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Towards quantification of protective antibody responses by passive transfer of the 1st WHO International Standard for Ebola virus antibody in a guinea pig model

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ABSTRACT

Ebola virus (EBOV) represents a major concern to global health due to the unpredictable nature of outbreaks. Infection with EBOV can cause a severe viral haemorrhagic fever with no licensed vaccine or treatment, restricting work with live EBOV to Containment/Biosafety Level 4 facilities. Whilst the magnitude of recent outbreaks has provided an impetus for vaccine and antiviral development, establishing the efficacy of candidate vaccine materials relies on EBOV challenge models and advanced human trials should outbreaks occur and where logistics and funding allow. To address these hurdles in vaccine development, we investigated whether a recently established serological reference standard, the 1st WHO International Standard for Ebola virus antibody, could be used to provide a quantifiable correlate of immune protection *in vivo*. Dilutions of the International Standard were inoculated into naïve guinea pigs 24 h before challenge with a lethal dose of Ebola virus. Only subjects receiving the highest dose of the International Standard exhibited evidence of delayed progression. Due to it being a WHO established reagent and available globally upon request, this standard allows for effective comparisons of data between laboratories and may prove valuable to select the candidate vaccines that are most likely to confer humoral immune protection ensuring the most promising candidates progress into efficacy studies.

1. Introduction

Ebola virus (EBOV) is one of the most recognised viral haemorrhagic fever viruses, and belongs to the Filoviridae family. The genus *Ebolavirus* taxonomy (revised in 2011 [1]) contains five species: *Bundibugyo ebolavirus* (Bundibugyo virus), *Reston ebolavirus* (Reston virus), *Sudan ebolavirus* (Sudan virus), *Taï Forest ebolavirus* (Taï Forest virus), and *Zaire ebolavirus* (EBOV). Since ebolaviruses were first identified in 1976, only Bundibugyo, Sudan and EBOV have been associated with disease outbreaks in humans. The unprecedented outbreak in West Africa from 2013 to 2016 resulted in more than 28,000 confirmed cases and 11,000 deaths [2]. This has now been followed by another major outbreak in the Democratic Republic of Congo (DR Congo) where an outbreak of EBOV was declared by the Ministry of Health during August 2018 in the North Kivu province which subsequently spread and remains ongoing [3].

Increased efforts have been focused towards the development of interventions. Several vaccine candidates against Ebola virus have been produced based on different recombinant vaccine platforms including DNA, recombinant proteins, virus-like particles, and replicating and non-replicating viral vectors. Most express the viral glycoprotein as the main immunogen [4,5]. Some of these candidates have been evaluated in clinical trials, including those based upon recombinant adenoviruses [6] and recombinant vesicular stomatitis virus (rVSV) [7]. Whilst it is not straightforward to compare the serological data presented in the two studies, antibody responses with chimpanzee adenovirus reduced after 6 months [8] whereas responses after a single dose with rVSV remained high for at least 2 years [9]. Whilst anti-EBOV responses primed with the recombinant chimpanzee adenovirus vaccine may be boosted with a recombinant modified vaccinia Ankara (MVA)-based vaccine expressing the same Ebola transgene [10], this approach of multiple immunisations combining different constructs represents significant challenges both in gaining regulatory approval and in their use in an outbreak situation. Therefore, the single-dose rVSV EBOV vaccine has progressed into further clinical

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testing in the ongoing outbreak in the Democratic Republic of Congo [11].

The development and selection of the most promising vaccine candidates for an EBOV vaccine is made more difficult as there is no defined correlate of protection. There is considerable evidence that antibody responses are critical. The humanised monoclonal antibody therapy ZMapp showed great promise in preclinical studies [12], although ZMapp treatment did not meet the prespecified statistical threshold for efficacy in human clinical trials in West Africa as the study target sample size could not be achieved as a result of the incidence of EBOV decreasing at the time of the trial [13]. As therapies which are based on monoclonal antibodies may be compromised by escape mutants, treatments with polyclonal antibody preparations have also been developed. For example, purified ovine immunoglobulin raised against EBOV antigen, EBOTAb [14], was shown to protect non-human primates (NHPs) post-exposure to EBOV [15]. Passive transfer studies with human convalescent sera have also been undertaken [16]. Furthermore, in the majority of cases EBOV causes an acute disease and these are more often controlled by antibodies whereas cytotoxic T-cells are generally more important in the control of chronic infections [17].

Studies of candidate vaccines have also indicated that the humoral arm of the immune system plays a pivotal role in protection. The efficacy of experimental EBOV vaccines in NHP challenge models most frequently correlate with the presence of anti-EBOV IgG detected by ELISA prior to challenge [18] including the leading vaccine candidate based on a recombinant vesicular stomatitis virus (rVSV) vector [19]. These data confirm earlier observations in genetically deficient mouse models where passive transfer of serum protected animals even in the absence of $CD8^+$ T-cells [20], as well as the analysis of guinea pig and macaque sera from vaccinated subjects that survived EBOV challenge [20]. Other candidate vaccine platforms, such as the rabies virus based bivalent vaccine concur [21].

The regulatory approval of vaccines benefits from the identification of methods that assure both the safety and efficacy of candidate vaccines. Since these methods are frequently bio-assays the comparison of the performance of the assays over time or between laboratories relies of the availability of reference reagents that can harmonise the data. The World Health Organisation has called for the development of reference materials for both molecular diagnostic and sero-diagnostic assays for EBOV.

The National Institute of Biological Standards and Control (NIBSC) responded and undertook studies to assess a panel of samples for comparing assay platforms for EBOV serology [22], and as a result of this work identified a pool of convalescent human sera that harmonised the measurement of anti-EBOV assays in a collaborative study between international expert laboratories. This material was subsequently established as the 1st International Standard (IS) for EBOV antibodies by the World Health Organisation (WHO) Expert Committee on Biological Standardisation [23].

However, to increase the value of this material for vaccine manufacturers, the team at NIBSC collaborated with colleagues at Public Health England (PHE) to establish whether the 1st IS for EBOV antibodies was able to protect subjects in a challenge model and thus provide a common reference standard for immunisation studies of current and novel candidate vaccines.

2. Results

2.1. Protective effects of the 1st International Standard for EBOV antibodies against EBOV infection

Guinea pigs (n = 5/group) received a dose of antibody via intraperitoneal delivery one day prior to challenge with EBOV. All animals met humane endpoints by the scheduled end of the study (14 days post-challenge), except for one animal in the group which received 3000 milli-International Units (mIU) antibody (Fig. 1a). The kinetics of survival for the group which received 3000 mIU antibody was significantly extended compared with the PBS control group (Log-Rank survival, P = 0.039). No other protective effects were observed amongst groups that received diluted international standard sera (P > 0.05). Similarly, delays in weight loss, temperature increase and clinical signs were observed only amongst the animals receiving 3000 mIU antibody compared with the other groups (Fig. 1b–d, respectively).

2.2. Pre-challenge antibody levels and increased protection against EBOV disease

Due to guinea pigs being outbred and the nature of biological systems, variabilities in the levels of antibody in the circulation were assessed. Uptake of the International Standard was established by measuring the serum levels of anti-EBOV antibodies for each subject immediately prior to challenge. Anti-EBOV antibodies were detectable in all those which received 3000 mIU antibody, whereas amongst subjects given a 300 mIU antibody, 60% had detectable levels of antibodies. Anti-EBOV reactivity was not detectable in the sera of the other treatment groups (Table 1). Comparing the levels of anti-EBOV antibodies pre-challenge and the time that animals met humane endpoints, a significant inverse correlation was observed (Pearson correlation 0.958, P < 0.001) (Fig. 2). The only subject that did not meet humane endpoint (ID 02055) exhibited the highest levels of anti-EBOV sero-reactivity prior to challenge.

Using the survival of naïve controls (group 6) as controls, then survival beyond day 10 was significantly associated with treatment with the International Standard (Log-Rank survival, day 10 P = 0.141 and P = 0.014). Using the day 11 cut-off then an antibody titre equivalent to 145 mIU/ml was strongly associated with extended survival.

3. Discussion

The EBOV outbreak in West Africa (2013–2016) has resulted in international investment in the development of effective prophylactic vaccines. The selection of the most promising candidates for progression into late stage clinical trials would be facilitated by an understanding of the scientific framework for vaccine protection and the availability of reference materials that harmonise the measurement of vaccines responses that are critical in protection. In response to a call from WHO, the 1st International Standard for anti EBOV antibodies was developed and established in 2017 [23]. The international collaborative study demonstrated that using this reference material to determine the amount of anti-EBOV antibodies in other serum samples as a relative potency in International Units (IU) harmonised the results from different laboratories and using a variety of different assays. However, it did not determine whether these antibody responses protected against virus challenge *in vivo*. The study described in this report demonstrated that the 1st International Standard for anti-EBOV antibodies does contain antibodies that alone are capable of extending survival significantly at 150 mIU/ml on day of sub-cutaneous challenge with 10^3 IU virus and would appear to prevent lethal challenge when serum levels are in excess of 250 mIU/ml. Whilst relatively small numbers of subjects were used to generate these data, they do provide a potential framework for the ongoing development and regulatory approval of prophylactic EBOV vaccines.

The approach of using an antibody reference standard as an indicator of vaccine efficacy provides many advantages, including

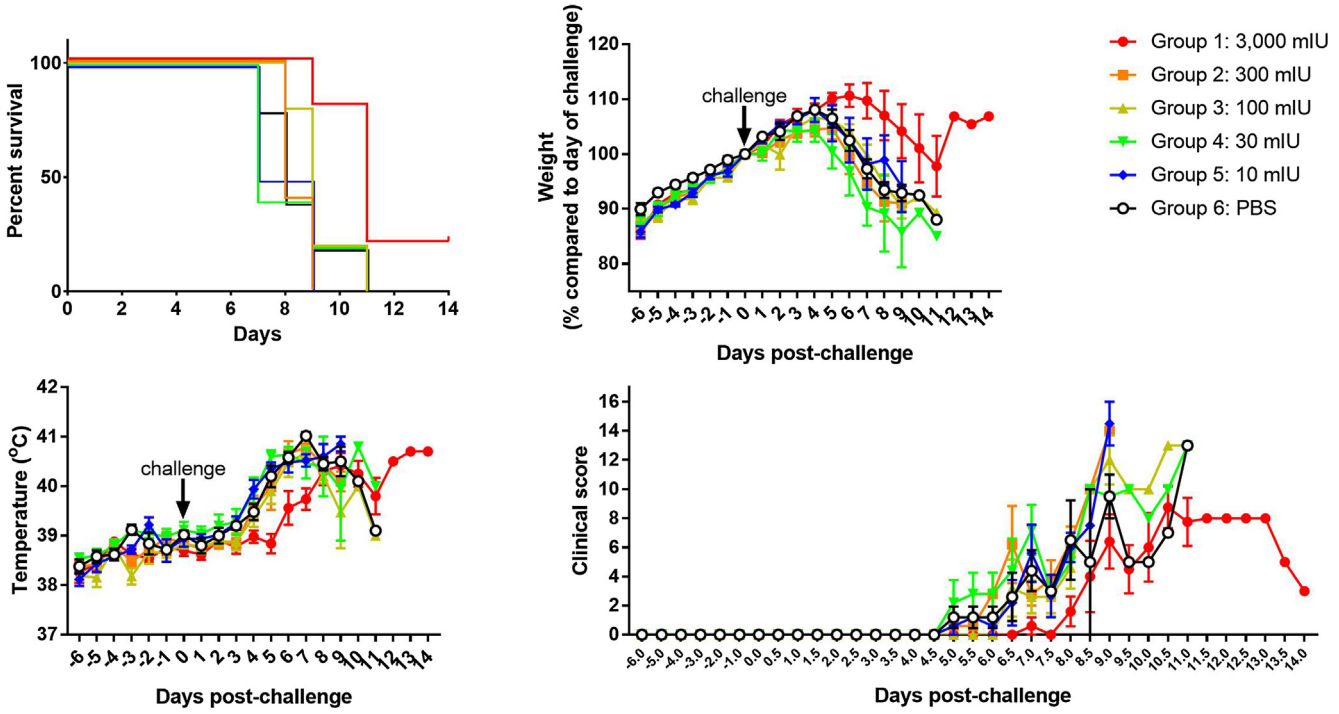

Fig. 1. Survival and clinical outcomes of animals receiving 1st International Standard for EBOV antibodies and challenged with EBOV. (a) Survival. (b) Changes in weight. (c) Temperature. (d) Clinical score. Graphs (b) to (d) show the mean value with error bars denoting standard error (n = 5/group).

Table 1
Antibody levels in blood samples taken at the time of EBOV challenge. UD; undetected. Animals receiving <300 mIU did not have measurable antibody levels pre-challenge.

Treatment	Animal ID	Pre-challenge antibody level (mIU/ml)	Mean (standard deviation)
3000 mIU	02055	266	163.6
	16873	122	(72.5)
	99712	78	
	16768	152	
	00725	200	
300 mIU	99763	UD	17.4
	16908	29	(15.9)
	17695	29	
	16993	UD	
	17685	29	

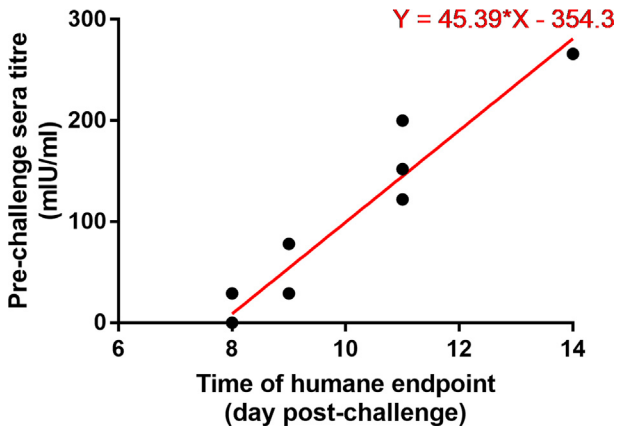

Fig. 2. Comparison of antibody titre pre-challenge with the time taken for animals to meet humane endpoints post-challenge with EBOV. Each symbol represents a single animal. Linear regression analysis is denoted by the red line and equation.

prioritising those with most promise to progress to efficacy testing in preclinical models, which due to the requirement of containment level 4 (or equivalent) facilities is both expensive and of limited capacity. Additionally, it would also refine the use of animals in vaccine research by ensuring that only the most promising candidates are progressed through *in vivo* models.

The evaluation of an antibody standard as a potential measure for vaccine efficacy was based upon data from different model systems. Studies in experimental mice, guinea pigs and NHPs have all reported a correlate between antibody responses and protection against experimental challenge [24]. Nevertheless, the mechanism of antibody immunity may not be solely due to neutralisation of the virus, as even antibody materials with low neutralisation activity have been shown to still be protective [18,24]. This indicates that other mechanisms may contribute to protection, such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-mediated or Fc-dependent mechanisms [25]. Thus, it is important that information from clinical studies are compared with pre-clinical models. Once again, the availability of the International Standard will enable data to be compared more directly as measurement of relative potency in International Units of an external and traceable reagent which harmonises the results of many different assays [23].

The outbreak of EBOV in West Africa from 2014 provided a huge stimulus for vaccine research. This progress means that there is hope that a licenced vaccine will soon be available. Nevertheless, more progress and optimisation is required. The widespread use of antibody standards is of utmost importance by allowing effective comparison between different vaccine candidates and the reproducibility of different batches of the same vaccine.

Our data demonstrated that a titre of >150 mIU/ml provides strong evidence of a statistical significant protective effect. Others have reported that an antibody titre of 1:3700 predicts 100% protection [18]. Understanding whether these two observations are congruent will help vaccine development. Unfortunately, the

material which was used to obtain the latter figure is not widely available and titres are frequently inherently variable between laboratories. By contrast the global availability of the 1st International Standard for anti-EBOV antibodies provides the basis of comparing and harmonising data between studies and laboratories. Similar approaches are underway for other emerging diseases of global importance. The availability of an increasing number of International Standards for the measurement of antibody responses will facilitate and accelerate vaccine development for these recrudescing infectious diseases.

4. Methods

4.1. Ethics statement

Animal studies with EBOV were performed under Containment Level 4 conditions with all procedures being undertaken according to the United Kingdom Animals (Scientific Procedures) Act 1986. Studies were conducted under Establishment Licence reference PEL PCD 70/1707 with Project Licence PPL 30/3247, approved by a UK Home Officer inspector. All animal procedures were approved by the PHE Animal Welfare and Ethical Review Body (AWERB).

4.2. Virus and antibody preparations

EBOV strain Yambuku-Ecran was passages five times in guinea pigs to achieve lethality, as previously described [26]. Virus was titrated by 50% tissue culture infective dose ($TCID_{50}$) assay in VeroE6 cells (European Collection of Cell Cultures, UK). The 1st International Standard for Ebola virus antibodies was obtained from the National Institute of Biological Standards and Control (NIBSC, cat. no. 15/262 [27]) Each vial was reconstituted with 0.5 mL sterile distilled water (Gibco, UK) to provide a pool of neat solution with an assigned potency of 1.5 IU/ml. Sterile phosphate buffered saline (PBS) solution (Gibco, UK) was used to further dilute the antibody where required.

4.3. Animal experiments

Female adult Dunkin-Hartley guinea pigs (Marshall BioResources, UK) were used for *in vivo* studies, with an average mean starting weight of 264 g (range 226–296 g) and housed in pairs. For procedures, guinea pigs were anaesthetised with 1.5–2% isoflurane in oxygen until full sedation was achieved. Food and sterile water were available *ad libitum*. Animals were weighed and temperatures recorded daily via an indwelling temperature chip. Clinical signs were monitored at least twice daily, and the following numerical score was assigned for analysis: 0 (normal); 2 (ruffled fur); 3 (lethargy, hunched and wasp waisted); and 5 (rapid breathing). Neat or dilutions (1:10, 1:30, 1:90, 1:270) of international serology standard were delivered via the intraperitoneal route in a volume of 2 mL equating to 3000, 300, 100, 30 and 10 mIU, respectively. A control group received 2 mL of PBS solution. Each group consisted of 5 guinea pigs. One day post-antibody administration, all animals were challenged with 10^3 $TCID_{50}$ EBOV in a volume of 0.2 mL via the subcutaneous route. The study was terminated at day 14 after virus challenge. Animals which reached humane clinical endpoints (defined as 20% weight loss or 10% weight loss alongside a moderate clinical sign) were immediately culled.

4.4. Sera collection

On the day of challenge, but prior to any EBOV being inoculated, 0.5 mL blood was taken from each animal and placed into a serum separation tube (Becton Dickinson, UK). Sera was prepared by centrifuging according to tube manufacturers instructions, and sera stored at −80 °C until required.

4.5. ELISA assay

Guinea-pig sera collected on the day of the challenge were tested for anti-EBOV IgG using the Human anti-Zaire Ebola virus GP IgG ELISA kit (Alpha Diagnostics Int., USA) following the manufacturer's instructions. Samples were diluted 1:200 with the diluent provided and tested in duplicate. A fresh vial of the 1st International Standard for Ebola virus antibodies was used to generate a standard curve by doubling dilutions, with a starting point of 1:100 (equivalent to 15 mIU/mL).

4.6. Statistical analysis

The Log-Rank test for nonparametric survival was used to compare differences between groups of animals using Minitab, version 16 and applying right-censoring with a time censor of 14 days. Linear regression was perfomed using GraphPad Prism, version 7. For all statistical tests, a p-value less than 0.05 was considered significant.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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