**Asymptomatic infection and unrecognised Ebola Virus Disease: seroprevalence of antibodies to Ebola virus in a large cross-sectional study in Ebola-affected households, Sierra Leone, using a new non-invasive assay**

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**Abstract**

**Background**

The frequency of asymptomatic infection with Ebola virus (EBOV) is unclear: previous estimates vary and there is no standard test. Asymptomatic infection with EBOV could contribute to population immunity, reducing spread. If people with asymptomatic infection are infectious it could explain re-emergences of Ebola virus disease (EVD) without known contact.

**Methods**

We validated a new oral fluid anti-glycoprotein IgG capture assay among survivors from Kerry Town Ebola Treatment Centre, and controls from communities unaffected by EVD in Sierra Leone. We then assessed the seroprevalence of antibodies to EBOV in a cross-sectional study of household contacts of the survivors. All household members were interviewed. Two reactive tests were required for a positive result, with a third test to resolve any discrepancies.

**Findings**

The assay had a specificity of 100% (339/339 controls tested negative) and sensitivity of 96% (93/97 PCR-confirmed survivors tested positive). Of household contacts not diagnosed with EVD, 48% had high level exposure (direct contact with a corpse, body fluids or a case with diarrhoea, vomiting or bleeding). Among the contacts, 12.0% (11/92) with symptoms at the time other household members had EVD, and 2.6% (10/389) with no symptoms tested positive. Among asymptomatic contacts, seropositivity was weakly correlated with exposure level.

**Interpretation**

This new highly specific and sensitive assay showed asymptomatic infection with EBOV was uncommon despite high exposure. The low prevalence suggests asymptomatic infection contributes little to herd immunity in Ebola, and even if infectious, would account for few transmissions.

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**Research in context**

**Evidence before this study**

We conducted a systematic review of studies of seroprevalence of antibodies to Ebola virus. We searched PubMed and Web of Science using the search string “ebola AND (asymptom\* OR antibod\* OR IgG OR immun\* OR ELISA OR serol\*) NOT vacc\* NOT immuniz\* AND (Humans[Mesh])”, as well as reference lists (including those of previous reviews) and conference reports from the West Africa epidemic. We last updated the search on 31 July 2016 and used no language restrictions.

 Different assays have been used and the specificity of the tests is frequently questioned. Of 50 studies, only 6 reported results for asymptomatic household contacts, with varying prevalence estimates: 2.5% in the first known Ebola virus outbreak using an immunofluorescence assay; and 1.0% in Uganda, 4.0% in Democratic Republic of Congo, 6.5% in Sierra Leone, and 21.4%, and 45.9% in Gabon, using different ELISAs.

**Added value of this study**

We present the first field validation of a new assay. It had very high specificity and sensitivity and has the added advantage of being non-invasive so was well accepted. Using this assay we showed that the prevalence of sero-positivity to Ebola virus in asymptomatic household contacts, many of whom were highly exposed, was only 2.6%. In addition, 12% of contacts with some symptoms but never diagnosed with Ebola Virus Disease were seropositive. In these Ebola-affected households, asymptomatic infections accounted for 2.3% and missed symptomatic infections for 2.6% of all Ebola virus infections.

**Implications of all the available evidence**

Asymptomatic infection with Ebola virus occurs but given the low seroprevalence seen even in highly exposed individuals, it would not be a major contributor to herd immunity.

The availability of a reliable non-invasive assay that is easy to administer and highly acceptable in the field will greatly aid future investigations and interventions, including testing and targeting of vaccines.**Introduction**

It is not known how frequently asymptomatic Ebola virus (EBOV) infection occurs, yet it could influence the course of epidemics. High rates of asymptomatic infection would reduce incidence through herd immunity, radically altering model predictions of epidemic spread.1 If those with asymptomatic infection are infectious, perhaps with persistent viral shedding, it would help explain some failures in control and the emergence of new chains of transmission.2

The extent of asymptomatic infection is unclear because previous findings have varied widely (e.g., from 1-46% of household contacts),3, 4 with positive results reported in some populations unlikely to have been exposed to filoviruses.5-7 This has led to questions about assay specificity and cross-reactivity for enzyme-linked immunosorbent assays (ELISA) as well as for the older immunofluorescence antibody techniques. There is no FDA-approved assay, and the need for caution in interpreting EBOV antibody serosurveys continues to be emphasised.8

A reliable serological test could also help identify missed cases with minor symptoms. Asymptomatic infections and missed symptomatic cases might explain the apparent lower incidence of Ebola virus disease (EVD) in children.9, 10 Diagnosis may be missed in young children,11 and older children could be less susceptible to developing EVD if infected.12

A test for EBOV antibodies with high sensitivity and specificity is needed. Taking blood is difficult in an Ebola epidemic, due to both the infection risk and population suspicion. We describe the field validation of a new Capture ELISA which detects IgG to EBOV glycoprotein in oral fluid,13 and the results of a large seroprevalence study in Ebola-affected households.

**Methods and materials**

*Participants and data collection*

All survivors from Kerry Town Ebola Treatment Centre, who were discharged between November 2014 and March 2015, and their household members (people eating from the same pot), were sought for this study. Interviews were conducted in July-September 2015, encouraging household members to tell their story as a group, as described elsewhere.12 For each person in the household who was ill or died of EVD we asked who had helped them and had contact with them. We also asked about exposures outside the household. With additional probing questions, we established the maximum exposure level for each person, including those who had not been ill and those who had died, using predefined levels.12 The highest level was touching the body of someone who died of EVD, then direct contact with body fluids of a “wet” case (i.e. an EVD case with diarrhoea, vomiting or bleeding); direct contact with a wet case (including nursing and personal care, sharing a bed); direct contact with a dry case (i.e. an EVD case without “wet” symptoms); indirect contact with a wet case (e.g. washing clothes/bed linen); indirect contact with a dry case; minimal contact (e.g. shared meals); and no known contact.

Individuals who did not report EVD were asked about symptoms at the time that others in the household had EVD. Those reporting symptoms were classified using the Sierra Leone case definition for “probable” EVD14 (i.e. contact plus fever or miscarriage or unexplained bleeding; or contact plus three or more symptoms (of fatigue, headache, loss of appetite, nausea or vomiting, abdominal pain, diarrhoea, muscle or joint pain, sore throat or pain on swallowing, hiccups).

Swabs (Oracol, Malvern Medical Developments Limited) for oral fluid collection were demonstrated by the field staff and then self-administered, with adults helping children. Each swab was rubbed firmly on the gums for 90 seconds, sealed, put in a cool box, and transferred daily to a -20°C freezer for storage prior to processing.

In addition, we recruited community controls in three neighbourhoods of rural Western Area Sierra Leone without known EVD cases (Kent, Tokeh and York). Through community leaders with megaphones, we asked for volunteers of all ages, excluded any with exposure to Ebola, and collected oral swabs as described above.

Individual written informed consent was obtained from all participants (or their parents/guardians for those <18 years) before interview and sample collection. Permission for the study was granted by the Sierra Leone Ethics and Scientific Review Committee and the Ethics Committee of the London School of Hygiene & Tropical Medicine.

*Laboratory analysis*

Oral fluid samples were tested for Ebola virus glycoprotein IgG using a new IgG Capture assay based on the EBOV Mayinga GP antigen (rGPδTM, IBT Bioservices Inc. USA cat.0501-016) as described elsewhere.13 Two positive controls (plasma from a UK EVD survivor infected in Sierra Leone) and four negative controls (plasma from UK donors) were included in each plate. The cut-off for a reactive result was defined per plate as the mean optical density (OD) of the negative controls plus a fixed OD measure (0.1). (Since the mean negative OD varied between 0.049 and 0.067 per plate, this is equivalent to 2.5-3 times the mean negative OD.) We present “normalised ODs”, i.e. the ratio of the test OD to the cut-off, so results >1 are reactive. All reactive samples from household members and controls, all unreactive samples from survivors, and a selection of other samples including those closer to the cut-off, were repeated. Samples with discrepant results were re-tested.

Using this assay, results from paired oral fluid and plasma samples have previously been shown to correlate well in: 76 participants in an early-phase Ebola vaccine trial in the UK (r=0.68, p< 0.0001, 2-tailed non-parametric Spearman’s correlation);13 10 EVD survivors tested in Connaught Blood Bank, Sierra Leone (r2=0.83, linear regression); and 80 EVD survivors from Sierra Leone tested in the UK (r2=0.78, linear regression) (Tedder et al submitted). Using the same cut-off as in our study, 78/80 samples from the EVD survivors were positive on serum, of which 76 were positive on oral fluid, giving a sensitivity compared to serum of 97.4% (76/78). The two samples negative on both oral fluid and plasma were also negative on competitive and double-antigen bridging assay ELISAs. All 44 paired oral fluid and plasma samples from The Gambia were negative on the capture ELISA using the same protocol (Tedder et al submitted).

*Statistical analysis*

We assessed the sensitivity and specificity of the assay under field conditions using samples from PCR-confirmed Kerry Town EVD survivors and from the community controls.

For further analyses individuals were defined as having been infected if their sample was reactive on two or more tests, uninfected if their sample was unreactive on one or more tests, and indeterminate if their sample had an equal number of reactive and unreactive tests. Two reactive tests were required to define infection to maximise specificity and hence positive predictive value, which is important as the prevalence of asymptomatic infection could be low. The confidence intervals for the proportion positive were calculated using exact methods because of small numbers.

We assessed risk factors for infection among asymptomatic and symptomatic household members using Chi squared or Fisher’s exact test as appropriate. We assessed confounding by age using logistic regression; further multivariable analysis was limited by the small number of events. Linear regression was used to assess the association of level of reactivity in the samples from survivors with time since admission and with age.

*Role of the funding source*

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

**Results**

The households of 123 of 151 Kerry Town survivors were included in the study. One survivor had subsequently died15 but the household included another survivor so was visited. Of the remaining survivors, eight lived outside Western Area, three had died,15 16 were unavailable or uncontactable, and one refused to take part (Figure 1).

The participating Kerry Town survivors lived in 91 households with 814 household members, of whom 242 had died (227 from EVD, 11 from probable EVD, and four from unknown causes) and 45 were survivors from other facilities ( Figure 1). Of the 527 other household members, 96 had some symptoms around the time others in their household had EVD, and 431 were asymptomatic. We collected 639 oral swabs from 153 survivors and 486 living household members of which 633 (99.6%) could be analysed; only seven people (1.0%) refused to give a swab (Figure 1). The mean age of the household members was 16.7 years (range <1 year to 84 years); 57% were female. The age and sex distribution of participating survivors and household members was similar to non-participants.

Oral swabs were collected from 663 community controls. Three people with possible EBOV exposure (two Ebola intervention workers and one funeral attendee) were excluded. Due to availability of test kits, we analysed a random sample of 339, mean age 19.0 years (range <1 year to 76 years), 53% female.

*Validation of test*

The distribution of normalised ODs (NODs) in the Kerry Town survivors and the community controls on the first test is shown in Figure 2. From the Kerry Town survivors, 113/116 (97.4%) samples were reactive on the first test. Ninety-seven samples were retested: the three unreactive samples remained unreactive; one reactive sample was unreactive on retesting and on a third test (NODs 1.59, 0.97, 0.69) so considered negative; another reactive sample was unreactive on retesting and reactive on the third test (NODs 2.50, 0.76. 1.01) so considered positive. All remaining initially reactive samples were repeatedly reactive and considered positive (Table 1). Defining positive as two reactive tests gives a sensitivity of 95.9% (93/97; 95% CI 89.8-98.9%, Table 2).

Among the community controls, all but one sample were unreactive on the initial test (338/339, 99.7%). This sample was unreactive on second and third tests (NODs 1.41, 0.33, 0.32). There were no further reactive results among 25 samples that were retested. Since no control sample was considered positive, specificity was 100% (95% CI 98.9-100%).

Among those with duplicate tests NODs were in good agreement in the different participant groups (Appendix, pages 2-4). Overall, comparing the NODs of the first and second test using linear regression, r2 =0.88.

*Seropositivity of households members*

Among the survivors from other treatment centres (for whom we did not have documented evidence of positive EBOV PCRs) 86.1% (31/36) were positive for Ebola IgG. Forty of 481 samples from household contacts without diagnosed EVD were reactive on the first test. After subsequent tests, 21 were considered positive, 18 negative and 1 indeterminate ( Table 1, and Appendix, page 5). Among 389 asymptomatic contacts, 2.6% were seropositive, compared to 12.0% of 92 symptomatic contacts (p=0.004). The asymptomatic infections were from different households, whereas two people with symptomatic undiagnosed infections were from the same household.

Asymptomatic infection was only seen in those >12 years. By contrast among symptomatic contacts, seropositivity was highest in children <5 (26.7%) and in adults ≥30 (32.6%) but undetected in teens and young adults (Table 2).

Level of exposure to Ebola correlated with seropositivity among asymptomatic and symptomatic contacts (Table 2). Of the 12 individuals with direct contact with an EVD corpse who were not diagnosed with EVD themselves, four (33%) were infected, two asymptomatically. Among the 229 without known EVD with the three highest exposure levels (contact with corpse, body fluids or wet cases), 16 (7%) were infected, 7 asymptomatically. There were few socio-economic factors associated with positivity (Table 2). Associations with occupation and being household head were explained by age. Twenty-three contacts had spouses who were EVD survivors so could potentially have been infected by sexual transmission after recovery. Two of these contacts were seropositive; both were male and had been symptomatic. .

Among symptomatic contacts neither the number of symptoms, nor any individual symptom in the case definition, were associated with seropositivity, except “red eyes” which correlated weakly (p=0.07). The 11 seropositive undiagnosed symptomatic individuals were: a 1-year-old with multiple symptoms who was not tested or admitted due to a nurses’ strike; a 2-year-old and a 9-year-old with multiple symptoms who were not taken to a facility; three people with two symptoms (headache + fatigue, loss of appetite or muscle/joint pain); and five people with single symptoms, (abdominal pain, red eyes, hiccups, fever and headache).

*Contribution of asymptomatic and undiagnosed infections to total Ebola burden*

Overall in these households there were 168 survivors and 238 EVD deaths reported at interview (Figure 1), so, assuming seropositivity is a marker of EBOV infection, the 10 asymptomatic and 11 symptomatic seropositives contributed 2.3% (10/427) and 2.6% (11/427) of EBOV infections respectively. The contribution by age and exposure level is shown in Figure 3 and Appendix page 9. In all age groups the proportion of infections that were asymptomatic was low, but it was higher in 5-14 year-olds (6.3%, 4/64) than in the <5s (0%, 0/53) and people aged ≥15 (2.0%, 6/307, *p=*0.07). The proportion of undiagnosed symptomatic infections was higher in the <5s (7.5%, 4/53), than in 5-14 year-olds (1.6%, 1/64) and those aged ≥15 (2.0%, 6/307), *p*=0.07.

*Reactivity level*

Among those with positive tests the NOD was similar in survivors and in those with asymptomatic or missed symptomatic infections: *p=*0.9 and *p*=0.7 in Wilcoxon rank sum test, respectively, Appendix, page 6.

Among survivors, no relationship was seen between the magnitude of the NOD and the length of time since admission (Appendix, page 7) but the NOD was higher at younger ages (Appendix, page 8, r2 0.08, *p*<0.001).

**Discussion**

The oral fluid IgG Capture ELISA performed well in this field setting. The oral swabs were accepted by the population (only 1% refused) and were suitable for children and adults. The swabs required no processing before storage at -20◦C, making them easy to use in field conditions. We optimised specificity by using a high cut-off (Figure 2) and requiring two reactive results to confirm a positive; sensitivity remained high (96%).

Using this assay, 2.6% (10/389) of asymptomatic members of Ebola-affected households had evidence of EBOV infection. This is lower than some household contact studies, but few such studies restricted examination to asymptomatic contacts, different assays were used, and the definition of contact varied. Excluding any symptomatic individuals, previous estimates are 2.5% (10/404) in Yambuku, DRC, using an immunofluorescence assay;16 4.0% (4/101) in Kikwit;17 21.4% (12/56) in Gabon;18 45.9% (11/24) among highly-exposed contacts in Gabon;4 1.0% (2/210) in Uganda3, and 6.5% (12/185) in Kono, Sierra Leone,19 using different ELISAs. A preliminary report from Liberia studied 760 household members or sexual contacts; 13% were positive but it is not clear if all were asymptomatic or which contact group they belonged to.20

The higher proportion of asymptomatic infection in adolescents, and the higher reactivity levels in younger survivors are consistent with a lower risk of severe disease. Immunological differences between symptomatic and asymptomatically-infected individuals, and between adults and children, have been noted previously.[17](#_ENREF_17), [27](#_ENREF_27), [28](#_ENREF_28) The slight excess of missed symptomatic infections in children <5 is consistent with under-diagnosis in this group.[11](#_ENREF_11) There was no evidence that any of the seropositive results were due to late transmission via semen:[23](#_ENREF_23) only two spouses of EVD survivors were seropositive and both were male.

WHO guidelines for EVD survivor care[29](#_ENREF_29) suggest that a positive IgG test could help define survivors if certificates (issued on discharge from a Treatment Centre) are missing, so a highly specific test is essential. An acceptable, sensitive and specific assay would also assist vaccine studies, where knowledge of pre-existing immunity is important, and in identifying previously undiagnosed EVD cases who may have played a critical role in transmission.

*Limitations*

Testing the sensitivity assumed the Kerry Town survivors were correctly diagnosed. All four seronegative Kerry Town survivors were documented PCR-positive before admission; after admission, two (including the one with reactivity near the cut-off) had high-level PCR results, one had two low-level PCR results, and for the one with the lowest reactivity (Appendix, page 5), who was in her 80s, we have no post-admission record of positive PCR results. Oral fluid containing insufficient IgG will fail to signal; this can only be checked by determining IgG levels, which was not available in this setting. We did not have paired serum samples from these individuals, though good correlation with oral fluid results has been shown previously.

The oral fluid samples were collected up to 10 months after exposure. Decline in IgG levels is possible, though antibody persistence for several years has been noted previously,[17](#_ENREF_17), [21](#_ENREF_21), [22](#_ENREF_22) and we found no evidence of decline (Appendix, page 7). It is theoretically possible that low level infections may have led to low levels of IgG that were not detected, which would underestimate the proportion of asymptomatic infections. However in our study, above the cut-off, the NOD was similar in those with asymptomatic infection and in survivors (Appendix, page 6). We did not have enough test kits to re-test all those with initially unreactive results, but all 119 tested in duplicate remained unreactive.

Because our initial contact was through the community reintegration team we only investigated survivor households. Survivors may be less infectious than those who die,[12](#_ENREF_12), [24-26](#_ENREF_24) but 70% of households in the study had ≥1 EVD death and exposure levels were high: 48% of household contacts without diagnosed EVD reported contact with corpses, body fluids or ‘wet’ cases, yet only 7% of these were infected.

Accurate recall of symptoms is difficult. Forgetting or reluctance to admit previously unreported symptoms might overestimate the incidence of asymptomatic infection. Conversely, being in an EVD-affected household may have led to over-reporting of symptoms. During interviews family members would contribute details of the exposure and health of others, probably increasing recall accuracy.

*Conclusion*

Using a non-invasive assay, we have shown that asymptomatic EBOV infection occurs, but accounted for only a small proportion of infections, so would have little influence on herd immunity. It is unknown whether those with asymptomatic EBOV infection are infectious, or could harbour virus in the longer-term, like some survivors. In that respect, the low proportion of asymptomatic infections is reassuring as these transmissions would be challenging to prevent. We also identified missed symptomatic cases, some of which were mild. Many questions remain, including why some people escape infection and/or disease despite high exposure, and whether those asymptomatically-infected will have any immunity in future outbreaks.

**Authors’ contributions**

JRG, HB and FC designed the study with contributions from all other authors. RST and DS developed the assay, with SD, MGS and JTS. HB and SJ led the fieldwork with MSB, AJK, OK, SHM, DS, and CT. CH and CM led the laboratory work, with REGW and VC. HB and JRG did the analysis. JRG and HB led the writing with contributions from all other authors. All authors have approved the final manuscript.

**Declaration of interests**

Save the Children International operated the Kerry Town Ebola Treatment Centre during the period under study, and employed the field team members. One author (FC) was employed by Save the Children UK and was involved in commissioning the study and interpreting findings. There are no other conflicts of interests.

**Table 1**: Prevalence of Ebola IgG-positivity in samples from Ebola virus disease survivors, household contacts, and community controls, Sierra Leone 2015

**Table 2:** Sensitivity and specificity of the oral fluid EBOV antibody test. Results are presented in two ways: on the basis of a single test, and using the rule that all reactive results should be confirmed by a second test.

**Table 3**: Prevalence of Ebola IgG-positivity in asymptomatic and symptomatic household members of Ebola virus disease survivors, Sierra Leone 2015, by individual and household characteristics

**Figure 1**: Flow chart of study participants, Sierra Leone 2015.

Footnote to Figure 1: Households were defined as those who ate from the same pot. They included everyone who stayed there at the time Ebola was in the household, including those who were not normally resident.

**Figure 2**: Normalised optical densities of the first test in samples from 116 Kerry Town survivors and 339 Sierra Leone controls

**Figure 3**: Ebola manifestation and risk in households of EVD survivors (A) by age group in all members; (B) by exposure level (excluding the primary cases in each household).

 Footnote to Figure 3: The primary cases were excluded for (B) so that the outcomes for each type of contact in Ebola-affected households can be seen. Information on deceased household members was provided at interview by the surviving household members. Exposure levels were determined from the interviews with all household members. Exposure levels are defined as follows: “Corpse” touched body of someone who died of EVD; “Fluids” direct contact with body fluids of a “wet” case (i.e. an EVD case with diarrhoea, vomiting or bleeding); “Direct wet” direct contact with a wet case (including nursing and personal care, sharing a bed, breastfeeding an EVD positive child); “Direct dry” direct contact with a dry case (i.e. an EVD case without “wet” symptoms); “Indirect wet” indirect contact with a wet case (e.g. washing clothes/bed linen); “Indirect dry” indirect contact with a dry case; “Minimal/none” minimal contact (e.g. shared meals) or no known contact. See Bower et al[12](#_ENREF_12) for details.

**Appendix**

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