identification of ALKV-RNA in the cell culture was confirmed with RT-PCR and IFA. The virus titre was 3.2×10^6 tissue culture infective dose 50 ($TCID_{50}$) per mL. Three more viral passages were successfully made in the C6/36 cells. This is the first description of propagation of ALKV in mosquito cells.
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1. Introduction

Alkhumra virus (ALKV) infection is a new viral hemorrhagic fever (VHF) identified first in Saudi Arabia.[1–4] More recently, two unrelated travelers returning to Italy from southern Egypt were confirmed to have ALKV infection.[5] *Alkhumra* virus is a member of the tick-borne encephalitis (TBE) virus group in the genus *Flavivirus* of the family *Flaviviridae*. Even though some countries classify ALKV as a biosafety level (BSL) 4 agent, other countries, including Saudi Arabia, classify it as a BSL 3 agent.[6] The virus was first isolated in 1995 from six patients living in Alkhumra district in Jeddah, the main sea-port in the western border of Saudi Arabia.[1,2] In 2001–2003, Madani re-identified the disease and described 20 confirmed cases in the holy city of Makkah, 75 km from Alkhumra district in Jeddah, and proposed the name 'Alkhumra' be given to the virus after the geographic location from which it was originally isolated.[3] Unfortunately, Alkhumra virus was misnamed as 'Alkhurma' virus in many scientific publications due to a typographical error where the letters 'm' and 'r' were transpositioned.[3,4] From 2003 to 2007, eight confirmed cases of ALKV infections were

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sporadically reported from Najran in the southern border region of Saudi Arabia.[4] Subsequently, an outbreak of ALKV infection occurred in Najran in 2008-2009 with 70 confirmed cases reported by the authors.[4] In that outbreak, ALKV infection was confirmed mainly with reverse transcription polymerase chain reaction (RT-PCR).[4] In this study we describe the successful isolation and propagation of ALKV in C6/36 mosquito cells inoculated with sera taken from seven acutely febrile patients with clinically suspected ALKV infection during ALKV outbreak in Najran, Saudi Arabia.

2. Materials and methods

2.1. Patient population

The case definition previously developed by Madani was used to identify suspected cases of ALKV infection in Najran.[3] Blood samples were collected from seven acutely febrile patients with suspected ALKV infection in Najran from 18 March to 4 April 2009 when ALKV outbreak was initially recognized in this region. The blood samples were collected within 3–7 days after the onset of the illness when patients were still febrile. Details of this outbreak have been published by the authors.[4] Data of the patients presenting or referred to Najran hospitals with suspected ALKV infection were reviewed on admission, and data recorded on a standard case report form. Information collected included patients' demographics, risk factors for ALKV infection, clinical manifestations, laboratory results, complications and outcome.

2.2. Blood samples

Whole blood was separately collected in EDTA and plain vacutainers (BD Vacutainers®, Becton, Dickinson and Company, Plymouth, UK) from each of the seven patients. EDTA specimens were spun in a cooled centrifuge at 1500 rpm for 10 minutes and the plasma was collected and stored in 0.5 ml aliquots at −86 °C. The blood samples in the plain containers were left to clot and the serum was separated by low-speed cooled centrifugation and stored at −20 °C. The plasma and serum specimens were subsequently transported on dry ice in IATA-compliant containers from Najran to the Special Infectious Agents Unit, a BSL 3 virology laboratory, at King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia.

2.3. Detection of Alkhumra virus–ribonucleic acid by real time reverse transcriptase-polymerase chain reaction

2.3.1. Ribonucleic acid preparation

Human samples and cell culture supernatants were cleared by centrifugation in a tabletop centrifuge at 10 000 × g for 10 min. Viral RNA was extracted from 140 µl of the cell-free fluid using the QIAmp viral RNA kit (Qiagen, Hilden, Germany) without modification. RNA was eluted in 50 µl.

2.3.2. Primers and probe design

A pair of primers (ALKV S1: 5′ – GTG AGT GGC GCT TTG TTTG TA and ALKV R: 5′ – CCC CCT TTC CTT TAA GGA CG) and the corresponding 5′-nuclease detection probe (TBV TM: 6FAM-ACA GCT TAG GAG AAC AAG AGC TGG GGA XT—PH) were designed with Primer Express software (Applied Biosystems, Weiterstadt, Germany), and synthesized by Tib Molbiol (Berlin, Germany) based on ALKV sequence published by Charrel et al.[7] The 5′-nuclease probe was labelled with 6-carboxyfluorescein at the 5′ end and with 6-carboxy-N,N,N′,N′-tetramethylrhodamine at the 3′ end. The 3′ end of each probe was phosphorylated to prevent elongation during PCR as previously described.[8,9]

2.3.3. Real time reverse transcriptase-polymerase chain reaction conditions

The one-step real time RT-PCR system combining superscript reverse transcriptase with platinum Taq-polymerase (Life Technologies, Karlsruhe, Germany) was used in 5′-nuclease assay. The reaction mix contained 10 µl of master mix provided with the kit (including the basic level of MgSO4), 40 ng of bovine serum albumin (Sigma, Munich, Germany) per µl, and 2 µl of RNA. The 20-µl assay of the Light Cycler reaction capillary for ALKV RT-PCR with 5′-nuclease probe detection involved RT at 50 °C for 30 min, initial denaturation at 95 °C for 15 min, and 45 cycles at 95 °C for 1 second and then at 57 °C for 30 seconds.[6,7] Fluorescence was read at the combined annealing-extension step at 57 °C.

2.4. Cell culture

The widely used mosquito cell line, C6/36, derived from *Aedes albopictus* cells, was used to propagate the virus (kindly provided by Dr Matthias Niedrig from Robert Koch Institute, Berlin, Germany). Cells were grown in 25 cm^2 tissue culture flasks using Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% fetal bovine serum (FBS). The growth medium was decanted and 1 mL each of patients' sera and plasma diluted 1:10 in phosphate buffer saline (PBS) was added and incubated at 30 °C for 1 hour for adsorption. Maintenance medium, containing 2% FBS was then added. The flasks were observed daily under inverted microscope for cytopathic effect. Cells showing cytopathic effect were trypsined from the flasks, transferred to 15 ml tubes and centrifuged at 3000 rpm for 15 min. The supernatant was collected and tested with ALKV RT-PCR.

2.5. Virus titration

The isolated virus was titrated in the mammalian rhesus monkey kidney cells (LLC-MK2) cultured in DMEM supplemented with 10% FBS, penicillin (5 U/mL), and streptomycin (5 ug/mL). The cells were incubated at 37 °C in CO_2 atmosphere. The microtitration method was employed in 96-well cell culture microtitre plates as previously described.[10] The end point titre was calculated in tissue culture infective dose 50 ($TCID_{50}$) per mL as described by Reed and Muench.[11]

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2.6. Indirect immunfluorescence assay

Alkhumra virus infected C6/36 cells were deposited on Teflon coated 8-well slides. The slides were air dried inside a biosafety cabinet and fixed in chilled acetone/methanol (1:1) for 20 minutes. The wells were overlaid with 20 μl (1:200 dilution in PBS) of hyperimmune mouse ascitic fluid containing polyclonal antibodies against ALKV prepared using the same procedure previously described.[12] The slides were incubated in a moist chamber at 37 °C for 60 min before they were washed three times in PBS. The bound antibody was detected with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma, Chemicals Co.) with 0.2% Evans blue (Sigma Chemicals Co.). The slides were washed, mounted with Fluoprep (BioMerieux, Marcy L'Etoile, France) and finally, examined under a Leitz fluorescence microscope.

3. Results

3.1. Alkhumra virus–ribonucleic acid detection in patients' serum and plasma by reverse transcriptase-polymerase chain reaction

Of the seven specimens tested with ALKV RT-PCR, five specimens were positive for ALKV-RNA (Table 1). ALKV RT-PCR was positive both in plasma and serum in three patients (patients 1, 4, 5), in plasma alone in one patient (patient 2), and in serum alone in one patient (patient 3). Table 2 summarises the demographic, clinical, and laboratory characteristics of the five patients with detectable ALKV-RNA.

3.2. Alkhumra virus propagation in C6/36 cell culture

Inoculation of C6/36 cells with the serum and plasma of the five RNA-positive specimens successfully yielded virus isolates (Table 1). Cell rounding, aggregation, and degeneration were first noticed three days after inoculation of the C6/36 and gradually progressed forming multinucleated giant cells and syncytia of various sizes eight days after inoculation (Figure 1). The virus titre was 3.2×10^6 $TCID_{50}$ per mL and three more viral passages were successfully made in the C6/36 cells. ALKV-RNA was undetectable in the supernatant collected 3–7 days after inoculation of the C6/36 cells. However, on day 8 post inoculation, when the syncytia were evident, the supernatant was positive for ALKV-RNA. Figure 2 illustrates ALKV antigens in the

Table 1
Alkhumra virus RNA detection in serum and plasma and propagation in C6/36 cells for five infected patients from Najran, Saudi Arabia

Patient no.	Detection of *Alkhumra* virus RNA by RT-PCR		Propagation of *Alkhumra* virus in C6/36 cells	
	Plasma	Serum	Plasma	Serum
1	+	+	+	+
2	+	–	+	+
3	–	+	+	+
4	+	+	+	+
5	+	+	+	+

Table 2
Demographic, clinical, and laboratory characteristics at presentation of the five patients from whom *Alkhumra* virus was isolated

Patients	Age (y) sex, nationality	Occupation	Duration of illness (d)	Clinical features	Leukocyte count ($\times 10^9$/L)	Platelet count ($\times 10^9$/L)	PTT (s)	AST (U/L)	AlT (U/L)	LDH (U/L)	CK (U/L)
1	21 M Saudi	Student	5	Fever, malaise, headache, anorexia, nausea, vomiting, altered sensorium, convulsions, neck stiffness	2.38	61	51.5	33	60	222	163
2	57 M Saudi	Livestock trader	6	Fever, chills, malaise, headache, anorexia, abdominal pain, diarrhea, myalgia, arthralgia, backache, gingival bleeding, altered sensorium	3.30	45	49	857	516	1252	2346
3	33 M Saudi	Self-employed	7	Fever, malaise, headache, anorexia, nausea, vomiting, myalgia, arthralgia	2.50	147	43.0	152	80	1067	1186
4	23 M Saudi	Self-employed	4	Fever, malaise, headache, abdominal pain, diarrhea, myalgia, arthralgia, backache, epistaxis, altered sensorium	1.60	197	40.0	51	26	1153	1340
5	22 F Yemen	Housewife	3	Fever, malaise, headache, anorexia, nausea, vomiting, abdominal pain, diarrhea, myalgia, arthralgia, backache, altered sensorium	1.00	55	47	947	472	1136	1479

AlT: Alanine transferase; AST: Aspartate transferase; CK: Creatine phosphokinase; LDH: Lactate dehydrogenase; PTT: partial thromboplastin time.

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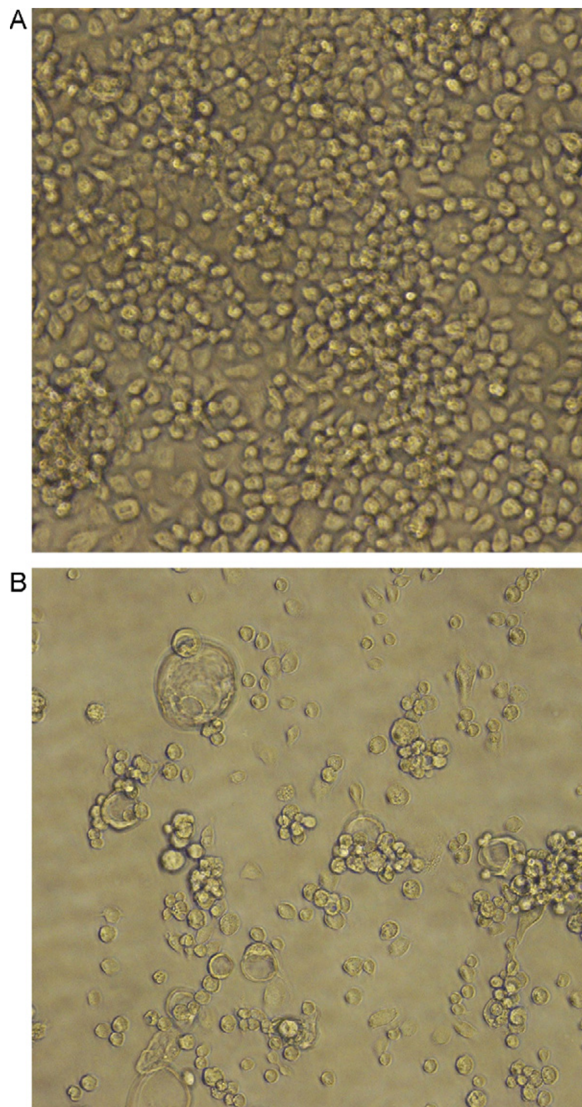

Figure 1. A: C6/36 mosquito cell line before inoculation with ALKV-RNA-positive serum (magnification 4x).
B: C6/36 demonstrating cell aggregation, cellular damage and degeneration, formation of syncytia and multinucleated giant cells 8 days after inoculation with ALKV-RNA-positive serum (magnification 20x).

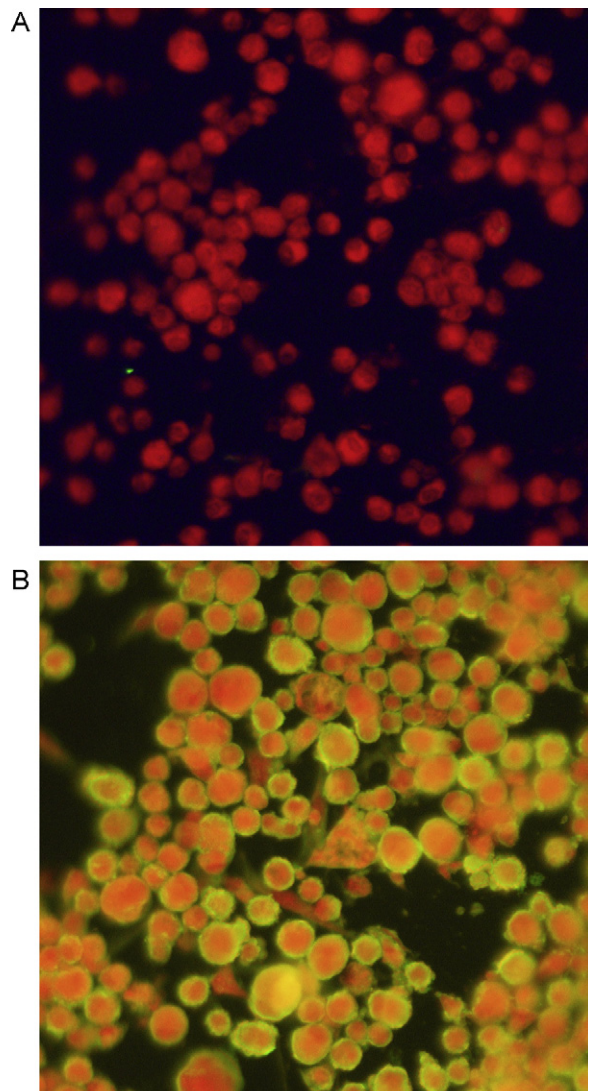

Figure 2. A: Non-infected (control) C6/36 cells (magnification 40x).
B: Indirect immunofluorescent antibody assay with ALKV-specific polyclonal antibodies prepared as hyperimmune mouse ascitic fluid diluted 1:200 in phosphate buffer saline, staining ALKV antigens in C6/36 tissue culture cells 8 days after inoculation (magnification 40x).

cytoplasm of C6/36 infected cells on day 8 after staining with ALKV-specific polyclonal antibodies using indirect IFA.

4. Discussion

Aedes albopictus clone C6/36 cell line is useful for the replication of flaviviruses and can be used to propagate dengue viruses to high titres.[13–17] The cells are non-anchorage dependent, non-tumorigenic, and maintain a diploid chromosome number.[13–17] In this study, ALKV was successfully propagated to high titres in C6/36 cells. Release of ALKV virus in the supernatant of infected cells was detected by RT-PCR only when syncytia formation was evident on the eighth day after inoculation. Since ALKV in this study was a wild virus from clinical specimens, delayed or absent cytopathic effect and perhaps also failure to yield positive RNA in the supernatant in the first passage were not totally unexpected. Generally speaking, isolation attempts of a wild field virus in cell culture may require several blind passages.[18–20] Culture of ALKV in C6/36 mosquito cells was more sensitive than viral RNA detection by RT-PCR in either plasma or serum. The plasma and serum of two culture-positive patients (patients 2 and 3) gave negative results when tested with RT-PCR, respectively, whereas the remaining three culture-positive specimens were also positive by RT-PCR. Thus, the sensitivity of RT-PCR in either plasma or serum compared to culture in C6/36 cells was 80%. Our results stand in contrast to what was previously reported by Zaki

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that this new flavivirus failed to grow in C6/36 cell culture either directly from patients' blood or brain suspension of experimentally infected mice.[2]

Due to the close phylogenetic similarity between ALKV and Kyasanur forest disease virus, it could be argued that ticks may play an important role in the transmission cycle of ALKV.[21] This argument is further supported by the PCR-based detection of a virus closely related to ALKV from one *Ornithodoros savignyi* (sand tampan) tick out of 124 ticks collected from camels and camel resting places in Jeddah, Saudi Arabia.[22] However, epidemiological information available thus far suggests that the virus is transmitted to humans from livestock animals (sheep, goats or camels) by direct contact or by mosquito bites, and that ticks do not seem to play an important role in the transmission; however, their role as a reservoir of the virus in its ecologic niche is conceivably possible.[3,4]

The genus *Flavivirus* consists of more than 70 virus species and subtypes, the majority of which are transmitted by mosquitoes or ticks, although some have no known vector. The ability of these viruses to infect cultured cells derived from mosquito or tick species offers a useful insight into the suitability of such vectors to harbour and replicate particular viruses but does not confirm that these viruses can be carried or transmitted by a specific arthropod. Whilst the mosquito cell line C6/36 is highly susceptible to infection with mosquito-borne flaviviruses such as yellow fever and dengue viruses, these cells are not infectable with several tick-borne viruses, or with the no-known-vector viruses Apoi or Modoc virus.[23–25]

To our knowledge, none of the arthropod-borne flaviviruses has been shown to be dually vectored by both mosquitoes and ticks. Further, none of the TBE viruses has been shown to be transmitted by mosquitoes. One possible exception to these observations is ALKV, where epidemiological data support transmission by mosquitoes, albeit, no biological experiments have been published to confirm that this virus can be carried and transmitted by ticks and/or mosquitoes. However, our current study showing the ability of ALKV to grow in C6/36 mosquito cells as well as other studies assessing the ability of other arthropod-borne viruses to replicate in mosquito and tick-derived cell lines provide preliminary support to our hypothesis that ALKV could be mosquito-borne.[24] For example, in a comparative study of the susceptibility of mammalian Vero cells, C6/36 mosquito cell line, and cell lines derived from the ticks *Ixodes ricinus* (L.) (IRE/CTVM18), *I. scapularis* (Say) (ISE6), *Rhipicephalus appendiculatus* (Neumann) (RAE/CTVM1) and *Amblyomma variegatum* (Fabricius) (AVL/CTVM17) to infection with 13 flaviviruses and one alphavirus (Venezuelan equine encephalitis virus; VEEV) using immunofluorescence microscopy and plaque assay techniques, the C6/36 mosquito cell line was infected by all the mosquito-borne flaviviruses tested but not by no-known-vector or tick-borne viruses, with the exception of *Langat* virus. The four tick cell lines were susceptible to infection by all of the tick-borne viruses tested, as well as two mosquito-borne viruses, West Nile virus (WNV) and VEEV, but not other mosquito-borne or no-known-vector viruses.[24]

The presumed mosquito-borne nature of ALKV is also supported by the fact that several mosquito-borne flaviviruses have been isolated from ticks, e.g., Saint Louis encephalitis virus and WNV; yet, ticks were not confirmed to be efficient biological vectors of such infections.[26,27] Additionally, even though most ALKV-closely related viruses are known to be associated with ticks, there is evidence that TBE and *Powassan* viruses are also found in different species of mosquitoes supporting the hypothesis that mosquitoes may well be mechanical or even biological vectors of such tick-associated viruses.[28–33] *Aedes albopictus*, the mosquito species from which C6/36 cell line is derived, exists in ALKV-endemic areas in Saudi Arabia, namely Jeddah, Makkah and Najran. Interestingly, these same areas are also endemic for dengue virus which is transmitted by *Aedes* species (*A. aegypti*, and *A. albopictus*).

In conclusion, this is the first report of successful propagation of ALKV in C6/36 mosquito cells to a high titre with sensitivity higher than viral RNA detection by RT-PCR in either plasma or serum. This may support the possibility of a mosquito-borne nature of transmission of this virus from animals to humans as suggested by previous epidemiological data. However, more biological studies are needed to confirm that mosquitoes are a vector of this presumably tick-borne virus.

Authors' contributions: TAM conceived and designed the study and clinically assessed the patients; TAM, EIA and EMEA collected the samples; MK performed the viral isolation on C6/36 mosquito cell line and the indirect IFA; EIA performed the real time RT-PCR; EMEA analysed the data; MK, EIA, EMEA, HMA, HA and TGK interpreted the data; TAM, MK, EIA and EMEA wrote the manuscript; EMEA, HMA, HA and TGK critically revised the manuscript. All authors read and approved the final version. TAM is guarantor of the paper.

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