**Review**

**TJPS**
**The Thai Journal of Pharmaceutical Sciences**
**38 (4), October-December 2014: 156-209**

# Plant produced therapies for ebola infection

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## Abstract

Ebola virus (EBOV) causes severe haemorrhagic fever in humans with high fatality rate up to 90 %. There is currently no approved drug or vaccine for human use, and treatment relies almost exclusively on supportive therapy. However, there are several potential candidates for EBOV treatment including antibody therapies. In the 2014 outbreak, non-licensed plant-produced monoclonal antibodies (mAbs) against EBOV were used to treat infected humans. This review provides an insight into the efficacy and potential of antibody therapies and the characterized EBOV mAbs produced in different platforms. Among these platforms, the plant system has notable advantages for antibody production over others, including high scalability, short production time, post-translational modification, and no human pathogen contamination. EBOV mAbs have been produced in plants and their protective efficacy demonstrated in nonhuman primates. Cocktails of EBOV mAbs are potential candidates for the treatment of EBOV infection. Moreover, plants were also used to produce EBOV vaccines and these were shown to be robust in protecting animal models. In summary, we review the potential of plants to serve as a production system for recombinant therapeutic proteins targeted at this orphan disease.

*Keywords:* Ebola, Plant, Antibody

## Introduction

The ongoing Ebola outbreak of 2014 has already become the most severe ever recorded in terms of both disease prevalence and mortality. There is currently no licensed vaccine or specific treatment available against Ebola virus (EBOV). However, scientific studies have identified several potential treatments for Ebola. A plant made Ebola drug, ZMapp™, a cocktail of EBOV neutralising monoclonal antibodies (mAbs), was chosen to treat two American doctors who became infected with EBOV in Africa and has now been given to at least 5 patients with confirmed EBOV infection. Three out of five of these patients survived. The use of ZMapp™ in this scenario is particularly interesting for two reasons. First, although the drug had shown promising results in animal studies, it had not been previously tested for safety or efficacy in humans. Secondly, the drug was produced by means of plant biotechnology. This highlights such technologies as potential alternatives to established paradigms (such as mammalian cell bioreactors) with beneficial characteristics where others are less able to deliver. In this review, we provide an overview of the

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*Received: 29 August 2014*
*Revised: 15 October 2014*
*Accepted:26 November 2014*

*Academic Editor:* Suidjit Luanpitpong

disease and possible therapeutic approaches, with a particular emphasis on the role of plant based recombinant protein production, which may provide effective therapeutics and vaccines for this terrible disease.

***Ebola: epidemiology and pathology***

EBOV was first described in 1976 as the causative agent of two simultaneous outbreaks in Zaire (now the Democratic Republic of Congo) and Sudan [1-2]. The virus was named after the Ebola River in northern Democratic Republic of Congo where the first outbreak occurred. The strain of EBOV isolated from the first outbreak in Zaire (Zaire strain, ZEBOV) remains the most virulent yet reported, with a mortality rate of 88%. Since the initial report, there have been approximately 20 outbreaks associated with different EBOV strains [3]. The 2014 outbreak began in Guinea and spread to Sierra Leone, Liberia, and Nigeria. To date, this outbreak is the most severe recorded in regards to both the number of infected people and fatalities, owing in part to global travel. Until now the Centers for Disease Control and Prevention (CDC) has reported 5,006 confirmed infections and 4,493 suspected case deaths [4].

Generally, EBOV is transmitted through direct contact with infected bodily fluids (e.g., blood, semen, and vaginal fluid) of infected persons or primates [5]. Fruit bats have also been identified as a possible zoonotic reservoir and typically do not show symptoms of infection [6]. The incubation period is between 2-21 days [7]. The virus targets mononuclear phagocytic cells such as macrophages and monocytes by binding receptors including T-cell immunoglobulin and mucin domain 1 (TIM-1) [8-11]. After infecting these primary target cells in the blood, virus replication and cell lysis causes high viremia. This allows the virus to be disseminated throughout the bloodstream and to infect secondary target cells, such as endothelial cells in the liver, spleen, pancreas, lungs, and kidneys. Viral infection of various organs leads to the disease symptoms associated with multi-organ failure such as fever, extreme fatique, diarrhea, abdominal pain, hemorhagic rash, and coma.

The molecular mechanism of Ebola pathogenesis is difficult to study due to the rapid onset of disease symptoms and death both in natural infections and in laboratory animal models. Research on this virus requires Biosafety Level 4 (BSL-4) facilities—the most stringent degree of laboratory protection. BSL-4 laboratories are designated for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections, cause severe or fatal disease in humans, and for which there are no vaccines or treatments available. The low number of laboratories designated BSL-4 in the world is a significant limit to Ebola research. Several laboratory animals are sui models for the study of the EBOV infection including mouse, guinea pig, and nonhuman primates, but the "gold standard" animal model for pathogenesis, treatment, and vaccine studies are rhesus and cynomolgus macaques [12]. Only these animals are lethally infected with non-adapted human isolates, and the resulting pathology closely mirrors the pathology described in humans.

***Treatment options***

Currently, there is no approved vaccine or treatment available for human use. The current protocol for Ebola infected patients is to quarantine and provide supportive management and palliative care. Support care for patients includes oral fluid rehydration, oral medication, nutritional supplementation and psychosocial support [7]. Treatment of Ebola infection with passive transfer of antibodies is a potential therapy. However, there are conflicting results from animal studies. Administration of hyper-IgG serum from horses immunized with EBOV delayed the onset of viremia and disease.[10] However, it failed to protect cynomolgus monkeys against EBOV challenge. Moreover, mAb KZ52, which showed good neutralizing activity in vitro and protected guinea pigs [13], could not protect rhesus macaques from EBOV challenge when it is administered either 1 day before challenge or 4 days after challenge [14].

In addition, there are various anti-viral therapies using several agents such as a vesicular stomatitis virus (VSV) [15], an anticoagulant protein [16-17], phosphorodiamidate morpholino oligomers [18-19], and small interfering RNAs (siRNAs) [20-21]. Typically, these candidates reduce the mortality when administered to nonhuman primates up to 1 hour after Ebola challenge. TKM-Ebola, a treatment based on RNA interference mediated by a pool of EBOV specific siRNAs delivered using lipid nanoparticles, has entered Phase I clinical trials in humans [22]. In an interesting recent development, the US food and drugs agency (FDA) has acted to partially revoke a hold order on the trial that was originally enforced to allow time for the drug's manufacturer Tekmira to answer questions concerning the drug's mode of action. This action by the FDA, along with the authorisation to use ZMapp™, amounts to an unprecedented level of access to experimental anti-infective drugs and underlines the severity of the current outbreak.

***Antibody immunotherapy***

The passive transfer of neutralising antibodies remains one of the most promising approaches for treating an established EBOV infection. Previous studies have demonstrated that plasma from sheep and goat infected with live EBOV effectively protects guinea pigs from lethal Ebola challenge if it is administered within 48 hours after infection [23]. Moreover, equine anti-EBOV immunoglobulins were also effective in a challenge study in baboons [23]. In the 1995 Kikwit outbreak, antibody therapy against Ebola infection was first reported as a potential treatment in human after the transfusion of crude blood containing Ebola antibodies from convalescent patients significantly reduced the observed fatality rate (79.4 %), 7 of 8 treated patients survived (12.5 %) [24]. These studies suggested the Ebola immunoglobulin as an effective treatment for Ebola virus. However, these reports were counterbalanced by several consequent studies that showed the failure of the antibody treatments [14, 25-26].

**Table 1** A summary of monoclonal antibodies against Ebola virus

| Ebola mAb | Subtype | Epitope | Source | Reference |
|---|---|---|---|---|
| 13F6† | IgG2a | GP1 | H, CHO, *N. benthamiana* | [28, 38-39, 52] |
| 6E3 | IgG1 | GP1 | H | [28] |
| 6D8† | IgG2a | GP1 | H, CHO, *N. benthamiana, lettuce* | [28, 39, 53] |
| 12B5 | IgG1 | GP1 | H | [28] |
| 13C6† | IgG2a | GP1, sGP | H, CHO, *N. benthamiana* | [28, 39] |
| 12E12 | IgG3 | GP1, sGP | H | [28] |
| 6D3 | IgG2a | GP1, sGP | H | [28] |
| 8C10 | IgG2a | GP1, sGP | H | [28] |
| 3H8 | IgG2a | GP1, sGP | H | [28] |
| KZ51 | IgG1 | NP | H | [27] |
| KZ52 | IgG1 | GP | H | [27] |
| KS5 | IgG1 | ND | H | [27] |
| KS14 | IgG1 | GP | H | [27] |
| KS56 | IgG1 | GP | H | [27] |
| KS518 | IgG1 | GP | H | [27] |
| LS4 | IgG1 | GP | H | [27] |
| LZ51 | IgG1 | GP | H | [27] |
| ELZ510 | IgG1 | NP | H | [27] |
| 1H3* | IgG2a | GP | H | [29] |
| 2G4* | IgG2b | GP | H | [29] |
| 4G7* | IgG2a | GP | H | [29] |
| 5D2 | IgG2a | GP | H | [29] |
| 5E6 | IgG2a | GP | H | [29] |
| 7C9 | IgG2a | GP | H | [29] |
| 7G4 | IgG1 | GP | H | [29] |
| 10C8 | IgG2a | GP | H | [29] |

† : MB-003, *: ZMab, H: hybridoma cells

**Table 2** Comparison of antibody production in different platform

| Platforms | Yield | Time from gene to protein | Scalability | Fidelity of PTM | Support human pathogen? | Recent Review Reference |
|---|---|---|---|---|---|---|
| Mammalian cell (eg. CHO) | +++ | 8 weeks | + | +++ | Y | [54] |
| Bacteria (eg *E.coli*) | +++ | 1 week | ++ | - | Y | [55] |
| Yeast | +++ | 1 week | ++ | + | Y | [56] |
| Transgenic plant | ++ | 6 months- 1 year | +++ | ++ | N | [57] |
| Transient expression *N. benthamiana* | +++ | 1 week | ++ | ++ | N | [58] |

Monoclonal antibodies (mAbs), which can bind and neutralise EBOV, have been identified. Maruyama et al. generated and characterized several mAbs that can bind to Ebola Zaire nucleoprotein (NP), glycoprotein (GP) and secreted glycoprotein (sGP) [27]. Among all mAbs characterized in this study, KZ52 has the highest GP affinity and potent viral neutralization activity. In 2000, Wilson and colleagues identified several protective mAbs against epitopes on Ebola GP and classified them into five groups on the basis of competitive binding assays [28]. These mAbs conferred protection when administered to non-human primates 1 day before challenge, but protection was also observed for some of the mAbs when they were administered 2 days after exposure. In 2011, Qiu, et al identified 8 mAbs against Ebola GP, which improved the survival rates by 33-100% against a high dose lethal challenge with mouse-adapted EBOV [29]. The identification of several sets of protective mAbs has been invaluable for current studies to develop vaccines and therapies for EBOV. A summary of published mAbs against EBOV is given in Table 1.

In 2012, Dye, et al. demonstrated protection of rhesus macaques from Ebola challenge using polyclonal IgG isolated from macaques that had survived a previous infection [30]. However, attempts to neutralise EBOV *in vivo* using cocktails of recombinant mAbs have revealed mixed results. In 2012, Marzi and co-workers showed that a 50 mg intravenous dose of two mAbs with strong *in vitro* neutralizing activity, human-mouse chimeric ch133 and ch226, protected only one of three rhesus macaques from the Ebola challenge when the animals were treated 1 day before the challenge [31]. Also in 2012, Qiu, et al. showed a combination of three neutralizing mAbs against Ebola GP, dosed 3 days apart starting 24 hours after the challenge, protected all four challenged cynomolgus macaques with no disease symptoms [32]. Nonetheless, the same treatment protected only two of four cynomolgus macaques when they were administered at 48 hours after the challenge. Thus, treatment time is likely to be a critical factor in an effective antibody immunotherapy.

Recently, Qiu, et al. studied a combination of mAbs administered with adenovirus-vectored interferon in the cynomolgus and rhesus macaque challenge model [33].

This treatment protected 75 % (3 of 4) cynomolus macaques and 100 % (4 of 4) of rhesus macaques when the treatment was administered 3 days post-infection. However, the treatment protected only 50 % (2 of 4) if adenovector-interferon and mAbs were administered at 1 and 4 days after infection, respectively. This study suggested that the treatment is effective even if it is given after the animal showed symptoms but further reinforces the requirement for timely treatment.

***Plants as a production platform for antibodies***

Plants have been used as bioreactors for antibody production as they offer several potential advantages over other conventional production systems, including using bacteria, yeast or mammalian cell culture (for a recent review see ref. [34]). Plant production facilities are cheaper than equivalent bioreactors, and offer a rapid gene to protein turnaround time and high scalability. They are not susceptible to contamination with mammalian-tropic pathogens. Post-translational modification (PTM) in plants is controllable [35] and represents an important advantage over using bacteria since many proteins, including most antibody formats, do not fold correctly and have limited functionality when expressed without PTM.

To produce antibodies in plants, plants must be transformed with genes encoding antibody proteins. Typically, the bacterium *Agrobacterium tumefaciens* is used to transfer recombinant regions of DNA encoding for the genes of interest into the plant nucleus through the activity of the *vir* (virulence) operon. These DNA regions are termed transfer DNAs (T-DNA). T-DNA is capable of integrating into plant chromosomes, generating a stable transgenic cell that can be regenerated into a whole plant. However, a high level of transcriptional activity occurs before integration takes place. This burst of transcription can be utilised to produce large amounts of recombinant protein without the need for time-consuming regeneration steps. Furthermore, the rate of transcription can be significantly enhanced through the simultaneous delivery of viral genes encoding proteins directing the replication of RNA or even permitting cell-to-cell spread of message

[36]. The process of producing an antibody using a transient expression system is represented in Figure 1.

A comparison of these two plant-based approaches with other methods of producing recombinant proteins is provided in Table 2. Crucially, transient expression allows antibodies to be expressed with faithful PTMs at scale and within an extremely short time frame, without the need for expensive bioreactors or product-dedicated production facilities. Transgenic plants require no specialised equipment for growth or antibody production except that required for the control of genetically modified organisms and can be grown at agricultural scale. Downstream processing is similar for both approaches, and protein A or G matrices are commonly used to purify mAbs from plant extracts.

***Plant mAbs for Ebola***

Following the isolation of protective mAbs against epitopes on Ebola glycoprotein [28], Mapp biopharmaceutical Inc. reengineered the sequences for expression via *A. tumefaciens* mediated T-DNA transfer to *N. benthamiana* plants. Ebola 6D8 mAb was produced in leaves using an expression cassette based on the ssDNA virus Bean Yellow Dwarf Virus, a geminivirus [37]. The 6D8 mAb, against Ebola GP1 protein, was produced at 0.5 mg of mAb per gram of leaf fresh weight within 4 days, which is considered a high yield and compares well with other production approaches (CHO cells typically yield up to 10mg/l culture volume). Zeitlin et al. produced 13F6 mAb in plants and investigated the influence of the plant N-glycan in the Fc region [38]. It was found that the plant glycan was associated with improved protective efficacy compared with mammalian (CHO cell) glycans, and antibody-dependent cellular cytotoxicity (ADCC) was implicated as an important mode of action for this antibody.

***Antibody Cocktails***

Two significant drawbacks to the use of antibody monotherapy in the treatment of infectious disease are incomplete coverage of circulating strains and the emergence of escape mutants that are no longer sensitive to neutralization. To avoid these shortcomings, it is preferable that a combination of antibodies recognizing different epitopes is used as an immunotherapy.

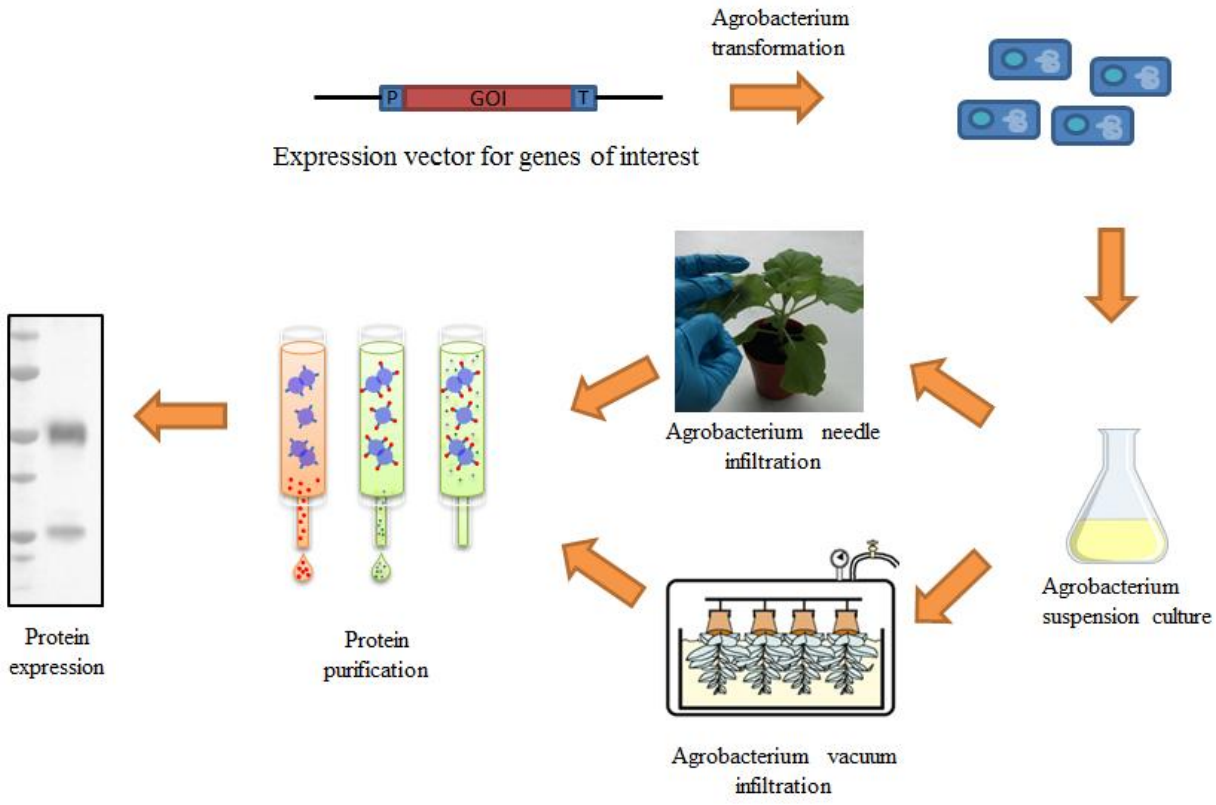


**Figure 1** The process of producing an antibody using a transient expression system. Both heavy chain and light chain genes are inserted into the transfer DNA (T-DNA) region of a plant expression vector and this vector is then used to transform *Agrobacterium tumefaciens*. *A. tumefaciens* has been engineered as tool for plant biotechnology to deliver the T-DNA region into the plant cell through the action of the *vir* gene products encoded on a separate helper plasmid. The *A. tumefaciens* suspension culture is infiltrated into leaves by either manual syringe infiltration for laboratory scale production or vacuum infiltration for commercial/ clinical production. After infiltration, the plants are kept in the greenhouse for 4-10 days as determined by the stability of the transgene product. After the extraction process, the antibody is purified from plant proteins using conventional protein A or protein G affinity chromatography.

In a pivotal study concerning the antibodies produced by Mapp biopharmaceutical Inc., Olinger, et al. compared the protective efficacy of humanized mAbs 13C6, 13F6, and 6D8, produced from CHO cells and plants (*N. benthamiana*) and the mixture of these three mAbs (MB-003) in rhesus macaques [39]. This study concluded that MB-003 produced from both CHO cells and plants protected rhesus macaques from lethal EBOV challenge when administered 1 hour after infection. Moreover, the animals showed little viremia and few clinical symptoms. Pettitt, et al. demonstrated that the MB-003 prevented death in 43 % of rhesus macaques from EBOV infection after appropriate diagnostic indicators became positive, whereas all the untreated animals succumbed to the infection [40]. This study was important, as previous work has focused on pre-exposure treatment, or treatment within a short window after infection, which is not an appropriate model for EBOV infection in a developing country outbreak scenario. This study ultimately paved the way for the use of the ZMapp™ antibody cocktail in infected humans. ZMapp™ is a cocktail of three antibodies, including at least one of the components of MB-003, and at least one of the antibodies isolated by Qiu et al. and commercialized by Defyrus Inc. of Canada (ZMab). Limited information is available on the ZMapp™ cocktail although it has shown efficacy in the non-human primate challenge model and Mapp Biopharmaceutical Inc. will shortly publish these data (K. Whaley, pers. comm.). All component antibodies are believed to bind EBOV GP. Licenses to develop both sets of antibodies have been granted to Leaf Biopharmaceutical Inc., the commercialization partner of Mapp Biopharmaceutical Inc., who has made a limited supply of ZMapp™ available at no cost. The production of ZMapp™ has also been scaled up to supply those with a legitimate need for the experiment therapy, and to demonstrate the potential of transient expression platform to provide 'a cost effective rapid response system to meet global health challenges of emerging pathogens'.

***Ebola Vaccines***

Many approaches to creating a vaccine for EBOV have been proposed, including DNA vaccines [41-42] and viral-based vectors [43-47]. Among these candidates, EBOV pseudotyped Venezuelan equine encephalitis virus (VEE) is one of the most advanced candidates with promising pre-clinical results. VEE expressing EBOV GP in place of the structural polyprotein protected guinea pigs and mice from Ebola challenge [43]. This vaccine induced both antibodies and cytotoxic T lymphocytes in the vaccinated mice [44]. However, when vaccinated non-human primates (both cynomolgus and rhesus macaque models) were challenged 49 days after three subcutaneous doses, all animals succumbed from the EBOV challenge [48]. However, Herbert et al. recently showed that VEE replicon administered via the intramuscular route could protect cynomolgus macaques from EBOV challenge 28 days after the vaccination [49]. These studies therefore suggest that timing and route of vaccination is critical for achieving robust protection.

The ability of plants to produce high levels of EBOV specific antibodies has been used as the basis for a novel approach to EBOV vaccine design. Ebola glycoprotein was genetically fused to the heavy chain of mAb 6D8 and expressed in *N. benthamiana* [50]. Driven by self-affinity, these chimeric antibody-antigen structures were capable of forming immune complexes when purified from plant tissue. Subcutaneous administration of plant-produced Ebola immune complex induced EBOV-specific antibody responses in mice. Moreover, when adjuvanted with polyinosinic:polycytidylic acid, the EBOV immune complexes could protect mice from challenge [51]. Plant-produced mAb 6D8-GP1 complexes are still subject to ongoing tests in non-human primates.

***Perspective***

There is a clear lack of effective pharmacological management strategies for EBOV infection. The nature of the disease (fast, historically self-limiting outbreaks) and the geographical distribution of cases have both contributed to the slow progress of drug development. There has been little or no contribution made by established pharmaceutical sector, and funding for drug development has largely come instead from public sources. The lack of "Big Pharma" involvement has given early stage biotechnology and pharmaceutical enterprises an opportunity to develop and ultimately supply drugs to combat the disease outbreak without addressing clinical trials. These drugs include Tekmira's TKM-Ebola, an siRNA based approach, and Leafbio's ZMapp™, an optimised cocktail of three mAb targeting EBOV glycoprotein produced in plants. As it is impossible to draw valid conclusions regarding the efficacy of these treatments in such a setting, the motivation for supplying these experimental drugs is founded on largely humanitarian goals.

For Mapp Biopharmaceutical Inc., as a company with a significant interest in plant biologics production (commonly known as 'Molecular Farming'), there may be another driving force at play. Using plants to produce antibodies and therapeutic proteins is not new. Andy Hiatt and colleagues made the first report of an antibody from a transgenic tobacco plant in Nature in 1989. However, to date the only plant made drug approved by FDA for human use is ELELYSO™, an enzyme replacement therapy for Gaucher's disease made in carrot cells. As yet no antibody-based therapeutic has proceeded past Phase II clinical trial. The authorisation of ZMapp™ for emergency use in the current EBOV outbreak by the WHO is potentially a major breakthrough in the field, as it will serve as an endorsement of the technology to potential investors and grant funding agencies.

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