The serology of *Ebolavirus* – a wider geographical range, a wider genus of viruses or a wider range of virulence?

Magdalena Formella and Derek Gatherer

Viruses of the genus *Ebolavirus* are the causative agents of Ebola virus disease (EVD), of which there have been only 25 recorded outbreaks since the discovery of Zaire and Sudan ebolaviruses in the late 1970s. Until the west African outbreak commencing in late 2013, EVD was confined to an area of central Africa stretching from the coast of Gabon through the Congo river basin and eastward to the Great Lakes. Nevertheless, population serological studies since 1976, most of which were carried out in the first two decades after that date, have suggested a wider distribution and more frequent occurrence across tropical Africa. We review this body of work, discussing the various methods employed over the years and the degree to which they can currently be regarded as reliable. We conclude that there is adequate evidence for a wider geographical range of exposure to *Ebolavirus* or related filoviruses and discuss three possibilities that could account for this: (a) EVD outbreaks have been misidentified as other diseases in the past; (b) unidentified, and clinically milder, species of the genus *Ebolavirus* circulate over a wider range than the most pathogenic species; and (c) EVD may be subclinical with a frequency high enough that smaller outbreaks may be unidentified. We conclude that the second option is the most likely and therefore predict the future discovery of other, less virulent, members of the genus *Ebolavirus*.

**Ebola virus disease outbreaks**

*Zaire ebolavirus* (EBOV: WHO, 1978b), *Sudan ebolavirus* (SUDV: WHO, 1978a) and *Bundibugyo ebolavirus* (BDBV: MacNeil et al., 2010) (all family *Filoviridae*; genus *Ebolavirus*) have together caused 25 outbreaks of high-mortality haemorrhagic fever that have been officially recognized as such by the World Health Organization (WHO) (Table 1). This figure is open to interpretation, as many of the outbreaks are temporally and geographically clustered, and some clusters may represent recurrent flare-ups of outbreaks with a single origin. Until the Ebola-Makona strain (Kuhn et al., 2014) outbreak beginning in Guinea in late 2013, collection of virus genome data was relatively sporadic, so data are not available to answer some of the questions that Table 1 might beg. The repeated minor recurrences of Ebola virus disease (EVD) after the end of the main wave of the west African outbreak (e.g. Blackley et al., 2016; Diallo et al., 2016), which we know from genome data to have all been Ebola-Makona in origin, might in the past have been classified as a cluster of separate outbreaks. Therefore, 25 outbreaks since 1976 must be seen as a ceiling rather than a precise value.

These EVD outbreaks have ranged in size from single cases to the 11,310 official fatalities associated with EBOV-Makona in west Africa between December 2013 and April 2016. Two additional species in the genus, *Reston ebolavirus* (RESTV: Geisbert et al., 1992) and *Tai Forest ebolavirus* (TAFV: Le Guenno et al., 1995), have not been associated with transmission between humans, although TAFV has produced one non-fatal clinical case. Based on the distribution of EVD outbreaks by species before 2013, it is possible to define a geographical range for each virus: EBOV in the Congo Basin and westward to the Atlantic Ocean, SUDV in Uganda and northward into what is now South Sudan and BDBV in an intermediate zone between the two (Fig. 1). The appearance of EBOV-Makona in eastern Guinea in December 2013 presented an additional geographical locus which disturbed the pre-2013 view of EVD as a disease limited to central Africa. The location of RESTV in Asia and TAFV in Ivory Coast did not previously affect this picture as neither had been responsible for human-to-human EVD transmission. Defining the true geographical extent of EVD is of great importance, since the absence of west Africa from the previously accepted account of EVD incidence was a factor in the failure to recognize the disease until the outbreak was already spreading widely (Moon et al., 2015).
Methods employed in Ebola serology

Since the first recorded EVD outbreak, caused by strain EBOV-Mayinga in Yambuku, DRC (then Zaire) in 1976 (WHO, 1978b), sporadic efforts have been made to assess seropositivity in human and animal populations across Africa and occasionally elsewhere. A variety of techniques, sample sizes and study designs have been used, together defining a larger area of tropical Africa where ebolaviruses have left their serological traces (Fig. 1 and Table 2).

Table 2 shows that, of the 30 studies we were able to identify in the literature, 24 consisted of samples collected before 1990. Two studies (Becker et al., 1992; Tignor et al., 1993) used archive samples stored for up to two decades. All pre-1992 studies, with one exception (Boiro et al., 1987), used immunofluorescence (IF). Subsequent studies have all used ELISA. Both of these techniques rely on cross-reaction of serum samples with an antigen immobilized on a slide or in a well. The antigens used for this purpose have also been highly variable: some papers specifying the strain as well as the species (e.g. Gonzalez et al., 1989; Meunier et al., 1987; Nakounné et al., 2000; Tignor et al., 1993; Van der Waals et al., 1986), with others merely the species (e.g. Blackburn et al., 1982; Mathiot et al., 1989; Rodhain et al., 1989), and a third category with even fewer details (e.g. Paix et al., 1988; Saluzzo et al., 1980). Many of the studies were also performed in the field, often in remote areas and presumably with limited facilities for preventing degradation of both serum samples and laboratory materials. All studies focused on immunological reactivity, and neutralization of virus was not studied. Under such circumstances, scepticism concerning results is justified and a further examination of techniques is warranted.

Immunofluorescence

The early IF-based methods are described in detail by Johnson et al. (1981b). Virus-infected Vero cells in suspension were UV irradiated to inactivate viral infectivity and then dried onto Teflon-coated microscope slides which were fixed in acetone and then gamma irradiated to destroy any residual infectivity and further sterilize both the slides and the inside of the slide box. The infection and dilution process was titrated such that an average of less than 10% of cells per slide were infected, thus providing an internal negative control. Test samples reacting to all the surface of the slide could therefore be discarded as false positives and only those slides displaying the predicted fluorescence from 5 to 15% of cells would be scored as positive reactions. Negative

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Table 1. The 25 officially declared outbreaks of EVD from 1976 to 2016

Data from WHO (2016). CFR, case fatality rate; EBOV, Zaire ebolavirus; SUDV, Sudan ebolavirus; BDBV, Bundibugyo ebolavirus.

<table>
<thead>
<tr>
<th>Year</th>
<th>Country</th>
<th>Species</th>
<th>Cases</th>
<th>Deaths</th>
<th>CFR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013–2016</td>
<td>Guinea, Liberia,</td>
<td>EBOV</td>
<td>28616</td>
<td>11310</td>
<td>40</td>
</tr>
<tr>
<td>2014</td>
<td>DRC (Zaire)</td>
<td>EBOV</td>
<td>66</td>
<td>49</td>
<td>74</td>
</tr>
<tr>
<td>2012</td>
<td>DRC (Zaire)</td>
<td>BDVB</td>
<td>57</td>
<td>29</td>
<td>51</td>
</tr>
<tr>
<td>2012</td>
<td>Uganda</td>
<td>SUDV</td>
<td>7</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>2012</td>
<td>Uganda</td>
<td>SUDV</td>
<td>24</td>
<td>17</td>
<td>71</td>
</tr>
<tr>
<td>2011</td>
<td>Uganda</td>
<td>SUDV</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2008</td>
<td>DRC (Zaire)</td>
<td>EBOV</td>
<td>32</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td>2007</td>
<td>Uganda</td>
<td>BDVB</td>
<td>149</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>2007</td>
<td>DRC (Zaire)</td>
<td>EBOV</td>
<td>264</td>
<td>187</td>
<td>71</td>
</tr>
<tr>
<td>2005</td>
<td>Congo</td>
<td>EBOV</td>
<td>12</td>
<td>10</td>
<td>83</td>
</tr>
<tr>
<td>2004</td>
<td>Sudan</td>
<td>SUDV</td>
<td>17</td>
<td>7</td>
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<tr>
<td>2003</td>
<td>Congo</td>
<td>EBOV</td>
<td>35</td>
<td>29</td>
<td>83</td>
</tr>
<tr>
<td>2003</td>
<td>Congo</td>
<td>EBOV</td>
<td>143</td>
<td>128</td>
<td>90</td>
</tr>
<tr>
<td>2001–2002</td>
<td>Congo</td>
<td>EBOV</td>
<td>59</td>
<td>44</td>
<td>75</td>
</tr>
<tr>
<td>2001–2002</td>
<td>Gabon</td>
<td>EBOV</td>
<td>65</td>
<td>53</td>
<td>82</td>
</tr>
<tr>
<td>2000</td>
<td>Uganda</td>
<td>SUDV</td>
<td>425</td>
<td>224</td>
<td>53</td>
</tr>
<tr>
<td>1996</td>
<td>South Africa</td>
<td>EBOV</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>1996</td>
<td>Gabon</td>
<td>EBOV</td>
<td>60</td>
<td>45</td>
<td>75</td>
</tr>
<tr>
<td>1996</td>
<td>Gabon</td>
<td>EBOV</td>
<td>31</td>
<td>21</td>
<td>68</td>
</tr>
<tr>
<td>1995</td>
<td>Zaire</td>
<td>EBOV</td>
<td>315</td>
<td>254</td>
<td>81</td>
</tr>
<tr>
<td>1994</td>
<td>Gabon</td>
<td>EBOV</td>
<td>52</td>
<td>31</td>
<td>60</td>
</tr>
<tr>
<td>1979</td>
<td>Sudan</td>
<td>SUDV</td>
<td>34</td>
<td>22</td>
<td>65</td>
</tr>
<tr>
<td>1977</td>
<td>Zaire</td>
<td>EBOV</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>1976</td>
<td>Sudan</td>
<td>SUDV</td>
<td>284</td>
<td>151</td>
<td>53</td>
</tr>
<tr>
<td>1976</td>
<td>Zaire</td>
<td>EBOV</td>
<td>318</td>
<td>280</td>
<td>88</td>
</tr>
</tbody>
</table>
false positives, they were able to differentially score EBOV-
However, having implemented this procedure for reducing
well as positive and negative controls and blind scoring.
sity for pre-incubation of serum with uninfected cells, as
Liberia in 1981
describe some of the associated limitations. Surveying in
of Johnson
ried out in the field used slides prepared similarly to those
line: range of three fruit bat species implicated as EBOV reser-
results in a population survey prior to outbreak in that area; dotted
breaks in the same region are not shown (for which, see Table 3).

control sera were also used to differentiate slides producing
non-specific reactions and antisera raised against the virus
in the laboratory were used as positive controls. In the lab-
atory setting, Johnson et al. (1981b) stored their IF slides at
−70 °C prior to use, which would be impossible in a field
setting.

It must be assumed that most of the early IF studies car-
ried out in the field used slides prepared similarly to those
of Johnson et al. (1981b). Van der Waals et al. (1986)
describe some of the associated limitations. Surveying in
Liberia in 1981–1982 (Table 2), they emphasize the neces-
sity for pre-incubation of serum with uninfected cells, as
well as positive and negative controls and blind scoring.
However, having implemented this procedure for reducing
false positives, they were able to differentially score EBOV-
Mayinga against SUDV-Boniface (11.8 % seropositivity vs
1.6 %, respectively) and both against other viruses (Lassa
fever: 1.3 %; Rift Valley fever: 0.4 %; Crimean-Congo hae-
morrhagic fever: 4.4 %; Marburg virus: 1.3 %). This capa-
city, shown in several of the early IF surveys, to diffe-
tentially score for, and therefore by implication different-
entially detect, *Ebolavirus* species, provides a plausible
internal control for the method. For instance, Ivanoff et al.
(1982) found 6 % seropositivity to EBOV in their Gabon-
eses samples but ‘little or no’ seropositivity to SUDV and
none to Marburg or Lassa viruses. Likewise, Mathiot et al.
(1989) produced a similar result – 4–13 % seropositivity to
EBOV versus 0 % to SUDV in Madagascar.

Providing the IF slides satisfied the required controls before
leaving the laboratory, and the field surveyors applied posi-
tive and negative serum controls in parallel with patient
samples; while discarding samples which produced non-
specific fluorescence across all cells on the slide, there is no
*a priori* reason to reject completely the findings of the early
studies. Reservations must remain about preservation of
slides and control sera outside the laboratory in potentially
hot climates.

**ELISA**

From the early 1990s, IF methods fell into disuse in Ebola
serology and were replaced with ELISA. Boiro et al. (1987)
were the first to implement ELISA in this context. Only brief
details are given in their paper, but ‘l’antigène du virus Ebola’
without species or strain specification was bound to polysty-
rene microplates and incubated with guinea pig IgG to
decrease non-specific binding prior to the addition of the
study samples. The secondary antibody was peroxidase conju-
gated. IF was also performed, and the antigen in that case
specified as EBOV. Unlike the earlier IF studies, many of
which were performed in the field, Boiro et al. (1987) carried
out their work in the laboratory where their ELISA results
could be analysed spectrophotometrically, and the reduction
in the number of Ebola serology studies since the 1990s has
probably partly been a consequence of this necessary addi-
tional technical requirement. Boiro et al. (1987) also present
their results very briefly, simply recording that four serum
samples judged positive by ELISA also tested positive using IF,
out of a total of 138 ELISA-tested samples and 79 IF-tested
samples, within which there were 11 (8.0 %) and 15 (19 %)
positives, respectively. The difference in positivity between
methods is not relevant since the IF tests were done on conva-
lescent patients only, whereas the clinical status of the ELISA
test subjects is not specified. It is also not clear, apart from the
case specifically cross-checked samples, whether there is any
overlap between the two sets.

No other comparative study of IF against ELISA for Ebola
on the same field sample set has been recorded in the litera-
ture. Subsequent studies using ELISA have dealt with the
problem of false positives by the use of positive and negative
control sera and, in some cases, by sending the samples to
other centres for independent cross-checking (Gonzalez
Table 2. Sero-surveys with positive results conducted in regions of Africa where no EVD outbreak had occurred or prior to the occurrence of EVD in that region

Where a variety of locations were sampled, the range of seropositivity is given. Where EBOV and SUDV were assayed separately, corresponding values are given. Species or strain of *Ebolavirus* antigen used is also given when specified in publication. 'Patients' indicates that the subjects were suffering from haemorrhagic fever at the time of the test. Where no specific date for the survey is given in the paper, submission date of the paper is presented as 'pre-19nn'. CAR, Central African Republic; DRC, Democratic Republic of Congo. IF, immunofluorescence; WB, Western blot.

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Region</th>
<th>Method</th>
<th>% seropositive</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1972–1991</td>
<td>Germany</td>
<td>ELISA/IF/WB†</td>
<td>Filovirus 7, EBOV 1</td>
<td>Becker et al. (1992)</td>
</tr>
<tr>
<td>1979</td>
<td>Bangassou, CAR</td>
<td>IF</td>
<td>3</td>
<td>Saluzzo et al. (1980)</td>
</tr>
<tr>
<td>1980</td>
<td>Franceville, Gabon</td>
<td>IF – EBOV</td>
<td>6</td>
<td>Ivanoff et al. (1982)</td>
</tr>
<tr>
<td>Pre-1983</td>
<td>Six locations, Kenya</td>
<td>IF – Mayinga/SUDV</td>
<td>EBOV 1–7, SUDV&lt;1</td>
<td>Johnson et al. (1983a)</td>
</tr>
<tr>
<td>1983</td>
<td>Awash Valley, Ethiopia</td>
<td>IF – Mayinga</td>
<td>30</td>
<td>Tignor et al. (1993)</td>
</tr>
<tr>
<td>1984</td>
<td>Four locations, Uganda</td>
<td>IF – EBOV/SUDV</td>
<td>EBOV 3, SUDV 3</td>
<td>Rodhain et al. (1989)</td>
</tr>
<tr>
<td>1985</td>
<td>Nkongsamba, Cameroon</td>
<td>IF</td>
<td>2</td>
<td>Paix et al. (1988)</td>
</tr>
<tr>
<td>1987</td>
<td>Mongoumba, CAR</td>
<td>IF – EBOV/SUDV</td>
<td>18</td>
<td>Johnson et al. (1993a)</td>
</tr>
<tr>
<td>Pre-1992</td>
<td>Four locations, CAR</td>
<td>IF – Mayinga/Boniface</td>
<td>EBOV 1–9, SUDV 19–27</td>
<td>Johnson et al. (1993b)</td>
</tr>
<tr>
<td>2002</td>
<td>Watsa, DRC</td>
<td>ELISA – EBOV</td>
<td>19</td>
<td>Mulangu et al. (2016)</td>
</tr>
<tr>
<td>2006–2008</td>
<td>Kenema, Sierra Leone</td>
<td>ELISA – EBOV</td>
<td>9 (patients)</td>
<td>Schoepf et al. (2014)</td>
</tr>
<tr>
<td>2011–2014</td>
<td>Kenema, Sierra Leone</td>
<td>ELISA – Mayinga</td>
<td>22 (patients)</td>
<td>Boisen et al. (2015)</td>
</tr>
</tbody>
</table>

*Becker et al. (1992) used Mayinga (EBOV), RESTV and Musoke (*Marburgvirus*).
†Meunier et al. (1987) used Mayinga, Boniface (SUDV) and Musoke (*Marburgvirus*).
‡Purified antigens, otherwise whole virus.

et al., 2000) or titrating the threshold for scoring positivity by reference to a sample of unexposed individuals from the USA (Boisen et al., 2015) or France (Nkoghe et al., 2011). Heterologous incubation – e.g. when testing for EBOV, add Marburg-positive serum to remove Marburg-specific binding and vice versa – has also been used (Becker et al., 1992).

**Laboratory evidence for inter-specific cross-reactivity**

Although both IF and ELISA in the field show some evidence of specificity between different viruses within the genus *Ebolavirus*, several laboratory studies have indicated that some cross-reactivity is likely. A comparative study of ELISA methods using antigens prepared from all species of the genus *Ebolavirus* (Macneil et al., 2011) showed cross-reactivity to be considerable, but another study considered it to be more limited (Nakayama et al., 2010). In a clinical context, sera from survivors of outbreaks displayed cross-reactivity to recombinant proteins from other filovirus species, which had been bound to a protein microarray (Natesan et al., 2016), and mAbs raised against the glycoproteins (GPs) of BDBV, SUDV and EBOV exhibit some cross-specific *in vitro* binding. In the
Table 3. Sero-surveys with positive results conducted in regions of Africa subsequent to an EVD outbreak, divided into general population seropositivity, and contacts of known cases where available

Where a variety of locations were sampled, the range of seropositivity is given. ‘Patients’ indicates that the subjects were suffering from a haemorrhagic fever at the time of the test. IF, immunofluorescence; WB, Western blot.

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Region</th>
<th>Associated outbreak (see Table 1)</th>
<th>Method</th>
<th>% general population</th>
<th>% contacts</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1979</td>
<td>Nzara/Yambio, Sudan</td>
<td>SUDV 1976, 1979</td>
<td>IF</td>
<td>18</td>
<td>32</td>
<td>Baron et al. (1983)</td>
</tr>
<tr>
<td>1995</td>
<td>Bandundu, Zaire</td>
<td>EBOV 1995</td>
<td>ELISA 3</td>
<td>3</td>
<td></td>
<td>Rowe et al. (1999)</td>
</tr>
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<td>1997</td>
<td>Ogooue-Ivindo, Gabon</td>
<td>EBOV 1996</td>
<td>ELISA 1</td>
<td>1</td>
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<td>Heffernan et al. (2005)</td>
</tr>
</tbody>
</table>

Unexpected results from Ebola serology studies

Despite the technical and descriptive issues delineated above, and subsequent in vitro findings regarding cross-reactivity, the paper of Boiro et al. (1987) has become in retrospect significant in that it presents, along with the IF paper of Van der Waals et al. (1986), evidence for the occurrence of EVD in two of the three countries later affected by the 2013–2016 Ebola-Makona outbreak. If the conclusions of these two papers had been more widely known, it might have served to alert health authorities earlier to the potential cause of the outbreak and avoid the delay that Moon et al. (2015) identify as one of the main factors in the loss of control in the early stages of the epidemic. After the commencement of the Ebola-Makona outbreak, Schoepf et al. (2014) tested samples collected in the affected area from 2006 to 2008, using IgG and IgM capture ELISA methods. The IgM capture method coats the assay plates with anti-human IgM antibody rather than viral antigen. The serum samples are then added to allow the anti-IgM antibody to bind the IgM in the samples, after which the antigen is added. Pre-selecting in this way for the IgM within the study sample helps to reduce the potential for non-specific binding of viral antigen. The 8.2 % seropositivity rate for EBOV corresponds well to the 8.0 % detected by Boiro et al. (1987), suggesting that the original paper is believable. Boisen et al. (2015) using the same clinical source as Schoepf et al. (2014) – the Kenema General Hospital in Sierra Leone – found a higher figure of 22 % including samples taken up to March 2014, just prior to the arrival of the EBOV-Makona strain in Kenema.

One of the most surprising results in Ebola serology comes from a study performed in Germany (Becker et al., 1992) on an anonymized heterogeneous sample set collected over a 19-year period from 1972 to 1991, comprising contacts of the original Marburg virus outbreak patients, routine diagnostic samples from Marburg (western-central Germany),
blood donors and others of unspecified origin and a sample set from Greifswald (on the Baltic coast of north-east Germany). This is the only study to combine ELISA, IF and WB methods. ELISA was carried out in the first instance, and then the positive samples cross-checked with IF and WB analysis with confirmation rates in excess of 66% for all antigens tested. The initial ELISAs used heterologous incubation to address the problem of cross-specificity. RESTV has the highest positivity at 3.4% followed by Marburg at 2.6% and EBOV at 0.85%. The study provides no information about the travel history or birthplace of individuals, which means that the possibility that the signal represents travel-related exposure, rather than autochthonous transmission within Europe, cannot be discounted.

Candidate reservoir serology in Africa

Three fruit bat species (Hypsignathus monstrosus, Eumops franqueti and Myonycteris torquata) have been hypothesized to be the natural reservoir host of EBOV on the basis of detection of viral genomes (Leroy et al., 2005) but the human serological zone is wider than their incidence (Fig. 1), suggesting that our current knowledge of the animal reservoir is incomplete. Other species of fruit bat with a wider range may be involved, and a serological study of Eidolon helvum in Zambia between 2006 and 2013 showed annual seropositivity fluctuating between 0 and 6% for any one filovirus, with all species tested (EBOV, SUDV, RESTV, BDBV, TAFV and Marburg virus) occurring at least once over the study period, but different species dominating in different years, and EBOV and SUDV being jointly the most common (Ogawa et al., 2015). Several bat species in Ghana (Hayman et al., 2010, 2012) and Gabon (Pourrut et al., 2007, 2009) were also shown to be seropositive. Outside of Africa, seropositive bats have been detected in Bangladesh (Oival et al., 2013) and China (Yuan et al., 2012).

Of course, serological studies in bats share all the interpretational problems of those in humans. Not all studies have been positive – the first extensive investigation (Leirs et al., 1999) into seropositivity in African mammals, using ELISA, conducted following the 1995 EBOV outbreak in Kikwit, Zaire (Table 1), sampled 3066 specimens drawn from 2493 species and failed to find any seropositivity, including in the fruit bat Eumops franqueti from which a later study isolated a fragment of viral genome (Leroy et al., 2005).

It is known that apes are susceptible to EVD (Walsh et al., 2003) and also have signals of seropositivity (Becker et al., 1992; Johnson et al., 1981a, 1982; Leroy et al., 2004a, b; Nidom et al., 2012; Rouquet et al., 2005) but their status seems to be more similar to that of humans as occasional victims of epidemics, rather than to represent a reservoir population. Likewise, surveys of other mammals have shown seropositivity in domestic animals (Allela et al., 2005; Stansfield et al., 1982) and limited evidence of virus genomes (Morvan et al., 1999) or disease (Leroy et al., 2004; Rouquet et al., 2005) in small mammals.

Human serological and clinical ranges in Africa

However, if the serological signal in humans represents the true geographical range of the genus Ebolavirus, this begs the question as to why EVD outbreaks were not more widely distributed prior to 2013. The serological signal is consistent over time, some African countries having been sampled extensively in terms of number of studies, geographical range and variety of different reporting research groups. The Central African Republic has been the subject of seven published studies, carried out between 1979 and 1997 (Fig. 1, Table 2). Several sites have been sampled more than once, with maximum seropositivity at 23%, despite no recorded EVD outbreak having occurred in the Central African Republic. Prior to the first EVD outbreak in Gabon in 1994, that country was the subject of sero-surveys in 1980 and 1985–1987 indicating a maximum seropositivity of 22% (Table 2) and identifying seropositive subjects in some of the areas where EVD subsequently broke out in 1994–1996 and 2001–2002 (Fig. 1). All three countries involved in the 2013–2016 EBOV-Makona outbreak had given positive signals over four studies from 1981 to 2011, three of which involved patients with haemorrhagic fevers of unknown aetiology (Table 2).

We therefore next discuss three scenarios that could account for the discrepancy between EVD’s range when defined clinically versus serologically, and their implications both for our understanding of the biology of the genus Ebolavirus and future risk assessment.

Hypothesis 1: EVD outbreaks have been misidentified as other diseases in the past

Although the international response to the EBOV-Makona outbreak was criticized on several counts (Moon et al., 2015), there was much media interest from the beginning. A similar level of media interest was stimulated in 1995 by the EBOV-Kikwit outbreak (Garrett, 2001), and recognition of the potential seriousness of EVD outbreaks and consequent surveillance began almost as soon as EBOV and SUDV were discovered in the late 1970s (Table 3). Under these circumstances, it perhaps seems unlikely that an EVD outbreak could have passed completely unnoticed.

However, this would presumably not apply to outbreaks occurring before the discovery of EBOV in 1976. One potential event in that category was the 1961–1962 outbreak in Ethiopia of a disease described at the time as yellow fever (Tignor et al., 1993). Even after 1976, some of the population sero-surveys identified clusters of patients with fevers, for instance, in Kenya (Johnson et al., 1983b, 1986) and west Africa (Boiro et al., 1987; Boisen et al., 2015; Schoepf et al., 2014), which may represent small EVD outbreaks that escaped official classification, speculation being particularly focused on Guinea in 1982–1983 (Balde, 2014; Boiro et al., 1987). Corroboration of this hypothesis would require work to be done by medical historians to identify previous disease
outbreaks that may have been unrecognized EVD. A similar effort was undertaken in the 1980s to identify traces in the literature of potential cases of pre-1979 human immunodeficiency virus type 1 infection in Africa, without much success. Our knowledge of the pre-history of AIDS comes largely from retrospective serology and phylogenetic reconstruction, and this may also remain the case for EVD.

**Hypothesis 2: The genus *Ebolavirus* is larger than currently known, and includes milder species with a wider geographical range**

The finding of seropositive individuals does not necessarily indicate that those individuals have been exposed to EBOV, BDBV or SUDV. As reviewed above, reports of cross-specificity *in vitro* make it likely that some of the serological tests detect other members of the genus *Ebolavirus* or related filoviruses. Human population serology may simply indicate the geographical range of the genus or the family as a whole. The discovery of TAFV in 1994, still limited to a single case in humans, illustrates that very rare *Ebolavirus* species do exist, so it is plausible that more species diversity remains to be discovered. These hypothetical extra members of the genus *Ebolavirus* would presumably be relatively mild compared to EBOV, BDBV and SUDV, and thus have not produced any recognized outbreaks of EVD. TAFV produced a ‘dengue-like syndrome’ in its single human case (Le Guenno et al., 1995).

**Hypothesis 3: EVD may be subclinical with a frequency high enough that smaller outbreaks may be unidentified**

Post-outbreak sero-surveys (Table 3) conducted in the wake of prior EVD events (Table 1) have often shown localized high levels of seropositivity. Some settlements in the vicinity of the second Zaire outbreak of 1977 had seropositivity at 56% (Van der Groen & Pattyn, 1979), and 32% of the population was recorded in parts of Gabon exposed to the 2001–2002 outbreak (Nkoghe et al., 2011). These numbers would appear to be too large to be simply representative of known patients, and suggest a larger body of affected individuals, some of whom possibly might have been subclinical. However, Jezek et al. (1999), returning in the early 1980s to the scene of the 1976 and 1977 EBOV outbreaks in Zaire, detected 60% seropositivity in recovered patients but only 1% in the general population, suggesting that the bulk of seropositivity is due to those who have had a recognized previous EVD attack. On the other hand, these same authors also scored asymptomatic contacts at 18% seropositive. Other studies on asymptomatic contacts of known cases have given seropositivity scores as high as 32% for the 1979 SUDV outbreak (Baron et al., 1983) and 50% for the 1996 Gabon EBOV outbreak (Leroy et al., 2000). In the latter study, viral RNA was also isolated from 7 out of 11 seropositive asymptomatic contacts, but from none of 13 seronegative contacts. A meta-analysis by Dean et al. (2016) covering many of the studies in Tables 2 and 3 estimated that 14–40% of EBOV infections are asymptomatic. However, these studies do not conclusively prove that the asymptomatic contacts contracted subclinical EVD despite the circumstantial implication. Becquart et al. (2014) compared sera from asymptomatic seropositive individuals to equivalent samples from symptomatic survivors, showing that IgG responses were qualitatively different in each group. The asymptomatic group displayed greater response to EBOV VP40 (40 kDa protein), whereas the survivors known to have been infected with EBOV had their greatest IgG response to GP. This might be consistent with the seropositive individuals within the asymptomatic group having been previously infected with a non-EBOV filovirus, perhaps one with greater sequence/antigenic similarity to EBOV in its VP40 than its GP. Alternatively, it might indicate some variation in the immune response that contributes to the asymptomatic state.

The EBOV-Makona outbreak of 2013–2016 produced 28,616 official confirmed or suspected cases, allowing a far more extensive investigation of seropositivity in a human population than had previously been possible. Asymptomatic relatives of Ebola-Makona victims were routinely identified as seropositive, as high as 65% for the later stages of the outbreak in Sierra Leone (de La Vega et al., 2015). Of course, it must be noted that the stigma associated with EVD, recognized in both the EBOV 1995 and SUDV 2000 outbreaks (Kinsman, 2012) and emerging as a major factor in the EBOV-Makona outbreak (Karamouzian & Hategikiman, 2015), may have led to a reluctance among the contacts of EVD cases to admit their own symptoms. Those subsequently classified as asymptomatic contacts may therefore have been true survivors. Gignoux et al. (2015) used a statistical comparison of two databases of patients covering Montserrado, Liberia, from June to August 2014 to estimate that the true number of clinical EVD cases was threefold higher than the reported number. It is unclear, however, whether this is due to administrative deficiencies or deliberate under-reporting.

**Conclusions and future prospects**

The serological footprint of Ebola is wider than expected from our knowledge of EVD outbreaks. Each sero-survey must be considered on its own merits, as a variety of methods have been used over the years, with differing degrees of technical sophistication and attention to controls for false positives. Nevertheless, the most recent experiments remain generally supportive of the idea that contact between humans and some viruses of the genus *Ebolavirus*, although not necessarily any of the known ones, has occurred in tropical Africa outside of known outbreak zones. Caution must be exercised before making similar statements concerning Europe and Asia. The former has no recent study and seropositive signals in the latter may be accounted for by RESTV.

Each of the three scenarios listed here can draw on some support from the data. Hypothesis 3 – a widespread occurrence of asymptomatic EBOV infections – would perhaps be the most troubling, as this would imply that EBOV is far...
more common than previously appreciated, and across a wide area of Africa, presenting the possibility that full-blown EVD crises may arise at any time. It does, however, beg the question of why some EBOV outbreaks would consist largely of asymptomatic cases, in contrast to a more typical devastating EVD episode. Nevertheless, wherever efforts have been made to assess exposure to the virus among asymptomatic contacts, most studies have figures in excess of 18% (Table 3).

Hypothesis 1 – more clinically conventional, but nevertheless missed, EVD outbreaks – may therefore require fewer assumptions. However, it requires us to explain why our postulated extra EVD outbreaks have not been detected, especially after 1976 when surveillance for haemorrhagic fevers intensified. There may be evidence in the colonial medical literature of outbreaks that were classified according to the diseases known at the time, but which now in retrospect may seem more probably to be EVD outbreaks. However, the serological traces in modern human populations of such outbreaks would be confined to the very elderly, and we need to account for unexplained Ebola seropositivity in younger individuals too.

A compromise between hypotheses 1 and 3 may be possible, if EVD outbreaks are normally very small and localized with a high proportion of asymptomatic cases. The documented EVD outbreaks since 1976 would then represent the extreme end of a probability distribution, being only those outbreaks large enough to present sufficient fatal cases to attract attention. This, however, would require an answer as to why known EVD outbreaks, until 2013, were all in a relatively restricted region of central Africa comprising the Congo Basin and areas to its east and west, whereas the Ebola seropositivity signal is far wider.

Hypothesis 2 – the existence of other members of the genus Ebolavirus – may therefore be the least problematic albeit, as it does not require any revision of our understanding of EVD as caused by three virulent, and until 2013 solely central African, species in that genus. The relatively little that we know concerning RESTV and TAFV is consistent with the idea of reduced pathogenicity in humans of some species of Ebolavirus. Where cross-reactivity experiments have been performed, there appears to be an indication that exposure to one member of the genus Ebolavirus can produce antibodies that will bind other members to a greater (Flyak et al., 2016; Hashiguchi et al., 2015; Hensley et al., 2016; Macneil et al., 2011; Natesan et al., 2016; WHO, 2015) or lesser (Nakayama et al., 2010) degree.

There is therefore considerable justification for a renewal of the Ebola sero-survey research programme, which has atrophied since the 1990s. This should be coupled with a deep sequencing initiative in candidate reservoir hosts, especially bats. Discovery of new members of the genus Ebolavirus could account for the widespread seropositivity among humans. Since even current standard immunological tests based on antigen–antibody binding cannot distinguish different species of Ebolavirus with absolute reliability, methodological research is required to make the next generation of serology techniques as precise as those currently based on genome sequencing. The same concerns apply to other viral genera, for instance Flavivirus, where Zika and dengue exhibit cross-reactivity (Dejnirattisai et al., 2016; Priyamvada et al., 2016). Conclusions about Zika’s clinical occurrence prior to the beginning of the large Pacific/Americas outbreak are therefore both crucial to assessment of the degree of herd immunity to Zika and based on potentially unreliable data.

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References


