Research review paper

The case for plant-made veterinary immunotherapeutics☆

Edward Topp a, Rebecca Irwin b, Tim McAllister c, Martin Lessard d, Jussi J. Joensuu e, Igor Kolotilin f, Udo Conrad g, Eva Stöger b, Tsafir Mor i, Heribert Warzeka j, J. Chris Hall k, Michael D. McLean l, Eric Cox l, Bert Devriendt l, Andrew Potter m, Ann Depicker n, Vikram Virdi o, Larry Holbrook o, Ketan Doshi o, Marike Dussault p, Robert Friendship p, Oksana Yarosh r, Han Sang Yoo s, Jacqueline MacDonald s, Rima Menassa h, i, j, k

a Agriculture and Agri-Food Canada, London Research and Development Centre, 1391 Sandford St, London, ON N5V 4T3, Canada
b Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, 160 Research Lane, Guelph, ON N1G 5Z2, Canada
c Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, 5403 - 1 Avenue South, Lethbridge, AB T1J 4B1, Canada
d Agriculture and Agri-Food Canada, Sherbrooke Research and Development Centre, 2000 College Street, Sherbrooke, QC J1M 0C8, Canada

Vaccines and Antibodies to Protect Farm Animals From Diseases that Have Thus Far Been Managed with Antibiotics; VLPs, Virus-like particles

Key words: Veterinary vaccine; Immunotherapeutic; Antibody; Recombinant protein; Plant biotechnology; Molecular farming; Livestock production; Antibiotic resistance

The excessive use of antibiotics in food animal production has contributed to resistance in pathogenic bacteria, thereby triggering regulations and consumer demands to limit their use. Alternatives for disease control are therefore required that are cost-effective and compatible with intensive production. While vaccines are widely used and effective, they are available against a minority of animal diseases, and development of novel vaccines and other immunotherapeutics is therefore needed. Production of such proteins recombinantly in plants can provide products that are effective and safe, can be orally administered with minimal processing, and are easily scalable with a relatively low capital investment. The present report thus advocates the use of plants for producing vaccines and antibodies to protect farm animals from diseases that have thus far been managed with antibiotics; and highlights recent advances in product efficacy, competitiveness, and regulatory approval.

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Abbreviations: CT, cholera toxin; CTB, subunit of CT; ELP, elastin-like polypeptide; ETEC, enterotoxigenic Escherichia coli; IgG, immunoglobulin G; IgA, immunoglobulin A; sIgA, secretory immunoglobulin A; LT, thermolabile enterotoxin; PRRSV, porcine respiratory and reproductive syndrome virus; PWD, postweaning diarrhea disease; VHH, single variable domain on a heavy chain; VLPs, Virus-like particles.

☆ The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

Corresponding author at: Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario N5V 4T3, Canada.
E-mail address: Rima.Menassa@agr.gc.ca (R. Menassa).

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1. The looming challenges for food animal production

The development of resistance to virtually every clinically-important antibiotic currently available for the treatment of bacterial infection is an important global health challenge. In the worst case, the end of the “antibiotic era” would greatly increase human mortality, morbidity and health care costs. The primary driver for antibiotic resistance is thought to be the improper or excessive use of antibiotics in human medicine and in food animal production. Recently, the World Health Organization, the UK government and the G8 governments have emphasized the need for judicious use of antibiotics in agriculture as a key element of strategies to prevent or delay the onset of antibiotic resistance (G8 Science Ministers Statement, 2013; UK Department of Health, 2013; World Health Organization, 2012). These initiatives, coupled with a growing public demand for animal-based food which is “produced without antibiotics”, will undoubtedly constrain the availability and routine practice of using antibiotics for growth promotion and prophylaxis in livestock, poultry and fish production. Within this context, it is imperative to devise cost-effective strategies for the intensive production of livestock and fish using fewer antibiotics. Increased use of vaccines and immunotherapeutic agents will be a cornerstone of these strategies.

2. The need for efficacious vaccines and immunotherapeutic agents

Animal diseases have both direct costs — the immediate impact on livestock populations and agriculture — and indirect costs, such as mitigation or control efforts, losses in trade and other revenues, and impacts on human health. Zoonotic diseases are estimated to cause 75% of new emerging human infections, thus leading to significant morbidity and mortality, and creating costs in labor markets due to reduced trade and control measures. Diseases without zoonotic potential also impact human welfare costs through instability and increases in the cost of food. For example, the most recent estimate made in 2007 by the World Organization for Animal Health (OIE) of the direct impact of avian influenza is $43 billion annually, while indirect costs are expected to be around $1.5 trillion ([The World Organisation for Animal Health, 2007], and tables 24–25 therein).

A variety of interventions can be used to combat bacterial (and viral) disease in animals, each with its own advantages and disadvantages (Table 1). Of the available alternatives to antibiotics, vaccination is likely the most widely used and effective strategy. Vaccination against viruses can also contribute to lower therapeutic use of antibiotics by reducing the incidence of secondary infections (Glass-Kaastra et al., 2013). Yet, vaccines and immunotherapeutics are available for only a limited number of animal diseases; and while global sales of animal health products in 2013 were $23 billion, only $5 billion corresponded to veterinary vaccines (Dolcera, 2014; Health for Animals, 2014).

Among the important veterinary diseases where current vaccines are not effective is porcine reproductive and respiratory syndrome virus (PRRSV), one of the most economically significant swine diseases in the world. A serious consequence of PRRSV infection is the loss of alveolar macrophages and therefore the weakening of the respiratory tract defense system, allowing secondary bacterial superinfections. Bacterial pathogens such as Mycoplasma hyopneumoniae cause more severe disease when PRRSV is present, and for this reason PRRSV outbreaks are often treated with antibiotics (Glass-Kaastra et al., 2013). Therefore, development of effective vaccines against viruses can lead to a reduction in antibiotic use in livestock. Furthermore, vaccination or the use of targeted immunotherapeutic antibodies can contribute to the maintenance of animal health, and offer promise as a pre-slaughter treatment to reduce meat contamination with zoonotic pathogens.

To be competitive, veterinary vaccines need to have a number of desirable attributes, many of which are met using plant-based production (Table 2). Many candidate subunit vaccines have been produced in plants and tested in target animals with positive outcomes (Kolotilin et al., 2014). Table 3 lists platforms that have been used for veterinary subunit vaccine production and examples of successful trials. Key aspects of the advantages of plant-based versus other platforms are discussed in the following paragraphs.

3. Attributes of plant-made pharmaceutical proteins

Compared to other platforms, plant-based production of recombinant proteins offers enhanced safety, reduced capital investment in infrastructure, and easy scale-up (Floss et al., 2007; Stoger et al., 2014). In terms of safety, plants have the evolutionary advantage of not being host to any prions, viruses, bacteria, or mycoplasmas that are infective to animals or humans. Progress towards high yields and product quality has also been achieved through advances in fundamental knowledge of heterologous gene expression and development of robust expression methods such as the use of transient expression through agro-infiltration of binary or viral vectors (Salazar-Gonzalez et al., 2015; Vézina et al., 2009), chloroplast transformation (Jin and Danielli, 2015), subcellular targeting and the use of suppressors of post-transcriptional gene silencing (Alvarez et al., 2008; Alvarez et al., 2010). Plants also provide eukaryotic-type processing and post-translational modifications, and modified expression systems are being developed that provide functionally-improved therapeutic proteins especially in terms of N-glycosylation (Steinkellner and Castilho, 2015).

Numerous bacterial and viral antigens have been expressed in plants and tested with positive results in the target animal species (Table 3). Similar approaches have been employed for prototype vaccines for use in humans including influenza, hepatitis B, Norwalk virus, rotavirus, human papillomavirus, hepatitis C and others (Gomez et al., 2009; Hernandez et al., 2014; Landry et al., 2010; Thanavala et al., 2005; Yusibov et al., 2011). However, for human vaccines an absolute requirement is high product purity, which remains challenging with plant-based products, making veterinary vaccine production in plants more attractive (see Section 6).

While there are no studies comparing process economics in various production systems, the cost of unpurified therapeutic protein
production is expected to be lowest in plants. While field-production of transgenic plants would be the most cost-effective method of production, regulations as well as public perception about risks associated with contamination of the environment and the food chain with genetically modified organisms will likely limit production to greenhouses. Greenhouse production, although more costly than field production, allows for year-round yields and better-controlled growth environments with higher biomass productivity and more reproducible accumulation of recombinant proteins. These characteristics should allow greenhouse production to remain competitive over non-plant systems for the production of unpurified therapeutic proteins. As well, it is generally accepted that when lyophilized leaves or dried seeds can be directly orally administered, the cost of the final plant-made product would be lower than in alternative production systems (Xiao et al., 2015). Nevertheless, in cases where parenteral administration is required, the minimization of extraction, recovery and purification costs is critical for plant-made proteins to be economically competitive with other production systems (Wilken and Nikolov, 2012). To this end, protein fusion tags such as Zeta, ELP and hydrophobins could be particularly useful for the production of low-cost veterinary products, as they enhance accumulation while avoiding the need for costly affinity chromatography (Conley et al., 2011). Similarly, producing proteins in the chloroplasts has the potential to boost production levels and allow the product to be orally administered as capsules containing lyophilized plant tissue (Bock, 2014; Sherman et al., 2014).

tMP1: *Eimeria tenella* immune-mapped protein 1; CD40L: chicken CD40 ligand; SF9: *Spodoptera frugipera* cells; VLP: Virus-like particle; BTV-2, 4, 8: Bluetongue virus serotypes 2, 4 or 8; APCH: antigen-presenting cell homing molecule; CHO: Chinese hamster ovary cells; tE2: truncated E2 glycoprotein of bovine viral diarrhea virus; HEK293: human embryonic kidney 293 cell line; TGEV: transmissible gastroenteritis virus; scFv: single chain variable fragment antibody; VIHH-IgA: llama heavy chain-only antibodies fused to the fixed component of porcine IgA; NT-1: *Nicotiana tabacum*-1 cell line; GP85: main viral envelope protein of avian leucosis virus-J; EspA, EspB: *E. coli* secreted proteins A, B; E2: viral envelope glycoprotein of classical swine fever virus; GP90: 90 kDa envelope protein of reticuloendotheliosis virus; Bm86: antigen of the cattle tick; VP1, 2: viral proteins 1, 2 from several viruses; NS1, NS2 non-structural proteins 1 and 2 of bluetongue virus; P12A3C: capsid precursor P12A and protease 3C of foot and mouth disease; FedA: major adhesin of *E. coli* F18 fimbriae; VT2Eβ: B subunit of *E. coli* verocytotoxin; gd: glycoprotein D of Bovine herpes virus; GP5, M: glycoprotein S and membrane protein, respectively, of porcine reproductive and respiratory syndrome virus; and HA: Hemagglutinin of several viruses; FaeG: major adhesin of *E. coli* F4 fimbriae; IFN: interferon.

4. Induction of protective immunity through oral delivery of plant-made antigens

Most pathogens invade the host at mucosal surfaces, such as the intestinal epithelium and the respiratory tract. Protection against these pathogens requires primarily the induction of pathogen-specific secretory immunoglobulin A (SlgA) at the infection site (Snoeck et al., 2006). A topical/mucosal vaccination is necessary to elicit robust SlgA immune responses (Holmgren and Czerkinsky, 2005). As such, orally administered vaccines derived from plants have several advantages. One of them is that, depending on the plant species and the plant tissue in which the subunit vaccines are expressed, the plant matrix provides some degree of protection from the hostile environment of the gastrointestinal tract (Kwon et al., 2013a, 2013b; Rosales-Mendoza and Salazar-Gonzalez, 2014). For example, upon oral delivery, recombinant proteins expressed in rice or pea seeds were better protected from degradation than their purified counterparts (Nochi et al., 2007; Zimmermann et al., 2009). This protective effect can be further enhanced by incorporating the recombinant proteins into storage organelles such as oil bodies, protein storage vacuoles and artificial protein bodies which may also act as adjuvants (Bhatia et al., 2010; Conley et al., 2011; Khan et al., 2012; Torrent et al., 2009; Wakasa et al., 2013; Whitehead et al., 2014).

Besides protection from degradation, plant-derived vaccine antigens should cross the epithelial barrier in order to activate the intestinal immune tissues and bypass the default tolerogenic responses (Devriendt et al., 2012). With some exceptions such as Vibrio cholerae toxin (CT), ETEC-derived heat-labile enterotoxin (LT) and porcine-specific ETEC colonization factor (F4 fimbriae) (Elson and Ealding, 1984; Lycke et al., 1985; Takahashi et al., 1996; Van den Broeck et al., 1999; Kolotilin et al., 2012), most proteins are poor immunogens upon oral delivery. However, soluble antigens can be conjugated or fused with mucosal adjuvants such as *E. coli* heat labile enterotoxin B LTB or cholera toxin B (CTB) subunits, and have been demonstrated to yield an enhanced immune response (Baldauf et al., 2015; Wagner et al., 2004; Soria-Guerra et al., 2011). Fusing vaccine antigens to antibodies or antibody fragments can further target the subunit vaccine to antigen sampling routes at the mucosal surfaces, such as transcytotic epithelial receptors, thereby drastically improving oral vaccine efficacy (Joensuu et al., 2006; Van Molle et al., 2007). However, care must be taken with protein fusions not to negatively influence the immunogenicity of the antigen by affecting antigen folding, glycosylation and/or by interacting with the antigen and interfering with its capacity to target the mucosa (Joensuu et al., 2006; Van Molle et al., 2007).

As an alternative to fusions with adjuvants or antibodies, the ability of plants to produce correctly folded, functional particulate antigens should result in efficient uptake by the gut-associated lymphoid tissue

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**Table 1** Technologies for the control of bacterial pathogens in food animal production. Market penetration for biological products and availability to farmers can be constrained by a number of factors including regulatory approval and cost.

<table>
<thead>
<tr>
<th>Intervention method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Phage</td>
<td>Highly target specific, potentially highly effective</td>
<td>Potential resistance development, cost, shelf life, delivery method</td>
</tr>
<tr>
<td>Probiotics and prebiotics</td>
<td>Stimulate host immune response, change host microbiome to disadvantage pathogen establishment</td>
<td>Effectiveness variable, probiotics need to be viable, complexity of effects complicate identification of effective agents</td>
</tr>
<tr>
<td>Nutritional supplements, e.g. plant bioactives</td>
<td>Low cost, ease of administration</td>
<td>Variable efficacy, broad spectrum effects, potential health side effects</td>
</tr>
<tr>
<td>Antimicrobial peptides</td>
<td>Broad activity spectrum, Various effective mechanisms of action</td>
<td>Costly production, low specificity, potential toxicity to animal cells</td>
</tr>
<tr>
<td>Breeding</td>
<td>Selecting for broadly immune-competent animals, genomics tools will accelerate</td>
<td>Cost, time, limitation of germlapse, multifactorial basis for immune robustness</td>
</tr>
<tr>
<td>Antibodies</td>
<td>Potentially highly effective for specific pathogens, prophylactic or therapeutic use</td>
<td>Shelf life limitations, stability following administration, target specificity, cost and delivery method</td>
</tr>
<tr>
<td>Vaccines</td>
<td>Potentially highly effective, no side effects</td>
<td>Immune system must be mature, prophylactic use only, efficacy can be challenging, delivery method</td>
</tr>
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</table>
In addition to vaccination, passive immunization can contribute to lower reliance on antibiotics. This approach involves the application of infection-specific antibodies, conferring immediate but temporary protection (Virdi and Depicker, 2013). The potential of passive immunization is illustrated by its recent use during the West African Ebola virus outbreak with the ZMapp antibody cocktail produced in *N. benthamiana* plants. This cocktail was found to reverse disease in 100% of rhesus macaques in advanced stages of the disease (Qiu et al., 2014). Even though this antibody cocktail was developed for human treatment, it serves as an example for the capability of plant-produced immunotherapeutics. Such immediate protection is often needed in animal health care, for instance, in the case of post-weaning diarrhea (PWD) in piglets. PWD is an economically important multifactorial disease where infection with *ETEC* leads to diarrhea, weight loss, and potentially death (Fairbrother et al., 2005). Vaccines administered to suckling piglets before weaning run the risk of being neutralized by immunoglobulins in milk, whereas vaccines administered at weaning do not elicit immediate protection (Melenkebek et al., 2013). Several studies show that in the transition period immediately after weaning, administration of anti-ETEC antibodies in feed provides piglets with protection against *ETEC* and prevents PWD. These studies used anti-ETEC antibodies obtained from immunized animal plasma (Niewold et al., 2007), immunized hen egg powder (Yokoyama et al., 1992) or transgenic plants (Virdi et al., 2013). While polyclonal animal serum antibodies, and polyclonal egg-produced IgY antibodies have the inherent demerit of batch-to-batch variation of antibody composition, plants can produce specific monoclonal antibodies where features such as binding efficiency, glycosylation, and components can be specifically engineered to suit the therapeutic need. For instance, nanobodies derived from the antigen binding domain of heavy chain-only camelid antibodies (VHH) are known to resist harsh environmental conditions. These can be grafted to animal-specific Fc fragments to design customized antibodies intended to be functional in presumably harsh environments like the gastrointestinal tract. Thus far, a variety of engineered antibodies and antibody fragments against veterinary pathogens have been expressed in plant leaves or seeds, including ScFv (single chain variable fragments) (Zimmermann et al., 2009), VHH-IgG, VHH-IgA (Virdi et al., 2013) and secretory-IgA (SigA) (Virdi et al., 2013; Wieland et al., 2006).

(GALT) following oral administration, although there will likely be differences in the efficiency of this process among treated animal species. For example, immunogenicity can be improved if antigens are presented as part of virions or virus-like particles (VLPs), and intriguingly, viral coat proteins produced in plants can self-assemble into highly immunogenic VLPs (Bock and Warzecha, 2010; Landry et al., 2010; Scotti and Rybicki, 2013). The production of virus-like particles in plants has been successfully achieved for human vaccine candidates and the company Medicago (Medicago Inc., 2015) has taken products from its transient plant expression technology into human clinical trials for pandemic H5N1 and quadrivalent seasonal influenza (Landry et al., 2010; Le Mauff et al., 2015). Furthermore, a recent study investigated oral immunogenicity in mice of lyophylized lettuce leaves containing hepatitis B surface antigen VLPs, and found that a booster dose of lyophylized lettuce leaves containing 50 ng S-HBsAg VLP administered orally produced an equivalent immune response as a commercial Hepatitis B vaccine administered intramuscularly (Czyz et al., 2014). VLP-based veterinary vaccines are also being developed to battle among others avian flu, bluetongue disease, PRRSV, and Newcastle disease (McGinnes et al., 2010; Shen et al., 2013; Thuenemann et al., 2013; Uribe-Campero et al., 2015). The VLP platform has some additional advantages as it offers the opportunity to fuse heterologous genes to the viral coat subunits resulting in highly immunogenic chimeric VLPs (Kim et al., 2013; Shen et al., 2013; Zhai et al., 2013). Even though these studies did not target oral delivery of VLPs, they demonstrate that immunogenicity of vaccine antigens could be increased via multimerization of the subunits, an outcome that appears to be especially true for oral immunogens.

## 5. Passive immunization for animal health

In addition to vaccination, passive immunization can contribute to lower reliance on antibiotics. This approach involves the application of infection-specific antibodies, conferring immediate but temporary protection (Virdi and Depicker, 2013). The potential of passive immunization is illustrated by its recent use during the West African Ebola virus outbreak with the ZMapp antibody cocktail produced in *N. benthamiana* plants. This cocktail was found to reverse disease in 100% of rhesus macaques in advanced stages of the disease (Qiu et al., 2014). Even though this antibody cocktail was developed for human treatment, it serves as an example for the capability of plant-produced immunotherapeutics. Such immediate protection is often needed in animal health care, for instance, in the case of post-weaning diarrhea (PWD) in piglets. PWD is an economically important multifactorial disease where infection with *ETEC* leads to diarrhea, weight loss, and potentially death (Fairbrother et al., 2005). Vaccines administered to suckling piglets before weaning run the risk of being neutralized by immunoglobulins in milk, whereas vaccines administered at weaning do not elicit immediate protection (Melenkebek et al., 2013). Several studies show that in the transition period immediately after weaning, administration of anti-ETEC antibodies in feed provides piglets with protection against *ETEC* and prevents PWD. These studies used anti-ETEC antibodies obtained from immunized animal plasma (Niewold et al., 2007), immunized hen egg powder (Yokoyama et al., 1992) or transgenic plants (Virdi et al., 2013). While polyclonal animal serum antibodies, and polyclonal egg-produced IgY antibodies have the inherent demerit of batch-to-batch variation of antibody composition, plants can produce specific monoclonal antibodies where features such as binding efficiency, glycosylation, and components can be specifically engineered to suit the therapeutic need. For instance, nanobodies derived from the antigen binding domain of heavy chain-only camelid antibodies (VHH) are known to resist harsh environmental conditions. These can be grafted to animal-specific Fc fragments to design customized antibodies intended to be functional in presumably harsh environments like the gastrointestinal tract. Thus far, a variety of engineered antibodies and antibody fragments against veterinary pathogens have been expressed in plant leaves or seeds, including ScFv (single chain variable fragments) (Zimmermann et al., 2009), VHH-IgG, VHH-IgA (Virdi et al., 2013) and secretory-IgA (SigA) (Virdi et al., 2013; Wieland et al., 2006).
In contrast to monoclonal antibody production in other systems, the ability of plants to produce diverse N-glycoforms is a defining characteristic. The plant N-glycosylation pathway has been successfully engineered to remove natural plant-specific \(\alpha(1,2)\)-linked xylose and core \(\alpha(1,3)\)-linked fucose residues and to introduce complete mammalian glycosylation pathways (Bosch et al., 2013; Castilho et al., 2010). In addition, specific N-glycans have been designed to produce glyco-optimized antibodies with greater receptor affinity, and enhanced pharmacokinetic properties (Gadska et al., 2012).

As well as N-glycosylation, plants are able to perform O-glycosylation of serine, threonine and hydroxyproline residues. While the hydroxyproline modification is plant-specific, this pathway has been engineered in plants to produce mammalian mucin-type O-glycans (Strasser, 2013; Yang et al., 2012). Research suggests

<table>
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<tr>
<th>Platform</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Examples of successful subunit vaccine trials in host species</th>
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<tbody>
<tr>
<td>Bacteria</td>
<td>Well characterized, inexpensive, high yield, ease of genetic modification, systems well established</td>
<td>Limitations with protein size, folding, glycosylation and secretion.</td>
<td>E. coli-made Coli-Mallein chimeric toxoid for Coxiella burnetii in cattle (Cunha et al., 2014) E. coli-made C-terminal EIMPI (Yin et al., 2014) and EIMPI-CD40L chimeric protein for coccidiosis in chicken (Yin et al., 2015) E. coli-made VP2 for infectious bursal disease in chicken (Pradhan et al., 2012) E. coli-made GP85 for avian leucosis virus-J in chicken (Dou et al., 2013) E. coli-made EspA, EspB and intimin for E. coli O157:H7 in sheep (Yekta et al., 2011) Pichia pastoris-made E2 for classical swine fever in pigs (Lin et al., 2012) P. pastoris-made GP90 for reticuloendotheliosis in chicken (Li et al., 2012) P. pastoris-made Bm86 for cattle tick in bovines (Vargas et al., 2010) Sf9-made chimeric VLPs for rabies in dogs (Qi et al., 2015) Sf9-made VP2 (BTV-8), and NS1 (BTV-2) and E. coli-made NS2 (BTV-2) for bluetongue in ruminants (Anderson et al., 2013, 2014) Sf9-made VP-2 and APCH-VP2 (BTV-4) for bluetongue in ruminants (Legisa et al., 2015) CHO cell-made E2 for bovine viral diarrhea in cattle (Pecora et al., 2012) HEK293 cell transient production of P12A3C for foot and mouth disease of cattle (tested in mice) (Mignaqui et al., 2013) Alfalfa-made APCH-E2 for bovine viral diarrhea in cattle (Aguirrebrusallade et al., 2013) N. benthamiana-made Bluetongue (BTV-8) VLPs for ruminants (Thuenemann et al., 2013) Potato-made Spike protein for infectious bronchitis virus in chicken (Zhou et al., 2004) Arabidopsis-made VP2 for infectious bursal disease in chicken (Wu et al., 2004) Oral corn-made Spike protein for TGEV in swine (Lamphear et al., 2004) Chenopodium-made VP1 for foot and mouth disease in swine (Yang et al., 2007) Nicotiana-mades V protein for Bovine herpes virus in cattle (Perez Filgueira et al., 2003) Peanut-made HA for rinderpest virus of cattle (Khandelwal et al., 2003) Alfalfa-made Faeg for post weaning diarrhea in piglets (Joensuu et al., 2006) Strawberry-made IFN-alpha for gingivitis in dogs approved by PASC in Japan (Stoger et al., 2014) Pea seed-made scfV antibody for coccidiosis in chickens (Zimmermann et al., 2009) Arabidopsis seed-made VH3-IgA antibodies for post weaning diarrhea in piglets (Virdi et al., 2013) Tobacco seed-made FedA and VT2e for verocytotoxic E. coli in piglets (Rossi et al., 2014) Banana leaf-made GP3 for PRRSV in pigs (Chan et al., 2013) Corn seed M protein for PRRSV in pigs (tested in mice) (Hu et al., 2012) HA-NA for Newcastle disease in chicken approved by USDA (Vermij, 2006) NT-1 cell-made LTA-K63/LTB as vaccine adjuvants in chickens (Miller et al., 2012)</td>
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<td>Yeast</td>
<td>Can fold and glycosylate proteins, genetic modification systems well established</td>
<td>Natural yeast glycosylation pattern distinct from mammalian glycosylation affecting protein stability and function</td>
<td>Pichia pastoris-made Bm86 for cattle tick in bovines (Vargas et al., 2010) Sf9-made chimeric VLPs for rabies in dogs (Qi et al., 2015) Sf9-made VP2 (BTV-8), and NS1 (BTV-2) and E. coli-made NS2 (BTV-2) for bluetongue in ruminants (Anderson et al., 2013, 2014) Sf9-made VP-2 and APCH-VP2 (BTV-4) for bluetongue in ruminants (Legisa et al., 2015) CHO cell-made E2 for bovine viral diarrhea in cattle (Pecora et al., 2012) HEK293 cell transient production of P12A3C for foot and mouth disease of cattle (tested in mice) (Mignaqui et al., 2013) Alfalfa-made APCH-E2 for bovine viral diarrhea in cattle (Aguirrebrusallade et al., 2013) N. benthamiana-made Bluetongue (BTV-8) VLPs for ruminants (Thuenemann et al., 2013) Potato-made Spike protein for infectious bronchitis virus in chicken (Zhou et al., 2004) Arabidopsis-made VP2 for infectious bursal disease in chicken (Wu et al., 2004) Oral corn-made Spike protein for TGEV in swine (Lamphear et al., 2004) Chenopodium-made VP1 for foot and mouth disease in swine (Yang et al., 2007) Nicotiana-made gD protein for Bovine herpes virus in cattle (Perez Filgueira et al., 2003) Peanut-made HA for rinderpest virus of cattle (Khandelwal et al., 2003) Alfalfa-made Faeg for post weaning diarrhea in piglets (Joensuu et al., 2006) Strawberry-made IFN-alpha for gingivitis in dogs approved by PASC in Japan (Stoger et al., 2014) Pea seed-made scfV antibody for coccidiosis in chickens (Zimmermann et al., 2009) Arabidopsis seed-made VH3-IgA antibodies for post weaning diarrhea in piglets (Virdi et al., 2013) Tobacco seed-made FedA and VT2e for verocytotoxic E. coli in piglets (Rossi et al., 2014) banana leaf-made GP3 for PRRSV in pigs (Chan et al., 2013) corn seed M protein for PRRSV in pigs (tested in mice) (Hu et al., 2012) HA-NA for Newcastle disease in chicken approved by USDA (Vermij, 2006) NT-1 cell-made LTA-K63/LTB as vaccine adjuvants in chickens (Miller et al., 2012)</td>
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<td>Mammalian cells</td>
<td>Accurate recombinant protein folding, assembly, post-translational modification, excellent secretion</td>
<td>Risk of contamination with human/animal pathogens, high processing and scale-up costs.</td>
<td>Pichia pastoris-made Bm86 for cattle tick in bovines (Vargas et al., 2010) Sf9-made chimeric VLPs for rabies in dogs (Qi et al., 2015) Sf9-made VP2 (BTV-8), and NS1 (BTV-2) and E. coli-made NS2 (BTV-2) for bluetongue in ruminants (Anderson et al., 2013, 2014) Sf9-made VP-2 and APCH-VP2 (BTV-4) for bluetongue in ruminants (Legisa et al., 2015) CHO cell-made E2 for bovine viral diarrhea in cattle (Pecora et al., 2012) HEK293 cell transient production of P12A3C for foot and mouth disease of cattle (tested in mice) (Mignaqui et al., 2013) Alfalfa-made APCH-E2 for bovine viral diarrhea in cattle (Aguirrebrusallade et al., 2013) N. benthamiana-made Bluetongue (BTV-8) VLPs for ruminants (Thuenemann et al., 2013) Potato-made Spike protein for infectious bronchitis virus in chicken (Zhou et al., 2004) Arabidopsis-made VP2 for infectious bursal disease in chicken (Wu et al., 2004) Oral corn-made Spike protein for TGEV in swine (Lamphear et al., 2004) Chenopodium-made VP1 for foot and mouth disease in swine (Yang et al., 2007) Nicotiana-made G protein for Bovine herpes virus in cattle (Perez Filgueira et al., 2003) Peanut-made HA for rinderpest virus of cattle (Khandelwal et al., 2003) Alfalfa-made Faeg for post weaning diarrhea in piglets (Joensuu et al., 2006) Strawberry-made IFN-alpha for gingivitis in dogs approved by PASC in Japan (Stoger et al., 2014) Pea seed-made scfV antibody for coccidiosis in chickens (Zimmermann et al., 2009) Arabidopsis seed-made VH3-IgA antibodies for post weaning diarrhea in piglets (Virdi et al., 2013) Tobacco seed-made FedA and VT2e for verocytotoxic E. coli in piglets (Rossi et al., 2014) banana leaf-made GP3 for PRRSV in pigs (Chan et al., 2013) corn seed M protein for PRRSV in pigs (tested in mice) (Hu et al., 2012) HA-NA for Newcastle disease in chicken approved by USDA (Vermij, 2006) NT-1 cell-made LTA-K63/LTB as vaccine adjuvants in chickens (Miller et al., 2012)</td>
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that O-glycan structures may have adjuvant properties, or may increase serum stability (Gomord et al., 2010). Mammalian-like O-glycosylation could also improve the stability or function of proteins such as erythropoietin or secretory IgA (Castillo et al., 2012; Deshpande et al., 2010).

6. Regulatory requirements and commercialization

Plant-made veterinary therapeutics have already made their way through regulatory approval, facilitating the regulatory process for further products. A poultry vaccine against Newcastle disease purified from cultured tobacco cells was the first plant-derived vaccine to receive approval from the USDA; while in 2013 the Pharmaceutical Affairs and Sanitation Council in Japan awarded manufacturing and marketing approval for interferon alpha produced in strawberries for treatment of gingivitis in dogs (Stoger et al., 2014).

In Canada and the USA, vaccines and antibodies are classified as veterinary biologics, as opposed to veterinary drugs. As such, they are regulated differently than veterinary drugs. While Good Manufacturing Practices (GMPs) form the basis of regulatory requirements for human vaccines and veterinary drugs (US 21 CFR and Canadian Food and Drugs Regulations), and are required for veterinary vaccines in Europe, neither Canada (Canadian Food Inspection Agency [CFIA] – Health of Animals Regulations) nor the USA (USDA – Title 9 of the U. S. Code of Federal Regulations (9 CFR)) has such stringent requirements for veterinary biologics, and consequently, the costs of production are lower for veterinary vaccines than for human vaccines or other veterinary drugs. GMPs are being developed for human-targeted plant-made pharmaceuticals, resulting in the recent FDA approval of taliglucerase alfa (ElelysoTM), the first plant-made protein drug for the treatment of Gaucher disease in humans (Fischer et al., 2012; Mori, 2015). This accomplishment reinforces the suitability of plant-based production platforms for commercial applications and has encouraged clinical development for additional plant-derived pharmaceutical proteins by several companies (Caliber Biotherapeutics, 2015; Fraunhofer USA, 2015; Kentucky Bioprocessing LLC, 2015; Mapp Biopharmaceutical, 2015; Medicago Inc., 2015; Protalix Biotherapeutics, 2015).

Regulatory approval for veterinary vaccines is facilitated in many jurisdictions, because a veterinary injectable vaccine is not required to be as pure as a human vaccine. The CFIA’s definition of purity for a veterinary biologic is the following: “Purity means quality of a biologic prepared to a final form and relatively free of extraneous microorganisms and extraneous material, as determined by established test methods and approved in the production outline” (Canadian Food Inspection Agency, 2013). Therefore, the requirement for purity of injectable vaccines is simply that they must not have any detectable extraneous micro-organisms and the extraneous material (so-called debris such as residual RNA for plasmid DNA vaccines, or endotoxin molecules for vaccines from Gram negative bacteria) must be identifiable and quantifiable. For oral vaccines or oral veterinary biologics (e.g. colostrum, egg antibody product, plant material), the product would have to be negative for coliforms and for Salmonella, and would have to be within a maximum limit for total microorganisms (bacterial colony forming units). Any residual plant material that is in the vaccine would have to be shown to be safe to the animal, with no toxic effect at a ten-fold dose.

Oral vaccines delivered through feed will also face the issue of how to control dose, since not all animals will ingest the same quantity. This could be solved by setting broad minimum and maximum immunizing doses backed up by efficacy data for upper and lower dosages. One potential use of a feed-based system may be to use it as booster rather than as a primary immunization since the issue of dose would be less important. In addition, this would permit multiple immunizations to be given at times when animals are not readily accessible for individual handling, an important benefit to the animal health industry.

Despite the relative ease and favorable cost of regulatory approval for veterinary vaccines, the current industry average for research and development of a novel vaccine is currently 5–7 years (MacDonald et al., 2015). Technologies such as subunit vaccine production in plants that permit faster development are called for, particularly when managing emerging viral epidemics. While small-scale studies have demonstrated the promise of plant-made immunotherapeutics for livestock, they have not yet been adopted or commercialized. For this to occur, a company must be willing and able to invest in a first-to-market prototype, pass regulatory approval, and have the capacity to scale-up production, formulation and distribution to farmers. It is clear that vaccine candidates need to be identified that show a definite advantage for plant production before this technology becomes widely accepted.

7. Conclusions

Issues surrounding the presence of adventitious agents, especially prions, in mammalian cell production systems have been a concern with regulators and the public. The use of plants for the production of veterinary vaccine components for oral or parenteral delivery would circumvent these issues and can offer advantages in terms of safety, cost, and facilitated regulatory approval. While no plant-made veterinary vaccines or antibodies appear in the pipeline of regulators in Canada or the USA, research is actively pursued by several academic and government laboratories that may pave the way for new products in the near future. Strategies to improve yield and purification from plants have achieved significant progress; while advantages for oral delivery, a route that is the practical choice for convenience of animal mass immunization, include protection in the gastro-intestinal tract and the potential for incorporation into highly immunogenic, self-assembling VLPs. These advantages make plants an attractive platform for the production of cost-effective immunotherapeutics, which can contribute to lowering reliance on antibiotics in agriculture.

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