IgY antibodies against Ebola virus possess post-exposure protection and excellent thermostability

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Abstract

Ebola virus (EBOV) is the most virulent pathogens that cause hemorrhagic fever with high mortality rates in humans and nonhuman primates. The postexposure antibody therapies to prevent EBOV infection are considered efficient. However, due to the poor thermal stability of mammalian antibody, their application in the tropics has been limited. Here, we developed a thermostable therapeutic antibody against EBOV based on chicken immunoglobulin Y (IgY). The IgY antibodies demonstrated excellent thermal stability, which retained their neutralizing activity at 25°C for one year, in contrast to conventional polyclonal or monoclonal antibodies (MAbs). We immunized laying hens with a variety of EBOV vaccine candidates and confirmed that VSV Δ G/EBOVGP encoding the EBOV glycoprotein could induce high titer neutralizing antibodies against EBOV. The therapeutic efficacy of immune IgY antibodies *in vivo* was evaluated in the newborn Balb/c mice model. Lethal dose of virus challenged mice were treated 2 or 24 h post-infection with different doses of anti-EBOV IgY. The group receiving a high dose of 10^6 NAU/kg (neutralizing antibody units/kilogram) achieved complete protection with no signs of disease, while the low-dose group was only partially protected. In contrast, all mice receiving naïve IgY died within 10 days. In conclusion, the anti-EBOV IgY exhibits excellent thermostability and protective efficacy, and it is very promising to be developed as alternative therapeutic entities.

Author Summary

Although several Ebola virus therapeutic antibodies have been reported in recent years, however, due to the poor thermal stability of mammalian antibody, their application in tropical

endemic areas has been limited. We developed a highly thermostable therapeutic antibody against EBOV based on chicken immunoglobulin Y (IgY). The IgY antibodies demonstrated excellent thermal stability, which retained their neutralizing activity at 25°C for one year. The newborn mice receiving passive transfer of IgY achieved complete protection against a lethal dose of virus challenge indicating that the anti-EBOV IgY provides a promising countermeasure to solve the current clinical application problems of Ebola antibody-based treatments in Africa.

Introduction

Ebola virus (EBOV) belongs to the Filoviridae family and the known cause of severe hemorrhagic fever in humans and nonhuman primates (NHPs). Since the epidemic of Zaire (now the Democratic Republic of the Congo, DRC) and Sudan in 1976, intermittent local epidemics persist in Africa, with a case-fatality rate of 25-90%. The most severe Ebola outbreak to date occurred in West Africa from 2014 to 2016, more than 28 000 people infected, and 11 323 patients died. Occasionally, cases have been exported into other countries through travel. The ongoing outbreak in the DRC is the second-largest Ebola epidemic on record, with 2 279 lives lost and 3 462 confirmed infections since August 2018, which prompted WHO to declare this epidemic a public health emergency of international concern. Pandemic potential, high mortality, high infectivity, and lack of preventive and therapeutic approaches make EBOV a Class A pathogen that seriously threatens public health.

The intermittent and continuous outbreak of Ebola disease (EVD) poses a challenge for

public health, which promoted research on vaccines and antiviral drugs. The most effective strategy to prevent and treat EVD is antibody immunotherapy [1-5]. However, antibody treatment was not optimistic at the beginning, although antibody KZ52 had high neutralizing activity but failed to play a role in NHPs [6]. Until 2012, it was discovered that convalescent plasma can protect NHPs from lethal dose of EBOV infection, and antibody therapy strategy rekindled researchers' interest [7]. The researchers tried to mix multiple MAbs against EBOV glycoprotein (GP) to form ZMapp (2G4, 4G7, 13C6), a new antibody combination based on cocktail therapy. It was used to treat seven infected persons, and 5 of them survived. ZMapp has an excellent protective effect in NHPs and patients and has greatly extended the window period of administration. The emergence of ZMapp has brought the peak of antibody treatment research [8]. Nowadays, antibody therapy becomes a promising approach to control EVD. Recently, anti-EBOV equine sera and ovine sera were confirmed that can protects rodent models against EBOV challenge [9-11]. These studies suggest that EBOV-specific antibodies from different species are expected to be developed as therapeutic agents.

Although EBOV therapeutic antibody has inspiring application prospects, it still has some application limitations. Firstly, the limited availability and security of convalescent plasma have hampered its global application. Secondly, MAbs have long preparation cycles and high production costs. Also, they all need strict transportation and storage conditions. However, the Ebola outbreak mainly occurred in the hot and less wealthy African regions, thus limiting the current application of antibodies. Poultry-derived IgY provides an alternative strategy for producing safe and inexpensive antibodies. IgY antibodies, the predominant serum

88 immunoglobulin in birds, reptiles, and amphibians, are transferred from the serum of females
89 to the egg yolk [12], where they offer passive immunity to embryos and neonates. As a potential
90 therapeutic antibody, IgY has lots of advantages over mammalian IgG due to its structural and
91 immunological properties [13]. It possesses excellent stability under various physicochemical
92 conditions and lower manufacturing costs. It cannot bind to mammalian Fc receptors or
93 complement components [14], which can avoid potential antibody-dependent infection
94 enhancement (ADE) and adverse immune reactions. Eggs can be used to produce large amounts
95 of yolk antibodies quickly and do not cause harm to animals. In recent years, the antibody
96 therapy strategy based on IgY has been widely recognized, and many promising results have
97 been reported in influenza virus [15-17], dengue virus [18], zika virus [19], hantavirus [20],
98 severe acute respiratory syndrome [21], rotavirus and norovirus [22], etc. Previous studies have
99 reported multiple Ebola vaccine candidates based on different platforms. These candidates
100 exhibit different immunogenicity. To prepare high-immunity IgY antibodies, we need to screen
101 for immunogens that can induce laying hens to produce the most robust humoral immune
102 response.

103 This study aimed to produce an anti-EBOV IgY antibody and assess its antiviral efficiency.
104 The IgY antibody was obtained from laying hens immunized with recombinant vesicular
105 stomatitis virus vector encoding EBOV GP (VSV Δ G/EBOVGP). The protecting efficiency of
106 the resulting IgY antibody against EBOV was evaluated by Enzyme-linked immunosorbent
107 assay (ELISA), pseudotyped virus neutralization assay, and a mouse challenge model. The
108 results show that the IgY antibody has excellent thermal stability and achieve protection against

lethal challenge in newborn mice. Our results suggest that the potent IgY warrant further development as prophylactic and therapeutic reagents for EVD.

Results

Preparation of immunogens

Vaccine-elicited neutralizing antibodies (NAbs) are associated with protection against Filoviridae family mediated disease. In order to obtain the most potent anti-EBOV antibody, we prepared several EBOV immunogens based on multiple different platforms, including DNA vaccine (pCAGGS/EBOVGP), recombinant protein (rEBOVGP) or virus-like particle (EBOV-VLP) subunit vaccines, and two viral vector vaccines (VSV Δ G/EBOVGP, Ad5/EBOVGP). Western blot confirmed that these immunogens could express or contain EBOV GP that can induce NAbs in animals (Fig 1). Due to the differences in humoral immune responses induced by different vaccines, we need to screen for the most suitable immunogen for IgY antibody production.

Immunogens elicited high titer of anti-EBOV IgY

To obtain high titer EBOV NAbs, 5-month-old laying hens were vaccinated with five different immunogens, including 10^3 or 10^4 $TCID_{50}$ VSVΔG/EBOVGP, 100 μg rEBOVGP, 100 μg pCAGGS/EBOVGP, 10 μg EBOV-VLP, and 10^{11} virus particles (vp) Ad5/EBOVGP (Fig 2a). Thirty-five laying hens were randomly divided into seven groups, which were immunized four times with each immunogen or PBS control intramuscularly (i.m.) at a 14-day interval. Eggs were collected at 0, 2, 4, 6, 8 weeks, and IgY antibodies were purified from egg yolk for ELISA and NAbs test. Both titers in all groups were gradually increased after the first immunization.

The results showed that all immunogens except DNA vaccine induced potent Gp-specific ELISA antibodies (Fig 2b). For the NAbs, the geometric mean titers (GMTs) in 10^4 TCID50 VSVΔG/EBOVGP group reached 1:3650 (VSV pseudoneutralisation, VSV-PsN) and 1:320 (lentiviral vectors pseudoneutralisation, LVV-PsN) after the third boost, which significantly higher than other groups (Fig 2c-d). VSVΔG/EBOVGP induced the strongest NAb titers than the other immunogens. In contrast, the GP-specific ELISA and NAb titers were not detected in the negative control group. Besides, to determine the correlation between the immune dosage and antibody level, two dosages of VSVΔG/EBOVGP were used to immunize laying hens. 10^4 TCID50 VSVΔG/EBOVGP induced stronger NAbs titer compared to 10^3 $TCID_{50}$ VSVΔG/EBOVGP, suggesting that the immunization dose is crucial for obtaining higher titers of NAbs. In order to exclude the possible interference of the antibody generated by the VSV vector backbone, we used two PsN assays, VSV-PsN and LVV-PsN, and the results showed that the two assays obtained consistent results.

Purification and characterization of IgY

After four immunizations with 10^4 TCID50 VSVΔG/EBOVGP, IgY antibodies were purified from these collected eggs and then verified by SDS-PAGE (Fig 3a) and Western blot (Fig 3b) assays. SDS-PAGE results showed that two bands with molecular weights of 66 and 22 kDa appeared, representing the heavy chain and light chain of IgY, respectively. The purified IgY antibodies exhibited EBOV GP-specific immunoreactivity to EBOV sGP expressed in *E. coli* expression system when tested with Western blot. Besides, about 50 mg of IgY antibodies with a purity greater than 95% can be obtained from an egg, suggesting that the production cost of

151 this antibody is relatively lower.

152 **Passive transfer of IgY protect newborn BALB/c mice from lethal challenge**

153 To determine whether the anti-EBOV IgY antibodies are protective against EBOV, passive
154 protection experiment was performed in newborn BALB/c mice (within the first 3 days of life).
155 Forty newborn BALB/c mice were divided into eight groups, which were challenged
156 subcutaneously (s.c.) with 10^4 TCID50 VSVΔG/EBOVGP. Two hours or 1 day post-infection
157 (dpi), each mouse was adoptively transferred with IgY twice daily for 3 days, and control group
158 mice treated with naive IgY (Fig 4a). To determine the correlation between the transferred IgY
159 dosage and therapeutic efficacy, three different dosages with 10^4, 10^5, or 10^6 NAU/kg (NAb
160 units/kilogram, VSV-PsN) were intraperitoneally (i.p.) transferred to six groups of challenged
161 mice, respectively. The results showed that all the mice receiving 10^6 NAU/kg IgY were healthy
162 with the gradually increasing bodyweights and without any clinical syndromes within 15 dpi
163 (Fig 4b-c). However, all the control mice died during 6-10 dpi, and the bodyweights began to
164 decrease rapidly from the second day until death. In the other two lower doses administration
165 groups, mice failed to obtain full protection and displayed bodyweight losses after 4 dpi. Only
166 one mouse receiving 10^4 NAU/kg IgY survived to day 15, and the mice of two timepoints
167 treatment groups increase 5% or 2% of bodyweight with a mean time to death (MTD) of 11.25
168 or 11 dpi (Fig 4d-e). The situation has improved when the injection amount is 10^5 NAU/kg. 4/5
169 mice receiving IgY 2 h post-infection survived to day 15 with an average weight increase of 6%
170 and an MTD of 12 dpi. Another group, 3/5 mice receiving IgY 1 dpi survived to day 15 with
171 an average bodyweight increase of 2% and an MTD of 11.5 dpi. These data indicated that anti-

EBOV IgY conferred complete passive protection to newborn mice against a high concentration of VSV vector-based EBOV recombinant chimeric virus challenge. Administrating high-concentration IgY immediately after infection or even one day later can prevent mice from dying by infection, suggesting that anti-EBOV IgY can be used for emergency prevention and treatment after exposure to EBOV.

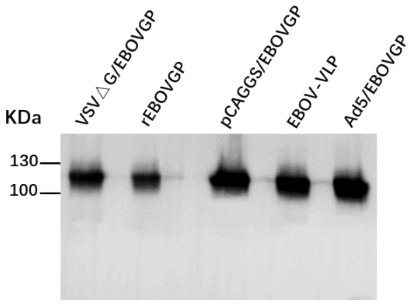
Bioavailability of IgY in guinea pigs

To more accurately confirm the protective efficacy of the anti-EBOV IgY, the metabolic level of antibodies in the body needs to be evaluated. The bioavailability of IgY *in vivo* was assessed, two groups of 12-week-old female guinea pigs received s.c. injection with 10^5 or 10^6 NAU/kg IgY, respectively. Sera were collected daily and tested by VSV-PsN assay within six days after antibodies injection. The NAbs titer in the guinea pig sera reached a high level. Then the antibody level gradually decreased and fell below the detection limit on the third (low dosage group) and fourth days (high dosage group), respectively (Fig 5). These results suggest that passive transfer of IgY can provide guinea pigs with 2-3 days of protection and that higher doses of antibodies can provide longer protection time.

Thermal Stability of IgY

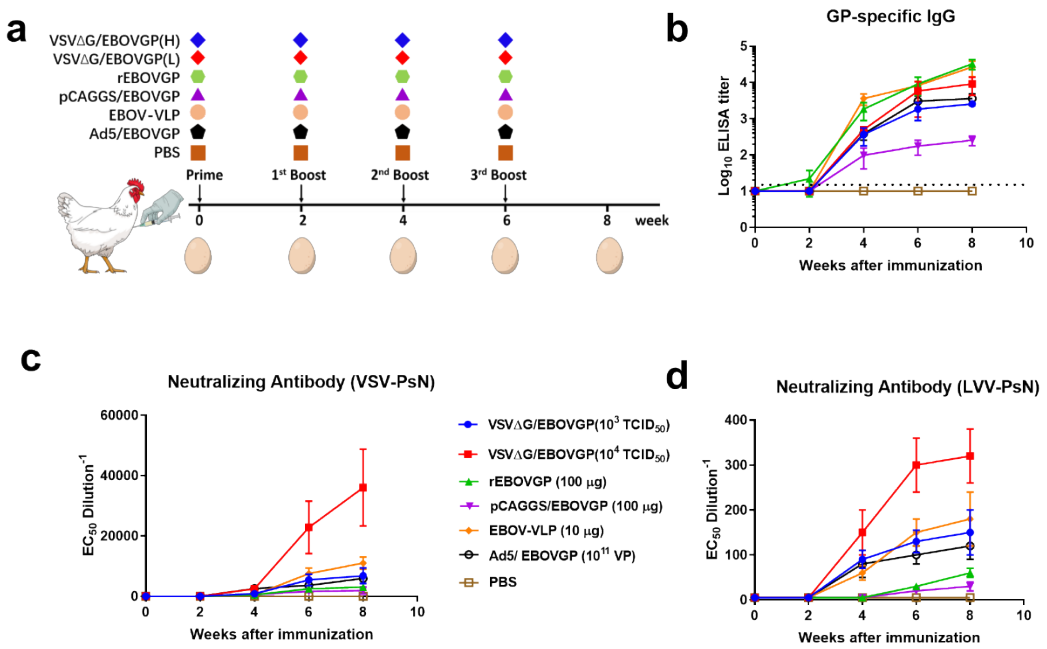
Considering that Ebola hemorrhagic fever mainly occurs in tropical regions, the thermal stability of antibody-based antiviral reagents is essential for practical applications. We stored the purified and filtered sterilized IgY in four different temperature environments, including 4℃, 25℃, 37℃, and 45℃. Then, the NAb titers were tested every month for one year. The results showed that the IgY NAb titers had no significant change at 4℃ and 25℃ within one

193 year. However, the antibody titer stored at 37℃ gradually decreased from the second month,
194 and only about 20% of the antibody activity remained by the end. In contrast, the activity of the
195 IgY stored at 45℃ is lost faster, and the NAbs titer cannot be measured by the third month.
196 These results proved that the anti-EBOV IgY has excellent thermal stability, can be stored at
197 room temperature (RT) for up to one year, and can maintain one month of activity at 37℃
198 without significant changes. Even at a high temperature of 45℃, it still can short-term retention
199 of activity (Fig 6). It is suggested that this anti-EBOV IgY can be used as an emergency
200 prevention/treatment reagent in endemic tropical regions.



201

202 **Fig 1.** Western blot analysis of EBOV immunogens. Recombinant protein, vector immunogens
203 and 293T cell lysates transfected with plasmid expressing EBOV GP were separated by SDS–
204 PAGE under reducing conditions, transferred to PVDF membrane, and detected using EBOV
205 GP1-specific MAb.



206

207 **Fig 2.** Immunogenicity of different EBOV immunogens in laying hens. (a) Seven groups of

208 laying hens (5-month-old, n=5 per group) were inoculated with VSV Δ G/EBOVGP,

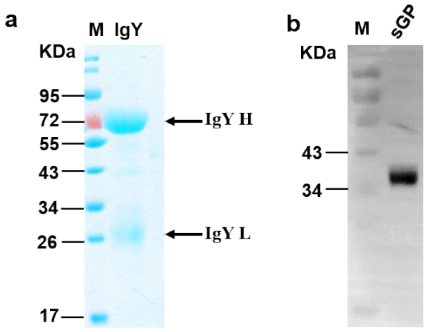
209 rEBOVGP, pCAGGS/EBOVGP, EBOV-VLP, Ad5/EBOVGP or PBS as control, respectively,

210 through i.m. route at 0, 2, 4, 6 weeks. The eggs were collected at 0, 2, 4, 6, 8 weeks. The yolk

211 IgY were purified and used to test the GP-specific ELISA antibody titers (b) and NAb titers (c,

212 d) by VSV-PsN and LVV-PsN assay. The dashed line in B indicates the detection limit. Data

213 are shown as means ± SEM.



214

215 **Fig 3.** Purity and immunogenicity analysis of anti-EBOV yolk antibody. (a) Purified IgY was

216 analyzed by SDS-PAGE. (b) Recombinant sGP expressed in *E. coli* was analyzed by Western

217 blot with anti-EBOV IgY.

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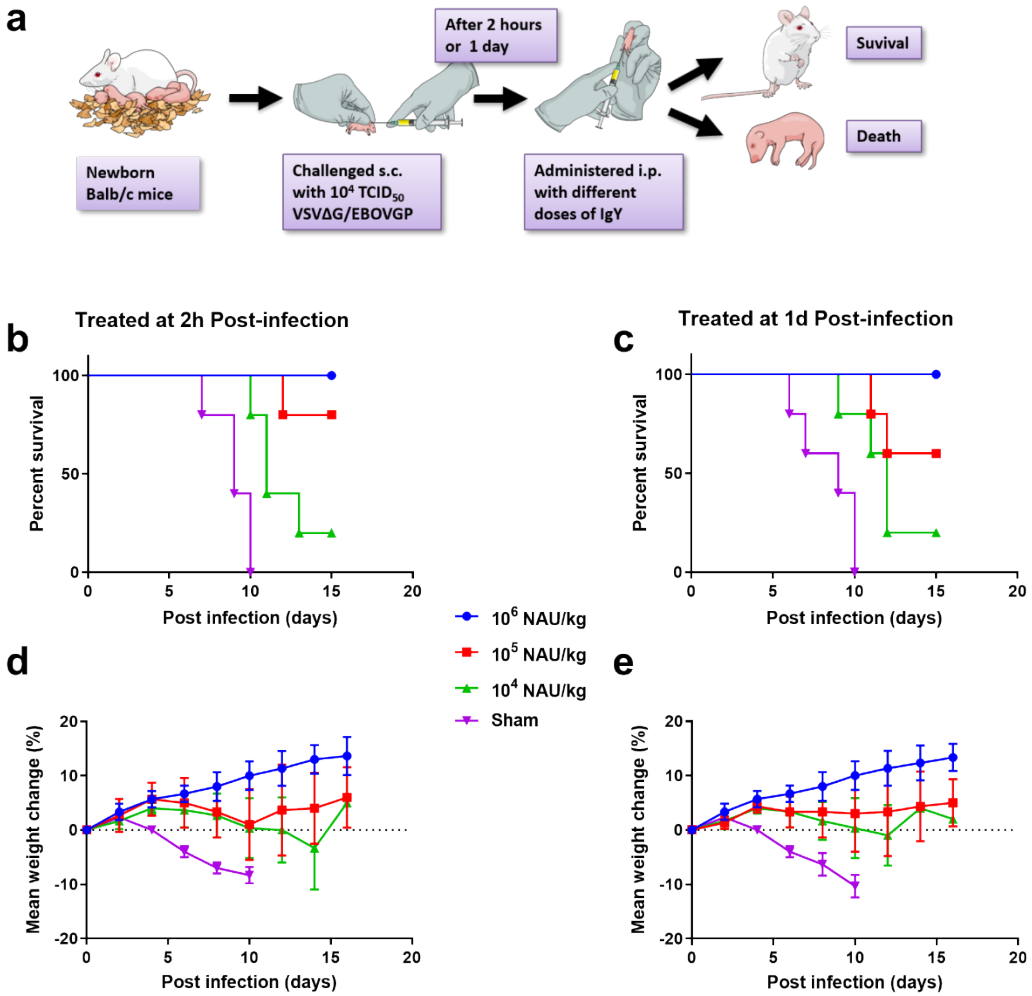


219

220 **Fig 4.** Passive protection of anti-EBOV IgY in newborn Balb/c mice. Experimental scheme (a).

221 Eight groups of newborn Balb/c mice (3-day-old, n=5 per group) were challenged s.c. with 10^4

222 TCID50 VSVΔG/EBOVGP, after 2 h or 1 day, each mouse was given different doses of IgY

or naïve IgY via i.p. route twice daily for 3 days. The survival rates (b, c) and bodyweight changes (d, e) were monitored every day. Data are shown as means ± SEM.

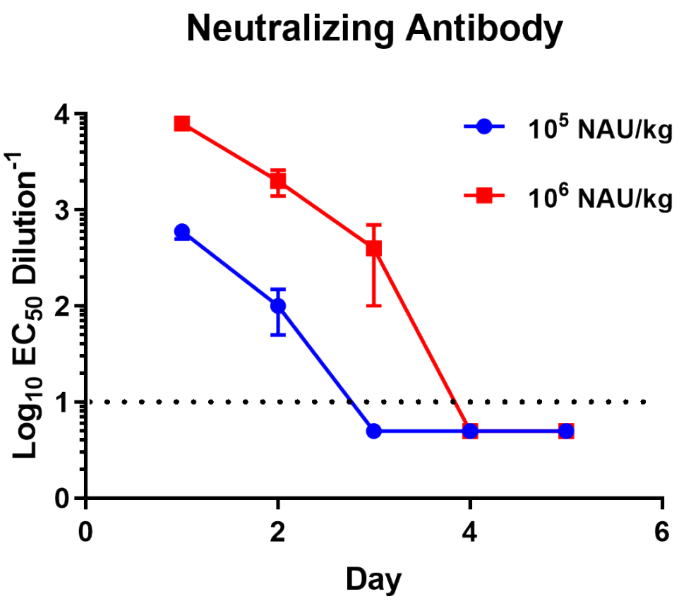
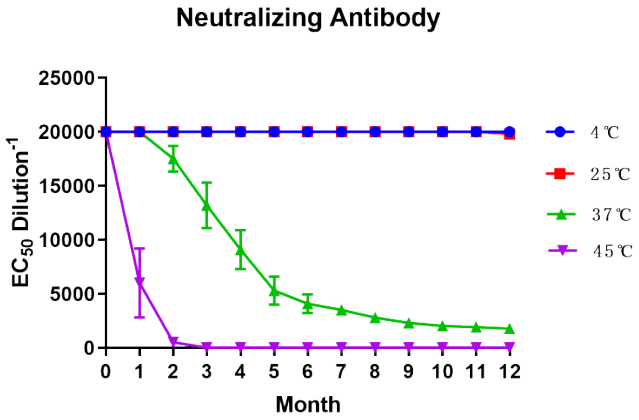


Fig 5. Bioavailability of anti-EBOV IgY in guinea pigs. Two Groups of guinea pigs (12-week-old, n=3 per group) were injected s.c. with 10^6 NAU/kg or 10^5 NAU/kg IgY. Sera NAb titers were tested daily by VSV-PsN assay. The dashed line indicates the detection limit. Data are shown as means ± SEM.



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232

233 **Fig 6:** Thermal Stability analysis of purified IgY. Purified IgY were placed under different

234 temperature conditions (4 °C, 25 °C, 37 °C, and 45 °C) for 1 year and neutralizing activity

235 were detected per month.

236 Discussion

237 Ebola hemorrhagic fever is associated with high mortality in humans and spread quickly among

238 populations in which medical facilities are scarce, antibody therapies is the only option for

239 EBOV-infected patients. An ideal therapeutic antibody should be highly efficacious, easy to

240 use, and inexpensive. Previous strategies to develop antibody against EBOV were derived from

241 mammals, although these antibody reagents have proved to have good protective efficacy, their

242 thermal stability is not good [7-11, 23-29]. Considering that EVD mainly occurs in Africa, due

243 to the hot climate and lack of cold chain transportation, the clinical application of these

244 antibodies may be hampered. Moreover, the sooner people receive treatment post exposure to

245 EBOV, the higher the survival rate. Therefore, an Ebola therapeutic antibody with high-

246 efficiency protection and good thermal stability has become a high priority. In recent years,

247 therapeutic antibodies based on avian IgY have begun to receive more and more attention, and
248 a variety of pathogen therapeutic antibodies based on IgY have proved to have very good safety
249 and therapeutic effects [15-19, 21, 22]. Compared with mammalian antibodies, avian antibodies
250 have several distinct advantages. First of all, IgY has higher target specificity, greater binding
251 avidity and longer circulating half-life, which could increase its efficacy against infections [30];
252 Secondly, IgY does not react with complement system, so the risk of causing inflammation *in*
253 *vivo* is less. In addition, previous studies have shown that EVD has an ADE effect, which can
254 enhance EBOV infection by human antibodies. Blocking of Fc-receptors reaction through
255 mutating the Fc fragment can abolish ADE, and can effectively enhance the protection efficacy
256 of EBOV antibody [31]. Because IgY does not bind to Fc receptors, so it can perfectly avoid
257 the EBOV ADE effect caused by antibodies. Furthermore, one hen can produce up to 22 grams
258 of IgY antibody a year, which means that the antibody production of two hens is equivalent to
259 one horse, and egg production has already been scaled up, making large-scale and low-cost IgY
260 production feasible [30]. The cheaper antibody drugs are also more suitable for applications in
261 Africa. In addition, compared with antibodies derived from mammalian serum, IgY is derived
262 from eggs, and therefore more in line with animal welfare principles. Finally, IgY has excellent
263 thermal stability, it can even be stored at RT for more than 6 months and at 37℃ for more than
264 1 month, while maintaining its activity [30].

265 Although multiple EBOV vaccine platforms have been established, several vaccine
266 candidates showed excellent protective efficacy, but a systematic comparison of the humoral
267 immunity levels of different candidates on an animal model has not been addressed. Herein, we

tested five different vaccine candidates in laying hens for eliciting yolk IgY level. We demonstrated that the VSV vector-based EBOV vaccine can induce laying hens to produce high-neutralizing IgY, while other vaccines failed to induce a strong humoral immune response. Interestingly, the rVSV vaccine has been widely used (> 40 000 recipients) to prevent the current Ebola outbreak in the DRC [32]. The Ebola vaccine candidates reported earlier have verified its immunogenicity in rodents and primates, but due to the differences in the immune systems of poultry and mammals, these studies cannot be used to direct guide immunization in poultry. The immunogenicity of these vaccines was verified on laying hens, confirming that there is a big difference in the immunogenicity of different vaccines on hens. Fortunately, we obtained an efficient immunization protocol, and prepared high titer neutralizing IgY antibody. To further confirm the protective efficacy of the anti-EBOV IgY, it needs to be verified by the challenge/protection animal model. However, EBOV needs to be operated in the BSL-4 laboratory. Due to limited conditions, we cannot operate live EBOV. We tried to establish an alternative solution. We injected high dose rVSV expressing EBOV GP into newborn mice. We were surprised to find that mice injected with rVSV achieved 100% death, while the control group injected with the same amount of PBS all survival, this phenomenon is limited to mice within 3 days of birth. We injected 1-week-old mice with even higher doses of rVSV and could not cause mice to die, suggesting that the infection model is limited to newborn mice. We used this animal model to evaluate our antibodies and proved that treatment with high-concentration IgY at 2 and 24 hours after challenge can protect mice from death, thus proving that we prepared IgY can protect mice from lethal dose of virus attack.

289 Antibody therapy played a critical role in antiviral over the past 100 years, humans have
290 exploited passive immunization for treating a variety of infectious diseases. In the West Africa
291 Ebola outbreak, recent evidence suggests therapeutic antibody treatment post-exposure can
292 affect the progression of EVD. The production of recombinantly manufactured MAbs can be
293 clinically useful. Therefore, antibody-based treatments should be further investigated for use in
294 humans. Given the economic and medical restrictions in Africa, all the antibody therapeutic
295 preparations currently have the disadvantages of high cost, long preparation period, and strict
296 need for cold chain transportation conditions. These features suggest there will be an ongoing
297 need for better EBOV therapeutic reagents. Poultry antibodies have the advantages of safety,
298 high efficiency, stability, easy scale production and low cost, which make it very good
299 candidate for global therapeutic use in the time of epidemic.

300 In this study, we utilized the avian antibodies platform to develop an EBOV therapeutic
301 formulation based on poultry IgY, which is effective when administered as a post-exposure
302 prophylactic in the newborn Balb/c mice model. The conversion of polyclonal antibody
303 products derived from eggs into clinical practice is a daunting challenge. However, ongoing
304 Phase III clinical trials have tested the avian polyclonal IgY against Pseudomonas aeruginosa
305 with the support of the European Medicines Agency. The efficacy of antibodies in the treatment
306 of cystic fibrosis suggests that new methods based on avian antibodies can be used to develop
307 therapies for prevention and treatment. The IgY antibody-based treatments have optimistic
308 research prospects. Compared with other mammalian antibodies, our anti-EBOV IgY antibody
309 has the advantage of greatly improved thermal stability, large quantities lower cost and avoiding

ADE reaction, so it is more suitable for African applications. Ebola epidemic areas are in Africa with high temperatures, lack of electricity, and many places do not have cold chain transport and storage conditions. Current mammalian antibodies have limited clinical application due to poor thermal stability. Because of the excellent thermal stability, our developed anti-EBOV IgY provides a promising strategy to solve the current application problems of Ebola antibody-based treatments in Africa.

Materials and Methods

Cells and animals

293T, Vero and BHK-21 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin (Gibco, Grand Island, NY, USA) at 37℃ in 5% CO_2. Sf9 insect cells were cultured in SF900 serum-free media at 28℃ CO_2- free incubator.

Pregnant Balb/c mice and guinea pigs were purchased from a commercial supplier (Charles River). Laying hens were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were kept in sterile, autoclaved cages and provided sterilized food and water.

Generation of Immunogens

Five EBOV immunogens based on different platforms were prepared as described previously [33-35], with some modification. All immunogens are designed based on EBOV GP of the 2014 Ebola Makona epidemic strain. Briefly, EBOV GP gene sequence was inserted an

330 additional A residue at position 1019 to 1025 results in a frameshift; thus the complete GP can
331 be expressed. Then the gene is optimized for enhanced transgene expression and then produced
332 synthetically. To obtain DNA vaccine, the GP coding sequence was cloned into a mammalian
333 expression plasmid pCAGGS, transgene expression was verified by western blot. Recombinant
334 EBOVGP (rEBOVGP) protein containing a C-terminal His-tag was obtained through using the
335 insect baculovirus expression system. The EBOV GP gene was first cloned into the pFastBac
336 vector, and the constructed plasmid was transformed into *E.coli* DH10Bac cells to generate a
337 recombinant bacmid. Then Sf9 cells were transfected with recombinant bacmid to generate
338 recombinant baculovirus. After three consecutive passages, the cell culture supernatants were
339 harvested and purified by Ni-NTA affinity chromatography (GE Healthcare, USA). Similar to
340 the preparation of rEBOVGP, to obtain EBOV-VLP, pFastBac Dual vector including EBOV
341 GP and VP40 genes was transformed into DH10Bac, and the resulting bacmid was transfected
342 into Sf9 cells to generate recombinant baculovirus co-expressing EBOV GP and VP40. Culture
343 supernatants were clarified and then pelleted by ultracentrifugation at 30 000 × g for 1 h at
344 4℃. The pellets were resuspended in PBS and further purified through a 10–50% (w/v)
345 discontinuous sucrose gradient at 25 000 × g for 1.5 h at 4℃. The visible band between 30%
346 and 50% density range was collected and resuspended in PBS. The resulting protein products
347 are EBOV-VLP. A recombinant E1/E3-deleted adenovirus type-5 vector-based EBOV vaccine
348 was created by displacing of adenovirus E1 gene with EBOVGP, forming a recombinant
349 Ad5/EBOVGP genome. The Ad5/EBOVGP was rescued by transfecting the genome into 293T
350 cells and further propagated and purified by CsCl density gradient centrifugation. The number

of virus particles (vp) was determined using optical density (260 nm) measurement. The resulting Ad5/EBOVGP cannot replicate inside human tissues. To engineer the recombinant vesicular stomatitis virus (VSV) expressing EBOVGP, the VSVGP gene in rVSV replicon vector pVSV-XN2 was replaced by EBOVGP to generate pVSV-XN2/EBOVGP. Then the recombinant VSVΔG/EBOVGP was recovered using reverse genetics by co-transfecting pVSV-XN2/ EBOVGP and pBluescript SK+ (pBS) plasmids expressing the VSV nucleocapsid (N), phosphoprotein (P) and large polymerase subunit (L) into BHK-21 cells. rVSV virions were plaque purified, and virus titers were determined by standard plaque assay using BHK-21 cells. All produced EBOV immunogens were verified by western blot.

Immunizations

Seven groups of 5-month-old laying hens (n =5 per group) were inoculated i.m with immunogens prepared as described above. The detailed scheme is 10^3 or 10^4 TCID50 VSVΔG/EBOVGP, 100 μg rEBOVGP, 100 μg pCAGGS/EBOVGP, 10 μg EBOV-VLP, 10^{11} VP Ad5/EBOVGP, or an equivalent volume of PBS as a sham control at weeks 0, 2, 4, 6 (4 times). Eggs were collected at weeks, 0, 2, 4, 6, 8 for ELISA and NAbs test.

Purification of yolk IgY antibody

IgY was isolated from the egg yolk using the water dilution method, a rapid and simple method was used to separate IgY from the yolk. The separation method is improved based on previous research. Yolks were isolated, removed the yolk membrane, and diluted the contents 1:8 with cold deionized water. Dilution was stirred uniformly, acidified to pH 5.0, keeping stable 10 h

371 then centrifuged at 10 000 × g for 15 min, and the supernatant was gathered. Further purifying
372 the IgY antibody, slowly add 1% (by volume) of the solution of n-octanoic acid with stirring to
373 the supernatant and centrifuge and filter in the same way. Finally, The IgY was further purified
374 by gel filtration (Superdex 200, GE Healthcare), eluted in PBS (pH 7.2) buffer, and
375 concentrated by ultrafiltration to approximately 20 mg/ml using 100-kDa cut-off membranes
376 (Millipore).

377 **SDS–PAGE and Western blot analysis**

378 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to
379 determine the purity of IgY. The samples were mixed with reducing sample buffer, heated at
380 98 °C for 10 min. Ten microliters of the sample were loaded into each well to SDS-PAGE (12%
381 gel, staining for 3 h and destaining for 2 h). The pre-stained protein standard (Fermentas,
382 Lithuania) was used as a molecular weight marker. The protein bands were visualized with
383 Coomassie Brilliant Blue R250 (Fluka USA). The gel was analyzed using Bio-Rad image
384 analysis software.

385 Western blot was performed to check the specificity of the prepared immunogens. VSVΔ
386 G/EBOVGP, rEBOVGP, EBOV-VLP, Ad5/EBOVGP and cell lysate transfected with
387 pCAGGS/EBOVGP plasmid were separated using SDS-PAGE on 15% polyacrylamide gels.
388 For Western blot analysis, the proteins were electrically transferred onto a polyvinylidene
389 difluoride (PVDF) membrane using a semi-dry blotting apparatus (15V, 40 min, RT), then
390 blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% non-fat dry
391 milk for 1 h at RT and was incubated overnight at 4 °C with a 1:5000 dilution of mouse anti-

392 EBOVGP1 specific MAb. After washed five times with TBS-T, the membrane was incubated
393 with HRP-conjugated goat anti-rabbit IgG diluted 1:2000 (Promega, Madison, Wisconsin, USA)
394 for 2 h at RT. The membrane was washed 4 times. Then specific binding bands were detected
395 by incubation in substrate buffer containing 4 mg 3,3′-diaminobenzidine tetrahydrochloride
396 (Aladdin, China) in 5 mL Tris–HCl and 15 μl hydrogen peroxide for 3–5 min. This reaction
397 was stopped by rinsing with distilled water.

398 **ELISA**

399 EBOVGP-specific ELISA was used to determine endpoint binding antibody titers of immune
400 yolk IgY. Endpoint titers were defined as the reciprocal serum dilution that yielded an OD450 >
401 2-fold over background values. Briefly, 96-well plates were coated with 10 μg/mL rEBOVGP
402 in carbonate-bicarbonate buffer, pH 9.6, at 4℃ overnight. The plates were then blocked with
403 5% skim milk in PBS (pH 7.4) at 37℃ for 1 h. IgY was added to the top row (1:40), and 2-
404 fold serial dilutions were tested in the remaining rows. The plates were incubated at 37℃ for
405 1 h, followed by five washes with PBST. Subsequently, the plates were incubated with 100 μl
406 of HRP-conjugated rabbit anti-chicken IgY working solution at 37℃ for 30 min and washed
407 with PBST five times. The assay was developed using 3,3',5',5-Tetramethylbenzidine HRP
408 substrate (TMB) with 100 μl each well stopped by the addition of 50 μl of 2 M H_2SO_4 for 10
409 min. Plates were measured at 450nm by a microplate reader using Softmax Pro 6.0 software
410 (Molecular Devices, CA, USA). PBS was used as a blank control. At the same time, a negative
411 control (IgY derived from PBS-immunized hens) was ascertained in each plate. All ELISA
412 measurements were repeated at least three times with each sample in triplicate.

Pseudotyped virus neutralization assay

Two pseudoneutralisation (PsN) assays were performed, based on non-replicating VSV pseudotype and lentiviral pseudotype, respectively. EBOV GP pseudotyped lentiviral and VSV virions with a luciferase reporter were produced as previously described [36, 37]. The resulting lentiviral particle can achieve a single-round infection. In brief, Vero cells were plated in 96-well plates and cultured overnight. IgY serial dilutions (1:10, 1:30, 1:90, etc., in DMEM) and pseudoparticles were mixed in a ratio of 1: 9 and incubated at 37 °C for 1 h, before addition to pre-plated target cells in 96-well culture plates (density of 10^4 cells/well) with 3 replicates. The luciferase activities of infected cells were examined 36 h post-infection. Sample dilutions which showed a 50% reduction in the number of fluorescing cells compared to controls were considered to neutralize antibody titers.

Adoptive transfer experiment

The yolk was collected and purified two weeks after the final vaccination. Eight groups of newborn BALB/c mice within three days (n=5 per group) were challenged by the s.c. route with 10^4 $TCID_{50}$ VSVΔG/EBOVGP, after 2 or 24 h, challenged mice were treated i.p. with 10^4, 10^5, or 10^6 NAU/kg anti-EBOV IgY twice daily for 3 days, respectively. Clinical symptoms, bodyweight change rate and survival rate were monitored within 15 days.

Metabolic studies in guinea pigs

Two Groups of guinea pigs (n=5 per group) were administrated s.c. with 10^6 or 10^5 NAU/kg purified anti-EBOV IgY. Sera were collected daily within 6 days for NAbs determination.

Ethics statement

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Institute of Microbiology, Chinese Academy of Sciences (IMCAS), and conducted in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the IMCAS Ethics Committee.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was jointly funded the National Key Technologies Research and Development Program of China (No. 2018YFC1200600, 2018YFC1200500) and the National Key Program for Infectious Disease of China (No. 2016ZX10004222-006, 2018ZX10101004, 2018ZX10734-404).

Author Contributions

Conceived and designed the experiments: YLM LWJ. Performed the experiments: ZY WYQ LYL LY. Analyzed the data: WX TDY JXJ. Contributed reagents/materials: GR. Wrote the paper: YLM ZY.

References

1. Levine MM. Monoclonal Antibody Therapy for Ebola Virus Disease. The New England journal of medicine. 2019;381(24):2365-6. doi: 10.1056/NEJMe1915350. PubMed PMID: MEDLINE:31774948.

2. Lucey DR. New treatments for Ebola virus disease. BMJ-British Medical Journal. 2019;366:2. doi: 10.1136/bmj.l5371. PubMed PMID: WOS:000485448300003.

3. Wec AZ, Herbert AS, Murin CD, Nyakatura EK, Abelson DM, Fels JM, et al. Antibodies from a Human Survivor Define Sites of Vulnerability for Broad Protection against Ebolaviruses. Cell. 2017;169(5):878-+. doi: 10.1016/j.cell.2017.04.037. PubMed PMID: WOS:000401515900013.

4. Zeitlin L, Whaley KJ, Olinger GG, Jacobs M, Gopal R, Qiu XG, et al. Antibody therapeutics for Ebola virus disease. Current Opinion in Virology. 2016;17:45-9. doi: 10.1016/j.coviro.2016.01.006. PubMed PMID: WOS:000378963300009.

5. Corti D, Misasi J, Mulangu S, Stanley DA, Kanekiyo M, Wollen S, et al. Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody. Science. 2016;351(6279):1339-42. doi: 10.1126/science.aad5224. PubMed PMID: WOS:000372397700047.

6. Oswald WB, Geisbert TW, Davis KJ, Geisbert JB, Sullivan NJ, Jahrling PB, et al. Neutralizing antibody fails to impact the course of Ebola virus infection in monkeys. Plos Pathogens. 2007;3(1):62-6. doi: 10.1371/journal.ppat.0030009. PubMed PMID: WOS:000248492500007.

7. Dye JM, Herbert AS, Kuehne AI, Barth JF, Muhammad MA, Zak SE, et al. Postexposure antibody prophylaxis protects nonhuman primates from filovirus disease. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(13):5034-9. doi: 10.1073/pnas.1200409109. PubMed PMID: 22411795; PubMed Central PMCID: PMC3323977.

8. Qiu X, Wong G, Audet J, Bello A, Fernando L, Alimonti JB, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. Nature. 2014. doi: 10.1038/nature13777. PubMed PMID: 25171469.

9. Dowall SD, Callan J, Zeltina A, Al-Abdulla I, Strecker T, Fehling SK, et al. Development of a Cost-effective Ovine Polyclonal Antibody-Based Product, EBOTAb, to Treat Ebola Virus Infection. The Journal of infectious diseases. 2016;213(7):1124-33. doi: 10.1093/infdis/jiv565. PubMed PMID: 26715676; PubMed Central PMCID: PMCPMC4779302.

10. Dowall SD, Bosworth A, Rayner E, Taylor I, Landon J, Cameron I, et al. Post-exposure treatment of Ebola virus disease in guinea pigs using EBOTAb, an ovine antibody-based therapeutic. Scientific reports. 2016;6:30497. doi: 10.1038/srep30497. PubMed PMID: 27465308; PubMed Central PMCID: PMCPMC4964638.

11. Zheng X, Wong G, Zhao Y, Wang H, He S, Bi Y, et al. Treatment with hyperimmune equine immunoglobulin or immunoglobulin fragments completely protects rodents from Ebola virus infection. Scientific reports. 2016;6:24179. doi: 10.1038/srep24179. PubMed PMID: 27067649.

12. Patterson R, Youngner JS, Weigle WO, Dixon FJ. Antibody production and transfer to egg yolk in chickens. Journal of immunology (Baltimore, Md : 1950). 1962;89:272-8. Epub 1962/08/01. PubMed PMID: 14484407.

13. Zhang WW. The use of gene-specific IgY antibodies for drug target discovery. Drug Discov Today. 2003;8(8):364-71. doi: 10.1016/s1359-6446(03)02655-2. PubMed PMID: WOS:000182277500010.

14. Barkas T, Watson CM. Induction of an Fc conformational change by binding of antigen: the generation of protein A-reactive sites in chicken immunoglobulin. Immunology. 1979;36(3):557-61. Epub 1979/03/01. PubMed PMID: 437844; PubMed Central PMCID: PMCPMC1457583.

15. da Silva MC, Schaefer R, Gava D, Souza CK, da Silva Vaz I, Jr., Bastos AP, et al. Production and application of anti-nucleoprotein IgY antibodies for influenza A virus detection in swine. Journal of immunological methods. 2018;461:100-5. Epub 2018/08/31. doi: 10.1016/j.jim.2018.06.023. PubMed PMID: 30158073.

16. Wen J, Zhao S, He D, Yang Y, Li Y, Zhu S. Preparation and characterization of egg yolk immunoglobulin Y specific to influenza B virus. Antiviral research. 2012;93(1):154-9. Epub 2011/12/01. doi: 10.1016/j.antiviral.2011.11.005. PubMed PMID: 22127067.

17. Yang YE, Wen J, Zhao S, Zhang K, Zhou Y. Prophylaxis and therapy of pandemic H1N1 virus infection using egg yolk antibody. Journal of virological methods. 2014;206:19-26. Epub 2014/06/01. doi: 10.1016/j.jviromet.2014.05.016. PubMed PMID: 24880066.

18. Fink AL, Williams KL, Harris E, Alvine TD, Henderson T, Schiltz J, et al. Dengue virus specific IgY provides protection following lethal dengue virus challenge and is neutralizing in the absence of inducing antibody dependent enhancement. PLoS neglected tropical diseases. 2017;11(7):e0005721. Epub 2017/07/08. doi: 10.1371/journal.pntd.0005721. PubMed PMID: 28686617; PubMed Central PMCID: PMCPMC5517069.

19. O'Donnell KL, Meberg B, Schiltz J, Nilles ML, Bradley DS. Zika Virus-Specific IgY Results Are Therapeutic Following a Lethal Zika Virus Challenge without Inducing Antibody-Dependent Enhancement. Viruses. 2019;11(3). Epub 2019/03/29. doi: 10.3390/v11030301. PubMed PMID: 30917523; PubMed Central PMCID: PMCPMC6466411.

20. Haese N, Brocato RL, Henderson T, Nilles ML, Kwilas SA, Josleyn MD, et al. Antiviral Biologic Produced in DNA Vaccine/Goose Platform Protects Hamsters Against Hantavirus Pulmonary Syndrome When Administered Post-exposure. PLoS neglected tropical diseases. 2015;9(6):e0003803. doi: 10.1371/journal.pntd.0003803. PubMed PMID: PMC4457835.

21. Fu CY, Huang H, Wang XM, Liu YG, Wang ZG, Cui SJ, et al. Preparation and evaluation of anti-SARS coronavirus IgY from yolks of immunized SPF chickens. Journal of virological methods. 2006;133(1):112-5. Epub 2005/12/06. doi: 10.1016/j.jviromet.2005.10.027. PubMed PMID: 16325277; PubMed Central PMCID: PMCPMC7112787.

22. Dai YC, Zhang XF, Tan M, Huang P, Lei W, Fang H, et al. A dual chicken IgY against rotavirus and norovirus. Antiviral research. 2013;97(3):293-300. Epub 2012/12/27. doi: 10.1016/j.antiviral.2012.12.011. PubMed PMID: 23267830; PubMed Central PMCID: PMCPMC3995418.

23. Zeitlin L, Whaley KJ, Olinger GG, Jacobs M, Gopal R, Qiu X, et al. Antibody therapeutics for Ebola virus disease. Current opinion in virology. 2016;17:45-9. doi:

10.1016/j.coviro.2016.01.006. PubMed PMID: 26826442; PubMed Central PMCID: PMCPMC4902774.

24. Reynard O, Volchkov VE. Characterization of a Novel Neutralizing Monoclonal Antibody Against Ebola Virus GP. The Journal of infectious diseases. 2015;212 Suppl 2:S372-8. doi: 10.1093/infdis/jiv303. PubMed PMID: 26232760.

25. Hiatt A, Pauly M, Whaley K, Qiu X, Kobinger G, Zeitlin L. The emergence of antibody therapies for Ebola. Hum Antibodies. 2015;23(3-4):49-56. doi: 10.3233/HAB-150284. PubMed PMID: 27472862.

26. Corti D, Misasi J, Mulangu S, Stanley DA, Kanekiyo M, Wollen S, et al. Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody. Science. 2016;351(6279):1339-42. doi: 10.1126/science.aad5224. PubMed PMID: 26917593.

27. Wang Y, Liu Z, Dai Q. A highly immunogenic fragment derived from Zaire Ebola virus glycoprotein elicits effective neutralizing antibody. Virus research. 2014;189:254-61. doi: 10.1016/j.virusres.2014.06.001. PubMed PMID: 24930448.

28. Qiu X, Audet J, Lv M, He S, Wong G, Wei H, et al. Two-mAb cocktail protects macaques against the Makona variant of Ebola virus. Science translational medicine. 2016;8(329):329ra33. doi: 10.1126/scitranslmed.aad9875. PubMed PMID: 26962157.

29. Qiu X, Audet J, Wong G, Pillet S, Bello A, Cabral T, et al. Successful treatment of ebola virus-infected cynomolgus macaques with monoclonal antibodies. Science translational medicine. 2012;4(138):138ra81. doi: 10.1126/scitranslmed.3003876. PubMed PMID: 22700957.

30. Abbas AT, El-Kafrawy SA, Sohrab SS, Azhar EIA. IgY antibodies for the immunoprophylaxis and therapy of respiratory infections. Human vaccines & immunotherapeutics. 2019;15(1):264-75. Epub 2018/09/20. doi: 10.1080/21645515.2018.1514224. PubMed PMID: 30230944; PubMed Central PMCID: PMCPMC6363154.

31. Kuzmina NA, Younan P, Gilchuk P, Santos RI, Flyak AI, Ilinykh PA, et al. Antibody-Dependent Enhancement of Ebola Virus Infection by Human Antibodies Isolated from Survivors. Cell Rep. 2018;24(7):1802-15 e5. Epub 2018/08/16. doi: 10.1016/j.celrep.2018.07.035. PubMed PMID: 30110637; PubMed Central PMCID: PMCPMC6697154.

32. Kasereka MC, Sawatzky J, Hawkes MT. Ebola epidemic in war-torn Democratic Republic of Congo, 2018: Acceptability and patient satisfaction of the recombinant Vesicular Stomatitis Virus - Zaire Ebolavirus Vaccine. Vaccine. 2019;37(16):2174-8. Epub 2019/03/18. doi: 10.1016/j.vaccine.2019.03.004. PubMed PMID: 30878249.

33. Ledgerwood JE, Costner P, Desai N, Holman L, Enama ME, Yamshchikov G, et al. A replication defective recombinant Ad5 vaccine expressing Ebola virus GP is safe and immunogenic in healthy adults. Vaccine. 2010;29(2):304-13. doi: 10.1016/j.vaccine.2010.10.037. PubMed PMID: 21034824.

34. Ye L, Lin J, Sun Y, Bennouna S, Lo M, Wu Q, et al. Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies. Virology. 2006;351(2):260-70. doi: 10.1016/j.virol.2006.03.021. PubMed PMID: 16678231.

579 35. Lawson ND, Stillman EA, Whitt MA, Rose JK. Recombinant vesicular stomatitis viruses
580 from DNA. Proceedings of the National Academy of Sciences of the United States of
581 America. 1995;92(10):4477-81. PubMed PMID: 7753828; PubMed Central PMCID:
582 PMC41967.
583 36. Whitt MA. Generation of VSV pseudotypes using recombinant DeltaG-VSV for studies
584 on virus entry, identification of entry inhibitors, and immune responses to vaccines. Journal of
585 virological methods. 2010;169(2):365-74. Epub 2010/08/17. doi:
586 10.1016/j.jviromet.2010.08.006. PubMed PMID: 20709108; PubMed Central PMCID:
587 PMCPMC2956192.
588 37. Chin AW, Perera RA, Guan Y, Halfmann P, Kawaoka Y, Peiris M, et al. Pseudoparticle
589 neutralization assay for detecting ebola- neutralizing antibodies in biosafety level 2 settings.
590 Clinical chemistry. 2015;61(6):885-6. Epub 2015/04/02. doi: 10.1373/clinchem.2015.238204.
591 PubMed PMID: 25829407; PubMed Central PMCID: PMCPMC7108194.

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