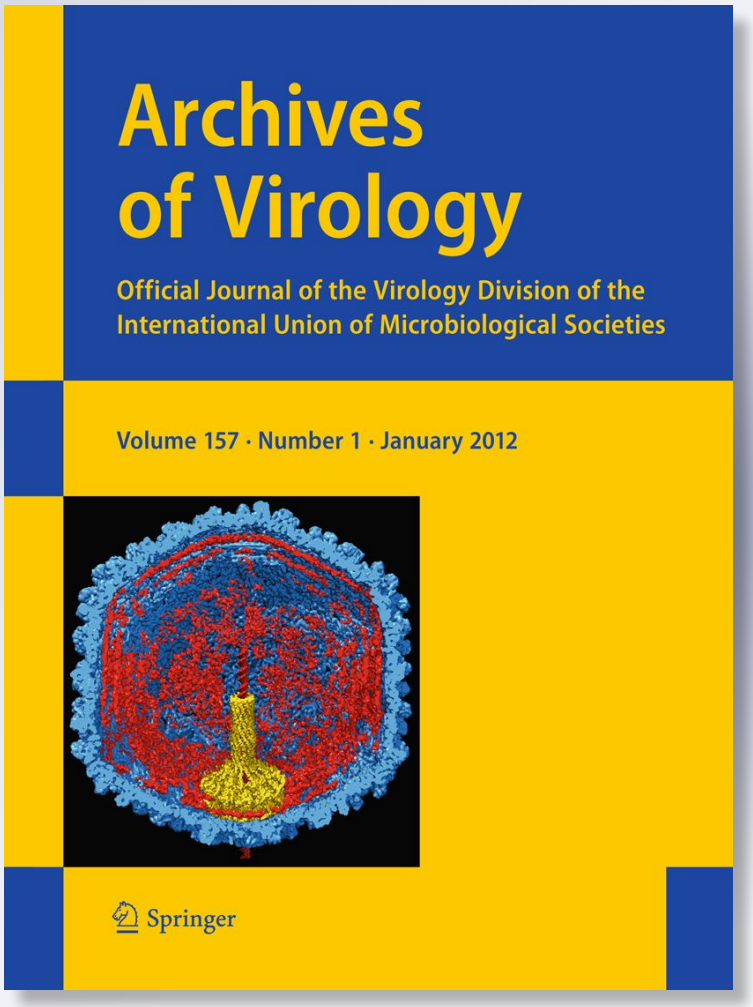
Superiority of the buffy coat over serum or plasma for the detection of Alkhumra virus RNA using real time RT-PCR

Tariq A. Madani, El-Tayeb M. E. Abuelzein, Esam I. Azhar, Moujahed Kao, Hussein M. S. Al-Bar, Huda Abu-Araki & Thomas G. Ksiazek

Archives of Virology
Official Journal of the Virology Division of the International Union of Microbiological Societies

ISSN 0304-8608

Arch Virol
DOI 10.1007/s00705-012-1237-7



Springer

Arch Virol
DOI 10.1007/s00705-012-1237-7

ORIGINAL ARTICLE

Superiority of the buffy coat over serum or plasma for the detection of Alkhumra virus RNA using real time RT-PCR

Tariq A. Madani · El-Tayeb M. E. Abuelzein · Esam I. Azhar · Moujahed Kao · Hussein M. S. Al-Bar · Huda Abu-Araki · Thomas G. Ksiazek

Received: 24 August 2011 / Accepted: 16 October 2011

Abstract RT-PCR to detect Alkhumra virus (ALKV) RNA in plasma or serum has been the standard practice to confirm this infection in the first seven days of illness. In this study, RT-PCR detection of viral RNA from the plasma, serum, and buffy coat (BC) was compared to virus isolation. Plasma, serum, and BC were obtained from seven patients with clinically suspected ALKV infection in Najran, Saudi Arabia. Baby hamster kidney (BHK-21) and rhesus monkey kidney (LLC-MK2) cell culture monolayers were used for virus isolation. Real-time RT-PCR was used to confirm ALKV infection and to detect viral RNA directly from plasma, serum, and BC. ALKV was isolated from five of the seven patients. The virus was isolated from all three specimen types (plasma, serum, and BC) of the five confirmed patients. ALKV RNA was detected directly by RT-PCR in BC in all five (100%) culture-positive patients and in plasma or serum in only four (80%) of the five patients. Three of the five patients for whom ALKV RNA was detected in BC also had detectable viral RNA in plasma and serum. In the remaining two patients with detectable ALKV RNA in the BC, the plasma was positive but the serum was negative in one patient, whereas the serum was positive and the plasma was negative in the other patient. The use of real-time RT-PCR to detect ALKV RNA in the BC was superior to using plasma and serum and equivalent to virus isolation.

T. A. Madani (✉)
Department of Medicine, Faculty of Medicine,
King Abdulaziz University, P.O. Box 80215,
Jeddah 21589, Saudi Arabia
e-mail: tmadani@kau.edu.sa

E.-T. M. E. Abuelzein · E. I. Azhar · M. Kao
Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

E. I. Azhar
Department of Medical Laboratory Technology,
Faculty of Applied Medical Sciences,
King Abdulaziz University, Jeddah, Saudi Arabia

H. M. S. Al-Bar
Department of Family and Community Medicine,
Faculty of Medicine, King Abdulaziz University,
Jeddah, Saudi Arabia

H. Abu-Araki
Laboratory Animals Unit, King Fahd Medical Research Center,
King Abdulaziz University, Jeddah, Saudi Arabia

T. G. Ksiazek
Galveston National Laboratory, Departments of Pathology and Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA

Introduction

Alkhumra virus (ALKV) is a new flavivirus that was originally reported from Saudi Arabia. It was first isolated in 1995 from six patients living in the Alkhumra district, located in the seaport Jeddah [1]. In 2001-2003, Madani re-identified the disease and described 20 confirmed patients in the holy city of Makkah, 75 kilometers from Alkhumra, and proposed the name "Alkhumra" be given to the virus after the geographic location from which it was originally isolated [2]. From 2003 to 2007, patients were sporadically reported from Najran, a region in the south of Saudi Arabia [3]. Since 2008, there has been a sharp increase in the number of reported patients from the Najran region, and the outbreak is currently still active [3]. We have recently published the results of outbreak investigations and the epidemiological, clinical, and

Published online: 01 February 2012

laboratory characteristics of the disease in Najran [3]. More recently, two travelers returning to Italy from southern Egypt were hospitalized with fever of unknown origin confirmed to be due to infection with ALKV [4]. Epidemiological data suggest that ALKV is transmitted to humans from livestock animals (sheep, goats, or camels) by direct contact with these animals or by mosquito bites [2, 3]. However, biological studies are still needed to confirm that mosquitoes are true vectors of this virus. Despite the close phylogenetic similarity between ALKV and Kyasanur Forest disease (KFD) virus, epidemiological data suggest that ticks do not seem to play an important role in the transmission of ALKV from animals to humans, although their role as a reservoir of the virus in its ecologic niche and as vectors transmitting the virus among animals is conceivably possible [2, 3].

Rapid laboratory diagnosis of ALKV depends mainly on reverse transcriptase polymerase chain reaction (RT-PCR) to detect viral RNA in the plasma and/or serum during the viremia that occurs in the acute febrile phase of the illness. Viral culture is not routinely performed because the virus is classified as a biosafety level 3 or 4 agent. In the outbreak that occurred in Najran, out of 148 patients with clinically suspected ALKV infection who were tested by RT-PCR, only 70 (47.3%) patients were positive for ALKV RNA [3]. Some of the possible reasons to explain the relatively low positivity of the RT-PCR among patients fulfilling the clinical case definition of ALKV infection include deterioration of viral RNA during collection and transportation of specimens to the reference laboratory in Jeddah, 700 kilometers from the epicenter in Najran, as well as variations in the virus content of serum, plasma, and buffy coat (BC) specimens. Since, we were able to isolate ALKV from five of seven patients at the outset of the epidemic in Najran, in this study, we compared the sensitivity of real-time RT-PCR for detection of ALKV RNA in BC, plasma, and serum with viral culture.

Materials and methods

Patient population

Blood samples were collected from seven acutely febrile patients with suspected viral hemorrhagic fever in Najran from March 18th to April 4th, 2009, when an 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. Patients presenting or referred to Najran hospitals with suspected ALKV infection were reviewed on admission, and data were recorded on a standard case report form. Information collected included patient demographics, risk factors for ALKV infection, clinical manifestations, clinical laboratory results, complications, and outcome.

Blood samples

Whole blood was collected separately in EDTA (ethylenediaminetetraacetic acid), and in plain vacutainers from each of the seven patients. EDTA specimens were spun in a cooled centrifuge at 1500 rpm for 10 minutes, and the plasma and BC were 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, BC, and serum specimens were subsequently transported on dry ice in IATA-compliant transport containers from Najran to the Special Infectious Agents Unit, a biosafety level 3 virology laboratory, at King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia.

Detection of ALKV RNA by real-time RT-PCR

RNA preparation

Viral RNA was extracted from plasma, serum, and BC and eluted in 50 µl using a QIAmp viral RNA Kit (QIAGEN, Hilden, Germany) without modification.

Primers and probe design

A pair of primers (ALKV S1: 5′ – GTGAGTGGCCGCTTT GTTTG TA and ALKV R: 5′ – CCCCCTTTCCTTTAAG GACG) and the corresponding 5′-nuclease detection probe (TBV TM: 6FAM-ACAGCTTAGGAGAACAAGAGCTG GGGAXT-PH) were designed with Primer Express software (Applied Biosystems, Weiterstadt, Germany) and synthesized by TIB-MOLBIOL, Berlin, Germany, based on the ALKV sequence published by Charrel et al. [5]. 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 described previously [6, 7].

Real-time RT-PCR 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 a 5′-nuclease assay. The reaction mix contained 10 µl of master mix provided with the kit (including the basic level of $MgSO_4$), 40 ng of bovine serum albumin (Sigma, Munich, Germany) per µl, and 2 µl of RNA. The 20-µl assay was

Table 1 Alkhumra virus RNA detection in the buffy coat, plasma, and serum of five patients with culture-proven infection using real-time RT-PCR and the corresponding cycle threshold (Ct)*

Patients	Buffy coat		Plasma		Serum	
	Result	Ct	Result	Ct	Result	Ct
1	Positive	30.67	Positive	31.07	Positive	30.6
2	Positive	32.77	Positive	38.2	Negative	–
3	Positive	37.71	Negative	–	Positive	37.34
4	Positive	36.1	Positive	37.23	Positive	36.28
5	Positive	31.25	Positive	37.39	Positive	36.14
% Positive	100%	Mean Ct 33.7	80%	Mean Ct 35.97	80%	Mean Ct 35.09

* The cutoff Ct value for a sample to be considered positive is <42 cycles

carried out in a Light Cycler reaction capillary for ALKV RT-PCR, with 5′-nuclease probe detection, using reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min, and 45 cycles at 95°C for 1 second followed by 57°C for 30 seconds [5, 6]. Fluorescence was read at each of the combined annealing-extension steps at 57°C.

Real-time RT-PCR for dengue and Rift Valley fever (RVF) viruses

The same samples were also tested for dengue and RVF viral RNA using real-time RT-PCR. The dengue and RVF viral RNA was amplified using oligonucleotide primers and probes as described by Drosten et al. [8]. A QIAGEN Quanti Test Probe RT-PCR Kit (QIAGEN, Hilden, Germany) was used for the real-time RT-PCR.

Virus isolation in cell culture

Baby hamster kidney (BHK-21) and rhesus monkey kidney (LLC-MK2) cell culture monolayers were grown in 15-ml cell culture tubes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (5 U/ml) and streptomycin (5 µg/ml). The cultures were incubated at 37°C in a 5% CO_2 atmosphere until they were 80% confluent.

Each sample of the BC, plasma and sera from the patients was diluted 1:10 in DMEM without FCS, and 0.1 ml was inoculated onto each cell culture tube after decanting of the medium. These were then incubated for 1 hour at 37°C to allow virus adsorption to occur. One ml of DMEM with 2% FCS was then added to each tube and incubated at 37°C. The monolayers were examined daily with an inverted microscope for the presence of discernible cytopathic effect (CPE). Cell monolayers showing 80% CPE were frozen at −86°C and subsequently thawed. The freezing and thawing process was repeated two more times. The cultures were subsequently centrifuged at 3000 rpm for 15 min, and the supernatant was collected and tested using the ALKV RT-PCR assay.

Results

Of the seven patients tested, five patients were confirmed to have ALKV infection by virus isolation and RNA detection in the plasma, serum, or BC. ALKV was isolated from plasma, serum, and BC of the five confirmed patients. Discernible CPE was observed in both BHK-21 and LLC-MK2 cell culture types from the third day following inoculation. The CPE was characterized by cell rounding and shrinkage of cells, which became refractile and eventually detached from the surface of the tubes. ALKV RNA was detected directly by RT-PCR in BC in all five (100%) culture-positive patients, and in plasma or serum of four (80%) of the five patients. All of the specimens from the seven patients were negative for dengue and RVF viral RNA. Table 1 shows the RT-PCR results obtained with the BC, plasma and serum samples from the five patients with culture-proven ALKV infection and the cycle threshold (Ct) value, representing the cycle at which the threshold of the real-time PCR was exceeded. The cutoff Ct value for a sample to be considered positive was less than 42 cycles. The RT-PCR assay was repeated five times, with the same results each time. Concordance between plasma and serum was 80% (ALKV RNA was detected in plasma but not in serum for patient number 2, whereas it was detected in serum but not plasma for patient number 3). The Ct in all specimen types was similar for patients 1, 3, and 4. However, the Ct for patient number 2 was lower with the BC (32.77) than with the plasma (38.2). Similarly, the Ct for patient number 5 was less in BC (31.25) than the plasma (37.39) and serum (36.14). Table 2 shows the demographic, clinical, and laboratory characteristics of the five patients with confirmed ALKV infection.

Discussion

In this study, ALKV was successfully isolated in BHK-21 and LLC-MK2 cell lines. However, the use of cell culture to confirm ALKV in clinical practice is very limited because the virus is classified as a biosafety level 3 or 4

Table 2 Demographic, clinical, and laboratory characteristics of the five patients with confirmed Alkhumra virus infection

Patient	Age (y), sex, nationality	Occupation	Duration of illness (d)	Clinical features	Laboratory results*						
					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, convulsion, 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

* Normal range: leukocyte count: 3.8-10.8 $\times$ 10^9/L; platelet count: 130-400 $\times$ 10^9/L; PTT: 25-35 seconds; AsT: 0-35 U/L; AlT: 0-35 U/L; LDH: 100-250 U/L; CK: 10-200 U/L

agent and considered to be one of the most dangerous hemorrhagic fever viruses, which is associated with a high case fatality rate that has been as high as 25%. It is thus crucial to have a safe and rapid diagnostic test for such a dangerous virus. It is also necessary to utilize the most appropriate samples that can give the most sensitive results in rapid diagnostic tests. To date, the safest and most reliable test for rapid diagnosis of ALKV is the RT-PCR, which utilizes plasma and serum from acute cases as the samples of choice. Although plasma and serum have served as the day-to-day diagnostic samples for ALKV, BC has neither been evaluated nor compared with plasma or serum for the detection of ALKV by RT-PCR [2–4].

The buffy coat contains the blood leukocytes. Functionally, these leukocytes are highly involved in viral infections and include antigen-presenting cells (APCs), macrophages, killer cells (antibody-dependent cytotoxic cells; ADCC), and natural killer cells. In many instances, viral tropism is directed toward these leukocytes, and thus they are often rich in virus content. For instance, dengue virus, another member of the genus *Flavivirus*, has been found to be highly associated with blood leukocytes [9, 10].

In our present study, BC specimens were utilized for the rapid detection of ALKV RNA using RT-PCR. The results confirmed that the rate of detection of viral RNA in BC was equivalent to virus isolation. Detection of viral RNA in BC was more efficient than in plasma or serum when these two types of specimens were used alone but equivalent when they were used in combination. When plasma or serum was used alone, one (20%) of the five ALKV-infected patients was missed. Since Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct level, the greater the amount of target nucleic acid in the sample), the finding that the Ct in the BC was lower than in the plasma and serum in two of the five patients also supports the greater sensitivity of BC over plasma and serum for the detection of ALKV RNA. Since previous studies utilized plasma or serum for the detection of the ALKV RNA and confirmation of the diagnosis, the actual number of confirmed cases could have been at least

20% higher than the reported number. The better yield of the BC was also described for dengue virus, where the BC was also found to be superior to the plasma and sera when the RT-PCR test was used for detection of dengue virus RNA [10, 11]. The BC is now routinely used in our laboratory for the differential diagnosis of suspected viral hemorrhagic fevers, including ALKV.

In conclusion, although the number of patients in this series is small, it appears that the use of real-time RT-PCR to detect ALKV RNA in the BC is equivalent in sensitivity to virus isolation and that BC offers greater sensitivity than plasma and serum for the detection of ALKV RNA.

Acknowledgments We thank Sheikh Mohammad Hussein Al-Amoudi for funding this research and the Scientific Chair for Viral Haemorrhagic Fever at King Abdulaziz University, Jeddah, Saudi Arabia. We thank the technologists Mr. Azad Godus, Mr. Badr Masri, and Mr. Raed Budaira for performing the real-time RT-PCR. The sponsor, Sheikh Mohammad Hussein Alamoudi, had no involvement in the study design, in the collection, analysis and interpretation of data, in the writing of the manuscript, or in the decision to submit the manuscript for publication.

Conflict of interest The authors declare that they have no competing interests.

References

1. Qattan I, Akbar N, Afif H, Abu Azmah S, Al-Khateeb T, Zaki A et al (1996) A novel flavivirus: Makkah Region 1994–1996. Saudi Epidemiol Bulletin 3(1):1–3 ISSN:1319-3965
2. Madani TA (2005) Alkhumra virus infection, a new viral hemorrhagic fever in Saudi Arabia. J Infect 51(2):91–97
3. Madani TA, Azhar EI, Abuelzein EME, Kao M, Al-Bar HMS, Abu-Araki H et al (2011) Alkhumra (Alkhurma) virus outbreak in Najran, Saudi Arabia: epidemiological, clinical, and laboratory characteristics. J Infect 62(1):67–76
4. Carletti F, Castilletti C, Di Caro A, Capobianchi MR, Nisii C, Suter F et al (2010) Alkhurma hemorrhagic fever in travelers returning from Egypt, 2010. Emerg Infect Dis 16(12):1979–1982
5. Charrel RN, Zaki AM, Fakeeh M, Yousef AI, de Chesse R, Attoui H et al (2005) Low diversity of Alkhurma hemorrhagic fever virus, Saudi Arabia, 1994–1999. Emerg Infect Dis 11:683–688
6. Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5′-3′exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc Natl Acad Sci USA 88:7276–7280
7. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 4:357–362
8. Drosten C, Göttig S, Schilling S, Asper M, Panning M, Schmitz H et al (2002) Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-RT-PCR. J Clin Microbiol 40(7):2323–2330
9. Wang HL, Lin KH, Yueh YY, Chow L, Wu YC, Chen HY et al (2000) Efficient diagnosis of dengue infections using patients peripheral blood leukocytes and serum/plasma. Intervirol 43:107–111
10. Scott RM, Nisalak A, Cheamudon U, Seridhoranakul S, Nimmannitya S (1980) Isolation of dengue viruses from peripheral blood leukocytes of patients with haemorrhagic fever. J Infect Dis 141:1–6
11. Klungthong C, Gibbson RV, Thaisomboonsuk B, Nisalak A, Kalayanarooj S, Thirawuth V et al (2007) Dengue virus detection using whole blood for reverse transcriptase PCR and virus isolation. J Clinic Microbiol 45(8):2480–2485