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REVIEW

Plant-made vaccines and reagents for the One Health initiative

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ABSTRACT

The One Health initiative is increasingly becoming a prominent discussion topic in animal and human health, with its focus on prevention of spread of zoonotic diseases, both in animals, and from animals to humans. An important part of One Health is that diagnostics and vaccines for diseases may be the same thing – and be used for both humans and animals. One potential problem standing in the way of wider adoption of One Health principles, though, is that use of conventional cell fermentation systems for production of the recombinant proteins that could be used as diagnostics or vaccines is often expensive and is not easily scalable. A solution to this may be the use of plants or plant cells as bioreactors: molecular farming, or the production of biologics in plants, is now a well-established science with many proofs of principle and important proofs of efficacy for especially animal vaccines. This review discusses how molecular farming could enable important advances in One Health, using as examples plant-made vacccines, reagents and therapeutics for influenza viruses, ebolaviruses, rabies virus, bunyaviruses and flaviviruses.

KEYWORDS

molecular farming; one health; plant-made vaccines; reagents

Introduction

The One Health initiative, formerly known as One Medicine, is "…dedicated to improving the lives of all species - human and animal - through the integration of human medicine, veterinary medicine and environmental science" (http://www.onehealthinitiative.com/mission.php). It is premised on the principle that the health of the Earth's people is connected to the health of its animals and of the environment, and encourages collaboration to achieve the best health for all of these. The initiative in its modern form dates from 2007, when a formal bond was established between the American Medical Association (AMA) and the American Veterinary Medical Association (AVMA), and when an International Ministerial Conference on Avian and Pandemic Influenza in New Delhi urged governments to build One Health linkages for pandemic preparedness and human health security. Adoption of the principles by the European Union and the US Centers for Disease Control & Prevention, and support from international bodies such as the World Health Organization, Food and Agriculture Organization and the World Organization for Animal Health, mean that while it is still a virtual initiative that does not provide funding, its influence on national and international health policies is significant.[1]

The importance of the approach can be seen when one realizes that 6 out of every 10 infectious diseases in humans are zoonotic, or spread from animals (https://www.cdc.gov/onehealth/), and 7 out of 10 of emerging or re-emerging infections are vector-borne or zoonotic (http://www.onehealthinitiative.com/). There is particular interest in developing low-cost reagents for point-of-care diagnostics that could also be used as vaccines for animals and possibly also for humans, as these diseases mainly affect people and animals in developing countries, meaning that resources to study them are often lacking.

The kinds of emerging zoonotic disease agent that are of concern to One Health include those that are transmitted directly from wild animals to humans (eg: Sin Nombre hantavirus; Lassa fever arenavirus); agents that originate in wild animals and then spread human to human (eg: HIV-1 and -2; ebolaviruses); agents that are transmitted from wild to domestic animals to humans (eg: Nipah and Hendra henipaviruses); and those that move from wild to domestic animals then go on to be transmitted between humans (eg: pandemic influenzaviruses, SARS and MERS coronaviruses).

While the One Health concept is far wider than simply considering vaccines and diagnostics – something that is shown very effectively here (http://www.onehealthinitiative.com/about.php) - provision of vaccines to combat the spread of disease agents, and of reagents to enable quick and inexpensive diagnosis of these, are an important component. A useful breakdown of the kinds of vaccines that would be useful is given here,[2] and is summarised below.

- Framework I vaccines may be used for protection of humans and domesticated animals, where neither is central to the transmission cycle (eg: West Nile flavivirus).
- Framework II vaccines are to be used in domesticated animals to prevent disease in both animals and humans, for disease agents transmitted directly or indirectly via arthropod vectors from animals to humans (eg: vaccines

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for brucellosis, *Escherichia coli* O157, anthrax; rabies, Rift Valley fever, Crimean-Congo hemorrhagic fever, Venezuelan equine encephalitis, and Hendra / Nipah viruses).

- Framework III vaccines are intended for use in wild animals, to prevent transmission of disease both to domesticated animals and to humans (eg: rabies virus vaccines in oral baits, *Mycobacterium bovis* and Lyme disease vaccines).

Anthrax and rabies are 2 good case studies for One Health. Anthrax – caused by *Bacillus anthracis* - is a disease of great antiquity; while the development of the live spore vaccine that is still used significantly reduced its incidence in livestock worldwide after 1937, outbreaks in domesticated animals are becoming increasingly common and outbreaks in wildlife are frequently underreported or undetected.[3] Humans are usually infected via exposure to infected livestock and to carcasses of wild animals, especially in Africa and other developing areas. There is also an increasing awareness of the potential use of *B. anthracis* spores as a biological weapon, which adds some urgency to the development of highly effective universal vaccines.

The control, and possibly even the global elimination of rabies virus disease, is also a good One Health test case.[4] While approaches such as the use of live vaccine baits have been effective in controlling the disease in Europe, for example, low-income countries generally require different control strategies, and both cheaper vaccines and therapeutics in the shape of virus-neutralising antibodies to rabies virus, to limit endemicity and especially human disease.

The production of the kinds of complex biologics that constitute modern vaccines and diagnostic reagents is a well-developed science, albeit highly expensive. Veterinary and especially human vaccine production requires Good Manufacturing Practice, and typically involves sterile cell-based systems that include bacterial, yeast or fungal, and animal cells cultured in highly controlled environments that are expensive to establish, and costly to maintain. An alternative production system that promises far cheaper production of active pharmaceutical ingredient, if not of finished product, is “molecular farming," or the use of plants and plant cells to make complex biologics. This field is in fact nearly 30 y old in 2017, and is becoming increasingly sophisticated: many proofs of concept and of efficacy for animal and human vaccines and therapeutics have been obtained, and several products are even licensed for human use (see reviews[5–8]).

This brief review will illustrate the potential applications of molecular farming to One Health, with examples drawn from the recent literature and from our lab’s work.

Influenza vaccines

Plant-made vaccines against influenza viruses are perhaps the poster children for molecular farming: many candidate vaccines made in plants have shown efficacy in animal models; candidate pandemic virus vaccines have been made to the scale of 10 million doses in less than a month, vaccines suitable for outbreak viruses similarly (see review[9]). Efficacy to homologous challenge has been shown in mice, ferrets and chickens; so too has efficacy to heterologous challenge with high pathogenicity avian influenza (HPAI) strain H5N1 in chickens.[10] While most of this work is directed toward protecting humans against potentially pandemic influenza viruses, it is often overlooked that the same vaccine candidates could be equally useful in birds and in swine: indeed, breaking the chain of recycling of influenza viruses that seems to occur in intensively farmed pigs is a prime goal of One Health.[11] Other targets for plant-made influenza vaccines include dogs[12] and potentially horses.

Our group investigated the potential for making influenza pandemic rapid response vaccines in South Africa by making influenzavirus A/Vietnam/1204/04 (H5N1) haemagglutinin by transient expression in *N. benthamiana*:[13] our success opened up the possibility of making H5 HA as a reagent and potentially as a vaccine, by means hitherto not available in Africa. We went on to use the HA2 portion of the protein as a virus-like particle (VLP)-based display vehicle in plant manufacture for the highly conserved M2e ectopic epitope as an elicitor of broadly neutralising antibodies to all influenzavirus A strains, as a candidate universal vaccine for humans and animals.[14]

Rabies vaccines and therapeutics

Vaccines to protect against rabies viruses were an early target of molecular farming: as early as 2003,[15] it was suggested that plant-made rabies vaccines could be a useful tool for wildlife immunisation, specifically in the context of fruit bats in the genus *Pteropus*. These are hosts of many potentially human-infecting viruses, including the henipaviruses Hendra and Nipah; Menangle and Tioman rubulaviruses, and Australian bat lyssavirus, which is a close relative of rabies virus.[15] Indeed, intraperitoneal immunisation of mice with purified extracts of transgenic tobacco (*Nicotiana tabacum*) expressing high concentrations of rabies virus G protein elicited comparable levels of immune response to the inactivated conventional vaccine, and complete protective immunity in mice against intracerebral lethal challenge with live rabies virus.[16] In a more recent study, sheep were protected from live virus challenge by oral immunisation with a single dose of transgenic maize kernels containing 2 mg of G protein.[17]

The Canadian biotech company Medicago Inc. has also patented a rabies virus-like particle vaccine made in in plants,[18] and announced an expansion of their vaccine pipeline to include the new product.[19]

It is also possible to produce effective anti-rabies monoclonal antibodies (MAbs) in plants:[20] The potential of such biologics to replace the rabies immunoglobulin (RIG) of either equine or human origin that is normally used for rabies immunotherapy, and which is prohibitively expensive for developing countries, is a highly attractive prospect for future development. In a response to what is seen as a pressing health need in a rabies-endemic developing country, the Council for Scientific and Industrial Research in South Africa is producing a soon-to-be commercialised anti-rabies MAb called Rabivir in transgenic tobacco, which they claim is 10 times cheaper than the conventional alternative.[21]

Ebolavirus vaccines and therapeutics

Ebolaviruses have been part of One Health thinking for some time: several reviews have, in recent years, discussed the control

of the viruses' emergence in the context of One Health, and in the context of zoonotic threats and the exotic animal practice.[22–24] An obvious target for their control, in humans but also possibly in other primates at risk like chimpanzees and gorillas, are vaccines – and this is one area of hope after the recent West African epidemic, where accelerated licensure and testing threw up several likely candidates, but one real success in recombinant vesicular stomatitis virus (rVSV-ZEBOV).[25] This notwithstanding, subunit vaccines may yet be useful, for Zaire and other ebola- and marburgviruses – and investigations have been done on the vaccine potential of different viral proteins to throw up useful candidates, including as well as the obvious GP1 envelope glycoprotein, VP24, VP30, and VP40.[26]

Toward this goal, several plant-made antigens have been shown to have potential. One novel approach was the expression of an Ebola immune complex (EIC) in *N benthamiana*, using a replicating geminivirus-derived vector.[27] The GP1 protein of Zaire ebolavirus was C-terminus fused to the heavy chain of a humanised 6D8 IgG mAb antibody, which specifically binds GP1: this was co-expressed with the 6D8 light chain and the assembled mAb::GP1 chimaera was purified by ammonium sulfate precipitation and protein G affinity chromatography. The mAb was functional in terms of binding C1q, and the chimaera formed a cross-linked immune complex with itself. BALB/C mice immunised subcutaneously with purified EIC produced anti-GP1 antibodies at levels similar to those elicited using a GP1 VLP vaccine. Another very recent approach was to make the ebolavirus matrix protein VP40 in plants:[28] this protein is highly multifunctional and elicits protective immunity in mice.[26,29] Transgenic tobacco plants expressed an ER-targetted VP40 at levels of ~3 mg/kg fresh weight of plant tissue. The protein was given orally or subcutaneously to BALB/C mice in 3 low-dose preparations (125 ng oral, 25 ng s/c) without adjuvants, and elicited reasonable responses. Both these proteins are candidate vaccines, and candidate low-cost reagents for ebolavirus diagnostic kits.

Perhaps the crowning achievement of molecular farming in recent years is one specifically related to the recent West African Zaire ebolavirus outbreak: this was the production and eventual accelerated clinical trial of the humanised mAb cocktail known as ZMapp™ by Mapp Biopharmaceutical, made by transient expression in *N benthamiana*.[30] This has been thoroughly described elsewhere,[9,31] and suffice it to say here that this one product has done more in terms of raising the profile of plant-produced pharmaceutical products in general, and therapeutics in particular, than any other. In the clinical trial in West Africa, 36 trial participants were randomly assigned to each of the 2 study arms: mortality in ZMapp™-treated participants was lower (8 of 36; 22 percent mortality) than in participants receiving standard-of-care alone (13 of 35; 37 percent mortality). While this was not statistically significant because the participant numbers were low, ZMapp™-treated participants eliminated virus from the bloodstream faster, had more rapid resolution of symptoms and were discharged from care earlier, than untreated participants. Given this apparent success, the US Government has funded Kentucky BioProcessing to stockpile the MAbs, and Mapp is going ahead to license ZMapp™ as a therapeutic for use in subsequent Ebola disease outbreaks.[30] It should be noted that the MAbs should also be highly useful in the diagnostic arena.

Reagents and vaccines for bunyaviruses

While inexpensive vaccines that can be made profitably at small scale are one of the drawcards of the use of molecular farming, it is not generally appreciated that reagents for some of the world's more dangerous zoonotic pathogens are also expensive and hard or dangerous to make. The need for reagents is exemplified by a recent large study of seroprevalence in humans in Nigeria against the tick-borne Crimean-Congo hemorrhagic fever bunyavirus (CCHFV).[32] In South Africa, routine detection of antibodies in animals or in humans to CCHFV is done using the nucleoprotein (N) in ELISA tests: making this protein is a hazardous and expensive process, because it involves fractionating it from live virus prepared from CCHFV infected cell cultures or brain tissue of inoculated suckling mice, under high biosafety conditions. While it can also be prepared as a recombinant protein from both insect and mammalian cells, these methods are both expensive and the protein yields and quality are often not good.

Our group accordingly used *Agrobacterium*-mediated transient expression in *N benthamiana* plants of a codon-optimised CCHFV *N* gene – which encodes the genome-associated nucleoprotein (NP) - to make a N-terminal 6xHis-tagged NP, that yielded ~2mg protein / kg fresh plant material, was soluble, and was easily purified by a combination of ammonium sulfate precipitation and immobilised metal ion chromatography. The protein was used in an anti-IgG ELISA with a standard panel of 13 serum samples collected 5–15 y post infection from patients confirmed to have anti-CCHFV IgG using commercial immunofluorescent antibody tests. Serum samples from 13 volunteers with no CCHFV infection history were used as a negative control panel. The plant-produced NP detected anti-CCHFV IgG in all positive serum samples, while all negative serum samples gave results below the cut-off value. The results suggest that recombinant NP expressed in plants has significant potential for use in both diagnosis and surveillance, while probably being a better, more easily produced and purified reagent than what is available presently.

Another innovative approach was the expression of a synthetic CCHFV envelope glycoprotein precursor (GcGn) polyprotein in leaves and induced hairy roots of transgenic tobacco plants: the material was immunogenic in mice via oral or parenteral immunisation routes, and GcGn purified from plants was an excellent ELISA plate coating antigen for detection of envelope glycoprotein-specific antibodies elicited by plant-made or conventional vaccines.[33]

Rift Valley fever bunyavirus (RVFV) is a mosquito-transmitted pathogen of livestock that causes abortion storms in sheep and goats, and potentially lethal in humans, that is expanding its geographical range out of Africa into Arabia and elsewhere.[34] It is a matter of concern in the One Health movement because while there are effective veterinary vaccines, these are not necessarily safe, and there is no licensed human vaccine,[35] although experimental candidates that are safer in animals and may be suitable for humans, have been described.[36] A plant-made approach to a RVFV vaccine published recently

described the expression in transgenic *Arabidopsis thaliana* of the N protein and a soluble version of the Gn glycoprotein.[37] Oral dosing of fresh transgenic plant material was immunogenic in mice, and elicited systemic antigen-specific IgG: this augurs well for similar experiments in larger animals. A spur to the deployment of human vaccines for RVFV could be the recent finding that the virus may be involved in human miscarriages in South Sudan.[38] Our laboratory is in the process of testing transiently-expressed RVFV N protein for its suitability as a diagnostic reagent, similar to the CCHFV case described above.

Vaccines and reagents for flaviviruses

West Nile flavivirus (WNV) – a mosquito-transmitted pathogen in the same family as Zika, yellow fever and Japanese encephalitis viruses – is also of concern to One Health, given its recent intercontinental transmission from Europe to the Americas and the fact that the virus is transmitted regularly to livestock and to humans, and can cause severe disease.[39] A potentially useful vaccine candidate consisting of the domain III of the E glycoprotein was produced by ER-targetted transient expression in *N benthamiana* at levels of 73 mg/kg, and was easily purified, bound MAbs recognizing a conformational epitope of the native protein, and elicited a potent systemic immune response after subcutaneous immunization in mice.[40] Other work from the same group provided evidence that a humanised mAb to WNV E protein (E16) could be produced in *N benthamiana* by transient agroinfiltration-mediated expression, and that it protected mice against WNV-induced mortality comparably to mammalian-cell-produced E16 MAbs.[41] Later work demonstrated that both the WNV domain III (DIII) protein and the E16 mAb could be rapidly produced at high levels in plants and easily purified, and that they could be used to identify WNV, and detect human IgM responses to WNV infection, in serological assays.[42] The fact that E16 mAb does not cross-react with other flaviviruses makes it useful as a diagnostic, but also potentially useful in therapy, as it is not likely to cause antibody-dependent enhancement (ADE) of infection by other related flaviviruses such as yellow fever, Zika and dengue viruses.

Dengue viruses (DENV) are a problematic vaccine target, given that antibodies directed against any one of the 4 dengue subtypes will not protect against infection by any of the others, and may in fact result in ADE of infection by them, which can result in dengue hemorrhagic fever. While there is now a licensed quadrivalent live vaccine,[43] new concerns over the interaction of dengue and other flaviviruses and in particular Zika virus, and the possibility of reciprocal ADE between them, have prompted caution over its use. For this reason, subunit vaccines consisting of E protein DIII – similar to the WNV example above, and which are serotype-specific and elicit neutralising antibodies which are not involved in ADE – have been trialled in monkeys, with good efficacy shown:[44] however, as with any other protein vaccine, production will probably be expensive. A group based in Korea has accordingly explored a variety of options of producing DIII-derived vaccines in plants, including fusion of a DIII with cholera toxin B subunit (CTB) and production in transgenic tobacco;[45] fusing a consensus DIII, which elicits neutralising antibodies against all 4 dengue virus serotypes, to M cell-targeting peptide ligand (Co1), producing it in transgenic rice calli and showing it was targeted to the mucosal immune system in mice;[46] and modifying the recombinant immune complexes (RIC) shown to work for Zaire ebolavirus GP1 by fusing the Ebola GP1 epitope binding the 6D8 mAb to the dengue virus consensus DIII domain, and then to the mAb, and producing it transiently in tobacco.[47] The purified hybrid dengue-Ebola RIC (DERIC) bound C1q, and elicited potent virus-neutralising Abs in mice without the use of adjuvants. Another group used transplastomic tobacco to produce the 4 serotype DIII proteins to moderate yield, without testing their immunogenicity.[48] This work shows that it is possible to produce candidate dengue subunit vaccines in plants that are at least the equivalent of conventionally-produced candidates.

A potentially therapeutic product for treatment of dengue infections is the mAb E60, which neutralizes all 4 serotypes of the virus:[49] a problem with production in mammalian cells, however, is that the mAb exhibits ADE, meaning treated animals are more susceptible to severe disease if infected with another serotype. Production of the mAb by agroinfiltration-mediated transient expression in *N benthamiana*, however, abrogated ADE activity without affecting antigen binding and neutralisation efficiency against DENV serotypes 2 and 4, presumably due to the different N-glycosylation pattern compared with the conventional mAb. This property could be exploited for other therapeutic MAbs, where modulation or reduction of Fc-mediated functions may be advantageous.

The live attenuated 17D yellow fever virus (YFV) vaccine has been both efficacious and regarded as safe since the 1930s, and is credited with saving possibly millions of lives. However, it is produced in eggs - meaning individuals with egg allergies may not safely receive the vaccine. There are other rare but serious risks for some, such as neurologic effects and organ failure. It is recommended that children under 6 months, the elderly and the immunocompromised, and pregnant or breastfeeding women should not receive the vaccine.[50] There are also concerns that, as with immunity to dengue, reactions to Zika virus infection could be exacerbated by prior immunity to YFV. While the existence of a live, monotypic, lifelong and highly successful vaccine has probably dampened most researchers' enthusiasm for researching a subunit alternative, it is interesting that the molecular farming contract manufacturer iBio Inc. has a collaboration with Fiocruz/Bio-Manguinhos of Brazil, to use their proprietary technology to manufacture a plant-made YFV vaccine.[51]

Conclusion

The technology we know as molecular farming has truly come of age recently: there are many products with proofs of efficacy in animal models, especially for veterinary use (for review of virus vaccines, see ref.[9]); however, there are proofs of principle for both animal and human products, and licensing for use in humans of therapeutic products including ZMapp™ and Elelyso, an enzyme replacement therapy for the glucocerebrosidase deficit that causes Gaucher's disease in humans.

I feel that it is in the sphere of interests of One Health, though, that molecular farming could truly make an immediate

impact: the application of plant-made proteins as inexpensive reagents could revolutionise point-of-care diagnostics, for example; use of farmed vaccines that could be the same proteins used in diagnostics could also make universal vaccination of livestock against certain diseases a reality. Where there are vaccines for animals but these are not safe for humans, such as in the case of RVFV and CCHFV, plant-made subunit vaccines may safely bridge the human-animal divide. Plant-made influenzavirus A vaccines, whether type-specific or universal, could soon be a reality – and may make vaccination of swine and poultry a much easier and cheaper and safer prospect. Rabies vaccines and especially therapeutics would be a most worthy target, given that most animal and human victims of the disease are in developing countries. It is also possible that molecular farming could allow more routine application of therapeutics in veterinary medicine, given the low cost of goods and the potential for oral dosing.

This brief review has hopefully illustrated both the existing arsenal of plant-made vaccines and reagents suitable for use in a One Health context, and also the potential of plants and plant cell systems to be used for other pathogens for these purposes.

Disclosure of potential conflicts of interest

In accordance with Taylor & Francis policy and my ethical obligation as a researcher, I am reporting that I am an inventor on numerous patents describing plant manufacture of subunit vaccines and other proteins, including H5N1 influenza virus and human papillomaviruses. The Biopharming Research Unit is presently funded for human papillomavirus vaccine development by Medicago Inc. of Canada, and receives patent licensing fees from Medicago.

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