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

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Abstract

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; 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

50 officially recognised as such by the World Health Organization (WHO) (Table 1). This figure
51 is open to interpretation, as many of the outbreaks are temporally and geographically
52 clustered, and some clusters may represent recurrent flare-ups of outbreaks with a single
53 origin. Until the Ebola-Makona strain (Kuhn *et al.*, 2014) outbreak beginning in Guinea in
54 late 2013, collection of virus genome data was relatively sporadic, so data is not available to
55 answer some of the questions that Table 1 might beg. The repeated minor recurrences of
56 Ebola virus disease (EVD) after the end of the main wave of the west African outbreak (e.g.
57 Blackley *et al.*, 2016; Diallo *et al.*, 2016), which we know from genome data to have all been
58 Ebola-Makona in origin, might in the past have been classified as a cluster of separate
59 outbreaks. Therefore, 25 outbreaks since 1976 must be seen as a ceiling rather than a
60 precise value.

61 These EVD outbreaks have ranged in size from single cases to the 11,310 official fatalities
62 associated with EBOV-Makona in west Africa between December 2013 and April 2016. An
63 additional two species in the genus, Reston ebolavirus (RESTV: Geisbert *et al.*, 1992) and Taï
64 Forest ebolavirus (TAFV: Le Guenno *et al.*, 1995) have not been associated with transmission
65 between humans, although TAFV has produced one non-fatal clinical case. Based on the
66 distribution of EVD outbreaks by species before 2013, it is possible to define a geographical
67 range for each virus: EBOV in the Congo Basin and westward to the Atlantic Ocean, SUDV in
68 Uganda and northwards into what is now South Sudan, and BDBV in an intermediate zone
69 between the two (Figure 1). The appearance of EBOV-Makona in eastern Guinea in
70 December 2013 presented an additional geographical locus which disturbed the pre-2013
71 view of EVD as a disease limited to central Africa. The location of RESTV in Asia and TAFV in
72 Ivory Coast did not previously affect this picture as neither had been responsible for human-
73 to-human EVD transmission. Defining the true geographical extent of EVD is of great

74 importance, since the absence of west Africa from the previously accepted account of EVD
75 incidence was a factor in the failure to recognise the disease until the outbreak was already
76 spreading widely (Moon *et al.*, 2015).

77

78 Methods Employed in Ebola Serology

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

84 Table 2 shows that of the 30 studies we were able to identify in the literature, 24 consisted
85 of samples collected before 1990. Two studies (Becker *et al.*, 1992; Tignor *et al.*, 1993) used
86 archive samples stored for up to two decades. All pre-1992 studies, with one exception
87 (Boiro *et al.*, 1987), used immunofluorescence (IF). Subsequent studies have all used
88 enzyme-linked immunosorbent assay (ELISA). Both of these techniques rely on cross-
89 reaction of serum samples with an antigen immobilised on a slide or in a well. The antigens
90 used for this purpose have also been highly variable, some papers specifying the strain as
91 well as the species (e.g. Gonzalez *et al.*, 1989; Meunier *et al.*, 1987; Nakounne *et al.*, 2000;
92 Tignor *et al.*, 1993; Van der Waals *et al.*, 1986), with others merely the species (e.g.
93 Blackburn *et al.*, 1982; Mathiot *et al.*, 1989; Rodhain *et al.*, 1989), and a third category with
94 even fewer details (e.g. Paix *et al.*, 1988; Saluzzo *et al.*, 1980). Many of the studies were
95 also performed in the field, often in remote areas and presumably with limited facilities for
96 preventing degradation of both serum samples and laboratory materials. All studies

97 focussed on immunological reactivity, and neutralization of virus was not studied. Under
98 such circumstances, scepticism concerning results is justified, and a further examination of
99 techniques is warranted.

100

101 ## Immunofluorescence

102 The early IF-based methods are described in detail by Johnson *et al.* (1981b). Virus-infected
103 Vero cells in suspension were ultraviolet-irradiated to inactivate viral infectivity and then
104 dried onto Teflon-coated microscope slides which were fixed in acetone, then gamma-
105 irradiated to destroy any residual infectivity and further sterilise both the slides and the
106 inside of the slide box. The infection and dilution process was titrated such that an average
107 of less than 10% of cells per slide were infected, thus providing an internal negative control.
108 Test samples reacting to all the surface of the slide could therefore be discarded as false
109 positives and only those slides displaying the predicted fluorescence from 5% to 15% of cells
110 would be scored as positive reactions. Negative control sera were also used to differentiate
111 slides producing non-specific reactions and antisera raised against the virus in the laboratory
112 were used as positive controls. In the laboratory setting, Johnson *et al.* (1981b) stored their
113 IF slides at -70^{o}C prior to use, which would be impossible in a field setting.

114 It must be assumed that most of the early IF studies carried out in the field, used slides
115 prepared similarly to those of Johnson *et al.* (1981b). Van der Waals *et al.* (1986) describe
116 some of the associated limitations. Surveying in Liberia in 1981-1982 (Table 2), they
117 emphasise the necessity for pre-incubation of serum with uninfected cells, as well as
118 positive and negative controls and blind scoring. However, having implemented this
119 procedure for reducing false positives, they were able to differentially score EBOV-Mayinga

against SUDV-Boniface (11.8% seropositivity versus 1.6% respectively) and both against other viruses (Lassa fever 1.3%, Rift Valley fever 0.4%, Congo-Crimean haemorrhagic fever 4.4% and Marburg virus 1.3%). This capacity, shown in several of the early IF surveys, to differentially score for, and therefore by implication differentially detect, *Ebolavirus* species, provides a plausible internal control for the method. For instance, Ivanoff *et al.* (1982) found 6% seropositivity to EBOV in their Gabonese 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% to 13% seropositivity to EBOV versus zero to SUDV in Madagascar.

Providing the IF slides satisfied the required controls before leaving the laboratory, and the field surveyors applied positive 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.

Enzyme-Linked Immunosorbent Assay

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 polystyrene 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-conjugated. 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 of the number of Ebola serology studies since the 1990s has probably partly been a consequence of this necessary additional 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 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 convalescent patients only whereas the clinical status of the ELISA test subjects is not specified. It is also not clear, apart from the four specifically cross-checked samples, if 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 literature. 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 sending the samples to other centres for independent cross-checking (Gonzalez *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

166 from all species of the genus *Ebolavirus* (Macneil et al., 2011), showed cross-reactivity to be
167 considerable, but another study considered it to be more limited (Nakayama *et al.*, 2010).
168 In a clinical context, sera from survivors of outbreaks displayed cross-reactivity to
169 recombinant proteins from other filovirus species, which had been bound to a protein
170 microarray (Natesan *et al.*, 2016) and monoclonal antibodies raised against the
171 glycoproteins of BDBV, SUDV and EBOV exhibit some cross-specific *in vitro* binding. In the
172 case of the anti-BDBV-GP (glycoprotein) antibodies, they were also protective against EBOV
173 infection in guinea pigs (Flyak *et al.*, 2016). Conversely heterologous vaccines expressing
174 recombinant EBOV and SUDV glycoprotein are protective against infection with BDBV in
175 macaques (Hensley *et al.*, 2010), and viral-like particles (VLPs) have also been used to
176 generate some cross-specific protection (Warfield *et al.*, 2015). Convalescent sera from the
177 EBOV-Makona outbreak contain antibodies which cross-react with commercial EBOV and
178 SUDV nucleoprotein antigens on Western Blot (WHO, 2015), and conversely sera from SUDV
179 patients reacts against EBOV antigens (Sobarzo *et al.*, 2015). The structural basis of cross-
180 reactivity between an anti-EBOV antibody and a Marburg virus antigen has also been
181 elucidated (Hashiguchi *et al.*, 2015).

182

183 ## Unexpected Results from Ebola Serology Studies

184 Despite the technical and descriptive issues delineated above, and subsequent *in vitro*
185 findings regarding cross-reactivity, Boiro *et al.* (1987) has become in retrospect a significant
186 paper, in that it presents, along with the IF paper of Van der Waals *et al.* (1986), evidence
187 for the occurrence of EVD in two of the three countries later affected by the 2013-2016
188 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, Schoepp *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 Schoepp *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 anonymised 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 Western blot methods. ELISA was carried out in the first instance, and then the positive samples cross-checked with IF and Western blotting with confirmation rates in excess of 66% for all antigens tested. The initial ELISAs used heterologous incubation to address the problem of cross-specificity. REBOV 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*, *Epomops franqueti*, and *Myonycteris torquata*) have been hypothesised to be the natural reservoir host of EBOV on the basis of detection of viral genomes (Leroy *et al.*, 2005), but the human seropositive zone is wider than their incidence (Figure 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 zero and 6% for any one filovirus, with all species tested (EBOV, SUDV, REBOV, 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 (Olival *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 *Epomops 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; 2004b; 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.*, 2004a; 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 (CAR) has been the subject of seven published studies, carried out between 1979 and 1997 (Figure 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 CAR. Prior to the first EVD outbreak in Gabon in 1994, that country was the subject of a 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 (Figure 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 criticised 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; Schoepp *et al.*, 2014) which may represent small EVD outbreaks that escaped official classification, speculation being particularly focussed 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 unrecognised EVD. A similar effort was undertaken in the 1980s to identify traces in the literature of potential cases of pre-1979 HIV-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 recognised 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 localised 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% 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 sub-clinical. 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 recognised 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 the 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 sub-clinical EVD, despite the circumstantial implication. Becquart *et al.* (2014) compared sera from asymptomatic seropositive individuals with 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, recognised in both the EBOV 1995 and SUDV 2000 outbreaks (Kinsman, 2012) and emerging as a major factor in the EBOV-Makona outbreak (Karamouzian & Hategekimana, 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 3-fold higher than the reported number. It is unclear however if 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 localised 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 answer, 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.*, 2010; 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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Figure Legend

Figure 1: Locations of EVD outbreaks and of seropositive human populations prior to known outbreaks. Black square: EBOV outbreak; ochre square: BDBV outbreak; purple

722 square: SUDV outbreak; blue square: TAFV case; red circle: seropositive result in a
723 population survey prior to outbreak in that area; dotted line: range of three fruit bat species
724 implicated as EBOV reservoirs. Squares mark epicentres of outbreaks, total range was often
725 larger. Seropositive populations sampled after EVD outbreaks in same region are not shown
726 (for which see Table 3).

727

728 ## Tables

729

Year	Country	Species	Cases	Deaths	CFR
2013-2016	Guinea, Liberia, Sierra Leone	EBOV	28,616	11,310	40%
2014	DRC (Zaire)	EBOV	66	49	74%
2012	DRC (Zaire)	BDBV	57	29	51%
2012	Uganda	SUDV	7	4	57%
2012	Uganda	SUDV	24	17	71%
2011	Uganda	SUDV	1	1	100%
2008	DRC (Zaire)	EBOV	32	14	44%
2007	Uganda	BDBV	149	37	25%
2007	DRC (Zaire)	EBOV	264	187	71%
2005	Congo	EBOV	12	10	83%
2004	Sudan	SUDV	17	7	41%
2003	Congo	EBOV	35	29	83%
2003	Congo	EBOV	143	128	90%
2001-2002	Congo	EBOV	59	44	75%
2001-2002	Gabon	EBOV	65	53	82%
2000	Uganda	SUDV	425	224	53%
1996	South Africa (ex-Gabon)	EBOV	1	1	100%
1996	Gabon	EBOV	60	45	75%
1996	Gabon	EBOV	31	21	68%
1995	Zaire	EBOV	315	254	81%
1994	Gabon	EBOV	52	31	60%
1979	Sudan	SUDV	34	22	65%
1977	Zaire	EBOV	1	1	100%
1976	Sudan	SUDV	284	151	53%
1976	Zaire	EBOV	318	280	88%

730

731 **Table 1: The 25 officially declared outbreaks of EVD from 1976 to 2016.** CFR: case fatality

732 rate. EBOV: Zaire ebolavirus; SUDV: Sudan ebolavirus; BDBV: Bundibugyo ebolavirus. Data

733 from WHO (2016)

734

Sampling date	Region	Method	% seropositive	Reference
1961-1962	8 locations, Ethiopia	IF – Mayinga	7-39	Tignor *et al.* (1993)
1972-1991	Germany	ELISA/IF/WB*	Filovirus 7, EBOV 1	Becker *et al.* (1992)
1979	Bangassou, CAR	IF	3	Saluzzo *et al.* (1980)
1980	Franceville, Gabon	IF – EBOV	6	Ivanoff *et al.* (1982)
1980	5 locations, Cameroon	IF	3-14	Bouree and Bergmann (1983)
1980	4 locations, Zimbabwe	IF – EBOV	1-3	Blackburn *et al.* (1982)
1980-1981	Nzoia, Kenya	IF – EBOV	1 (patients)	Johnson *et al.* (1983b)
1981-1982	Grand Bassa, Liberia	IF – Mayinga/Boniface	EBOV 12, SUDV 2	Van der Waals *et al.* (1986)
1982-1983	Madina-Oula, Guinea	ELISA/IF – EBOV	19 (patients), 8	Boiro *et al.* (1987)
pre-1983	6 locations, Kenya	IF – Mayinga/SUDV	EBOV 1-7, SUDV <1	Johnson *et al.* (1983a)
1983	Awash Valley, Ethiopia	IF – Mayinga	30	Tignor *et al.* (1993)
1984	4 locations, Uganda	IF – EBOV/SUDV	EBOV 3, SUDV 3	Rodhain *et al.* (1989)
1984-1985	13 locations, CAR	IF - filovirus†	Filovirus 4-23	Meunier *et al.* (1987)
1984-1985	Kenya	IF – EBOV/SUDV	10 (patients)	Johnson *et al.* (1986)
1985	Nkongsamba, Cameroon	IF	2	Paix *et al.* (1988)
1985-1987	N'Djamena, Chad	IF – Mayinga/Boniface	4	Gonzalez *et al.* (1989)
1985-1987	3 locations, Cameroon	IF – Mayinga/Boniface	2-11	Gonzalez *et al.* (1989)
1985-1987	Bangui, CAR	IF – Mayinga/Boniface	33	Gonzalez *et al.* (1989)
1985-1987	Bioco/Nsork, Equatorial Guinea	IF – Mayinga/Boniface	16	Gonzalez *et al.* (1989)
1985-1987	5 locations, Gabon	IF – Mayinga/Boniface	7-22	Gonzalez *et al.* (1989)
1985-1987	2 locations, Congo	IF – Mayinga/Boniface	6-8	Gonzalez *et al.* (1989)
pre-1987	Benue-Gongola, Nigeria	IF – EBOV/SUDV	2	Tomori *et al.* (1988)
1987	Mongoumba, CAR	IF – EBOV/SUDV	18	Johnson *et al.* (1993a)
pre-1988	Madagascar	IF – EBOV/SUDV	EBOV 4-13, SUDV 0	Mathiot *et al.* (1989)
pre-1992	4 locations, CAR	IF – Mayinga/Boniface	EBOV 1-9, SUDV 19-27	Johnson *et al.* (1993b)
1992-1996	4 locations, CAR	ELISA – Mayinga	4-7	Gonzalez *et al.* (2000)
1992-1997	3 locations, CAR	ELISA – Mayinga	2-13	Nakounne *et al.* (2000)
2002	Watsa, DRC	ELISA – EBOV	19	Mulangu *et al.* (2016)
2006-2008	Kenema, Sierra Leone	ELISA – EBOV	9 (patients)	Schoepp *et al.* (2014)
2011-2014	Kenema, Sierra Leone	ELISA – Mayinga‡	22 (patients)	Boisen *et al.* (2015)

735

736 **Table 2: Sero-surveys with positive results conducted in regions of Africa where no EVD**
737 **outbreak has occurred, or prior to the occurrence of EVD in that region.** Where a variety of
738 locations were sampled, the range of seropositivity is given. CAR: Central African Republic;
739 DRC: Democratic Republic of Congo. IF: immunofluorescence; ELISA: enzyme-linked
740 immunosorbent assay; WB: Western blot. Where EBOV and SUDV were assayed separately,
741 corresponding values are given. Species or strain of Ebolavirus antigen used also given
742 when specified in publication. "Patients" indicates that the subjects were suffering from a
743 haemorrhagic fever at the time of the test. Where no specific date for the survey is given in
744 the paper, submission date of the paper is used as "pre-19nn". *Becker *et al.* (1992) used
745 Mayinga (EBOV), RESTV and Musoke (*Marburgvirus*). †Meunier *et al.* (1987) used Mayinga,
746 Boniface (SUDV) and Musoke (*Marburgvirus*). ‡purified antigens, otherwise whole virus.

747

Sampling date	Region	Assoc. outbreak (see Table 1)	Method	% general pop.	% contacts	Reference
1972-1978	N.W. Zaire	EBOV 1977	IF	13-56		Van der Groen and Pattyn (1979)
1976-1977	Yambuku area, Zaire	EBOV 1976	IF	<1	2.5	WHO (1978b)
1978	Tandala area, Zaire	EBOV 1977	IF	4-10		Heymann *et al.* (1980)
1979	Nzara/Yambio, Sudan	SUDV 1976, 1979	IF	18	32	Baron *et al.* (1983)
1981	Tandala, Zaire	EBOV 1977	IF	5		Stansfield *et al.* (1982)
1981-1985	Sud-Ubangi, Zaire	EBOV 1976, 1977	IF	60 (patients), <1	18	Jezek *et al.* (1999)
1986	Juba, Sudan	SUDV 1976, 1979	IF	5 (patients)		Woodruff *et al.* (1988)
1995	Kikwit, Zaire	EBOV 1995	ELISA	3		Tomori *et al.* (1999)
1995	Kikwit, Zaire	EBOV 1995	ELISA	2-18		Busico *et al.* (1999)
1995	Bandundu, Zaire	EBOV 1995			3	(Rowe *et al.*, 1999)
1996	N. Gabon	EBOV 1996	ELISA/WB		50	Leroy *et al.* (2000)
1996	Ogooue-Ivindo, Gabon	EBOV 1996	ELISA	10		Bertherat *et al.* (1999)
1997	Ogooue-Ivindo, Gabon	EBOV 1996	ELISA	1		Heffernan *et al.* (2005)
2005-2008	Gabon	EBOV 2001-2002	ELISA	3-21		Becquart *et al.*

						(2010)
2005-2008	Gabon	EBOV 2001-2002	ELISA	<1-32		Nkoghe *et al.* (2011)
2014	Kailahun, Sierra Leone	EBOV 2013-2016	ELISA		20-65	de La Vega *et al.* (2015)

748

749 **Table 3: Sero-surveys with positive results conducted in regions of Africa subsequent to**
750 **an EVD outbreak, divided into general population seropositivity, and contacts of known**
751 **cases where available.** Where a variety of locations were sampled, the range of
752 seropositivity is given. IF: immunofluorescence; ELISA: enzyme-linked immunosorbent
753 assay; WB: Western blot. "Patients" indicates that the subjects were suffering from a
754 haemorrhagic fever at the time of the test.

755