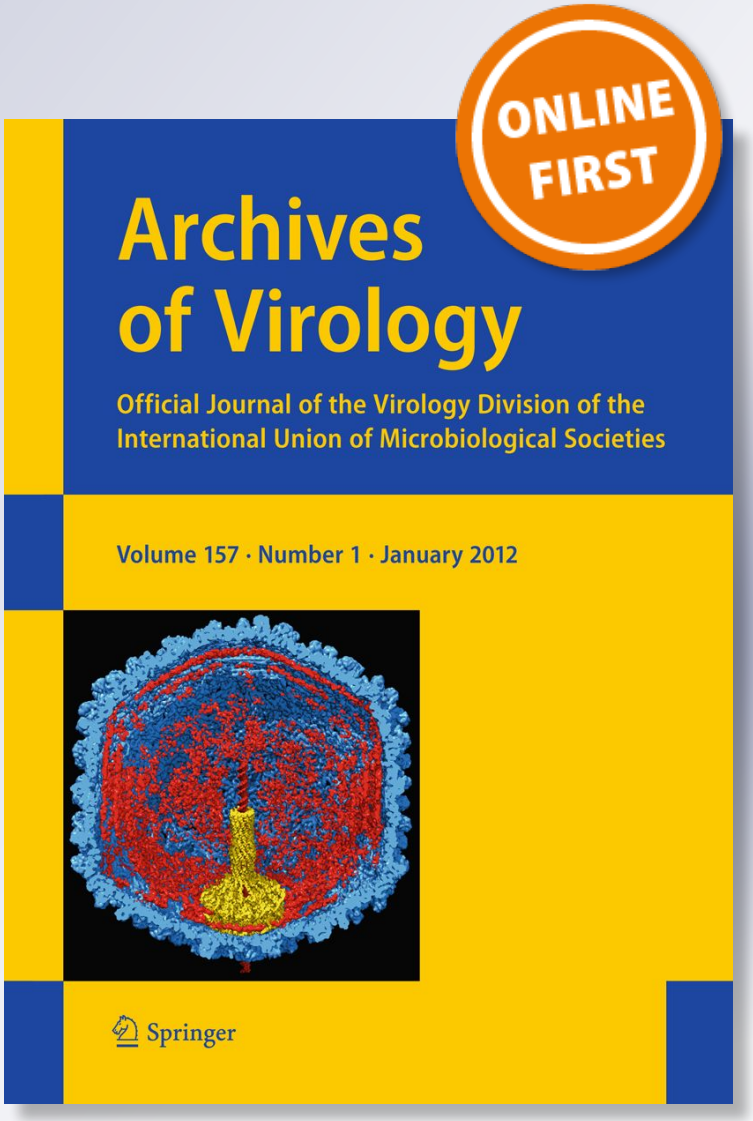
Trypsin-dependent hemagglutination of erythrocytes of a variety of mammalian and avian species by Alkhumra hemorrhagic fever virus

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

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

ISSN 0304-8608

Arch Virol
DOI 10.1007/s00705-012-1469-6



Springer

Arch Virol
DOI 10.1007/s00705-012-1469-6

ORIGINAL ARTICLE

Trypsin-dependent hemagglutination of erythrocytes of a variety of mammalian and avian species by Alkhumra hemorrhagic fever virus

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

Received: 16 June 2012 / Accepted: 30 July 2012

Abstract Alkhumra hemorrhagic fever virus (AHFV) is an emerging flavivirus that was discovered in 1994-1995 in Saudi Arabia. Clinical manifestations of AHFV infection include hemorrhagic fever, hepatitis, and encephalitis, with a reported mortality rate as high as 25 %. Biological characteristics of this virus have not been well defined. Agglutination of erythrocytes (hemagglutination) is a laboratory tool for studying the attachment of viruses to cellular receptors. The envelope protein contains sites for attachment to host receptors to initiate the process of infection and is thus an essential component of the virion. In the present study, we examined the ability of AHFV to agglutinate erythrocytes of 13 mammalian and avian species (human group O+, camel, cow, sheep, goat, rabbit, guinea pig, mouse, rat, chicken, duck, goose and turkey) with and without trypsin-treatment. Without trypsin treatment, AHFV failed to agglutinate erythrocytes of all examined species. Following trypsin treatment, AHFV agglutinated erythrocytes of five species, namely, goose, human group O+, rat, guinea pig, and mouse, in descending order of sensitivity. This trypsin-dependent hemagglutination test has potential for use in serological and functional studies of AHFV.

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

E.-T. M. E. Abuelzein
Scientific Chair of Sheikh Mohammad Hussein Alamoudi for Viral Hemorrhagic Fever, King Abdulaziz University, Jeddah, Saudi Arabia

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

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

E. I. Azhar
Department of Medical Laboratory Technology, Faculty of Applied Medical Science, 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

Introduction

AHFV is an emerging flavivirus that was first isolated in 1995 from six patients living in the Alkhumra district in Jeddah, the main seaport on the western coast of Saudi Arabia [1]. Between 2001 and 2003, 20 confirmed cases of AHFV infection were identified in the holy city of Makkah, 75 km from the Alkhumra district in Jeddah [2]. The name 'Alkhumra' was then proposed for the virus, after the geographic location from which it was originally isolated [2]. Unfortunately, Alkhumra virus was misnamed as 'Alkhurma' virus in many scientific publications due to a typographical error where the letters 'm' and 'r' were transposed [2–4]. The International Committee on Taxonomy of Viruses (ICTV) has recently corrected this mistake and approved the name "Alkhumra" as the correct name of the virus [5]. From 2003 to 2007, eight confirmed cases of AHFV infection were sporadically reported from Najran in the southern border region of Saudi Arabia [3]. Subsequently, an outbreak of AHFV infection occurred in Najran in 2008-2009, with 70 confirmed cases reported by the authors [3]. More recently, two unrelated travelers

Published online: 15 September 2012

returning to Italy from southern Egypt were confirmed to have AHFV infection [6].

AHFV was identified as a flavivirus on the basis of an immunofluorescence assay performed with the flavivirus-specific monoclonal antibody 4G2 and IgM capture ELISA using the peroxidase-conjugated flavivirus-specific monoclonal antibody 6B6C-1 [7]. Polymerase chain reaction (PCR) amplification of a 220-bp genome fragment showed 89 % nucleotide sequence homology with the non-structural gene 5 (NS5) of Kyasanur Forest disease virus (KFDV) [7]. The complete coding sequence of AHFV was determined and compared with other tick-borne flaviviruses, confirming that AHFV was most closely related to KFDV [8]. Genetic distances suggested that AHFV was a subtype of KFDV [8]. However, the epidemiological features and the mode of transmission of AFHV appear to be distinct from those of KFDV [2, 3, 9]. For example, AHFV is clearly associated with livestock animals and has not been associated with monkeys, porcupines, rats, squirrels, mice or shrews, which are thought to be associated with KFDV [2, 3]. Another important distinction is the possibility of transmission of AHFV by direct contact with livestock animals and/or by mosquito bites and not by ticks as typically described for KFDV [2, 3, 9].

The virological characteristics of this new virus remain to be elucidated. Agglutination of erythrocytes, known as hemagglutination (HA), is a laboratory tool for studying the attachment of viruses to cellular receptors. The envelope protein contains the sites for attachment to host cells receptors to initiate the process of infection. The possession of this envelope protein is thus a vital property of the virion. Historically, that property has been excessively utilized in HA and HA inhibition (HAI) tests in virus diagnosis and research. In general, not all viruses have the ability to agglutinate mammalian or avian erythrocytes. The agglutination ability of newly isolated viruses is frequently examined against erythrocytes of a spectrum of mammalian and avian species. In the present study, the agglutination ability of AHFV has been examined against erythrocytes of 13 mammalian or avian species, with and without trypsin treatment.

Materials and methods

The virus

The AHFV isolate (AHFV/997/Ng/09/SA) used in the HA tests was originally isolated from a patient during an AHFV outbreak in the Najran district in southern Saudi Arabia [3]. The virus was inoculated in an LLC-MK2 monkey cell line as described previously [10]. Seven days post-inoculation, the cytopathogenic effect (CPE) was complete, and the supernatant medium was collected and clarified by low-speed centrifugation. Three non-inactivated virus samples (A, B and C) were tested in the HA experiments. All experiments were performed under biosafety level 3 (BSL-3) containment.

Virus titration

The micro system for titration was followed employing 96-well tissue culture microplates. The virus suspension to be titrated was diluted in Eagle's minimum essential medium (EMEM) supplemented with 2 % fetal calf serum (FCS). To each well of the microtitre plate, 50 µl of EMEM, supplemented with 2 % FCS, was added. A ten-fold dilution series of the virus suspension was made in sterile vials, changing the tips with each dilution. For each virus dilution, five replicates were used. Starting from the highest to the lowest dilution, 50 µl per well was added to the relevant wells. This was followed by the addition of 50 µl per well of the relevant cell culture at a concentration of 10^6 per ml in EMEM supplemented with 2 % FCS. The plates were covered and incubated at 37 °C in a CO_2 incubator. The plates were examined for discernible CPE after 4 days, and the final reading was done after 7 days. The virus titer was calculated according to the method of Reed and Muench [11]. Control wells containing uninoculated cell culture were included in the tests.

The erythrocytes

Whole blood of human group O+, camel, cow, chicken, duck, goose, goat, guinea pig, mouse, rabbit, rat, sheep, and turkey was collected in Alsever's solution. The erythrocytes of each species were separated under cool centrifugation at 277 × g for 15 minutes, washed three times in phosphate-buffered saline (PBS), pH 7.4, and adjusted to a working dilution of 0.5 % in PBS, pH 7.4.

Trypsinization of erythrocytes

Erythrocytes of each species were treated with an equal volume of purified tissue-culture-grade trypsin (Difco Labs) at a concentration of 1 mg/ml for one hour at 37 °C. The trypsin was removed by centrifugation as described above, the erythrocytes were again washed three times in PBS, pH 7.4, and their working dilution was adjusted to 0.5 % in PBS.

Procedures for hemagglutination tests

V-shaped 96-well microtitre plates were used in the HA tests. Initially, each well of the plate received 50 µl of PBS, pH 7.4, followed by addition of 50 µl of un-inactivated

Table 1 Effect of trypsin on the hemagglutination activity (HA) of Alkhumra hemorrhagic fever virus (AHFV) on erythrocytes from various species

Erythrocyte source	Hemagglutination geometric mean titers of AHFV samples[a]					
	Trypsin-untreated virus samples			Trypsin-treated virus samples		
	A	B	C	A	B	C
Goose	<2	<2	<2	512	256	128
Human group O+	<2	<2	<2	256	128	128
Rat	<2	<2	<2	64	32	32
Guinea pig	<2	<2	<2	32	16	16
Mouse	<2	<2	<2	16	8	8
Camel	<2	<2	<2	<2	<2	<2
Cow	<2	<2	<2	<2	<2	<2
Chicken	<2	<2	<2	<2	<2	<2
Duck	<2	<2	<2	<2	<2	<2
Goat	<2	<2	<2	<2	<2	<2
Rabbit	<2	<2	<2	<2	<2	<2
Sheep	<2	<2	<2	<2	<2	<2
Turkey	<2	<2	<2	<2	<2	<2

[a] The experiments were repeated three times, and the same results were obtained

supernatant medium containing AHFV to each well of the first column of the plate, and a twofold dilution series was made across the plate until well number 12. At well number 12, 50 µl was discarded in disinfectant fluid. At each dilution level, the tips were changed to avoid a carryover effect. Duplicate wells were used for erythrocytes of each species. Trypsinized or untrypsinized erythrocytes of each species were added (50 µl per well) to their allocated wells in the plate. For each species of erythrocytes, wells containing only PBS, pH 7.4, and erythrocytes were included as controls. The plates were incubated at room temperature (22 °C) and read at 30, 45, and 60 minutes, and the geometric mean titers (GMTs) were calculated. The experiments were repeated three times, using fresh erythrocytes each time.

Results

The virus titers were $10^{+7.9}$/ml, $10^{+7.3}$/ml, and $10^{+6.9}$/ml for samples A, B and C, respectively. Table 1 shows the AHFV HA GMTs of the different erythrocytes preparations against the three virus samples (A, B and C). Without trypsin treatment, erythrocytes from the thirteen tested species were not agglutinated by AHFV. Trypsin-treated erythrocytes of five species were agglutinated by AHFV. The best results were obtained with goose (HA GMT range: 128-512) and human type O+ (HA GMT range: 128-256) erythrocytes, followed by rat (HA GMT range: 32-64) and guinea pig (HA GMT range: 16-32) erythrocytes. The least reactive erythrocytes were those from mice, which gave HA GMTs ranging between 8 and 16. The rest of the erythrocytes species were not agglutinated by AHFV before or after trypsin treatment. There was no difference in the HA GMT results after 30, 45, and 60 minutes. The experiments were repeated three times, using fresh erythrocytes each time, and the same results were obtained.

Discussion

Trypsin treatment of tissue culture cell lines is a widely used procedure for structure-function studies of virus-cell interactions [12–17]. Such studies on orthomyxoviruses were seminal in our understanding of the molecular basis for influenza virus pathogenicity [18–21]. Trypsin treatment of both mammalian and avian erythrocytes has been used to render these cells amenable to agglutination by viruses bearing appropriate surface peptides that can attach to unmasked receptors [22–26]. The resulting HA and HAI activity provides a powerful research and diagnostic tool for studies of viruses and their interactions with cellular receptors.

Many flaviviruses have been shown to agglutinate gander erythrocytes under carefully defined conditions of pH, temperature and buffers [27]. This procedure has stood the test of time; in fact, it has been the gold standard. However, not all flaviviruses agglutinate erythrocytes under these conditions. Moreover, this method is technically quite demanding and time-consuming. Nevertheless, the HA and HAI tests are extremely valuable tools for diagnostic and serological studies. We have exploited the principle of treating cells with trypsin to expose potential new receptor sites on the erythrocyte surface and then to test whether or not the treated cells can be used in technically simple and rapid HA tests. We believe a simple procedure of this type would not only be valuable for standard titrations of the sort described above with AHFV but would potentially expand the range of flaviviruses that could be studied even in the most basic laboratories.

In the present study, the HA activity of AHFV for a spectrum of mammalian and avian erythrocytes was examined. The erythrocytes of these species were chosen because some of them (e.g., human type O+, chicken, and guinea pig) are routinely employed in HA studies with some members of the family *Flaviviridae*, to which AHFV belongs [28]. Erythrocytes from the rest of the species are also used routinely with some other viruses (e.g., influenza virus, rubella virus, and hepatitis B virus) [29–31]. Our results clearly demonstrate that AHFV can agglutinate a variety of trypsin-treated erythrocytes that in the non-

treated form are not agglutinated by AHFV. Before trypsin treatment, AHFV failed to agglutinate erythrocytes of the thirteen species examined. Following trypsin treatment, AHFV agglutinated erythrocytes of five species. The effect of trypsin-induced enhancement of the sensitivity of HA activity of viruses has been well documented with many other viruses [22–26]. However, the previously described viruses caused a low level of HA with the relevant erythrocytes before trypsinization, and when trypsinized, the HA titres rose to high levels. In our study, HA was completely absent before trypsinization, indicating that the receptors on the surface of the erythrocytes were completely inaccessible to AHFV and that the HA activity was trypsinization-dependent in five of the thirteen species.

Several interesting observations emanate from the results of comparing different mammalian and avian species. Firstly, following trypsin treatment, the erythrocytes that were most sensitive to HA were from geese and human blood group O+; the remaining positive species (rat, guinea pig, and mouse) were successively less sensitive. Although this decline in sensitivity to HA could reflect experimental conditions such as pH or temperature, it more likely reflects decreasing availability of appropriate receptors for AHFV. All of the other species were negative in these tests. It is therefore tempting to suggest that these negative species represent animals that are unlikely to be susceptible to infection by AHFV, whereas the positive species might show respective reduction in susceptibility to infection by AHFV, with geese and humans being the most susceptible. This concept could initially be tested in appropriate cell culture systems and, perhaps in some cases, in laboratory animals.

In summary, we have described a simple procedure with which to render erythrocytes from humans and other species susceptible to agglutination by AHFV. This may provide a basis for developing an HAI test to detect antibodies against AHFV for diagnostic and research purposes. The method is much easier to use than the traditional method for arboviruses devised originally by Clarke and Casals [27]. It would be interesting to test whether or not the same procedure could be applied to all flaviviruses and perhaps to all arboviruses.

Acknowledgments We thank Sheikh Mohammad Hussein Al-Amoudi for funding this research and the Scientific Chair for Viral hemorrhagic Fever at King Abdulaziz University, Jeddah, Saudi Arabia. We also thank the technologist Mr. Ahmed Sharif for his assistance in the hemagglutination test. The sponsor, Sheikh Mohammad Hussein Al-Amoudi, 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.

Ethical approval King Abdulaziz University's policy on the care and use of laboratory animals was followed. Ethical approval was obtained from the Research Ethics Committee at the Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

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 Bull 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. Madani TA, Azhar EI, Abuelzein EME, Kao M, Al-Bar HMS, Niedrig M et al (2012) Alkhumra, not Alkhurma, is the correct name of the new hemorrhagic fever flavivirus identified in Saudi Arabia. Intervirology 55:259–260
5. Pletnev A, Gould E, Heinz FX, Meyers G, Thiel HJ, Bukh J, et al (2011) Flaviviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy ninth report of the international committee on viruses. Elsevier, Oxford, pp 1003–1020. ISBN: 978-0-12-384684-6
6. Carletti F, Castilletti C, Di Caro A, Capobianchi MR, Nisii C, Suter F et al (2010) Alkhurma hemorrhagic fever in travelers returning from Egypt. Emerg Infect Dis 16(12):1979–1982
7. Zaki AM (1997) Isolation of a flavivirus related to the tick-borne encephalitis complex from human cases in Saudi Arabia. Trans R Soc Trop Med Hyg 91:179–181
8. Charrel RN, Zaki AM, Attoui H, Fakeeh M, Billoir F, Yousef AI et al (2001) Complete coding sequence of the Alkhurma virus, a tick-borne flavivirus causing severe hemorrhagic fever in humans in Saudi Arabia. Biochem Biophys Res Commun 287:455–461
9. Madani TA, Kao M, Azhar EI, Abuelzein EME, Al-Bar HM, Abu-Araki H et al (2012) Successful propagation of Alkhumra (misnamed as Alkhurma) virus in C6/36 mosquito cells. Trans R Soc Trop Med Hyg 106(3):180–185
10. Madani TA, Abuelzein EME, Azhar EI, Kao M, Al-Bar HM, Abu-Araki H et al (2012) Superiority of the buffy coat over serum or plasma for the detection of Alkhumra virus RNA using real time RT-PCR. Arch Virol 157(5):819–823
11. Reed LJ, Muench H (1938) Simple method of estimating 50 % endpoints. Am J Hyg 27:493–497
12. Klenk HD, Rott R, Orlich M, Blodorn J (1975) Activation of influenza A viruses by trypsin treatment. Virology 68:426–439
13. Lazarowitz SG, Choppin PW (1975) Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 68:440–454
14. Maeda T, Ohnshi S (1980) Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. FEBS Lett 122:283–287
15. Skehel JJ, Bayley PM, Brown EB, Martin SR, Waterfield MD, White JM et al (1982) Changes in the conformation of influenza virus hemagglutinin at the pH optimum of in the virus-mediated membrane fusion. Proc Natl Acad Sci USA 79:968–972
16. Wiley DC, Skehel JJ (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu Rev Biochem 56:365–394
17. Kido H, Yokogoshi Y, Sakai K, Tashior M, Kishino Y, Fukutomi A et al (1992) Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A

possible activator of the viral fusion glycoprotein. J Biol Chem 267:13573–13579
18. Webster RG, Rott R (1987) Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell 50(5):665–666
19. Klenk HD, Rott R (1988) The molecular biology of influenza virus pathogenicity. Adv Virus Res 34:247–281
20. Klenk HD, Garten W (1994) Host cell proteases controlling virus pathogenicity. Trends Microbiol 2:39–43
21. Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC (1998) Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell 95(3):409–417
22. Biddle F (1971) The unmasking of receptors for rubella virus by trypsinization of red cells. Microbios 3:255–260
23. Quirin EP, Nelson DB, Inhorn SL (1972) Use of trypsin-modified human erythrocytes in rubella hemagglutination-inhibition testing. Appl Microbiol 24:353–357
24. Shortridge KF, HU LY (1974) Trypsinized human group O erythrocytes as an alternative hemagglutinating agent for Japanese Encephalitis virus. Appl Microbiol 27:653–656
25. Ponzi AN, Pugliese A, Pertusio P (1978) Rubella virus hemagglutination with human and animal erythrocytes: effect of age and trypsinization. J Clin Microbiol 7(5):442–447
26. Mahmood MS, Siddique M, Hussain I, Khan A (2004) Trypsin-induced hemagglutination assay for the detection of infectious bronchitis virus. Pak Vet 24:54–58
27. Clarke DH, Casals J (1958) Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am J Trop Med Hyg 7:561–573
28. Nagarkatti PS, MitziN RaoKM (1980) Development of a kit for the assay of hemagglutination inhibition antibodies to flaviviruses using formalinized goose erythrocytes. Trans R Soc Trop Med Hyg 74:22–25
29. Hussein M, Mehmood MD, Ahmad A, Shabbir MZ, Yaqub T (2008) Factors affecting hemagglutination activity of avian influenza virus subtype H5N1. J Vet Anim Sci 1:31–36
30. Morishita T, Nobusawa E, Nakajima K, Nakajima S (1996) Studies on the molecular basis for loss of the ability of recent influenza A (H1 N1) virus strains to agglutinate chicken erythrocytes. J Gen Virol 77:2499–2506
31. Nelson M, Phipps EJ, Watson PG, Watts JR, Zwolenski R (1973) An automated passive hemagglutination technique suitable for the detection of hepatitis B virus antigen and antibody in blood donors. J Clin Path 26:343–350