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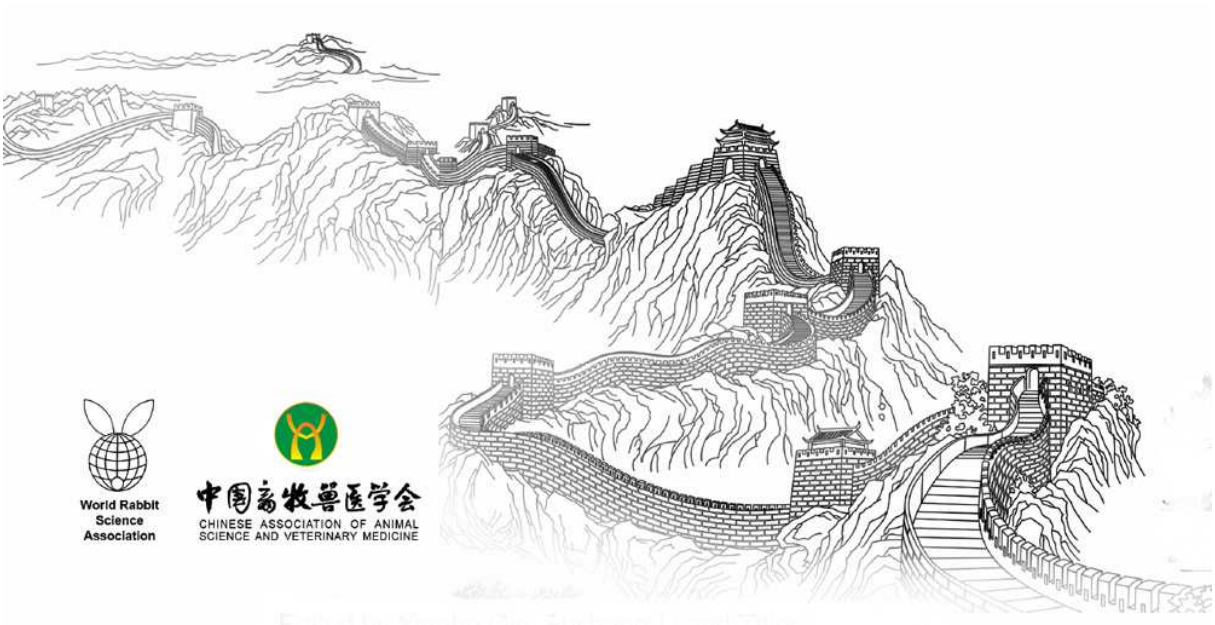

CONTROL OF RABBIT COCCIDIOSIS AND RABBIT HAEMORRHAGIC DISEASE : IMPACT OF RECOMBINANT DNA TECHNOLOGY

Xun Suo[1,2,3*], Fang Wang[4,5,6*], Xianyong Liu[1,2,3], Yanhua Song[4,5,6], Yunzhou Wang[1,2,3], Jiabin Xue[2], Geru Tao[1,2,3],Zhiyu Fan[4], Chao Li[1,2,3],Bo Hu[4], Xiaolong Gu[1,2,3], Houjun Wei[4], Rulong Qiu[4], Xing Liu[4]

[1]State Key Laboratory of Agrobiotechnology & College of Veterinary Medicine, China Agricultural University, Beijing, 100193 China;
[2]National Animal Protozoa Laboratory, China Agricultural University, Beijing, 100193 China;
[3]Key Laboratory of Animal Epidemiology and Zoonosis of Ministry of Agriculture, China Agricultural University, Beijing, 100193 China;
[4] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014 China;
[5] Key Laboratory of Veterinary Biological Engineering and Technology，Ministry of Agriculture, Nanjing, 210014 China;
[6] National Center for Engineering Research of Veterinary Bio-products, Nanjing, 210014 China

*Corresponding authors: Xun Suo, suoxun@cau.edu.cn; Fang Wang, rwangfang@126.com

ABSTRACT

The global demand for excellent sources of high-quality protein is sure to surge up for feeding 9 billion people in the near future. As domesticated rabbits can convert cellulose-rich plants into high-value animal protein and they can use less energy to produce more offspring, they will be among the most efficient sources of animal-derived protein when human population reaches its peak shortly. For securing rabbit production, however, two major rabbit infectious diseases, coccidiosis and viral hemorrhagic disease must be well controlled. In this review, we first describe rabbit coccidiosis and its control measures and then detail into a topic about using vaccination as an alternative strategy including protective immunity elicited by *Eimeria* infection in rabbits, vaccination by wild type strains and attenuated strains, and impact of recombinant DNA technology for the control of rabbit coccidiosis. Furthermore, we describe rabbit viral hemorrhagic disease and its control measures, with focus on vaccines developed using recombinant DNA technology.

Key words: rabbit coccidiosis, rabbit hemorrhagic disease, vaccine, recombinant DNA technology

Coccidiosis is caused by infection with one or several species of the genus *Eimeria* of apicomplexan parasites. *Eimeria* spp. are monoxenous and the domestic rabbit (*Oryctolagus cuniculus*) is the host for 11 species (Pakandl, 2009). Rabbits get infected by fecal-oral route, usually by consuming feed or water, contaminated with the environment-resistant stage, the oocysts. In liver or intestine, the parasites multiply through merogony. Merozoites from the last merogonic generation develop into macro- or micro-gamonts which develop into gametes. Fertilization of gametes leads to the formation of a new generation of oocysts which are excreted into the environment with the feces. Theoretically, one oocyst can produce 1.5 x10^5 -6 x 10^6 offspring oocysts depending different species (Coudert et al., 1995). The newly formed oocysts are unsporulated and non-infective, and become infective after the sporogonic stage in the presence of moisture, warmth (18-26^{o}C) and oxygen within 1-5 days (Coudert et al., 1995).

Infection with rabbit coccidia is very common in domesticated rabbits according to wide-ranging investigations around the world (Abdel-Baki & Al-Quraishy, 2013; Okumu et al., 2014; Qiao, 2012). Our investigation also showed that coccidia infections exist in at least one-third of the large-scale rabbit farms in China and the most prevalent species are *E. perforans*, *E. media* and *E. magna* (Jing et

al., 2012). Observation of co-infection with up to eight species suggests that a composition of multiple *Eimeria* species in the live vaccine against rabbit coccidiosis is necessary.
The 11 species of rabbit-infecting *Eimeria* vary in their predilection sites or niche for parasitism. *E. stiedai* parasitizes the liver; while the other ten propagate themselves in different parts of the intestine. A major character of rabbit coccidia is their extensive migration of sporozoites and/or merozoites (Pakandl et al., 1995). In example, , sporozoites of *E. stiedai* released in the duodenum have to migrate a long way to reach epithelial cells of biliary ducts in liver. Sporozoites of other ten species have to move from their release site in the duodenum to other parts of the intestine. For *E. flavescens*, first-generation schizonts develop deep in the glands of the lower small intestine, far from where the sporozoites are released; then, merozoites migrate to the caecum and colon where the remaining developmental stages take place (Norton et al., 1979). How many individuals disappear during their migration and the contribution of migration to the development of immunity would be interesting questions to tackle.

Not all the 11 species are equally virulent, and even field isolates of the same species vary in their pathogenicity. *E. flavescens* is the most pathogenic species, causing severe clinical signs and high mortality (Pakandl, 2009; Wang et al., unpublished data). An experiment in Dutch rabbits revealed that 10^2 oocysts cause growth retardation, and 10^5 oocysts a 100% mortality (Norton et al., 1979). *E. intestinalis* is highly pathogenic, but in China, a low pathogenic isolate was discovered recently (Shi et al., 2015). *E. magna* is usually recognized as a mildly pathogenic species (Pakandl, 2009), but in China, a highly pathogenic isolate of *E. magna* was discovered. It caused diarrhea in five out of six rabbits inoculated with 5×10^3 oocysts (Wang et al., unpublished data), while 1×10^4 oocysts of an European isolate caused diarrhea in one out of eight rabbits (Licois et al., 1995). Other mildly pathogenic species are *E. media, E. piriformis* and *E. irresidua*.

The main clinical signs of rabbit coccidiosis are low intake of feed and water, diarrhea, depression of weight gain/weight loss, and death (Lebas et al., 1997). The severity of the signs depends on complicated factors, such as the age of the animals and their nutrition/immune status or relative susceptibility, the dose of the infection, and the *Eimeria* species or strains. Diarrhea usually lasts about 7-10 days. However, aggravated diarrhea develops when pathogenic bacteria multiply in the damaged location (Lebas et al., 1997). Diarrhea ranges from intermittent type to profuse watery feces and mixed with mucus and blood. Usually, deaths start abruptly and last three to four days, or longer. Deaths are caused by heavy primary infection with pathogenic strains or complication with secondary infection. Infection by *E. intestinalis* and *E. flavescens* induces loss of water and sodium. The loss of sodium is compensated by the exchange of potassium from the blood, thereby leading to hypokalemia which causes the abrupt death of the animal (Licois & Mongin, 1980; Lebas et al., 1997).

2. CONTROL OF RABBIT COCCIDIOSIS BY MEDICATION

If there were no anticoccidial drugs, there would not have been modern intensive rabbitries. For the effective prophylactics of rabbit coccidiosis, anticoccidial drugs have to be ideally administered before the gamogony stage, as this stage causes the most of damage in rabbits and against which there are few effective drugs. In field, however, rabbits are continuously exposed to coccidia and this may lead to non-synchronized infection, so that treatment is often disappointing. Sulphonamides (sulfadimethoxine, sulfaquinoxaline and formosulfathiazole) are effective for treatment, usually administered in drinking water in two 7-day courses with one-week interval (Lebas et al., 1997). The bacteriostatic activity of sulphonamides is very effective in controlling coccidiosis complicated with secondary bacterial infection. In cases of persistent diarrhea, neomycin and tetracyclines are sometimes suggested (Lebas et al., 1997). A recent trend is the use of toltrazuril in drinking water or feed for treating the outbreak of rabbit coccidiosis. The advantage of toltrazuril in treating coccidiosis is the immediate reduction of clinical signs and in oocyst shedding, allowing the development of immunity against reinfection (Peeters & Greeroms, 1986).

The use of anticoccidial drugs as feed additives is the most effective method for preventing coccidiosis and subclinical coccidiosis. The ionophores are one of the major category of drugs

employed in the rabbit industry. The ionophores have a narrow range of safety for rabbits but the development of resistant strains is slow. For example, narasin at 8-12 mg/kg produces optimal food consumption and weight gain, while at higher dosage there was adverse effects on food intake, growth, relative liver and heart weights, and on serum level of potassium and glucose (Peeters et al., 1981). Other effective ionophores include salinomycin, monensin and lasalocid.

The other category includes the synthetic drugs. Effective synthetic drugs include diclazuril, decoquinate, a combination of clopidol and methyl benzoquate (Lerbek), and robenidine. How to choose and schedule an efficient medication procedure depends on its effect on the performance of rabbits. However, few reports are available for evaluating the efficacy of these drugs in the field. An important fact to remember is that not all drugs are effective against all species of *Eimeria* parasites. Robenidine significantly reduced oocyst output of *E. magna, E. intestinalis, E. irresidua, E. media* and *E. perforans*, whereas clopidol/methyl benzoquate reduced the oocyst shedding of the latter four species only, and it is least active against *E. magna* (Peeters et al., 1983).

Drug withdrawal time must be taken into consideration. As these data are not available for many drugs in rabbits, the withdrawal time for broilers, turkeys and calves may be used for reference. In China, related regulations demand withdrawal time for robenidine in rabbits to be 7 days, and 5 days for clopidal.

For the chemoprophylaxis of rabbit coccidiosis, the current challenges are: few drugs available for rabbits; drug resistance owing to large-scale and long-term use; drug toxicity, and increasing concerns about drug residue in the rabbit products and the environment. The extent of drug resistance in rabbit coccidia is not well documented (Peeters and Geeroms 1989). There are few reports on strains of rabbit coccidia that could establish infection in rabbits medicated with designated anticoccidial drugs. On account of the high prevalence of rabbit coccidia, even in rabbitries with chemoprophylaxis programs, it is assured that some species, or at least some strains of the most common species *E. media, E. magna* and *E. perforans*, have developed partial resistance to anticoccidial drugs (Jing et al., 2012).

3. VACCINATION AS AN ALTERNATIVE STRATEGY FOR THE CONTROL OF RABBIT COCCIDIOSIS

3.1 Protective immunity elicited by Eimeria *infection in rabbits*

In field observations and laboratory evidences demonstrated that vaccination will be a leading alternative for the effective control of rabbit coccidiosis. First of all, rabbits recovered from coccidiosis are resistant to re-infection, indicating a memory immunity is well developed after the primary infection. Secondly, rabbits inoculated with a small number of oocysts of mixed pathogenic species are in good health and are resistant to heavy infection, indicating that developing an effective vaccine against major pathogenic species is possible. Thirdly, infection with *Eimeria* spp. is self-limited and species-specific, indicating that live oocyst vaccines meet the safety requirement of veterinary authorities (Coudert et al., 1995; Suo et al., unpublished observation).

As according to the literature and our recent study, not all *Eimeria* species or strains are equally of high immunogenicity. Six oocysts of *E. intestinalis* was sufficient to minimize clinical signs and to reduce 60% of oocyst output following challenge with dose of immunization 500 times higher (Coudert et al., 1993). In our work, immunization with oocysts of *E. flavescens* failed to provide protection, demonstrating its low immunogenicity as reported previously (Norton et al., 1979; Zhang and Lin, 1994). An isolate of *E. magna* was highly immunogenic as showed in our recent study (Wang et al., unpublished), but Drouet-Viard et al., (1997) reported that this species is middle immunogenic, demonstrating the differences in immunogenicity among these strains.

3.2 Vaccination by wild-type strains

Many studies have been performed to test the immunogenicity of field strains isolated in Europe. Rabbits inoculated with 600 oocysts of *E. intestinalis* developed full immunity against the challenge

with an LD_{50} dose of 3×10^3 oocysts (Licois et al., 1990); similar results were also obtained in *E. magna* and *E. media* (Licois et al., 1994, Licois et al., 1995).
Work conducted by our group showed that New Zealand White rabbits vaccinated with 1×10^3 oocysts of either *E. intestinalis*, *E. magna*, *E. media*, or *E. irresidua* were well protected against homologous challenge with 50 times the vaccination dosage. Meanwhile, rabbits vaccinated with mixed oocysts of these four species (500 oocysts for each species) were well protected against infection with mixed oocysts (each of 100 times the vaccination dosage) of the four species, further indicating that rabbits can be well protected from coccidia infection by vaccination with live oocyst vaccines composed of pathogenic species which are commonly found in rabbitries. (Wang et al., unpublished)

3.3 Vaccination by attenuated strains

Currently, attenuation through precocious selection is a reliable way for the development of an anticoccidial vaccine for rabbits. The precocious line of *E. intestinalis* was first developed in France (Licois et al., 1990). Through only 6 consecutive passages with precocious selection, a line with characters of attenuated pathogenicity and high immunogenicity was obtained. The prepatent period was reduced from 215 h to less than 144 h. Subsequently, precocious lines of *E. media* (Licois et al, 1994), *E. magna* (Licois et al., 1995) and *E. flavescens* (Pakandl, 2005) were also successfully developed. Drouet-Viard et al., (1997) showed that if sucklings were vaccinated at 25 days of age using spray dispersion of oocysts of *E. magna* precocious line in the nest box, then complete protection against heavy challenge 9 days post vaccination could be established. Based on this trial, they proposed that if the challenge were performed later, rabbits would be protected after vaccination with even a lower dose.

We observed similar phenomena in our recent study. A precocious line of *E. intestinalis* (PEi8) was selected from an original strain (OEi) by 8 successive generations. The prepatent period of PEi8 shortened from 204 h to 132 h, and its multiplication rate was only 0.1~ 1% of that of OEi. PEi8 was much less virulent than OEi and rabbits immunized with PEi8 were protected against challenge with the parental strain. (Li et al., unpublished)

We do not know whether attenuated vaccine strains will occupy the niches of their wild corresponding strains. If yes, it would be a key step-wise strategy by vaccinating the suckling at an earlier age (25-27 days of age) leading to an effect of killing two birds with one stone: the attenuated vaccine strains occupy the niches preventing wild-type to multiply at the same time, giving the immune system the time to develop immunity in a period of 8-10 days. Thus, when the weanlings are susceptible at the age of 35 days, they are already immune to wild strains.

3.4 Impact of recombinant DNA technology for the control of rabbit coccidiosis

3.4.1 Subunit vaccines

Although CoxAbic®, an anticoccidial vaccine for chickens, is the only subunit vaccine against protozoan parasites reaching the marketplace, an anticoccidial subunit vaccine with comparable efficacy to an attenuated anticoccidial vaccine of live oocysts has an indefinite long run. Until now, it is not clear how many antigens from a 60-Mbp genome are needed for an effective anticoccidial vaccine, more difficult is to know how many antigens are needed in total for the three or four major *Eimeria* species.

A novel genetic approach was applied to identify regions within the genome of the highly immunogenic species *E. maxima* that encode protective antigens (Blake et al., 2006). The subsequent fine mapping of two of these regions identified two proteins, immune mapped protein-1 and apical membrane antigen 1 (AMA1), as partially immunoprotective antigens (Blake et al., 2011). However, which route of antigen delivery will realize the dream of eliciting an effective protective immunity using the two candidate antigens? Is eliciting protective cellular immunity a must-be event for anticoccidial vaccines or may a successful vaccine not necessarily need to elicit the protective response to eliminate natural infection (McDonald & Shirley, 2009)? CoxAbic® works through protecting broilers with antibodies transferred from egg yolk from immunized breeders, while cellular immunity is required for natural resistance against re-infection (Sharman et al., 2010). Hepatitis B and measles vaccines work by eliciting high levels of protective antibodies while protective cellular immunities are required in eliminating natural infections (Englar et al 2001; Permar et al., 2004).

"Thus the present scenario for the identification of putative immunogenic molecules of *Eimeria* represents a classic 'Catch 22' situation in which protective antigens may not be identified until a suitable method of delivery has been identified and an appropriate method of delivery may not be recognized until protective antigens have been isolated" (Shirley et al., 2005).

3.4.2 DNA vaccines

Although there are no licensed DNA vaccines in rabbits, the successful launch of three DNA vaccines licensed for veterinary use (i.e., a West Nile virus DNA vaccine for horses, a fish DNA vaccine against the Infectious Haematopoietic Necrosis virus, and a Canine Malignant Melanoma vaccine; (Meeusen et al., 2007)) will promote the development of gene vaccines in rabbits. The same question is how many antigens have to be included in an anticoccidial DNA vaccine to elicit good enough protective immunity against several *Eimeria* species in the field. Advanced technologies for improved vector design and vector/vaccine delivery will quicken the development of DNA vaccines against coccidian infection in rabbits.

3.4.3 Live viral vector vaccines

Viral vector vaccines have two major advantages, one being used without adjuvant, the other being able to elicit a robust cytotoxic T lymphocyte (CTL) response to eliminate pathogen-infected cells (Ura et al., 2014). Poxviruses, including vaccinia virus, fowlpox virus, and canarypox virus, have been successfully used as live viral vectors delivering exogenous antigens from other viruses including equine influenza virus, West Nile virus, rabies virus, canine distemper virus, and these live poxvirus-vectored vaccines have been licensed/commercialized (Meeusen et al., 2007). A transmissible virus-vectored vaccine was developed against myxomatosis and rabbit hemorrhagic disease (RHD). The vaccine was based on a recombinant myxoma virus (MV) expressing the RHDV capsid protein (Torres et al., 2000). Laboratory and field trials revealed that the vaccine was safe and effective against challenge with viruses and exhibited a limited horizontal transmission capacity (Torres et al., 2000). The possibility of the viral- vectored vaccine to include several *Eimeria* antigens eliciting protective cellular immunity will be an interesting research.

3.4.4 Live bacterial vector vaccine

Attenuated, especially genetically attenuated bacteria are being engineered to deliver heterologous antigens to stimulate mucosal, humoral and cellular systemic immunity. Presently, there are no licensed vaccines based on attenuated bacteria as vectors. Several papers reported the efficacy using recombinant non-antibiotic *Escherichia coli* (Yang et al., 2010), or recombinant *Mycobacterium bovis* BCG (Li et al., 2013) or live *Lactococcus lactis* (Ma et al., 2013) as vector expressing antigens from *Eimeria* parasites in chickens, but the efficacy was not comparable to live oocyst vaccines. The quality and quantity of protective immune responses elicited by these antigens are unknown. Novel strategies for enhancing immune responses are delivering DNA vaccines by bacterial vectors into the host cell (Du et al., 2005) and fusing the exogenous antigen to an exporting protein like ClyA reducing the metabolic burden of bacterial vectors (Chinchilla et al., 2007).

3.4.5 Live oocysts as a vaccine vector and beyond

One of the milestones in *Eimeria* research is the successful transfection of this enormously important group of parasites (Chapman et al., 2014). Transfection refers to the introduction of exogenous DNA or RNA into cells by chemical, biological or physical means; in transfection of *Eimeria*, a physical mean called electroporation is used to introduce the exogenous DNA or RNA (Chapman et al., 2013). Through transfection, the recipient cell can gain a new genetic trait, such as the ability to produce exogenous antigens. Expression of exogenous antigens is the basis for the development of transgenic *Eimeria* live oocysts as a vaccine vector. Two studies support this hypothesis: Huang et al. (2011) showed that a model antigen expressed by transgenic *Eimeria tenella* can elicit immune responses in the chicken; Clark et al., (2012) revealed that vaccination of specific pathogen-free chickens with transgenic *E. tenella* expressing *Campylobacter jejuni* antigen A induces a significant reduction in bacterial load following challenge with virulent *C. jejuni* compared with unvaccinated and wild-type *E. tenella* vaccinated controls.

In the development of a vaccine vector, a stable trait is a prerequisite. Fortunately, in the transfection of *Eimeria*, at least some of the introduced DNA can be integrated into the genome of the parasite,

leading to the establishment of a stable line after many generations of passages in the host chicken or rabbit (Yan et al., 2009; Shi et al., in press). Although fluorescent activated cell sorting, based on reporter genes like enhanced yellow fluorescent gene inserted in the transfection construct, helps selection of transfected parasites, a fast strategy is the use of drug resistant gene. In *Eimeria*, the only pyrimethamine-resistant gene of dihydrofolate reductase thymidylate synthase derived from *Toxoplasma gondii* (TgDHFR-TS) works, but in a vector to be used *in vivo*, this selection gene has to be deleted. The Cre-LoxP recombination technology, which can delete TgDHFR-TS after successful selection of transfected parasites, may be used to do the work.

Presently, transfection constructs are usually based on pre-constructed, commercially available plasmids, and contain *Eimeria*-derived elements including regulatory and signal sequences (Yin et al., 2011). Regulatory and signal sequences from related apicomplexan parasites, like those from *Toxoplasma*, work in *Eimeria*, and vise versa (Zou et al., 2009). This has greatly promoted the development of genetic manipulation in *Eimeria*. Although several promoters have been tested in *Eimeria*, a strong promoter, which enhances expression of "enough" exogenous antigens, has not been found. Another aspect to be considered in the development of a vector is that more signal sequences have to be tested, as it is not known the best location for an exogenous antigen to elicit an immunity of high quality and quantity. The third is the capacity of the *Eimeria* parasites to tolerate the exogenous protein.

In the transfection of bacteria like *E. coli*, a few hundred base pairs of replication sequence referred to as the origin of replication (*ori*) is on the plasmid, which helps replication of the plasmid in the bacterium. During the transfection of *Eimeria*, how the plasmids replicate or whether replication of the plasmid is needed remains a mysterious topic; no publication is available reporting the study of replication sequences from *Eimeria*.

The stage for introducing DNA into *Eimeria* is the sporozoite stage. Recovery of sporozoites requires a complicated process including purification and sterilization of oocysts, physical break of oocysts to release sporocysts, chemical break of sporocysts to release sporozoites, and finally purification of sporozoites by DE-52 anion-exchange chromatography. Transfection of sporocyst and/or oocyst stage is a straightforward way, which is waiting for a breakthrough as early as possible. Realization of oocyst-stage transfection will also allow analysis of expression of introduced gene during sporulation. I*n vitro* unsporulated oocysts in PBS or 2-4% potassium dichromate undergo meiosis and produce sporocysts and sporozoites, in a process which takes 1 to 5 days.

One of two essential factors for establishing transgenic *Eimeria* as a vaccine vector is how much expression of the exogenous antigen is enough to elicit enough protective immune responses, both in high quality and high quantity. Solutions include, but are not limited to, the use of strong regulatory sequences, the insertion of multiple copies by techniques such as piggyBack (Su et al., 2012) and P2A, and optimizing codon usage. The most difficult task is the manipulation of *Eimeria* to tolerate high-level expression of an exogenous protein. A solution may be the regulation of the exogenous protein secrete into parasitophorous vacuoles or even into the host cytoplasm. Of course, a related question is whether dynamics of expression controlled by stage-specific regulatory sequences will influence the immune responses. Proteomics and immune-proteomics may help resolve this issue.

The second essential factor is the location or compartmentalization of exogenous antigens. It was found that antigen compartmentalization affects the magnitude of the immune response with microneme-targeted EYFP stimulating a higher IgA response than cytoplasm-targeted EYFP (Huang et al., 2011). More work to be done in this aspect includes studies on the effect of antigen compartmentalization on quality and quantity/magnitude of humoral, cellular and mucosal protective immunity.

Realization of developing transgenic *Eimeria* parasites into a licensed vaccine vector has a long way to run. However, rapid development in the areas of "omics", including genomics, transcriptomics, proteomics, metabolomics, and of "ologies", including vaccinology, immunology, parasitology, and molecular and cell biology, will surely shorten the length of the way.

4. RABBIT HEMORRHAGIC DISEASE (RHD)

Rabbit hemorrhagic disease (RHD), caused by rabbit hemorrhagic disease virus (RHDV), is a highly contagious and fatal disease of domestic and wild rabbits, with relevant economic and ecologic

importance (Cooke et al., 2002; Ohlinger et al.,1990; Parra et al., 1990). RHD first emerged in 1984 in China, and nowadays the disease has a worldwide geographical dissemination (Abrantes et al., 2012). Furthermore, various outbreaks have been recorded from diverse and rather unusual geographical areas in recent years (Farnos et al., 2007; Forrester et al., 2008; McIntosh et al., 2007). RHDV, the prototype strain of *Lagovirus,* belongs to the Caliciviridae family (Cooke et al., 2002). The virus is nonenveloped with a polyadenylated positive-sense (+) single-stranded RNA genome of approximately 7.5 kb. Mature RHDV virions are spherical, non-enveloped particles with a T=3, icosahedral capsid whose outer diameter varies between 32 and 44 nm (Barcena et al., 2004; Prasad et al.,1994a; Prasad et al., 1994b).

RHDV strains are usually divided into two genotypes, termed RHDV and RHDVb (Dalton et al., 2012). The subclusters in the distinct RHDV groups have been termed genogroups 1–6 [G1–G6] (Le Gall-Recule et al., 2013; Le Gall-Recule et al., 2003; Le Gall et al., 1998; Nystrom et al., 2011; Wang et al., 2012). The G6 cluster is also referred to as RHDVa (Capucci et al., 1998) while the RHDVb branch is also identified as RHDV2 (Dalton et al., 2012; Le Gall-Recule et al., 2013; Puggioni et al., 2013). RHD is characterized by high mortality, 70–90% for RHDV/RHDVa and 5–70% for RHDV2. This is in particularly true in the case of RHDV2 with the poor cross-protection induced by classical vaccines based on RHDV/RHDVa.

5. STRATEGIES FOR THE CONTROL OF RABBIT HEMORRHAGIC DISEASE (RHD)

5.1 Traditional vaccine produced from tissue suspensions

Prevention and control of RHD through vaccination is critical. Due to the lack of a cell culture system for efficient virus propagation, commercially available vaccines against RHDV are produced from tissue suspensions of experimentally infected rabbits (Huang et al., 1991; Smid et al., 1991; Arguello et al., 1991). Solid protective immunity against RHDV infection is established soon (within 7–10 days) and will last for an extended period (Smid et al., 1991; Arguello et al., 1991).

5.2 Impact of recombinant DNA technology for the control of RHD

The recombinant-DNA technology provided an elegant solution for the effective vaccine of RHDV, which could not use cell culture technology for vaccine production directly. With the development of recombinant-DNA technology, there has been a spurt in the development of recombinant-DNA-based vaccines, some of which are effectively working (López-Vidal J et al., 2015; Yang et al.,2015; Spibey et al., 2012; Farnos et al., 2005; Torres et al., 2001). These have several characteristics: they are economical to be produced and safe to be handled; they are highly effective, and convenient and feasible for industrial production. For RHDV, recombinant-DNA technology could be intended to avoid two major drawbacks of the tissue-inactivated vaccine: the spread of the virus and the body immune rejection caused by tissue inactivated vaccine (López-Vidal J et al., 2015; Spibey et al., 2012). Meanwhile, recombinant-DNA technology could solve the difficulties of obtaining livers for RHDV2 vaccine with the lower mortality. The DNA-based vaccine technology represents a powerful and novel entry into the field of immunological control of RHDV.

5.2.1 The capsid protein of RHDV and its recombinant systems

The capsid protein (VP60) of RHDV is the exclusive structural protein and it can make animals produce neutralized antibodies, directly related to immune response. The humoral immunity clearly provides protection against RHDV when present (Parra et al., 1990; Laurent et al., 1994). Studies demonstrated that VP60 capsid protein can natural polymerized into virus-like particles (VLPs) not wrapped with a nucleic acid, and similar to natural RHDV virus particles in physical (Farnos et al., 2005). As a new type of vaccine, VLPs may be considered superior, as it contains no genetic materials, it can be prepared in high quantity and it has a good security and immunogenicity (Dong et al., 2006; Keim et al., 2007). RHDV VLPs has good immunogenicity, it enters into the antigen-presenting cells via endocytosis or pinocytosis, inducing humoral and cellular immune responses, respectively (Farnos et al., 2005).

Several heterologous expression systems or recombinant animal viruses have been developed to produce recombinant versions of the VP60 protein, such as *Escherichia coli* (Boga et al., 1994), insect cultured cells (Sibilia et al., 1995; Nagesha et al., 1995; Gromadzka et al., 2006; Marin et al., 1995), yeast (Boga et al., 1997; Farnos et al., 2005), plants (Mikschofsky et al., 2009; Castanon et al., 1999; Mikschofsky et al., 2009; Fernandez-Fernandez et al., 2001; Gil et al., 2006), insect larvae (Perez-Filgueira et al., 2007) and recombinant animal-derived viruses (Fernandez et a.l, 2011; Bertagnoli et al., 1996; Bertagnoli et al., 1996; Fischer et al., 1997; Barcena et al., 2000 ; Wang et al., 2012). While the fusion protein expressed in *E. coli* is highly insoluble and of low immunogenicity, active immunization can be

achieved with VLPs obtained in the baculovirus system or by using recombinant vaccinia, myxomavirus and canarypox, administered either intramuscularly or orally. Most of these systems were shown to be immunogenic and to confer protection against lethal doses of RHDV by eliciting a humoral response indicating that they are good substitutes for the tissue vaccines. Features such as low cost, high yield and ease of scaling up are amongst the most important factors for their commercial viability.

5.2.2 Recombinant DNA vaccines developed

Several vaccines have been developed, and results show that they efficiently protect the rabbits against RHDV. Rabbits vaccinated with recombinant myxomavirus expressing the RHDV capsid protein were protected against lethal RHDV and myxomavirus challenges (Bertagnoli et al., 1996; Barcena et al., 2000; Spibey et al., 2012; Torres et al., 2001; Torres et al., 2000). This type of recombinant vaccine has been developed and registered and is commercially available in several countries for administration by parenteral route. A vaccine developed in France and then marketed in some European countries is a combination of a traditional inactivated liver-derived RHD vaccine and a live attenuated myxomavirus vaccine, and can be administered by intradermal route. In addition, the VP60 structural protein has also been expressed in transgenic plants, either with a new plum pox virus (PPV)-based vector (PPV-NK), or in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter (Castanon et al., 1999; Fernandez-Fernandez et al., 2001). In both cases the immunization of rabbits with extracts of *Nicotiana clevelandii* plants infected with the PPV-NK VP60 chimera or with leaf extracts from potatoes carrying this modified 35S promoter, respectively, induced an efficient immune response that protected animals against a lethal challenge with RHDV (Castanon et al., 1999; Fernandez-Fernandez et al., 2001).

6. THE RECOMBINANT-DNA-Based RHDV VACCINE DEVELOPED BY OUR TEAM

We have developed an inactivated vaccine based on the recombinant baculovirus system expressing VP60 (BAC-VP60), named as Rabbit Haemorrhagic Disease Virus Baculovirus Vector Vaccine, Inactivated (Strain BAC-VP60).

Firstly, the recombinant protein was expressed effectively. We constructed the recombinant baculovirus plasmid containing VP60 referred as Bacmid-VP60. Then we transfected it into sf9 cells. VP60 expressed efficiently and could self-assemble into RHDV virus-like particles (VLPs), similar in size to the wild type VP60 (40 nm). The recombinant VP60 protein synthesis was confirmed by indirect immunofluorescent assay, Western blot and hemagglutination test. Therefore, this recombinant protein was used as vaccine antigen in next study.

Secondly, we also made great efforts to study the production process. The processes were optimized for the culture medium for cells, the initialinoculated concentration of cells, the proper harvest time for vaccine antigen, the satisfactory culture method, the methods of inactivation and vaccine storage. The initial concentration of Sf9 cells was no less than 1×10^6 cells per milliliter. Then, Sf9 cells were about 2×10^6 cells per milliliter after culturing for 24 hours and they were inoculated with the recombinant baculovirus BAC-VP60. After another continuous culture of 5 days with roller bottle cell culture or suspension culture, vaccine antigen was harvested. The vaccine antigen titre was determined before inactivation by calculating the HA titre, which should be higher than 1/256. Vaccine antigen was inactivated by formaldehyde at the final concentration of 0.2%, at 37 °C for 24 hours. Then it was mixed with Al(OH)3 and stored.

Meanwhile, the recombinant baculovirus BAC-VP60, RHDV isolate WF/China/2007 and Sf9 insect cells for vaccine production were serially passaged and comprehensively identified, respectively. The recombinant baculovirus BAC-VP60 as virus seeds for vaccine production showed stability and strong immunogenicity. Meanwhile, this isolate as seed for vaccine verification also showed the stability of toxicity after serial passages. Biological characteristics of Sf9 cells within 60 generations were stable, and they showed no oncogenicity.

Based on the above research, five batches of final vaccine were prepared in the laboratory. Tests were carried out to check their physical characteristics, their sterility, and to assess the volume and the formaldehyde residual quantity, and they all passed inspection. The safety and potency tests of the vaccine were also carried out on each batch of final vaccine.

For safety tests, rabbits were given one millilitre doses of the vaccine by subcutaneous injection. Moreover, the following tests, in particular, were carried out: the safety of the administration of one

dose; the safety of the administration of an overdose (at least two doses of vaccine); the safety of the repeated administration of one dose. Results showed that the vaccine was safe to all breeds of rabbits, namely 2 ~ 3 month healthy meat rabbits, rex rabbits, wool rabbits, pregnant rabbits and 20-30 day old rabbits. Vaccinated rabbits did not show any changes in their general conditions or abnormal local or systemic reactions for the whole test duration. Furthermore, the feed conversion efficiency (feed conversion ratio) of the immunized rabbits and the control rabbits showed no significant difference.

Next, the efficacy of the five batches of vaccines was determined in rabbits in a RHDV challenge. The vaccines could all provide protection against RHDV. Meanwhile, in the unimmunized and challenged group, five rabbits all died after RHDV challenge. There was no difference of efficacy in the different batches of vaccines, thus demonstrating that the prepared vaccine was stable. Our results also showed that the recombinant-DNA-based vaccine and the tradition tissue vaccine showed equal immunizing effects, all gaining good immunizing protection against RHDV.

Tests for the duration of immunity were also carried out using a batch of vaccine. Vaccinated animals quickly produced solid protective immunity against RHDV infection within 7 days, and experimental data indicated that protection could last for a long period of 7 months. The vaccine should be stored at 2–8°C up to 24 months.

Like inactivated vaccine, genetically engineered vaccine could induce effective humoral immunity and cellular immunity and effectively prevent RHD. The antibodies (IgG and IgM) and cytokines (IL-2, IL-4 and IFN-γ) in serum increased significantly ($P<0.05$) compared with those of the control group post immunization. After challenge, no motility occurred in rabbits immunized with genetically engineered vaccine and inactivated vaccine.

Finally, six batches of intermediate trial production of vaccine were immunized in six warrens respectively in Jiangsu, Shandong, Zhejiang provinces. In field, the safety and potency of the vaccine in rabbits were tested by evaluating their daily health, reproductive performance and the protective rate. Results showed the vaccine was safety, quite effective and the protection could last for 7 months.

7. PERSPECTIVE

Coccidiosis remains a major disease affecting rabbits. Despite drug resistance by *Eimeria* spp. is widely distributed, anticoccidial drugs will still be used to control the disease for a long time. It is predictable that the live attenuated vaccines will be developed rapidly and commercially available in a few years. For the attenuated vaccines based on precocious lines are low-productive and not cost-effective (Blake et al., 2014), vaccines based on recombinant DNA technology are focused on by many researchers now; and great progress is promising with the advance of related technologies especially the sequencing and annotation of the genomes of *Eimeria* species.

The recombinant-DNA-based vaccine producing VLPs could provide very strong immunogenicity and overcome the disadvantage of vaccines produced using the organs of infected rabbits, including the biological safety, contaminant residues and animal welfare. Recombinant DNA vaccines are demonstrating their advantages when more immunoprotective antigens are being found and reliable vectors are being tested. Though it is a longer way for recombinant DNA vaccines against rabbit coccidiosis, the baculovirus vector-based vaccine for viral hemorrhagic disease is on its final step to the market.

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