ORIGINAL ARTICLE 10.1111/1469-0691.12075

Molecular diagnostic and genetic characterization of highly pathogenic viruses: application during Crimean–Congo haemorrhagic fever virus outbreaks in Eastern Europe and the Middle East

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Abstract

Several haemorrhagic fevers are caused by highly pathogenic viruses that must be handled in Biosafety level 4 (BSL–4) containment. These zoonotic infections have an important impact on public health and the development of a rapid and differential diagnosis in case of outbreak in risk areas represents a critical priority. We have demonstrated the potential of a DNA resequencing microarray (PathogenID v2.0) for this purpose. The microarray was first validated *in vitro* using supernatants of cells infected with prototype strains from five different families of BSL-4 viruses (e.g. families *Arenaviridae, Bunyaviridae, Filoviridae, Flaviviridae* and *Paramyxoviridae*). RNA was amplified based on isothermal amplification by Phi29 polymerase before hybridization. We were able to detect and characterize Nipah virus and Crimean–Congo haemorrhagic fever virus (CCHFV) in the brains of experimentally infected animals. CCHFV was finally used as a paradigm for epidemics because of recent outbreaks in Turkey, Kosovo and Iran. Viral variants present in human sera were characterized by BLASTN analysis. Sensitivity was estimated to be 10^5–10^6 PFU/mL of hybridized cDNA. Detection specificity was limited to viral sequences having ~13–14% of global divergence with the tiled sequence, or stretches of ~20 identical nucleotides. These results highlight the benefits of using the PathogenID v2.0 resequencing microarray to characterize geographical variants in the follow-up of haemorrhagic fever epidemics; to manage patients and protect communities; and in cases of bioterrorism.

Keywords: Crimean–Congo haemorrhagic fever virus, differential diagnosis, microarray, viral haemorrhagic fevers, viral zoonoses
Original Submission: 13 June 2012; **Revised Submission:** 24 August 2012; **Accepted:** 3 September 2012
Editor: E. Gould

Clin Microbiol Infect

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Introduction

Viruses recognized as highly pathogenic for humans must be manipulated in a Biosafety level 4 (BSL-4) laboratory. They include viruses associated with encephalitis and respiratory infections, such as recently emerged members of the genus *Henipavirus*, family *Paramyxoviridae* and haemorrhagic fever viruses in the families *Arenaviridae, Filoviridae, Bunyaviridae* and *Flaviviridae* [1]. Infections with these viruses lead to a wide spectrum of clinical outcomes, from flu-like and malaria-like symptoms to vascular complications that may cause death [1,2]. Most members of the genus *Flavivirus* (family *Flaviviridae*) are arthropod-borne, as are those of the family *Bunyaviridae*, except for the genus *Hantavirus* which is rodent-borne or insectivore-borne [2,3]. Viruses of the family *Arenaviridae* are also rodent-borne [2]. Those of the genus *Henipavirus* have bat

reservoirs but may also infect humans through contact with infected horses or pigs [4]. Recent data indicate that bats are also probable reservoirs and vectors for viruses of the family *Filoviridae* [5,6]. Interhuman transmission and nosocomial infections also contribute to spreading the diseases [2,7].

Development of vaccines to prevent infection by these emerging zoonotic viruses is limited and only ribavirin has been used as an efficacious treatment for several of them [1], so early, rapid and specific diagnosis is critically important for disease control. At-risk areas should possess the necessary facilities and equipment, as well as rapid tests, to be prepared for public health emergencies [2–8]. Accurate diagnoses have traditionally relied on specific serological and virological analyses, which include western blotting, ELISA, immunofluorescence staining, genome detection by PCR and quantitative PCR, and ultimately, virus isolation [9–13]. Molecular methods are rapid and specific but are limited by the high genetic variability among different viral strains. To overcome this limitation, macroarray and microarray technology platforms have been developed to detect and identify a large number of pathogens in a single assay [14–20]. Long oligonucleotide probes have been used previously for the detection of viruses associated with haemorrhagic fevers [16]. Low-density macroarrays allowed different variants of Crimean–Congo haemorrhagic fever virus (CCHFV) to be rapidly detected [17], but were complicated by a requisite reverse transcription (RT-) PCR step. High-density resequencing microarrays not only detect pathogens but also determine nucleic acid sequences to single base-pair resolution. A large panel of viral genome sequences from different geographical origins can be characterized in a single test. The high-density resequencing DNA microarray, PathogenID v2.0, has been shown to be useful for rapid diagnosis during emerging viral infections, such as the 2009 influenza pandemic [18], and was useful for genotyping members of the family *Rhabdoviridae* [19].

Here, we used the PathogenID v2.0 microarray to detect highly pathogenic viruses. We first validated the microarray with *in vitro* samples by analysing supernatants from cells infected by prototype virus strains and variants belonging to five families of BSL-4 agents (*Arenaviridae, Bunyaviridae, Filoviridae, Flaviviridae, Paramyxoviridae*). We then evaluated its performance during a health emergency situation by testing human sera from CCHFV outbreaks in Turkey (2009), Kosovo (2001) and Iran (2009). CCHFV belongs to the genus *Nairovirus*, family *Bunyaviridae* and has the largest geographic distribution among haemorrhagic fever viruses [21,22]. Zoonotic infection occurs either directly through its vectors, which are various tick species from the genus *Hyalomma*, or indirectly through contact with infected livestock. Hospital environments are also vulnerable to inter-human transmissions [23]. CCHFV infection is associated with several clinical outcomes, some of which can become life threatening [22]. CCHFV outbreaks or sporadic cases have occurred in Mauritania [24], Iran [10], Turkey [25], Kosovo [26] and Sudan [23].

Materials and Methods

Ethics statement

This work includes a retrospective study on 12 human sera from clinical specimens submitted to France National-WHO-OIE Reference Centres for diagnosis during CCHF epidemics in Kosovo, Turkey and Iran.

The collection of the remaining samples to be used for scientific purpose was declared to and approved by the Comité de Protection des Personnes, Ile-de-France I and the French Research Ministry (no. DC 2011-1471) according to French regulations.

Animal experimental methods were approved by the Région Rhône Alpes Ethics Committee (France).

Viruses

Viral strains and geographical variants (Table 1 and Table 2) were cultured and isolated in permissive Vero-E6 cells as previously described [11,27]. To simulate the complexity of clinical samples, we pooled RNA samples from different Vero-E6 cell cultures that had each been infected by a single virus. Twelve pooled RNA samples of one to three viruses each were prepared. For Junin virus (family *Arenaviridae*) and Sin Nombre virus (genus *Hantavirus*, family *Bunyaviridae*) synthetic cDNA sequences (Eurofins MWG Operon, Ebersberg, Germany) were used as templates for the amplification step.

Human sera from CCHFV outbreaks

Sera from 12 infected humans were collected during CCHFV outbreaks (2003–09) in the Balkans (five from Kosovo, 2001 and two from Turkey, 2009) and the Middle East (five from Iran, 2009).

Animal biopsies

One non-human primate, a New World squirrel monkey (*Saimiri sciureus*) was experimentally infected intravenously with 10^3 PFU UM-MC1 Malaysian isolate of Nipah virus [28] as previously described [29]. It was imported from a breeding colony in French Guiana and housed in the BSL-4 animalcare facility in Lyon. The animal was observed daily for signs of disease onset; disease symptoms appeared at day 10 and lasted for 3 days before the moribund monkey was humanely euthanized. A brain biopsy was taken at necropsy and frozen at −80°C.