Diseases with Limited Research of Plant-Based Vaccines

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Abstract There are a number of diseases which are important globally in terms of the effect they have on livestock but for which the development of recombinant plant-produced vaccines is preliminary. For many of these diseases such as bovine viral diarrhoea (BVD), bovine rotavirus (BRV), bovine herpes (BoH), transmissible gastroenteritis (TGE) in pigs, infectious bronchitis (IB) in chickens, bluetongue (BT) in sheep, Rift Valley fever (RVF) in sheep and coccidiosis in chickens, commercially available live-attenuated or killed vaccines are available. Although most are effective to varying degrees, there are numerous issues with manufacture and potential reassortment of the vaccine strains. For some diseases such as bovine papillomavirus (BPV) infections and Crimean-Congo haemorrhagic fever (CCHF), there are no commercially available vaccines, and limited studies have been conducted on their development. This chapter discusses some of the research developments in plant-produced vaccine candidates which have potential for further development towards commercialisation.

Keywords Preliminary · BVD · BRV · BoH · TGE · IB · BT
RVF · CCHF · BPV

1 Viral Diseases

1.1 Bovine Viral Diarrhoea (BVD) in Cattle

Bovine viral diarrhoea (BVD) is an infectious disease occurring worldwide. The causative agent Bovine viral diarrhoea virus (BVDV) belongs to the *Pestivirus* genus of the *Flaviviridae* family and affects mainly cattle. It is a spherical enveloped virus with a single-stranded RNA genome encoding at least 4 structural

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proteins: the capsid protein (C), E^{rns} (envelope glycoprotein with RNAse) and two external glycoproteins (E1 and E2) (Choi et al. 2004). It is persistent in cattle, causing a range of immunosuppressive symptoms as well as affecting respiratory functions and fertility (Lanyon et al. 2014). This leads to herd morbidity, and subsequent financial impact on farmers and the livestock economy. A particular variant of BVD causes 'mucosal disease' which can be transmitted in utero to the foetus by an antibody-negative mother. Such antibody-negative animals are referred to as persistently infected (PI). PI animals do not show symptoms of BVD but they shed large amounts of virus and their young most often succumb to the disease after making it desirable to terminate any such animal (Brock et al. 2008). Transmission of BVDV occurs vertically and horizontally by aerosol or through the fecal/oral route. The virus usually enters mucosal tissue lining the mouth or nose and replicates in the epithelial tissue. It can then be spread via the blood stream and enters most other types of tissue cells using this route (Lanyon et al. 2014).

Currently used vaccines comprise modified-live viruses (MLV) and killed virus (KV). MLVs are preferred as they give a broader and longer lasting effect but there are disadvantages to using them such as the induction of post vaccinal mucosal disease and immunosuppression (Ridpath 2013). Immunity induced by recommended vaccination programmes at the herd level is effective, but problems with the heterogeneity of the virus leading to genetic drift and subsequent variation in strains have led to the constant demand for new vaccine strains. This in turn has led researchers to look into the development of recombinant vaccines (Fulton 2008). Recombinant vaccines against BVDV have focussed on E2, the major glycoprotein of the virus. E2 has been shown to be immunogenic and induce neutralising antibodies (Ridpath 2013). Recombinant E2, or modifications of it, provide cattle with up to 100% protection against BVDV when produced in animal cells (Pecora et al. 2012; Thomas et al. 2009).

There has also been some work carried out on the development of recombinant vaccine production in plants. Nelson et al. (2012) cloned a truncated version of the immunogenic E2 protein (tE2) in *N. tabacum*. The proteins accumulated up to 20 μg of tE2 per gram of fresh leaves and serum from guinea pigs immunised subcutaneously with 20 μg doses mixed with adjuvant showed the presence of neutralizing antibodies. In addition, Aguirreburualde et al. (2013) made transgenic alfalfa plants which produced a recombinant fusion protein [tE2 fused to a single chain variable fragment (scFv)] that targets proteins to antigen presenting cells. Protein yields of up to 1 μg/g fresh weight were reported. Immunogenicity evaluation of serum from guinea pigs immunised intramuscularly with leaf extracts containing 0.2–0.4 μg recombinant E2 showed the induction of a strong neutralising antibody response. In addition, cattle immunised intramuscularly with 3 μg doses of the product also showed a neutralising antibody response and subsequent complete protection against infection when challenged with live virus. Interestingly, Gao et al. (2015) have developed transgenic ginseng hairy root cultures which express BVDV glycoprotein $E2^{rns}$. Sika deer were immunised subcutaneously with transgenic hairy root extracts and serum was shown to contain E2-specific antibodies. There was also a specific cell-mediated response induced as measured by an increase in phytohaemagglutinin-induced lymphocyte proliferation.

1.2 Bovine Papillomavirus (BPV) in Cattle

Bovine papillomavirus is caused by a group of double-stranded DNA bovine papillomaviruses (BPV) belonging to the family *Papillomaviridae* of which there are 13 types described to date (Munday 2014). The DNA encodes 3 viral oncoproteins (E5, E6 and E7) as well as the two structural proteins L1 and L2 which form the virion capsid (Chen et al. 1982). Generally, BPVs have been shown to be specific to bovines, but BPV-1 BPV-2 and BTV-13 have been reported to infect equids as well as bovines (Hamad et al. 2016). The virus is endemic worldwide, causing benign or malignant tumours in infected mucosa or squamous epithelium, or in the mesenchyme of embryos. Such symptoms can lead to loss of milk production, infertility, and immunosuppression, causing a decline in general animal health (Love et al. 2012). BPV is spread between cattle by direct or indirect contact and can also be transmitted by insects. Infection is thought to occur with microtrauma and subsequent entry into various tissues including cutaneous and mucosal epithelium with subsequent disruption of the cell life cycle effected by the BPV E5 protein (Munday 2014).

In 1995, Campo et al. (1995) showed that prophylactic vaccination of animals with live BPV, stimulated the production of neutralising antibodies and provided complete protection against live virus challenge. The same group of researchers also showed that subunit vaccines comprising L1 and L2 antigens of BVP-2 provided effective prophylaxis and resulted in production of neutralising antibodies and protection. Another research group describes the vaccination of calves which induced virus neutralising antibodies and their subsequent protection from infection when challenged (Borzacchiello and Roperto 2008). The co-expression of L1 and L2 or L1 alone in mammalian cells results in the assembly of virus-like particles (VLPs) which are immunogenic, stimulating neutralising antibodies and providing protection (Kirnbauer et al. 1992). Despite this, there is no commercially available vaccine against BPV as of yet.

The production of such a recombinant vaccine in plants would be more appealing as it abrogates the possibilities of contamination with other animal derived proteins as high sterility tissue culture conditions are required. Love et al. (2012) have tested the production of BPV VLPs in plants and shown that similar L1 VLPs can be expressed transiently in *N. benthamiana* on infiltration with *Agrobacterium* harbouring BPV-1 cloned into the pEAQ-HT plant expression vector. Yields were reported to be up to 183 mg/kg fresh weight. Rabbits immunised with 150 μg of L1 VLPs mixed with Freund's incomplete adjuvant produced L1-specific IgG.

1.3 Bovine Rotavirus (BRV) Group A Gastroenteritis

Rotavirus A infection of cattle is caused by a double stranded RNA virus belonging to the *Rotavirus* genus, family *Reoviridae* and occurs worldwide. It causes acute

gastroenteritis and diarrhoea, particularly in young animals which are 1–3 weeks old, which progresses to increases in morbidity with severe enteritis and mortality as well as a reduction in growth rate (Dhama et al. 2009). Together with extensive treatment costs, these factors have lead to serious economic losses for livestock owners and affected countries. Although clinical signs do not last long, permanent outbreaks during certain seasons often occur as a result of viral shedding which can last for up to 3 weeks after infection. Virions are made up of an inner core mostly of the VP2 capsid protein, a middle layer comprised of the most abundant protein VP6 and 2 other proteins VP7 and VP4 forming the outer layer. The major capsid protein VP6 is highly immunogenic and contains the common antigens for typing serogroups of which there are 7 (Papp et al. 2013). The virion is very stable in the environment and is transmitted by the faecal-oral route and spread by viral shedding through faeces and water; very low doses are required for infectivity.

Most adult cattle populations are generally BRV positive, and naturally occurring passive immunisation of newborn calves is common by oral ingestion of colostrum and milk (Saif and Fernandez 1996). However, in modern times, intensive farming methods, exposure of animals to high concentrations of virus, early weaning and supplementation of feed with calf concentrates which dilutes milk antibodies has influenced and reduced the extent of immunity of the herd, leading to the requirement for vaccines. There are currently commercially available BRV maternal vaccines on the market which allow for the parenteral immunisation of females, which subsequently facilitate passive immunity in newborn calves. These vaccines comprise of attenuated-live viral strains or inactivated virus which have been shown to stimulate increased antibody production in mammary secretions of vaccinated females, which protects feeding calves from BRV challenge. However, limitations to attenuated-live vaccines such as the presence of adventitious agents and the gradual emergence of different serotypes as well as to inactivated vaccines such as alteration of the immunogenic form, make the generation of recombinant vaccines more favourable. There has also been some development in generating recombinant BRV vaccines in the form of VLPs. In 1991, Labbé et al. (1991) were able to produce rotavirus VLPs consisting of the VP2 and VP6 capsid proteins in a baculovirus expression system.

The production of BRV VLPs in plants seems to have yielded greater success and there are several examples of research into developing recombinant plant-produced BRV vaccines (Hammond and Nemchinov 2009; Ruiz et al. 2015). In 2005 Dong et al. made recombinant VP6 in transgenic alfalfa and orally gavaged mice with 24 μg purified VP6 adjuvanted with CpG (Dong et al. 2005). Serum from treated mice was shown to have anti-VP6 IgG antibodies and anti-VP6 IgA antibodies isolated from homogenised small intestine, faeces and saliva. Interestingly, pups born from the immunised mice and subsequently challenged with live rotavirus showed reduced symptoms of diarrhoea, and reduced intensity and duration of diarrhoea indicating that immunity had been passively transferred to the pups.

In another study recombinant rotavirus capsid protein VP6 was expressed in *Chenopodium amaranticolor* as a result of infection with engineered beet black scorch virus (BBSV) (Zhou et al. 2010). Partially purified doses of plant leaf extract

containing 25 μg VP6 and CpG adjuvant was used to gavage mice and analysis of their mucosal IgA and IgG titres showed high titres when compared to control mice. Pups born from these immunised mice were challenged with rotavirus and showed a significant reduction in incidence, severity and duration of diarrhoea indicating that the plant-produced VP6 provided passive protection against the rotavirus.

In 2006, Saldaña et al. described the production of VP2/VP6 VLPs in transgenic tomato (*Lycopersicon esculentum* L.) fruits albeit fairly low (Saldaña et al. 2006). Serum from mice immunised with lyophilised tomato extract containing 1 μg of rotavirus proteins and Freund's adjuvant was shown to contain antibodies against the proteins but no protection studies were carried out. Yang et al. (2011) co-expressed rotavirus VP2/VP6 or VP2/VP6/VP7 in transgenic tobacco plants. All proteins were expressed in leaves and both combinations of proteins resulted in the assembly of VLPs. Oral delivery of semi-purified preparations of VLPs administered with CT as an adjuvant showed that although both vaccine candidates induced raised titres of rotavirus-specific antibodies (IgA and IgG), those mice immunised with VP2/VP6/VP7 VLPs had higher titres than VP2/VP6 VLPs.

Lentz et al. approached the rotavirus vaccine differently by testing the expression of VP8* (Lentz et al. 2011). VP4 is one of the outermost capsid proteins and on infection this is cleaved to produce VP8*—a N-terminal non-glycosylated sialic acid-recognising domain—and VP5—a C-terminal fragment which remains associated with the virion. VP8* is a major determinant of viral infectivity and one of the neutralising antigens. VP8* was expressed in transplastomic tobacco leaves and shown to form insoluble aggregates. Both soluble and insoluble fractions of crude preparations containing 2 μg of VP8* and Marcol adjuvant were used to immunise mice intraperitoneally. VP8* IgG antibody titres from mice immunised with both samples were shown to be induced. In addition, both immunogens were shown to induce virus neutralising antibodies. They were also shown to passively immunise their offspring as demonstrated by the presence of high levels of IgG antibodies in pups. Eighty to 100% of the challenged pups were protected from rotavirus challenge as determined by the absence of diarrhoea.

Wigdorovitz et al. (2004) report the expression of an immunogenic peptide having a neutralising epitope originating from VP4—eBRV4—in transgenic alfalfa. This was fused to ß-glucuronidase serving as a carrier molecule which, when injected intraperitoneally with 3 doses into mice as a crude extract adjuvanted with Freund's incomplete adjuvant containing 0.5 μg eBRV4, was shown to induce rotavirus-specific antibodies. Moreover, when fresh leaves containing 6 μg eBRV4 were fed orally to mice once a week 8 times, rotavirus-specific antibodies were shown to be induced and mice were protected from oral virus challenge.

Matsamura et al. (2002) have reported the expression of rotavirus capsid protein VP6 in transgenic potato tubers. Intraperioneal immunisation of mice with 2 doses of adjuvanted potato tuber extract containing 750 ng each of VP6 showed that anti-VP6-specific antibodies were stimulated.

These very positive results all contribute to the feasibility of producing subunit rotavirus vaccines in plants.

1.4 Bovine Herpes (BoH) in Cattle

Bovine herpes virus 1 (BoHV-1) causes a worldwide disorder in cattle referred to as infectious bovine rhinotracheitis. BHV is a double-stranded DNA virus belonging to the *Varicellovirus* genus of the *Herpesviridae* family. It causes an extensive range of symptoms including rhinotracheitis, infectious pustular vulvovaginitis, enteritis, general respiratory disease, encephalitis, decreased milk production, weight loss and abortion in pregnant cows which is why it is of great concern with regard to causing severe economic losses (Graham 2013). It affects both adult and young animals. BoH infects animals through the mucous membranes of either the upper respiratory or genital tract (Muylkens et al. 2007). Transmission between animals requires direct contact. The BoH virion encodes 10 glycoproteins, of which glycoprotein D (gD) is responsible for permissive host cell receptor binding and this and 3 others are responsible for cell entry.

Current vaccines employed to combat this virus consist of inactivated or modified live virus (van Drunen 2006). It has been shown that these vaccines are efficacious in reducing symptoms of infection after challenge, but the challenge virus can remain latent in host tissue and be re-activated and subsequently excreted, thereby not eradicating the infection in the herd. In addition, the efficacy of these types of vaccines in neonates is not as efficacious as in adult animals due to the functional immaturity of their immune system and difficulty in particular in mounting a cellular response against BoH which is essential for immunity. This which has led to the development of recombinant vaccines. Subunit vaccines are comprised of glycoprotein antigens as these have been shown to be excellent targets for neutralising antibodies (Alves Dummer et al. 2014)—and have been expressed in baculovirus, adenovirus and mammalian cell culture systems (Muylkens et al. 2007).

Perez-Filguiera et al. (2003) have expressed glycoprotein D in *N. benthamiana* from a TMV-based expression vector. Mice immunised with approximately 4 μg crude leaf extract mixed with IFA showed specific humoral responses to glycoprotein D after a single dose. Cattle, which were immunised with approximately 100 μg crude leaf extract also showed a specific antibody response after 2 vaccinations. In addition analysis of lymphocytes from PBMCs indicated positive proliferation indices suggesting that the cellular immune response was also stimulated by this vaccine. Secreted nasal fluids from immunised animals which were subsequently challenged with live virus showed that there were similar amounts of virus in that of the mice, but that the period of virus shedding in the cattle was by up to 5 days indicating that the vaccine was protective.

1.5 Rinderpest in Cattle

Rinderpest, otherwise known as cattle plague, is caused by a single-stranded RNA *Morbillivirus* from the *Paramyxoviridae* family. The RNA encodes the phosphoprotein (P), nucleoprotein (N) and polymerase (L) which are found on the inside of the virion as well as the matrix protein (M) which forms the outer capsid and the haemagglutinin neuraminidase (HN) and fusion proteins (F) which form the outer envelope of the virion (Martin 1986). The virus is highly communicable and is spread by direct contact, contaminated drinking water and sometimes by aerosol. It affects mammals including man, but affects mostly ungulates. Symptoms are characterised by fever, ocular and nasal discharges and morbidity and mortality rates are high as these primary symptoms lead on to oral and gastrointestinal tract ulceration, dysentery, diarrhoea, dehydration, and lymphocyte depletion which causes protein loss and immunosuppression (Roeder et al. 2013). It is thought that rinderpest originated in Central or South Asia and as a result of human activity, spread to Europe and Africa. Rinderpest virus (RV) replicates very quickly and is inactivated quite easily by heat or direct sunlight. After infection, it targets the lymphatic system as well as the epithelial cells of the respiratory system and gastrointestinal tract (OIE 2008a).The virus moves very quickly and symptoms can be seen as soon as three days post infection.

The first vaccine developed against rinderpest was an attenuated virus cultured in bovine kidney cells (tissue culture rinderpest vaccine—TCRV) and was extremely successful in combatting all clades of the virus with lifelong immunity to cattle with only a single dose (Plowright and Ferris 1962). Subsequent to that, a more temperature resistant vaccine ThermoVax was developed, and this was used to finally eradicate rinderpest by 2011 (Roeder et al. 2013). Leading up to the declaration of final global eradication in 2011, there was some development of recombinant vaccines against rinderpest. The envelope proteins H (haemagglutinin) and F (fusion) have been expressed in cattle immunised with a disabled human adenovirus vector Ad 5. These vectors have been shown to result in the expression of rinderpest-specific proteins which stimulated the production of neutralising antibodies and subsequently the protection of cattle from infection when challenged with live virus (Cosby and Yamanouchi 2006).

Prior to the declaration of final eradication in 2011, Khandelwal et al. described the generation of transgenic peanut plants (*Arachis hypogea* L.) expressing the RPV H protein (Khandelwal et al. 2004). Mice immunised intraperitoneally with 10 μg H together with IFA elicited H-specific antibodies which neutralised virus in vitro. Oral immunisation of mice elicited H-specific IgG and IgA antibodies. The same group carried out oral immunisation of cattle with transgenic peanut leaves expressing H protein. Serum from immunised cattle was shown to contain H-specific antibodies as well as neutralising and cross-neutralising activity (Khandelwal et al. 2003).

Around the same time, Sathyavathi et al. (2003) published a report on the expression of RPV H protein in transgenic pigeon pea plants (*Cajanus cajan* (L.)

Millsp.), yielding levels of 0.1–0.49% of total soluble protein. However, no immunogenicity tests were carried out.

1.6 Transmissible Gastroenteritis (TGE) in Pigs

Transmissible gastroenteritis is a highly contagious disease causing severe acute diarrhoea in newborn piglets which results in very high mortality rates of less than two-week old animals (Saif et al. 1994). It is caused by transmissible gastroenteritis virus (TGEV), a single-stranded RNA virus which belongs to the *Coronavirus* genus of the family *Coronaviridae*. It is widespread, causing severe economic losses to pig farmers. The virus infects the enterocytes of the small intestine where it multiplies and causes enteritis (OIE 2008b). The M protein in an integral membrane protein and the gS (spike) glycoprotein occurs on the surface of the virion (Laude et al. 1990).

The current commercially available vaccine used to treat TGEV is a modified live virus. The gS glycoprotein is responsible for inducing neutralising antibodies. However, further development has progressed on recombinant vaccines produced in mammalian expression vectors which have been shown to promote systemic and mucosal immunity as well as passive immunity to suckling piglets (Hammond and Nemchinov 2009).

There are also several examples of plant-produced proteins which have been tested as vaccines against TGEV. Gómez et al. (1998) made transgenic *Arabidopsis* encoding the full-length gS protein or the N-terminal domain of gS shown to be neutralising (N-gS). Serum from mice inoculated with these proteins showed virus-specific neutralisation and immunoprecipitation in vitro. Gómez et al. (2000) also made transgenic potato plants expressing N-gS. Tuber extracts inoculated into mice induced TGEV-specific IgG. Oral immunisation of mice with the same antigen was also shown to stimulate serum antibodies specific to gS. Tuboly et al. (2000) similarly expressed truncated TGEV protein regions expressed in transgenic tobacco and immunised pigs which showed TGEV-specific immune responses. Lamphear et al. (2002, 2004) describe the production of an oral TGEV vaccine candidate produced in corn which when milled and fed to pigs, boosted antibody levels in their serum, colostrum and milk.

1.7 Infectious Bronchitis (IB) in Chickens

Infectious bronchitis in young chickens is an acute and highly contagious respiratory disease caused by infectious bronchitis virus (IBV), a gamma *Coronavirus* belonging to family *Coronaviridae* (Jackwood 2012). Apart from affecting the upper respiratory tract, it can also affect the reproductive tract, and some strains can cause nephritis. Infections cause weight loss, decreased egg production, general

poor performance of flocks and high morbidity (Bande et al. 2015). Inhaled IBV infects the respiratory tissues and replicates mainly in the upper respiratory tract (Raj and Jones 1997). The virus is then disseminated to other tissues and is epitheliotropic, entering the epithelial cells of primarily respiratory organ tissue but can move on to reproductive regions, kidneys and the intestine, causing permanent damage and dysfunction of the infected organs. IBV consists of 3 major structural proteins, S (spike), M (membrane) and N (nucleoprotein). The S protein is cleaved into the S1 and S2 forms which associate to form spikes on the virus surface. This is the protein which has been shown to be immunogenic.

IB is typically controlled with serotype-specific vaccines which are either live-attenuated or killed (Bande et al. 2015). But the problem with IBV is that there are more than 20 different serotypes, and even more variants, and there is little cross-protection between the vaccines. The constant evolution of new variants can cause the disappearance of others, requiring a constant demand for new serotype specific vaccines. The potential risk of reversion to virulence by live-attenuated vaccine strains and the weaker immune response and subsequent requirement for multiple dosing schedules has led to some development of recombinant vaccines involving expression of the S protein as this has been shown to have stimulate virus neutralisation (Cavanagh et al. 1986). Some research being carried out on the production of recombinant vaccines (Bande et al. 2015) include S1 protein production using a baculovirus expression system (Song et al. 1998) as well as a recombinant fowl adenovirus (Johnson et al. 2003) that has been shown to stimulate virus-neutralising antibodies and provide protective immunity in chickens.

Zhou et al. (2004) have shown that 2.5–5 μg transgenic potato tuber extract expressing the full length IBV S1 protein induced the production of virus neutralising antibodies and provided up to 85% protection in chickens when challenged with live IBV. In addition, cell mediated immunity which is considered to contribute to protective immunity against IBV was also stimulated, as measured by T-cell proliferations and an increase in chIL-2 levels.

1.8 Bluetongue (BT) Disease in Sheep

Bluetongue disease is caused by the Bluetongue virus (BTV) belonging to the *Orbivirus* genus of the *Reoviridae* family (Sperlova and Zendulkova 2011). It is a double-stranded RNA virus with the virion having a double capsid composed of 3 protein shells: the inner layer composed of capsid protein VP3, the intermediate layer composed of capsid protein VP5 and the outer layer composed of capsid protein VP2 and VP7 (Mertens et al. 2004). It is a non-contagious, infectious disease affecting ruminants and camelids and is transmitted by midges of the *Culicoides* genus. Up until 2014, 26 different serotypes had been recorded (Maan et al. 2012) but a 27th serotype has more recently been identified and sequenced (Jenckel et al. 2015). The presence of BTV was first recorded in South Africa at the end of the 18th century. Traditionally it has been known to be present in a region

spanning 40°N and 35°S which corresponded to the geographical distribution of the midges responsible for its transmission (Carpenter et al. 2008). It has become much more of a concern recently however, due to global warming which is thought to have favoured the survival of the midges over the colder winter periods. Thus the current distribution of BTV outbreaks has recently shown to extend beyond the previously recorded region and has been predicted to broaden in Africa, Russia and the United states with future-climate predictions (Samy and Peterson 2016). Bluetongue affects sheep the most with acute, chronic or subclinical conditions. Clinical signs are usually lameness, swelling, infertility and in severe cases, death. Goats are less frequently infected, rarely showing clinical signs of the disease although if they do, symptoms are less severe. Cattle act more as reservoir hosts and clinical signs of infection are rare, except those infected with BTV serotype 8 which has been reported to have caused elevated numbers of morbidity, fertility and mortality (Elbers et al. 2009). Virus is introduced into animals through the bite of an infected midge and directed to the lymph nodes which are the site of initial viral replication. Virus is then circulated in the blood, infecting secondary organs including additional lymph nodes, the spleen and lungs. Pathogenesis is characterised by small blood vessel injury to the target tissues which leads to increased vascular permeability and subsequent oedema and effusions (Maclachlan et al. 2009).

There are currently two types of vaccines available for prophylactic immunisation of animals: live attenuated vaccines and inactivated vaccines. The vaccines are serotype-specific so it is important to establish which serotype is responsible for circulation in a specific area. Until recently, live attenuated vaccines were the only ones available, and multivalent preparations are currently still used in regions such as South Africa. Although these are effective, they are temperature sensitive and show poor protection against heterologous BTV serotypes. More concerning however, is the fact that these vaccines can result in negative clinical signs such as bluetongue, abortion, a reduction in milk production and foetal malformation in pregnant ewes (Bhanuprakash et al. 2009). There is also the possibility of reversion to virulence or viral reassortment which could generate a more virulent strain virus (Sperlova and Zendulkova 2011). Inactivated vaccines are reliable and protective and prevent clinical disease from developing but are fairly expensive to produce and animals require re-vaccination. They have been predominantly used in various outbreaks experienced in Europe (Zientara et al. 2010).

Recombinant vaccines could help abrogate the use of live inactivated virus and provide a less expensive alternative to killed vaccines. Such vaccines include recombinant viral vector vaccines, subunit vaccines and virus-like particles (VLPs). French et al. (1990) and Roy (1992) were able to show that co-expression of the 4 BTV capsid proteins in insect cells using a baculovirus expression system resulted in the production of assembled VLPs, with the major immunogenic determinant VP2 being presented on the outer shell. Inoculation of animals with these and those representing other BTV serotypes showed that they were immunogenic and protective (Stewart et al. 2012) against BTV challenge. However, this product is not very scalable and is fairly costly to implement.

There has been some progress with developing the production of a BTV serotype 8 VLP vaccine in plants using transient expression in *N. benthamiana* (Thuenemann et al. 2013; van Zyl et al. 2016). The 4 BTV capsid proteins were cloned into a pEAQ-HT expression vector (Sainsbury et al. 2009) and co-infiltrated into *N. benthamiana* leaves using recombinant *Agrobacterium*. All four proteins were expressed and TEM analysis of purified extracts from harvested leaves showed that all 4 proteins assembled into VLPs. It was estimated that yields were approximately 70 mg VLPs per kg fresh leaf weight. Purified preparations of the VLPs at 50 μg per dose combined with IFA injected into sheep with a second booster dose at 28 days post initial injection induced a strong immune response and provided protective immunity in sheep challenged with a BTV serotype 8 strain. However, this method of purification of VLPs (centrifugation) is not very scalable.

1.9 Rift Valley Fever (RVF) in Ruminants

Rift Valley Fever (RVF) is a zoonotic infectious disease which primarily affects ruminants. It is caused by Rift Valley fever virus (RVFV) which was first identified in 1930 in the Rift Valley in Kenya when it caused an outbreak of disease in sheep. RVFV is an enveloped negative-stranded RNA virus with a tripartite genome belonging to the *Phlebovirus* genus in the *Bunyaviridae* family (Pepin et al. 2010). It has a lipid bilayer composed of glycoproteins G_n and G_c, encapsidating the RNA-associated ribonucleoproteins (RNPs) and RNA polymerase (L). The virus was restricted to sub-Saharan Africa prior to the middle 1970s but has now become endemic to parts of Africa and the Arabian Peninsula and is considered an emerging virus. (Pepin et al. 2010). Symptoms of infection in ruminants vary between different animal species but include the occurrence of near simultaneous abortions in pregnant animals, high neonatal mortality, hepatic damage and deformed young. RVFV is predominantly spread by mosquitoes although it can be harboured by a few other vectors such as ticks and sandflies. Infections in animals occur predominantly as a result of bites from mosquitoes carrying the virus but can also be spread by direct contact with infected animal tissues and fluids. This is the main route by which humans are infected, severe cases of which lead to jaundice, neurological disease or haemorrhagic complications and possible fatalities (Pepin et al. 2010).

The spread of RVFV can be prevented by vaccination of animals and humans but development of safe and effective vaccines has been difficult (Bouloy and Flick 2009; Ikegami and Makino 2009). Live attenuated vaccines such as the Smithburn strain have been used extensively for livestock vaccination programmes, but still cause undesirable symptoms in pregnant animals and could easily revert or mutate to a more virulent strain. Research has been carried out using inactivated viral strains but these are expensive to produce and require multiple doses and boosters for immunity to be maintained in animals. The formalin-inactivated vaccine TSI-GSD-200 is only used for veterinary workers in endemic regions, high

containment lab workers and others at high risk for contracting RVFV. It is not commercially available, but is used for veterinarians who work in areas endemic to RVFV as well as for others who are at high risk of contracting the disease including lab workers in high containment areas. Like the other live attenuated vaccines, this vaccine is expensive, difficult to make and requires multiple doses and boosters for efficacious immunity, making routine immunisation of animals impractical (Bouloy and Flick 2009).

The RVFV glycoproteins G_c and G_n, have been shown to be the antigenic determinants for stimulating neutralising antibodies which provide immunity to the disease. This discovery has enabled several recombinant vaccine candidates to be developed. Expression vectors such as the Venezuelan equine encephalitis (VEEV) vector and the lumpy skin disease virus (LSDV) vector (Wallace et al. 2006) have been used to generate RVFV glycoproteins which have elicited RVFV-specific immune responses in and provided protection against RFVF challenge in animals.

RVFV virus like particles (VLPs) are also a possible candidate for vaccination against RVF as they are stable, they may be more immunogenic than recombinant proteins alone and they maintain their conformational epitopes which induce neutralising antibodies. By expressing RVFV nucleocapsid (N) and glycoproteins (G_n-G_c) together in a dual baculovirus expression vector, (Liu et al. 2008) showed that enveloped VLPs resembling wildtype RVFV virions could be produced. VLPs were also produced by the dual baculovirus vector system with N and G_c alone. These VLPs were more pleomorphic than the VLPs comprised of both glycoproteins. Habjan et al (2009) have produced RVFV VLPs in mammalian cells by co-expressing recombinant RVFV polymerase and nucleocapsid protein together with a minireplicon RNA and additional expression of the viral glycoproteins (Habjan et al. 2009). These have been shown to protect mice from a lethal challenge of RVFV (Näslund et al. 2009).

Interestingly, De Boer et al. (2010) have shown that RVFV VLPs can be produced in a *Drosophila* insect cell system expressing the G_n and G_c proteins alone. Furthermore, these VLPs have been shown to provide 100% protection of mice when challenged with wildtype virus. This type of vaccine lacking the N protein is desirable as this can help with distinguishing between infected and vaccinated animals (DIVA) using diagnostic kits. De Boer et al. also tested the effect of soluble G_n in vaccinated mice and were able to show that full protection was afforded from lethal challenge with RVFV.

Some preliminary studies have been carried out on the immunogenicity of plant-produced RVFV antigens. Kalbina et al. made transgenic *Arabidopsis* plants expressing RVFV G_n (deletion mutant) and RVFV N (Kalbina et al. 2016). Leaves containing these proteins were fed to mice orally and mouse serum was shown to have elevated titres of antigen-specific IgG, suggesting that they are immunogenic.

1.10 Crimean-Congo Haemorrhagic Fever (CCHFV)

CCHF is a zoonotic disease caused by Crimean-Congo haemorrhagic fever virus (CCHFV) belonging to the genus *Nairovirus,* family *Bunyaviridae* (Whitehouse 2004). Similar to RVFV, it is an enveloped RNA virus with a lipid bilayer of glycoproteins G_n and G_c encapsidating the RNA-associated ribonucleoproteins (RNPs) and polymerase (L). CCHF is a highly contagious disease which infects a large variety of vertebrates including sheep, goats, cattle, large wild herbivores, hares and hedgehogs (Bente et al. 2013). There are also numerous bird species that have been shown to have antibodies to CCHFV although they are refractory to infection, as well as one instance of CCHFV antibodies found in a tortoise (reptile). However these animals generally show no symptoms of the disease, but develop sufficient viraemia to support transmission of the virus to uninfected ticks which then bite and infect humans. The distribution of the disease tends to follow the geographical range of this vector. Humans are the only host of CCHFV in which disease is manifested except for newborn mice. Humans also become infected by handling crushed, infected ticks and by direct contact with infected blood or tissue of animals or humans harbouring the virus. The disease is endemic in more than 30 countries in Africa, Asia, southeast Europe and the Middle East and it has recently emerged in areas previously free from the disease such as Turkey. CCHF is a notifiable disease as outbreaks have epidemic potential which constitutes a public health threat, they have a high fatality ratio, nosocomial outbreaks are extremely prevalent, the virus is potentially a bioterrorism agent and treatment and prevention of the disease is difficult. Ticks harbouring CCHFV bite animals and humans and the virus is transported in the bloodstream and absorbed into permissive cells (Bente et al. 2013).

There are currently no commercial vaccines available against CCHFV. However, due to the desire for a vaccine which is universally acceptable for use, and that can be used to inoculate animals which are the main carriers of the virus and responsible for the disease outbreaks and thereby reducing the CCHFV numbers within their populations, some progress has been made in their development.

Initially, a formalin-inactivated vaccine which was developed in Russia in the 1960s from suckling mouse brains for humans (Hoogstraal 1979). It was shown to induce neutralising antibodies in humans. This vaccine was licensed in Bulgaria for prophylactic use on people who work in army units, medical workers, agricultural workers and people living in CCHF endemic regions. Use of this vaccine is reported to have reduced CCHF cases in Bulgaria (Christova et al. 2010) however, this vaccine is not used in any other country in Europe due to concerns that its production in mouse brains may induce autoimmune or allergic responses in humans. In addition, it requires maximum containment facilities to generate virions for inactivation, and this is costly. Another type of treatment used in Russia, Bulgaria and several other countries is administration of anti-CCHF immunoglobulin derived from convalescent serum by intramuscular injection or

intravenously (Keshtkar-Jahromi et al. 2011). CCHF patients treated with convalescent serum recovered from their illness.

Despite these measures of vaccination and treatment, there is currently no universally accepted method of prophylaxis against CCHF and not much work done on the prevention of CCHFV in animals. Development of alternative vaccines has been hampered by the lack of information on immunogenic epitopes of CCHFV as well as the lack of a CCHF animal model to test efficacy. The use of newborn mice which are naturally affected as models are questionable, as they are very susceptible to a wide array of pathogens and may not be that useful as a disease model. However, recent research in the USA has yielded 2 animal models which have the potential for use in testing vaccines. These models are both knockout mice models which are sensitive to CCHFV infection (Keshtkar-Jahromi et al. 2011) and should encourage further development in the CCHFV vaccine research field.

Very little research on alternative vaccine platforms has been published. Zhou et al. (2011) report the production of CCHF N protein virus like particles (VLPs) in a baculovirus-insect cell expression system with the intention of testing their immunogenicity in mice. Spik et al. (2006) have shown that a DNA vaccine containing the CCHF M segment (encoding G_c and G_n) elicits neutralizing antibodies in vaccinated mice as well as antibodies that are able to react with G_n and G_c proteins.

A small amount of work has been conducted on the production of CCHFV vaccine candidates in plants. Ghiasi et al. (2012) report the production of a CCHFV glycoprotein (G_nG_c) in transgenic tobacco plants. Serum from Balb/C mice which were orally immunised with leaves or roots containing 5–20 μg of recombinant glycoprotein combined with Freund's adjuvant showed an increase in anti-glycoprotein antibodies, although these are not ideal models for the disease.

1.11 Rabbit Haemorrhagic Fever (RHF)

Rabbit haemorrhagic fever is a lethal disease of rabbits caused by the virus rabbit haemorrhagic disease virus, a member of the *Lagovirus* genus of the *Caliciviridae* family (Abrantes et al. 2012). It is a non-enveloped RNA virus having an outer capsid made up of the major structural protein VP60 which encapsidates several non-structural proteins including RND dependent polymerase, helicase and a protease. The virions are extremely resistant and stable in the environment and cause acute necrotising hepatitis, as well as haemorrhaging in other organs. Infection causes death within 48–72 h of necrotising hepatitis. Outbreaks of this disease consequently have a severe effect on the rabbit meat and fur industry. It enters through the oral, nasal or conjunctival routes in animals and usually infects host cells through the upper respiratory or digestive tracts (Abrantes et al. 2012).

Currently, rabbits can be vaccinated using formalin-inactivated liver homogenates of liver-infected individuals. RHV capsid protein VP60 has been shown to be immunogenic and injection with recombinant baculovirus-produced VP60 or

VP60 fusion proteins has been shown to protect immunised rabbits from lethal challenge with the virus (Hammond and Nemchinov 2009).

Fernandez et al. showed that the VP60 polypeptide could be produced in *Nicotiana clevelandii* using a plum pox potyvirus (PPV) based vector (Fernández-Fernández et al. 2001). Rabbits injected subcutaneously with leaf extracts containing recombinant VP60 showed an immune response and were protected when intranasally challenged with RHDV. Castanon et al. (1999) made transgenic potato plants expressing VP60 and immunised rabbits parenterally with leaf extract containing 12 μg recombinant VP60 mixed with Freund's adjuvant. High anti-VP60 titres were measured in the rabbit serum and rabbits were fully protected when challenged with live virus. In addition, Martin-Alonso et al. (2003) showed that VP60 could be expressed in transgenic potato tubers yielding up to 3.5 μg VP60 per mg total soluble protein (TSP). Oral immunisation of rabbits with lyophilised potato extract containing up to 500 μg per dose was only partially effective in inducing immunity and protection when rabbits were challenged with live virus.

2 Parasitic Diseases

2.1 Coccidiosis in Chickens

Coccidiosis is a disease of chickens caused by protozoan parasites which belong to the *Eimeria* genus (Shirley et al. 2005). It is a diarrhoeal disease, causing large poultry losses, particularly in the US. Transmission of the parasite is via the oral-faecal route. Oocysts of *Eimeria* are ingested by chickens and infect the gut where they undergo asexual and sexual reproductive phases. Developing oocysts are shed in the faeces, whereupon they undergo meiosis on contact with oxygen and moisture, and are then re-ingested by animals for sporozoite release in the intestine (Shirley et al. 2005).

Interestingly, infection with *Eimeria* has been shown to lead to lifelong immunity to the particular species of *Eimeria* infecting the host. Hence, commercially available live attenuated vaccines comprising specific *Eimeria* genera have been developed and are routinely used in chick hatcheries (Price 2012), although they are not cross-protective (Shirley et al. 2005). However, efficacy, stability issues, quality control and the cost effectiveness of making live attenuated vaccine strains have led to some developments in recombinant Eimeriid vaccines. The highly complex nature of the Eimeriid life-cycle and the difficulty in identification of specific antigens that protect against infection as well as the method of delivery of the vaccine have presented problems however with the development of recombinant vaccines (Shirley et al. 2007). To date, the most studied recombinant vaccine antigens have been those associated with the most motile and functionally important parasite cycle stages. Proteins associated with the subcellular organelles

micronemes, EtMIC1 and EtMIC2 are some of the most extensively studied (Shivaramaiah et al. 2014).

Sathish et al. (2011) have successfully transiently expressed the *E. tenella* microneme (MIC) protein EtMIC2 in *N. tabacum*. Immunisation of chicks with 50 μg of adjuvanted EtMIC2 with one primary and 2 boosts 2 weeks apart showed the stimulation of high serum-specific IgG titres as well as induction of a specific IFN-Y response. Immunised birds were also challenged and showed an increase in weight, compared to control birds. The same group have also produced recombinant EtMIC1 and tested its ability to stimulate an immune response in birds as a monovalent vaccine as well as in combination with plant-produced EtMIC2 (Sathish et al. 2012).

Zimmerman et al. (2009) describe the development of a plant-made recombinant antibody to be used against coccidiosis. They identified an anti-*Eimeria* scFv from a mouse phage display library when panning with proteins extracted from various developmental stages of the parasite. The scFv-encoding gene was cloned into a vector for the generation of transgenic pea plants (*Vicia faba*—Fodder pea). Pea seeds from the transgenic plants were dried, ground up and force-fed to chickens infected with *Eimeria* containing 1 mg scFv in pea seed flour doses.

3 Conclusion

There are several vaccine candidates targeting domestic animals that could potentially be produced in plants. Most candidates discussed in this chapter were successful in not only producing neutralising antibodies in vaccinated animals but protecting animals challenged with the corresponding virus, suggesting that the plant expression route could have potential for production of vaccines, thereby circumventing the biosafety levels required for live virus handling as well as other problems such as reassortment.

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