Infectious Bursal Disease Virus

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Abstract Infectious bursal disease is an acute, highly contagious, immunosuppressive disease that affects young birds causing important economic losses in the poultry industry. Its etiological agent is the *Infectious bursal disease virus* (IBDV), a non-enveloped bi-segmented double stranded RNA virus which belongs to the Genus *Avibirnavirus* from the Family *Birnaviridae*. Currently, control of IBDV is normally achieved by vaccination programs with inactivated and live attenuated viruses. However, conventional vaccines have a number of disadvantages due to their viral nature and, in many cases, fail to provide sufficient protection against very virulent and variant strains of IBDV. Several new vaccines have been developed as alternatives to solve these problems. Among these rationally designed vaccines live viral-vectored, immune complex and subunit vaccines are found. In this chapter, the contribution of these new technologies to the field will be addressed, with special focus on plant-made vaccines candidates against IBDV. The rationale, efficacy, and yield of these plant-based developments, as well as the comparison to established vaccines or alternatives will be discussed.

Keywords Molecular farming · IBDV · Chicken · VP2 · Recombinant vaccine Transient expression

1 Infectious Bursal Disease Virus and the Disease It Causes

Infectious bursal disease virus (IBDV) is the causative agent of Infectious bursal disease (IBD), also known as Gumboro disease. It is a non-enveloped bi-segmented double stranded RNA (dsRNA) virus which belongs to the Genus *Avibirnavirus*

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from the Family *Birnaviridae* (Dobos et al. 1979; Müller et al. 1979). From the two segments that compose the viral genome, segment B is the shortest and encodes for the viral protein VP1, a RNA-dependent-RNA-polymerase (Morgan et al. 1988; von Einem et al. 2004). Segment A, on the other hand, is slightly larger and consists of two partially overlapping open reading frames (ORFs). One of them encodes for a polyprotein (PP) that undergoes an autoproteolytic cleavage in early stages of an infection giving place to a precursor of VP2 (pVP2), VP3 and VP4. pVP2 is further cleaved into mature VP2 (VP2), the main capsid protein, and several C-terminal peptides. VP4 is the viral protease that cleaves the PP (Da Costa et al. 2000). VP3 is a scaffolding protein that interacts with VP1, pVP2, VP2 and with itself during morphogenesis. VP3 also has RNA-binding activity and is responsible of capsid stability (Mertens et al. 2015). The other ORF of segment A encodes the smallest of the viral proteins, VP5 (Mundt et al. 1995), which has been assigned with a role in viral progeny release (Wu et al. 2009; Méndez et al. 2017).

There are two serotypes of IBDV and, while both can infect chickens, only serotype I is pathogenic in this species (Jackwood et al. 1985; Ismail et al. 1988). The strains belonging to serotype I are traditionally classified as classical (cIBDV), variant (varIBDV) and very virulent (vvIBDV), although there also exists the "vaccine strain" category, which comprises classical strains with different degrees of attenuation for their use as vaccines against IBDV. It is well characterized that less attenuated vaccine strains are able to overcome higher levels of anti-IBDV maternally derived antibodies (MDA), but also to cause immunosuppression in vaccinated chicks (Müller et al. 2012). These vaccine strains have also been implicated in the generation of reassortant IBDV strains (Chen et al. 2012a; Raja et al. 2016).

IBD is a highly contagious disease which is regarded as endemic throughout the world (Fig. 1), causing considerable economic losses both directly, through clinical signs and mortality, and indirectly, due to failure in vaccination programs and incremented susceptibility to other pathogens (Kegne and Chanie 2014; Alkie and Rautenschlein 2016). IBD affects mainly chicks between 3 and 6 weeks of age, although the virus can also infect younger chicks. Because IBDV targets IgM-bearing B-lymphocytes, the infection will cause different degrees of immunosuppression (Sharma et al. 2000). The age and breed sensitivity of the birds, the virulence of the viral strain and the level of maternal antibodies constitute the main factors that will determine the outcome of an IBDV infection (Ahmed and Akhter 2003; Aricibasi et al. 2010; Tippenhauer et al. 2013; Zhao et al. 2016).

Three disease forms are most common in the field: classical, immunosuppressive and acute (Van den Berg et al. 2000). The classical form is associated with the presence of cIBDV and usually comes after a decline in maternal antibodies titers in vaccinated flocks. It has worldwide distribution, being endemic in most of the regions. It is often subclinical and courses with low specific mortality (0–5%). The signs, when a clinical manifestation occurs, include vent picking, trembling, ruffled feathers, watery diarrhoea, anorexia, depression, severe prostration and death. As there are no characteristic signs of IBDV infection, necropsy is where most of the information can be obtained (Van den Berg et al. 2000; Eterradossi and Saif 2008; Kegne and Chanie 2014). The immunossuppresive form is related to the emergence

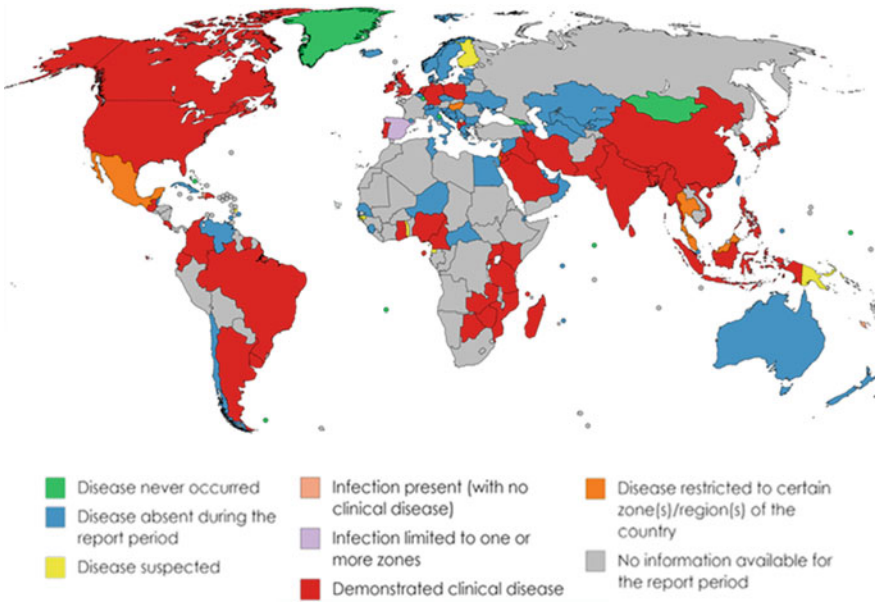

Fig. 1 Worldwide distribution of IBD and status of the disease according to the latest available reports (July–December, 2016) in the World Animal Health Information System (WAHIS) from the World Organisation for Animal Health (OIE). http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statuslist. Image created with https://mapchart.net

of varIBDV strains, which are able to evade the circulating neutralizing antibodies (Snyder et al. 1992), mainly found in USA, Canada and Australia (Snyder et al. 1992; Sapats and Ignjatovic 2000; Kurukulsuriya et al. 2016) because of the predominance of varIBDV strains in those regions. Although the immunocompetence of the chickens is severely diminished, this form is asymptomatic in the majority of the cases (Van den Berg et al. 2000; Kegne and Chanie 2014). However, the economic losses associated to varIBDV-related immunodeficient flocks are considerable (Zachar et al. 2016), mainly due to decreased effectiveness of vaccination programs and increased susceptibility to opportunistic pathogens (Ingrao et al. 2013). Finally, the acute form is caused by vvIBDV strains, which are able to infect in the presence of maternal antibodies and cause higher-than-expected mortality rates (Van den Berg 2000). The first reports of this form of disease took place in Europe, but vvIBDV is currently present in many regions mainly from Africa, Asia and South America (Eterradossi and Saif 2008). The clinical signs are those from the classical form, but they are described to have a more intense manifestation and to be more generalized within the affected flock. The mortality rates can range between 50 and 100% (Van den Berg 2000; Eterradossi and Saif 2008).

As there is no specific treatment for IBD, the attention should be focused on preventive measures. Hygienic measures are important but often insufficient. This makes the vaccination of the flocks the most important action to prevent IBDV

entry into any poultry production facility (Müller et al. 2012). The contribution of plant-based vaccines to this field will be discussed in the next sections.

2 Mechanism of IBDV Infection

Horizontal transmission of IBDV occurs through the ingestion of food and water contaminated with infectious feces. IBDV initiates replication in lymphocytes and macrophages of the gut-associated lymphoid tissues. This stage of viral replication marks the primary viraemia. Within 5 h post-infection, IBDV reaches the liver, where it is phagocytized by resident macrophages. Virus then enters the bloodstream where it is distributed to other tissues including the bursa of Fabricius. The bursa of Fabricius is an oval sac located dorsally to the cloaca, exclusive to avian species, and it is the site where B-cell lymphopoiesis, lymphocyte maturation and differentiation and development of the antibody repertoire take place. Bursal follicles represent the structural, functional and pathological bursal unit (Oláh et al. 2013). Virus ability to spread from the bursa to other lymphoid organs depends on the virulence of the infecting IBDV strain (Alkie and Rautenschlein 2016). By 13 h post-inoculation (hpi), most bursal follicles are positive for the virus and by 16 hpi a second, more pronounced, viraemia occurs, with secondary replication in other B-lymphocyte-containing tissues leading to disease. Clinical signs and death may result from the acute phase (7–10 days) of IBD. As previously mentioned, factors such as pathogenicity and virulence of a strain, as well as the chicken's age, breed, and immune status can influence the outcome and severity of the infection (Van den Berg et al. 2000; Harris 2010). The virus infects and destroys actively dividing immunoglobulin M (IgM)–bearing B cells in the bursa of Fabricius resulting in a prolonged suppression of the primary antibody response (Rodenberg et al. 1994; Sharma et al. 2000). In chickens that survive the acute disease, virus replication subsides and almost all bursal follicles become repopulated with IgM + B cells. The primary antibody response is gradually restored to near normal levels. Although the destruction of B lymphocytes may be one of the main inhibitors of humoral immunity, the involvement of other mechanisms such as altered antigen-presenting and helper T cell functions has been also proposed (Sharma et al. 2000). Together with B lymphocyte depletion in the bursa, an infiltration of activated CD4 + and CD8 + T lymphocytes occurs. Although T-cells are not susceptible to IBDV infection, the cellular immune response is also compromised (Sharma et al. 2000). Evidences suggest that T cells may modulate IBDV immunopathogenesis by restricting IBDV replication in the bursa in the early stage of the disease. Through their release of cytokines and cytotoxic effects, T-cells may enhance bursal tissue destruction, suppress immunity and delay recovery of bursa follicles. At the same time, T-cells may promote clearance of IBDV (Sharma et al. 2000).

Cells of the monocyte-macrophage lineage can be infected in a persistent and productive manner and play a crucial role on dissemination of the virus (Burkhardt and Müller 1987; Inoue et al. 1994) and on the onset of the disease

(Kim and Sharma 2000). Increased macrophage infiltration into the bursa may cause higher expression of proinflammatory cytokines [interleukin (IL)-6, IL-1β and IL-18] and inducible nitric oxide synthase, playing a specific role in the pathology of the disease (Khatri et al. 2005; Lee et al. 2015).

Mechanisms and strategies involved in IBDV life cycle are not clear enough. Nevertheless, it is well known that the virus early life cycle comprises cell surface attachment, internalization and penetration, leading to virus replication in the cytoplasm. Different host cell receptors or structures such as N-glycosilated polypeptides (Luo et al. 2010), heat shock proteins like cHSP90 (Lin et al. 2007), ∝4β1 integrin (Delgui et al. 2009) or lipid raft endocytic pathways (Yip et al. 2012) have been proposed as putative receptors for IBDV. Also, it has been proposed that endocytosis is required for IBDV entry and internalization, followed by the release of Pep46, a capsid-associated peptide which induces pores in the endosomal membrane allowing the release of viral ribonucleoproteins (RNPs) into the cytosol (Galloux et al. 2007). Then, RNPs would associate with the endosomal membrane, through the VP3 membrane-targeting ability, where viral genome replication occurs. Afterwards, the RNPs associated with the endocytic vesicles could traffic along microtubules to reach the perinuclear region, establishing physical contact with the Golgi complex where viral assembly takes place (Delgui et al. 2013). Finally, two independent releasing mechanisms were proposed. The first one, dependent on VP5 expression, allows the non-lytic release of infectious particles from live and metabolically active cells. The second one is associated to cell lysis and facilitates the release of the remaining progeny together with the intracellular content (Méndez et al. 2017).

3 Plant-Made Vaccine Candidates Against IBDV

When IBDV infects a chicken, a humoral response against structural proteins VP2 and VP3 is mostly found. pVP2/VP2 and VP3 are the major proteins present in 780 and ∼450 copies per capsid respectively, while VP1 is present in approximately 12 copies (Luque et al. 2009). Although an antibody response against VP3 exists and neutralizing epitopes were identified (Whetzel and Jackwood 1995), VP3 fails in promoting a protective response (Pitcovski et al. 1999). Conversely, antibodies raised against VP2 have neutralizing capability and elicit protective immunity. Therefore, along the last years, several attempts have been made to generate VP2 subunit vaccines (Ghafari et al. 2010). VP2 neutralizing epitopes are located in the hypervariable region of the protein, between amino acids 206 and 350 (Bayliss et al. 1990). The hypervariable region is highly conformational and comprises four loops named P_{BC} (aa 219–224), P_{HI} (aa 316–324), P_{DE} (aa 249–254) and P_{FG} (aa 279–284) (Coulibaly et al. 2010). P_{DE} and P_{FG} loops are responsible for virus-cell receptor binding and virulence, whereas P_{HI} and P_{BC} loops contain the neutralizing epitopes and have been proved to be suitable sites for foreign peptides display (Brandt et al. 2001; van Loon et al. 2002; Qi et al. 2009).

After infection with IBDV or recombinant expression of VP2, icosahedral T = 1 subviral particles (SVP) of ~23–26 nm in diameter formed by 20 trimers of VP2 are found (Coulibaly et al. 2005; Garriga et al. 2006; Taghavian et al. 2013). These particles were produced in different expression systems like *Pichia pastoris*, *Escherichia coli* and insect cells, for many purposes such as: subunit vaccines against IBDV (Rogel et al. 2003; Ho et al. 2010; Taghavian et al. 2013; Jackwood 2013), carrier of epitopes of non-related viruses (Remond et al. 2009; Caballero et al. 2012; Pascual et al. 2015), serological diagnosis (Dey et al. 2009) and life viral cycle studies (Lin et al. 2007; Delgui et al. 2009).

Due to the importance of the disease worldwide and the beneficial features of plant expression of valuable molecules, some groups have reported the production of VP2 in model plants and cereal crop evaluating in each case their performance as vaccine against IBD.

The first study appeared in 2004 when molecular farming, in particular the conception of edible vaccines, was in full swing. A*rabidopsis thaliana* expressing VP2 in the foliar area was the plant species of choice. Authors reported a percentage of total soluble protein (TSP) for VP2 ranging between 0.5 and 4.8%. These % TSP values were probably underestimated as authors considered in the calculations that VP2 represented 20% of IBDV TSP (Wu et al. 2004a), when nowadays it is known to be about 60% according to crystallographic and stoichiometry analyses (Luque et al. 2009). Nevertheless, these percentages are among the highest obtained in stably transformed plants for subunit vaccines.

Crude extract from leaves of the best transgenic line was evaluated by oral and subcutaneous routes in a prime/boost scheme at 1 and 3 weeks of age. Those animals immunized orally with VP2 received 5 doses at 3-days intervals. Chickens receiving subcutaneous immunization had moderate levels of antibodies compared to the live intermediate commercial vaccine (Bursine-2) group, and protection after challenge with a variant strain was 60%, measured as a bursa-to-body weight ratio. In spite of generating similar antibody levels, the oral route seemed to be more efficient than the subcutaneous route with 80% of protection. Chickens primed with the commercial vaccine at 1 wk followed by an oral booster with VP2 expressed in plants at 3 wk of age showed 90% protection while animals receiving two doses of Bursine-2 at the same time interval had 78% protection (Wu et al. 2004b).

Overall, these first approaches to a plant derived vaccine indicated that plants were capable of synthesizing IBDV VP2 and that both routes of vaccination were effective in generating protective response. Moreover, VP2 expressed in plants could be effectively used to prime or boost a previous response.

Later, another group drove the expression of VP2 to rice endosperm with the aim of producing a mucosal vaccine for IBDV (Wu et al. 2007). The strategy was to clone the coding sequence of VP2 under the promoter of Glutelin A, a very strong and specific promoter leading the expression of the most abundant protein in rice seeds.

The average of VP2 protein in the highest expressing transgenic line was 4.521% of seed TSP, which accumulated up to 56.12 μg of VP2 per grain, while the lowest presented 0.678%

Two-week old chickens fed with 1, 3 or 5 g of seeds of a line expressing 40.21 μg of VP2 per grain on days 0, 7, 14 and 21 developed a specific immune response. Unfortunately, the vaccination doses (μg VP2/g seeds) have not been informed although it was estimated elsewhere that 5 g contained 10 mg of VP2 (Mason and Herbst-Kralovetz 2012). The protection was recorded as the number of chickens with a bursal score of zero. The bursal score is a measure of how affected the bursa of Fabricius becomes after infection with IBDV and it is based on the percentage of follicles with lymphoid depletion in addition to the observation of specific lesions. The scale ranges between 0 and 4/5 (depending on the literature source) with lower score meaning less bursal damage. Results demonstrated a dose dependent response as animals fed with 5 g showed the highest rate of protection (83.3% vs. 33.33% showed by animals intranasally inoculated with a commercial attenuated vaccine strain B87 at days 0 and 21) after challenge with a very virulent strain. The neutralizing antibody levels were similar to that of the group vaccinated with the commercial vaccine and was also influenced by the vaccination dose.

Altogether, results indicated that VP2 was resistant to gut degradation and that the use of adjuvants was unnecessary. Moreover it showed an effective, safe and inexpensive vaccine with no requirements of needle/syringe or a cold chain to its commercialization. This work showed for the first time the efficacy of a rice-based vaccine in the natural host. Nevertheless, more detailed studies regarding stability of VP2 over time, storage conditions, mucosal response and the possibility of inclusion in balanced diet, would have been interesting towards the obtainment of an edible vaccine against IBDV.

Rice provides little energy per cost unit which makes it a very expensive cereal to use in birds feeding. Conversely, maize is the cereal that provides the highest amounts of metabolizable energy/kg. Also, it is a source of zeaxanthin and lutein, two carotenoids that provide color to the egg yolk and the chicken' skin, very desirable characteristics in the poultry industry. For these reasons maize would have been a better choice as an edible vaccine for chickens.

Gradually, plant transient expression of vaccine candidates has taken a prevailing place over stable expression mainly because the developing time and yields were improved with the arrival of new technologies. In this sense, Chen et al. described in 2012 the generation and immunogenicity of a chimeric Bamboo mosaic virus harboring the coding sequence of loop P_{BC} (18 aa), of a vvIBDV VP2, fused to the N terminal of the viral coat protein (CP) (Chen et al. 2012b). Bamboo mosaic virus is a filamentous potexvirus consisting of 1300 identical CP subunits so it was expected that the chimeric virus also contained 1300 IBDV epitopes. Authors reported a production of recombinant CP of 2.6–2.8 μg/mg of total soluble protein that represent 0.26 and 0.28% of TSP, respectively. Chimeric viruses were produced in *Chenopodium quinoa* and then purified for animal experiment. Three SPF chickens received an intramuscular injection of isolated chimeric viruses (600 μg) formulated with Freund's incomplete adjuvant and twenty eight days later they were challenged with a very virulent strain of IBDV. Two out of three animals of the control group died while chickens of the chimeric or commercial vaccine survived after challenge. Also, specific antibodies of the chimeric virus vaccinated

animals reached similar levels to the commercial vaccine group. This work demonstrated that a single region of VP2, the loop containing the neutralizing epitopes, was able to induce a specific response even in a single dose. A large scale experiment in field conditions without adjuvant would have been desirable to prove efficacy of the vaccine.

Our group has focused on the transiently production of VP2 in *Nicotiana benthamiana* plants and its subsequent application in different vaccination approaches in susceptible chickens (Gómez et al. 2013; Lucero et al. 2016; Richetta et al. 2017) (Fig. 2). This plant expression system has allowed us the obtainment of high levels of VP2 in a short time period. In addition, this plant expression system is suitable for a rapid response in case of a field outbreak where other sequences of VP2 might be required.

We have chosen the expression of mature VP2 (VP2 of 441 aa) because it has already proved to be immunogenic and to form subviral particles in other

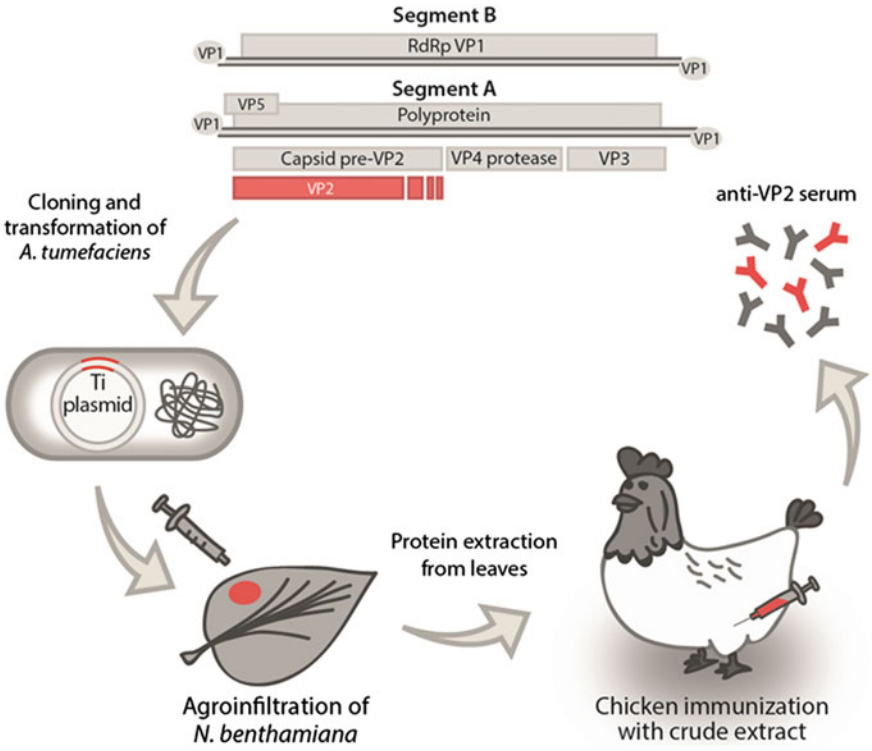

Fig. 2 The coding sequence of mature VP2 is cloned in a binary vector under a strong promoter for plant expression and introduced into *Agrobacterium tumefaciens* by electroporation. Transient expression is performed by infiltrating *Nicotiana benthamiana* leaves with a suspension of the recombinant bacteria. Four or five days later, agroinfiltrated leaves are harvested and total proteins are extracted in chilled buffer. This crude extract is used to vaccinate chickens in a prime/boost scheme which induces a humoral response against VP2

expression systems. Firstly, the strategy was to clone the coding sequence under the control of the rubisco small subunit promoter, said to be 8 times stronger than the 35S promoter. Then leaves of *N. benthamiana* were infiltrated with a suspension of recombinant agrobacteria harboring the sequence of interest followed by the collection of the leaves 4 days later. The yields were in average 1% TSP. Five animals were vaccinated intramuscularly with 200 μl of crude extract containing 12 μg, formulated with Freund's adjuvant, on days 0, 22 and 35 post inoculation. Animals were weekly bled and 18 days after the last vaccination they were challenged with a high dose of an intermediate IBDV strain. Results demonstrated that the extract was able to elicit a humoral response as early as 15 days, with neutralizing activity reaching high titers by the end of the experiment. Also, chickens vaccinated with VP2 and challenged showed a decrease in the frequency of T-cell infiltration into the bursa of Fabricius, from 2.7 to 22.6 times lower than the control group, indicating that the humoral response prompted by the experimental vaccine was efficacious in preventing the entrance of the virus in that organ (Gómez et al. 2013). Later, we showed that a more welfare-friendly immunization scheme with fewer injections and without adjuvant was also able to elicit a protective response. In four out of six animals primed and boosted with 7.5 μg of VP2, IBDV was not detected in the bursa of Fabricius while the other two animals presented a reduced viral titer of approximately 10^5 times regarding the control group. In addition, animals vaccinated with VP2 presented a bursa with normal morphology and nine times fewer infiltrating T cells than the control group (Lucero et al. 2016). We believe that the success of our antigen by parenteral route is not only related to the physical and chemical properties of the protein but also to the adjuvant capacity of the plant extract. It is possible that the plant extract contains PAMPS of toll like receptors (like LPS from *Agrobacterium*) and vegetal compounds that might help to prompt an innate response which in turn contributes to the establishment of the adaptive response (Licciardi and Underwood 2011). It is worth mentioning that when we assayed the mucosal vaccination with the same dosage, we found less encouraging results. Neither intranasal nor oral vaccinations were able to produce an effective immune response in chickens. Specific antibodies were not detected and chickens were not protected from IBDV challenge (Lucero et al. 2016). One possibility is that the immunization scheme applied was inappropriate as mucosal stimulation might require more and/or frequent boosts. Another option is that an adequate mucosal adjuvant or higher doses of immunogen could also be needed.

We also performed vaccinations with the extract in prime/boost schemes along with vectored vaccines based on recombinant Modified Ankara Virus harboring the coding region of VP2 (Richetta et al. 2017). Results showed that the extract can be used alone, as demonstrated earlier, and to prime or boost a vaccination with other types of recombinant immunogens. Finally, using the pEAQ vectors (Sainsbury et al. 2009) for VP2 plant expression we recovered SVP from plant material indicating that these nanoparticles can also be produced in plant-based expression systems (unpublished results).

4 Plant-Made Vaccines Against IBDV Versus Established Vaccines and Other Developments

IBDV was identified for the first time more than 50 years ago; still, this virus remains a significant threat to commercial poultry worldwide. Many advances have been made in the development of new recombinant vaccines, however, live-attenuated and inactivated vaccines, along with strict hygiene management of poultry farm, continue to be the most common practices to control IBDV.

Live viral vaccines can replicate and are effective in inducing both cellular and humoral immunity without the use of adjuvant (Müller et al. 2012). Besides, they are suitable for mass administration to chickens since they can be given with the drinking water. However, they present a number of disadvantages due to their viral nature. They can revert to virulence (Yamaguchi et al. 2000; He et al. 2009; Jackwood 2012), they usually produce a period of immunosuppression in young chickens and might interfere with response to other vaccines (Mazariegos et al. 1990; El-Yuguda et al. 2007); they exhibit poor efficacy in the presence of certain levels of maternally derived antibodies (MDA) (Kumar et al. 2000; Rautenschlein et al. 2005); and most importantly, they may not fully protect chickens against infection by the very virulent and variant IBDV strains (Rautenschlein et al. 2005; Alkhalaf 2009). Furthermore, although drinking water vaccination would seem to be the least labor intensive, there are major concerns regarding inconsistencies of vaccine dosage depending on water consumption within the flock and viral inactivation by traces of disinfectants or chlorine in the drinking water.

Live viral-vectored and immune complex vaccines, seem to be attractive candidates to replace the traditional live attenuated one and are already being commercialized by different animal healthcare companies. The two viral vectored vaccines available, VAXXITEK® HVT + IBD (Merial) and Vectormune® HVT IBD (Ceva) use the turkey herpesvirus (HVT) carrying IBDV antigens to stimulate immunity against Marek's disease and IBD simultaneously. On the other hand, immune complex Gumboro vaccine, Cevac® Transmune IBD (Ceva) consists of a well-defined mixture of IBDV-specific antibodies and infectious IBD vaccine virus. Both types of vaccines have proven to be effective in the presence of maternally derived antibodies (MDA) causing protection against different pathotypes of IBDV when inoculated in a single dose *in ovo* or subcutaneously to 1 day old chickens (Haddad et al. 1997; Kelemen et al. 2000; Perozo et al. 2009; Prandini et al. 2016; Gelb et al. 2016). Although *in ovo* or subcutaneous vaccination allow a more automated and systematic administration of these new vaccines, they require egg-injection machines, which are not available in all farms, or trained personnel. This, together with the fact that both live viral-vectored and immune complex vaccines are more expensive than the live attenuated ones, might be some of the reasons why the last ones have not been replaced yet.

IBD inactivated vaccines consist of virus that has been rendered incapable of replicating, so they cannot cause disease, but maintain the ability to induce a protective immune response. They are costly due to the treatment processes

involved in inactivating the virus and require strict quality control to ensure that killed organisms are fully inactivated and harmless before used for vaccination (Delrue et al. 2012). Moreover, they lack efficient immunogenicity unless they are combined with adjuvants and administered in repeated injections, or follow a prime with a replicating antigen (Müller et al. 2012). Since inactivated IBD vaccines are mostly formulated as water-in-oil emulsions and inoculated through the intramuscular route, they do not stimulate mucosal immunity. Normally, their use is constrained to breeder birds just before laying in order to provide passive immunity to the offspring by means of MDA (Maas et al. 2001).

As an alternative, many proteic subunit vaccines based on recombinant VP2 expression have been assessed in the laboratory against IBDV infection with diverse results. Generally, as they are non-replicating antigens, they have similar disadvantages to inactivated vaccines regarding efficiency. To date, there is only one commercially available recombinant subunit vaccine against IBDV, Gumbin®VP2 (Phibro Animal Health Corporation), which consists of inactivated NDV and recombinant VP2 produced in yeast (Pitcovski et al. 2003). It is intended for vaccination of all chicken breed after priming with live Newcastle disease and Infectious bursal disease vaccines. Nonetheless, these subunit vaccines exhibit great promise since the lack of inactivation procedures might render them cheaper (depending on the expression system), than inactivated whole virus formulations. In addition, some of these recombinant vaccines, depending on how they are formulated, could be administrated through the oral route to stimulate mucosal immunity.

VP2 has been expressed in a number of heterologous systems such as *E. coli*, yeast, baculovirus/insect cells and plants (reviewed in Lucero et al. 2012). All of them have different characteristics that are summarized in Table 1. Although mammalian/avian cultures have not been used to express recombinant VP2, they are used to propagate live attenuated or live viral-vectored vaccines; hence, they are also included in the table for comparison.

When compared to other expression systems, the disadvantages of plant expression systems have been related to protein yield and time of development. It is difficult to compare VP2 protein yield obtained in plants vs. other expression systems since not all reports include this information or is expressed in different units. Still, some of the higher yields were obtained in *E. coli* (1.178 g/l of culture or 0.19 g/g of bacteria) (Rong et al. 2007) and yeast (0.5 g/l of culture) (Pitcovski et al. 2003). Achieving this kind of VP2 yields in plants is one of the challenges of this platform, however, their almost unlimited scale up capacity has the potential to provide plenty biomass in order to accumulate sufficient quantity of the antigenic protein even when expression levels are not very high. On the other hand, although the development and selection of suitable transgenic lines expressing adequate amounts of VP2, like the case of *Arabidopsis* and rice (Wu et al. 2004b, 2007) its laborious and can take many months, VP2 transient expression approaches using *Agrobacterium* (Gómez et al. 2013; Lucero et al. 2016) and/or plant viral vectors (Chen et al. 2012b) are able to reduce developing times and can be as fast as producing a recombinant bacteria or yeast.

Table 1 Comparison of different production systems for expression of recombinant proteins

Expression system	Bacteria	Yeast	Plants	Plant viral vectors	Baculovirus/insect cell cultures	Mammalian/avian cell cultures
Time effort	Low	Medium	High (stable transformation) Low (transient expression)	Low	High	High
Production cost	Medium	Medium	Low	Low	High	High
Scale up capacity	High	High	Very high	Very high	Medium	Low
Production scale	Limited	Limited	Worldwide	Worldwide	Limited	Limited
Cost of maintenance	Inexpensive	Inexpensive	Inexpensive	Inexpensive	Expensive	Expensive
Protein yield	High	High	Medium	Very high	High	Medium-high
Gene protein size	Unknown	Unknown	Not limited	Limited	Limited	Limited
Contamination risk	Endotoxins	Low	Low	Low	Low	Viruses

As described in detail in the previous section, VP2 has been produced in different plant platforms. The efficacy as a subunit vaccine has been demonstrated both by intramuscular (Chen et al. 2012b; Gómez et al. 2013; Lucero et al. 2016) and oral routes (Wu et al. 2004b, 2007) although several doses of the experimental plant-based vaccines were needed in order to achieve a protective immune response against IBDV. These are some of the few studies in which VP2 protein delivered orally has been successful in achieving protection against IBDV challenge. While oral administration of *Kluyveromyces lactis* expressing VP2 (1–3 mg of recombinant protein in total) in a 2/2/2 scheme (two weeks feeding, two weeks break, two weeks feeding) only achieved a 10% rate protection (Arnold et al. 2012), 4 doses of orally-administered *Pichia pastoris* producing VP2, containing 400 μg or 4 mg of viral protein, induced a protective immune response against IBDV in chickens which increased survival rates to 60–100% compared to 40% of the control groups (Taghavian et al. 2013).

Subunit vaccines are safer than traditional IBDV vaccines; however, they are less immunogenic than live attenuated, viral-vectored or immune complex vaccines which can induce a strong immune response with only one dose in young birds. However, it may be beneficial to use a plant-derived VP2 as a booster vaccine in chickens that have been primed with live vaccines. Hence, taking into account the problems with inactivated vaccines and the benefits of plants as expression systems, we believe that a plant-based subunit vaccine against IBDV represents a viable alternative to the inactivated vaccine given to breeder hens before the laying period.

5 How Far Are We from an Anti-IBDV Commercial Vaccine?

Developments of plant-based vaccines against IBDV are nowadays in early stages, however they seem to be very promising strategies. An edible vaccine seems feasible since VP2 expressed in Arabidopsis and rice invoked an immune response when given orally, showing that this protein is resistant to gut degradation (Wu et al. 2004b, 2007). Additionally, as mentioned before, the rice-based vaccine does not need any type of protein extraction or purification prior to delivery and cold chain is not required. However, both experimental vaccines implicate the use of transgenic plants, so they would have to gain regulatory approval from the corresponding organism as all genetically modified (GM) crops in order to reach the market. Moreover, the production of an IBDV vaccine in food crops such as rice might have to overcome concerns regarding the safety of the food chain from cross-contamination with the GM organism (Naderi and Fakheri 2015). On the other hand, transient expressions of VP2 by means of *Agrobacterium* (Gómez et al. 2013; Lucero et al. 2016) or plant viruses (Chen et al. 2012b) have become attractive manufacturing systems since they might be able to overcome some of the regulatory issues and public concerns for genetically modified organisms (Chen and

Lai 2015). Besides, these systems are very rapid, generating recombinant proteins within days and avoiding the long times required to generate a transgenic plant. It has been speculated that it may be difficult to maintain a high efficacy of live vaccines against IBDV due to the complication of adapting them to cover the emergence of highly virulent or variant strains of the virus. In this sense, transient approaches, which allow a fast replacement of the VP2 gene, would be the most appropriate vaccines to deal with this situation (Saif 2004). For many years transient expression systems remained restricted by laborious scale-up limitations. However, with development of new platforms optimized to facilitate a scale-up production in a short time period, transient expression of large quantities of recombinant proteins in plants may become feasible (Gleba et al. 2005; Peyret and Lomonossoff 2013; Jin et al. 2015).

There is still a way to go from the evaluation of the products in the laboratory to the achievement of a finished plant based vaccine against IBDV. To date none of these vaccines have been scaled up and evaluated in large field trials with broiler chickens. Both safety and efficacy tests need to be carried out before an IBDV plant-based vaccine reaches the market. Nonetheless, we believe that a vaccine with the characteristics enumerated before would be of easy adoption in the veterinary field.

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