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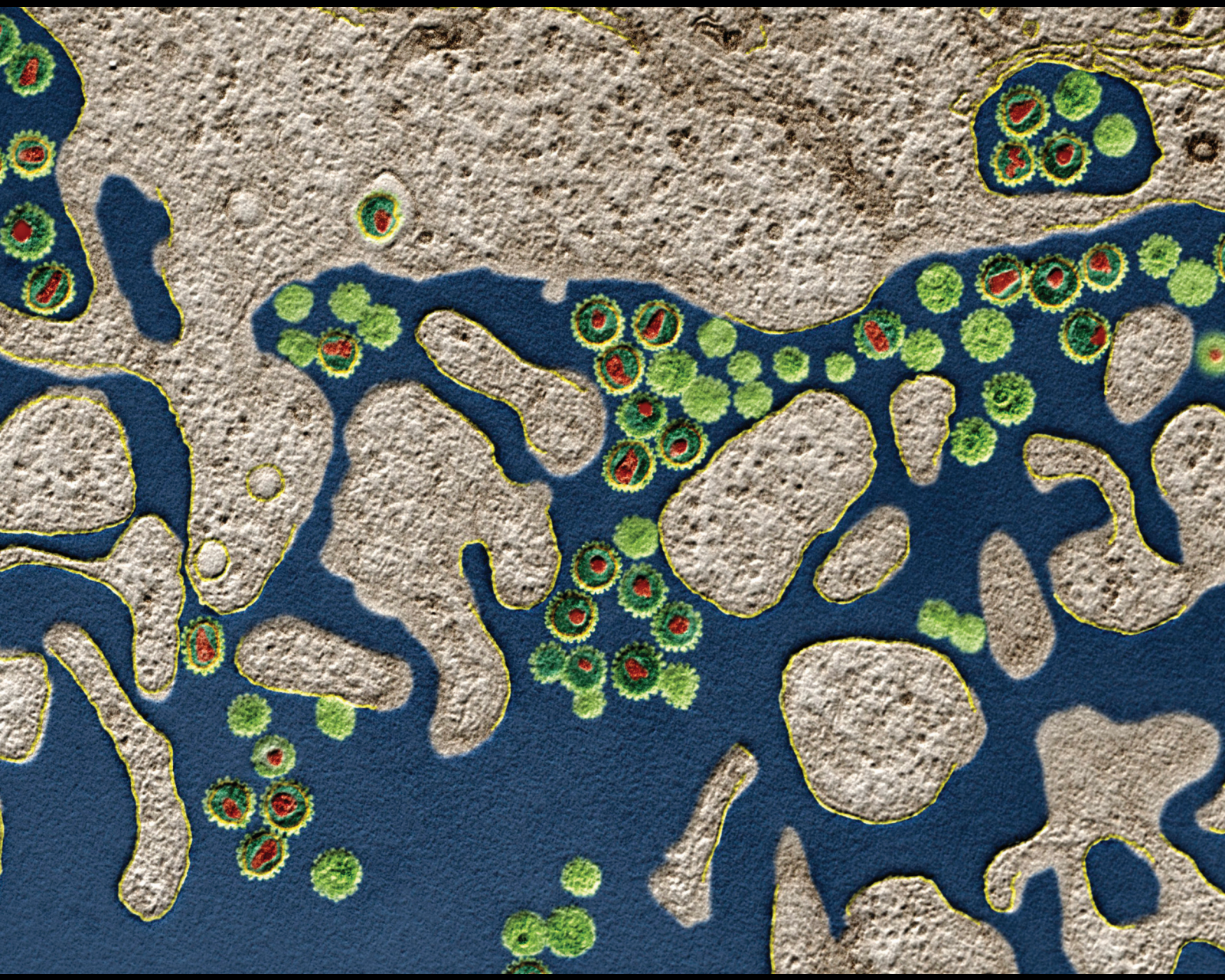
Guest Editors: Xiaofeng Ren, Hyun Lillehoj, Juergen Richt, Mohammed Mahbub Alam, Yong-Suk Jang, and Georg Herrler



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Review Article

Current Status for Gastrointestinal Nematode Diagnosis in Small Ruminants: Where Are We and Where Are We Going?

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Gastrointestinal nematode (GIN) parasites pose a significant economic burden particularly in small ruminant production systems. Anthelmintic resistance is a serious concern to the effective control of GIN parasites and has fuelled the focus to design and promote sustainable control of practices of parasite control. Many facets of sustainable GIN parasite control programs rely on the ability to diagnose infection both qualitatively and quantitatively. Diagnostics are required to determine anthelmintic efficacies, for targeted treatment programs and selection of animals for parasite resistant breeding. This review describes much of the research investigated to date to improve the current diagnostic for the above practices which is based on counting the number of parasite eggs in faeces.

1. Introduction

Small ruminant (goats and sheep) production systems worldwide are significantly constrained by gastrointestinal nematode (GIN) parasites, reducing meat, milk, and fibre production [1–3]. Anthelmintic treatment is the most cost-effective current control method in many farm enterprises. However, the ability of parasites to quickly develop resistance to these compounds, particularly if animals are under-dosed or treated under preventative and suppressive treatment regimes, suggests alternative and/or complementary sustainable control programs require adoption [4–8].

Sustainable control programs and guidelines (Table 1) have been introduced to small ruminant producers to prolong the effectiveness of anthelmintics whilst reducing the production loss caused by GIN parasite infections (reviewed by [9–13]). These programs/guidelines involve a combination of chemical and nonchemical strategies to adequately control GIN parasites; however, the success or otherwise of these programs is reliant on an ability to diagnose the parasitic infections qualitatively and quantitatively to estimate the severity of the infection and the potential cost to production traits [14, 15].

Alternatives and/or complementary solutions to anthelmintic use are also under investigation with breeding for genetic resistance of the host to infection already in commercial application (http://www.nsip.org; http://www.sheepgenetics.org.au/; http://www.signetfbc.co.uk/). Diagnostic assays that reliably measure the level of genetic resistance are required for such applications and a range of indicators have been or are being investigated and will be reviewed below.

2. Ideal Characteristics of Diagnostic Markers for GIN Parasite Infections

The ideal diagnostic test for GIN parasite infections has been described as having the following characteristics [14, 16]:

(1) reliability in terms of accuracy and repeatability
(2) ease of measurement

Table 1: Programs/guidelines promoting sustainable GIN parasite control.

Programs	Country	Resource
Wormboss	Australia	http://www.wormboss.com.au/
Wormwise	New Zealand	http://www.beeflambnz.com/farm/tools-resources/wormwise/
Sustainable control of parasites (SCOPS)	United Kingdom	http://www.scops.org.uk/

(3) cost effectiveness

(4) the ability to be used on-farm.

Additional characteristics for diagnostics that could be used in programs aimed at breeding for resistance include

(5) neutral or positive correlations with production traits,

(6) moderate to high heritability.

Although these parameters are easily defined, in practice, there is no universal marker currently that meets these characteristics for GIN parasite diagnosis. The most common test in use currently is the Worm Egg Count (WEC). The WEC test is promoted by government and nongovernment agencies in many commercialised small ruminant industries (Table 1). WEC has many technical disadvantages (Section 2.1) and poor adoption rates by farmers and is, therefore, considered an underutilised diagnostic in many small ruminant production systems [17]. As such, substantial research is being undertaken to find a more usable and accurate measure of infection intensity to assist the development and adoption of sustainable GIN parasite control programs. This review will examine diagnostic markers currently in use and those that are being considered or are currently under development with the potential to replace/improve WEC as a diagnostic marker. These diagnostic markers often involve components of the animals immune system and can be categorised under three major areas: infection-related, immune-related, and inherent markers.

2.1. Infection-Related Diagnostic Markers of GIN Parasitic Infection. Infection-related markers have been characterised by their dependence on current infection and are related to parasite induced pathology such as blood loss or a measure of parasite burden such as number of eggs. Table 2 summarises the markers associated with infection investigated to date.

2.1.1. Worm Egg Counts. WEC involves counting parasitic eggs in freshly collected faeces. Distinctive eggs from GIN parasites such as thin-neck intestinal worm (*Nematodirus* spp.), tape worm (*Moniezia expansa*), and whip worm (*Trichuris ovis*) can be easily identified. However, the worm species that have been identified to cause the largest economic impact on small ruminant production: *Trichostrongylus* spp., *Haemonchus contortus*, and *Teladorsagia circumcincta* are difficult to distinguish by egg morphology and, therefore, require further processing. Identification to at least genus level is important for correct anthelmintic selection but less important for the use of diagnostics as a selection marker in breeding the host for resistance because resistance is usually expressed to a range of parasite species [18–20].

Previously, identification of eggs to species level has involved larval culture requiring at least 7–10 days for egg hatch; however, recently, polymerase chain reaction (PCR) techniques have transformed testing, allowing species identification in less than 24 hours [21–23]. This is an exciting development particularly its potential to identify GIN parasite resistant to anthelmintic classes directly from the faeces as farmers perceive the current protocol to detect anthelmintic resistance involving mini-sheep trials as too difficult and time-consuming [17].

Other research involved in transforming WEC diagnostic into a more time and cost efficient method is to use fluorescently labelled lectins which bind differentially to different GIN parasite species eggs [24–26]. A lectin test for the identification of *H. contortus* eggs is now commercially available through the Australian Government (http://www.sheepcrc.org.au/management/worms-flies-lices/rapid-laboratory-test-for-haemonchus-in-worm-egg-counts.php). Both improvements (DNA technology and lectin staining) do not overcome the collection of faeces and both must be performed off-farm by laboratory experts; however, there are reports for the potential of some DNA-technology platforms to become on-farm diagnostics in the future [27].

Other drawbacks of WEC tests commonly cited in the literature include the low correlation between eggs and worm burden for low fecund worms such as for *T. circumcincta* and *T. colubriformis,* inability to detect worms in hypobiosis, and the high variability of eggs between individual subsamples due to aggregation [15, 28]. In addition, collection of faeces is time and labour intensive and often an unpalatable technique for many farmers and animal health professionals. Despite these disadvantages, WEC tests are promoted commercially as a diagnostic tool to determine if anthelmintics are still effective on farming properties, for targeted anthelmintic treatment strategies and for the selection of animals for breeding parasite resistance through estimated breeding values in many countries [15].

2.1.2. Blood Loss. Measurement of blood loss can indicate the presence of infection with a blood-feeding GIN parasite, such as *H. contortus*, and this parameter has been utilised as a diagnostic tool to target animals for anthelmintic treatment and selection of parasite resistant sheep. Tools available to measure blood loss include packed cell volume (PCV), the Haemonchus dipstick, and FAMACHA©. PCV is an indicator of anaemia and is usually used in conjunction with WEC to diagnose *H. contortus* infections for research purposes. PCV involves taking blood samples and measuring the percentage of red blood cells. The measurement of blood loss has an

Table 2: Potential and commercial infection-related markers for the diagnosis of GIN parasite infection.

Trait	Description	Advantages	Disadvantages	Application
Worm egg counts (WEC)	Phenotypic, WEC test where the amount of eggs in the faeces is an indicator of adult infection. Eggs counted by microscopy.	(i) Direct measure of infection (ii) On-farm readout (after training)	(i) Labour intensive and involves collection of faeces from the rectum (ii) High variability in counts (influenced by diet, age, degree of infection exposure, hypobiosis, and adult density)	Commercial; http://www.wormboss.com.au/tests-tools/tests/worm-egg-counting.php
	Genotypic, egg counts in faeces detected by DNA analysis.	(i) Direct measure of infection (ii) Quicker than phenotypic WEC (iii) Potentially cheaper as trained experts in worm egg counting are not required	(i) Qualitative only, can't determine infection level and hence resistance status (ii) Involves collection of faeces (iii) Off-farm	Research
	Phenotypic, lectin assay, use of antibodies to detect eggs in faeces.	(i) Direct measure of infection (ii) Can distinguish between species by egg morphology	(i) Involves collection of faeces (ii) Off-farm (iii) Currently only specific only for *H. contortus*	Commercial: http://archive.sheepcrc.org.au/management/worms-flies-lices/rapid-laboratory-test-for-haemonchus-in-worm-egg-counts.php
Blood loss	Phenotypic, packed cell volume (PCV); involves calculating the percentage of red blood cells from a blood sample.	(i) Direct measurement of infection (ii) Earlier detection of infection compared to WEC	(i) Invasive (ii) Not specific to *H. contortus* infection-other causes of blood loss (iii) Off-farm	Research
	Phenotypic, haemonchus dipstick; blood loss measured by amount detected in faeces.	(i) Quick (ii) No need for highly trained laboratory technician (iii) On-farm	Blood loss is nonspecific therefore recommended to be used in conjunction with WEC	Commercial: http://archive.sheepcrc.org.au/management/worms-flies-lices/haemonchus-dipstick-test.php
	Phenotypic, FAMACHA©; involves matching eye lid colour with coloured chart indicating level of anaemia.	(i) Noninvasive (ii) Quick (iii) No need for highly trained laboratory technician (iv) On-farm	(i) Nonspecific (other disease induce anaemia) (ii) Subjective measurement	Commercial: http://www.acsrpc.org/Resources/famacha.html
Faecal odour	Phenotypic, infected and uninfected faeces have differential odours detectable by canines.	(i) Detects infection earlier than WEC (ii) Potential for on-farm testing	Can odour be used to determine infection levels? (i) Cost and time to train canines or development of artificial nose?	Research
Animal behaviour	Phenotypic, global positioning systems (GPS) to detect reduced movement found in resistant animals compared to susceptible.	(i) Potential for on-farm testing (ii) Noninvasive	(i) Cost of technology (ii) Indirect measure	Research
Weight loss	Phenotypic, performance based marker which involves calculation of body weight gain based on food efficiency rates.	(i) Potential for on-farm testing (ii) Non-invasive	(i) Cost of technology (ii) Indirect measure	Research HappyFactor
Worm burden	Phenotypic, measuring the number of GIN parasites in the stomach at post mortem.	(i) Distinguish nematode species (ii) Direct measurement of burden (includes exsheathed L3, L4 and adult GIN parasites)	(i) Measurement is terminal (ii) Time consuming (iii) Impractical	Research
Worm weight	Phenotypic, weight of the total amount of GIN parasites collected at post mortem.	Direct measurement of burden (less intensive than counting worm numbers)	(i) Measurement is terminal (ii) Time consuming (iii) Impractical	Research

advantage over WEC in that it enables early detection of *H. contortus* infection as blood loss occurs prior to egg production [15]. However, blood sampling is labour intensive for farmers and blood loss can be due to infections other than *H. contortus* such as a severe coccidial or bacterial enteritis infections [14, 29].

To overcome the labour-intensive nature of measuring PCV, the Australian Government commercialised a product, the Haemonchus Dipstick, which measures blood loss by quantifying the amount of blood in the faeces [29]. Although this is a much more practical way of measuring blood loss for producers and can identify infection earlier than WEC to prevent sudden disease outbreaks in high risk periods [11], the concern of the exact origin for the blood loss remains [29]. Therefore, a WEC test is recommended to be used to confirm that blood loss is due to *H. contortus* infection and not another underlying physiological problem or infection [29].

FAMACHA© is a *H. contortus*-specific diagnostic tool developed in South Africa, which uses the colour of the eye as an indicator of anaemia due to the parasite's blood feeding activity [30–32]. FAMACHA© is a five-point scoring system in which goats or sheep scored at 3–5 are deemed at risk of disease and require treatment [30]. Trials using FAMACHA© as a selection tool in targeted selection anthelmintic treatment programs have shown reductions in the number of anthelmintic treatments with minimal production losses [31–33]. The major drawback of this tool as with the Haemonchus Dipstick is that anaemia is not exclusively caused by *H. contortus* infections. Despite this, a recent report describes high adoption rates in the southern states of the United States, with 5,000 small ruminant producers being trained through workshops to diagnose anaemic sheep based on FAMACHA© and the purchase of 20,000 FAMACHA© cards (reviewed by [13]).

FAMACHA© can also be applied as a tool to breed *H. contortus* resistant sheep. Trials in United States and South Africa have shown moderate heritability values similar to WEC and PCV and positive associations with increased production traits [34, 35]. The relative ease and low cost of the FAMACHA© system are advantageous for implementation, but it is only effective in areas where livestock are dominantly infected by *H. contortus,* and, as outlined above for the Haemonchus Dipstick, it lacks specificity.

2.1.3. Faecal Odour. Only one research group has investigated the use of odour to detect GIN parasite infections, by training canines to detect the scent [36]. The limited research in odour detection of GIN parasites is surprising, given anecdotal evidence of producers being able to smell GIN parasite infections, in combination with the routine use of canines to detect explosives, illegal drugs, and human remains and detection of humans with ill-health (reviewed by [37, 38]). The work indicated that this method for detecting GIN parasite infection has high sensitivity, detecting *T. circumcincta* infections in sheep as early as seven days post oral infection with an 85% accuracy [36], potentially meaning that the odour diagnosis of GIN parasite infections could occur before egg laying and before clinical symptoms appear. Richards et al. [36] suggested that further work should focus on defining the chemical composition of the detectable odour to transform this knowledge into a detection device. The potential of this device to operate on-farm is promising, however, whether level of odour correlates strongly with infection level, a prerequisite for likely commercial success, requires investigation.

2.1.4. Animal Behaviour. Parasite infections influence animal behaviour and some studies have been conducted to determine whether changes in behaviour would allow identification of parasite resistant or susceptible animals [39–41]. A recent study using a global positioning system (GPS) tracking device to monitor the behaviour of sheep under natural field infection conditions found that animals with higher WEC (more susceptible) travelled significantly greater distances than animals with lower WEC [42]. Theories as to why animals with heavier infections travelled greater distances included that these animals need to graze for longer periods to cope with protein loss due to infection and visit water sources more frequently due to an increased thirst [42]. GPS devices are still too expensive to be considered as a commercial tool for diagnosing GIN parasite resistant sheep, but they are an excellent research tool and further studies would be of interest.

2.1.5. Weight Loss. The Happy Factor is a performance based marker which involves calculation of body weight gain based on food efficiency rates [43]. Animals which do not reach the predicted target are treated with anthelmintics. An advantage of using body weight scores is that condition loss is an early symptom of infection and is an economically important trait [43]. A recent study based in a commercial setting in the United Kingdom showed that this approach resulted in a 50% decrease in anthelmintic treatment [44]. However, animals needed to be weighed fortnightly, which the authors acknowledge may not be practical in all livestock production settings.

2.1.6. Worm Number and Weight. Worm number and weight is the most direct measurement of determining GIN parasite infection levels and is consequently considered the gold standard for estimating parasitic worm burden [14]. Worm number involves counting the parasites in the gastrointestinal tract; a proportion of the worm population is usually measured as an estimate of the total [45]. Infective larval stages (L3, L4) and adult female and male GIN parasites can be enumerated and differentiated by morphology providing important information on the target of host resistance and are required for testing the activity of new anthelmintics [14]. Measuring worm weight involves collecting the parasites and recording their bulk weight. However, these markers can obviously only be taken at necropsy, are labour intensive, and are consequently only useful for research purposes.

2.2. Immune-Related Diagnostic Markers of GIN Parasitic Infection. It has long been established that the immune system plays a major role in resistance to GIN parasite infection

[46, 47]. Immune cell depletion and cytokine profile studies have shown that resistance to infection is dependent on the induction of the type two (T2) or the "allergic" phenotype response [48–50]. The Th2 immune response is characterised by the differentiation of T cells that produce the cytokines IL-4, IL-5, IL-9, and IL-13, the proliferation and recruitment of effector cells, and eosinophils, mucosal mast cells and globular leukocytes, along with increased mucus secretion and generation of parasite-specific antibodies such as IgA, IgG_1 and IgE [48, 51].

However, it is now recognised that manifestations before and after the T2 "allergic" immune response are also vitally important for successful control of infection [52]. Detailed studies in mice have shown the importance of correct innate receptor expression and functioning to recognise pathogen-associated molecular patterns and damage-associated molecular patterns (reviewed by [53]). Furthermore, the downregulation of the immune response through the recruitment of T regulatory cells is important in dictating disease outcomes. Innate receptors such as the toll-like receptors and alarmins have recently been identified in sheep [54, 55] and the suppression of the T regulatory pathways has been described as a mechanism for susceptibility in Scottish Black face sheep infected with *T. circumcincta* [56].

Thus, given the involvement of the abovementioned cells and mediators identified in development of immunity to GIN parasites, many of these immune parameters have been investigated as selection tools for identification of GIN parasite resistant sheep. However, due to the diverse and complex nature of the immune response, few of these parameters have as yet been substantiated as GIN parasite resistant markers [57, 58]. Table 3 outlines the immune-related selection parameters investigated to date.

2.2.1. Antibodies. Local and peripheral antibody production is associated with GIN parasiteinfection with high levels of parasite-specific IgG_1, IgE, and IgA correlating with low parasite burden [50, 59]. The role of each antibody isotype in resistance is not fully understood but IgA has been consistently correlated with reduced worm length and fecundity in sheep infected with *T. circumcincta* [60–62]. In *H. contortus* infections, serum IgA and IgG_1 were consistently higher in genetically resistant sheep compared to randomly bred animals indicating the potential for selection of resistance based on high parasite specific antibody titre [50]. However, the procedures for measuring antibodies in the periphery, while accessible, often give an inaccurate representation of antibody levels (e.g., mucosal) at the infection site [63–66]. Additionally, there is little evidence to support a role for serum antibodies in resistance as opposed to localised antibody production at the site of infection which is more likely to influence the resistance status of an animal [67].

To overcome these issues, other sources of antibodies besides blood have been examined. In faeces, IgG_1 and IgA can be detected in resistant-bred animals based on low WEC for *H. contortus* [50]. However, false positive readouts due to nonspecific antigen-protein binding terminated development of an assay [68]. The revelation that protective antibodies against larvae potentially derived from the gut associated lymphoid tissue in the intestinal mucosa could be easily detected in the saliva has resulted in the commercialisation of a diagnostic test, the CarLA Saliva Test, for selection of GIN parasite resistant animals [58, 66, 69]. Detection of salivary antibodies is advantageous in comparison to WEC due to earlier detection (at the L3 stage rather than the adult) and saliva from sheep is more appealing for producers to collect than faeces. However to develop an antibody response animals require prior and repeated exposure (not suitable in young animals less than six months which are at most risk) and a certain infection threshold for optimal detection [58], and these limitations may make it impractical for widespread industry adoption.

2.2.2. Eosinophilia. Peripheral blood eosinophilia is associated with GIN parasite infections and has been consistently reported to be higher in sheep resistant to *H. contortus*, *T. circumcincta*, and *T. colubriformis* [70–73]. Such findings led to the evaluation of peripheral blood eosinophilia as a potential marker of GIN parasite resistance. Results correlating blood eosinophilia during *H. contortus* infections with low WEC have been inconsistent (reviewed by [28]), but promising correlations have been found in other GIN parasite infections including *T. colubriformis* and *T. circumcincta* [74]. An early study showed that sheep which responded strongly to vaccination with irradiated *T. colubriformis* had higher blood eosinophilia than low-vaccine responder sheep [71]. This was supported by an extension study which showed that, following vaccination and challenge with the mitogen phytohaemagglutinin, blood eosinophilia was highly correlated to resistance to *T. colubriformis* in random-bred sheep [72]. However, the estimated heritability of blood eosinophilia for selection of resistant sheep was found to be only 43% as effective as using WEC as a selection parameter [75]. Animal behavioural studies have identified that resistant sheep which had increased locomotive patterns also had higher basal circulatory eosinophilia concluding that parasite resistant sheep were also resistant to stress [41]. These results supported those of earlier research in which it was noted that animal handling in cattle generated increased blood basal levels of eosinophils [76]. A more recent study in Scottish Black-faced sheep infected with *T. circumcincta* also found a strong correlation between eosinophilia and resistance and found that the relationship had similar heritability as WEC [60]. However, this relationship with eosinophilia and resistance was age-dependent, existing only in lambs aged 3–7 months [60]. In general, like serum antibodies, the value of peripheral eosinophils as a marker of GIN parasite resistance is confounded by the dynamic nature of the immune system and the changing relationship between the host and parasite interaction [77].

2.2.3. Ghrelin. Ghrelin is a satiety-regulating hormone, stimulating appetite and the release of growth hormones [78]. In sheep, reduced appetite is a symptom of GIN parasitic infection. Recent work has shown that *H. contortus* and *T. colubriformis* resistant and susceptible lines of Merino sheep

Table 3: Potential and commercialised immune-related markers of GIN parasite resistance.

Trait	Description	Advantages	Disadvantages	Application
Serum antibodies	Phenotypic, enzyme-linked immunosorbent assay (ELISA).	Routine laboratory procedure	(i) Invasive sampling (ii) Off-farm (iii) Not sensitive to infection level (iv) Transient up regulation	Research
Salivary antibodies		(i) Relatively easy collection (ii) Routine laboratory procedure	(i) Off-farm (ii) Requires certain level of infection for detection	Commercialised http://www.kelso.co.nz/partners/carla%c2%ae-saliva-test-measuring-parasite-immunity-in-sheep/
Faecal antibodies		(i) Relatively easy collection (ii) Routine laboratory procedure	(i) Off-farm (ii) Involves faecal collection (iii) Low accuracy	Research
Blood eosinophilia	Phenotypic, morphological cell differentiation after staining.	Routine laboratory procedure	(i) Invasive sampling (ii) Off-farm (iii) Trained technician required to count eosinophils (iv) Transient up regulation	Research
Ghrelin levels in blood	Phenotypic, ELISA platform. Higher levels in susceptible sheep following infection.	Routine laboratory procedure	(i) Invasive collection (ii) Off-farm (iii) Transient up regulation (iv) Only tested in resistant and susceptible lines	Research
Cutaneous hypersensitivity reactions	Phenotypic, cutaneous injection of sensitised antigen to measure immune function.	(i) Tests responds to a range of diseases (ii) Noninvasive readouts (iii) Potential for on-farm development	(i) Involves injection of antigens into animals (ii) 2–24 hr time delay for readout	Research

are divergent in their ghrelin expression [79]. Resistant sheep were observed to have lower basal levels, but following GIN parasite challenge the resistant sheep had higher ghrelin expression (gene and protein) early postinfection than susceptible animals [79]. The function of ghrelin in resistance to GIN parasites is still under investigation but it is believed that the interaction could be direct as ghrelin has previously been shown to have anti-inflammatory properties [80, 81] and immune cells circulating in the blood were found to express ghrelin receptors [79]. A competitive ELISA to detect circulating ghrelin levels has been developed [79] but heritability and the association with increased animal productivity will need to be investigated to order to determine its effectiveness as a marker of GIN parasite resistance.

2.3. Diagnostic Based on Inherent Markers. Inherent markers of GIN parasite resistance are categorised as innate markers independent of infection and age of the animal. Consequently, markers based on inherent traits are only useful for breeding of parasite/disease resistant animals. Inherent markers which have been investigated are discussed below.

2.3.1. Blood Type. Early work suggested a link between sheep blood type and resistance to GIN parasites [82]. In sheep, haemoglobin is controlled by two major alleles A and B [9]. Studies have shown that sheep with blood type HbAA are more resistant to *H. contortus* and *T. circumcincta* infections than blood types HbAB and HbBB [82–84]. A similar study investigated blood type as a factor contributing to the responder/nonresponder phenomenon in vaccinated sheep against *T. colubriformis* [85]. Results showed that blood type could not be used to predict if a sheep would respond to vaccination as no associations were found between blood type and WEC during either primary or secondary infections [85]. Further research in this area has not supported the relationship between blood type and resistance to GIN parasites [9].

2.3.2. Immune Cell Markers and Cytokines: Major Histocompatibility Complex and Interferon Gamma. The major histocompatibility complex (MHC) and interferon gamma (IFN-γ) genes have had the most attention as candidate genetic markers of GIN parasite resistance. Investigations of polymorphisms within the genes that control MHC class I and II expression stemmed from mice and guinea pig studies in which associations between MHC class II polymorphisms and susceptibility to *Trichinella spiralis* and *T. colubriformis* were identified [86, 87]. Ovine MHC differs from humans and mice in that the MHC class II is only encoded by two genes, *HLA-DQ* and *HLA-DR* [88]. Most attention has been focused on finding associations between *HLA-DR* isoforms and resistance as it is more polymorphic than *HLA-DQ* and is highly expressed on antigen-presenting cells [87]. The translation from rodent models to sheep produced inconsistent results with some researchers finding significant associations between gene variants of MHC and parasite resistance [89–93], whereas other researchers did not [87, 94].

A recent study combining quantitative trait loci (QTL) from cattle, mice, rats, humans, and sheep associated with resistance to internal parasite identified 14 common pathways, four directly involving MHC class II expression [95]. This study also reported the INF-γ pathway to be associated with parasite susceptibility supporting earlier genomic work [96–98].

Identifying single gene markers associated with resistance to GIN parasites is difficult as resistance to parasites is considered to be polygenic with hundreds to thousands mutations responsible for the resistant phenotype [99, 100]. However, research continues in the area of genetic markers as they have the advantage over phenotypic markers of measurement prior to birth [87], meaning that producers can make productivity decisions early. Traditionally, application of genetic tools to the selection of animals has been hampered by costs. However, genetic testing for the selection of enhanced animal production traits has now become relatively inexpensive with the development of the ovine single nucleotide polymorphism (SNP) CHIP (OvineSNP50 genotyping BeadChip, Illumina). While the expense of genomic technology has reduced, substantial and continuous investment is essential as large reference and validation animal flocks which closely represent the within and across breed diversity for given traits are required to increase the accuracy of genomic predictions before new genetic traits can enter the industry [101].

2.3.3. Markers of Immunocompetence/Disease Resistant Animals. Breeding for resistance to one infection may result in susceptibility to other pathogens. This statement is based on the theory that natural selection has stabilised intermediate levels of antibody and cell mediated responses to enable an organism to survive against a range of diseases [102]. Consequently, work is now being focused on finding immune traits that give an indication of the overall responsiveness of the immune system. This has been termed immunocompetence.

Recent work in the pork industry has focused on the identification of immunocompetence using traits that are easily measured and heritable [103, 104] and has identified a range of measurable immune traits that are strongly heritable by measuring the type and level of immune cells in blood samples as well as the immune cell's ability to respond to *in vitro* stimulation. Whether these traits can predict disease resistance is still under development.

Cutaneous hypersensitivity reactions are routinely used in humans to determine allergic responses and involve injection of antigens to stimulate a localised inflammatory response. An extension of these studies is whether these inflammatory responses to certain antigens predict the susceptibility or resistant status of animals. In the Canadian dairy industry, researchers have measured delayed hypersensitivity reactions after cutaneous antigen injections to create individual estimated breeding values of cell mediated immunity [105]. These values and estimated breeding values for antibody responses have been correlated to the prevalence of diseases such as mastitis [106]. Additionally, several immune traits measured in the serum have been associated with dairy cattle health in Scotland with higher ratios of CD4+ : CD8+ T lymphocytes associated with reduced occurrences of subclinical mastitis during the lactation period [107].

Cutaneous hypersensitivity reactions have also been investigated in the small livestock industry. An early study examined cutaneous hypersensitivity reactions as a diagnostic for the bacterial infection, *Chlamydia psittaci*, with sheep giving a positive wheal reaction (an increase in eyelid skin thickness), correlating with a decrease in spontaneous lamb abortions [108]. Cutaneous hypersensitivity reactions have been assessed as a potential tool for the identification of sheep resistance to *T. colubriformis* and *H. contortus* infection [72, 109, 110]. Rothwell et al. [72] investigated immune responsiveness in *T. colubriformis*-resistant and susceptible lines of sheep, measuring blood eosinophilia following an intradermal injection of exsheathed L3 (exsheathed L3) and suggest that cutaneous hypersensitivity reactions may be a reliable way to measure the immune system's ability to respond effectively to disease and potentially distinguish between disease resistant and susceptible animals.

Cutaneous hypersensitivity reactions may be a valuable research tool for identifying differential immune responses to various stimuli, due to the relative ease of data collection and sample site monitoring. As research has now implicated an array of immune pathways responsible for resistance to GIN parasites [97, 98], cutaneous hypersensitivity reactions have the potential to explore these mechanisms in more detail in a nonterminal manner and with further development, potential on-farm application. However, limited research to date has focused on cutaneous hypersensitivity reactions to discern parasite resistant animals and the potential of this approach remains unknown. The cost-benefit ratio for producers will also need to be explored and may only be suitable for certain animal production industries.

3. Conclusion

Currently there are two primary reasons for use of a diagnostic marker to detect GIN parasites in small ruminants:

(1) conserving the effectiveness of anthelmintics,

(2) breeding animals with resistance to infection.

Advances in this field have provided a number of diagnostics that are excellent for laboratory-based research with recent molecular advances improving the accuracy and cost-effectiveness of larval identification. However, advances in practical on-farm diagnostics suitable to replace WEC have been limited with many commercialised products being recommended to complement rather than replace WEC. However, a number of immune-based diagnostics show some promise and further understanding of the parasite epidemiology; infection and immune responses of the host will hopefully provide further advancements in the area of practical diagnostics for parasite control in small ruminants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Multiplex Evaluation of Influenza Neutralizing Antibodies with Potential Applicability to In-Field Serological Studies

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The increased number of outbreaks of H5 and H7 LPAI and HPAI viruses in poultry has major public and animal health implications. The continuous rapid evolution of these subtypes and the emergence of new variants influence the ability to undertake effective surveillance. Retroviral pseudotypes bearing influenza haemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins represent a flexible platform for sensitive, readily standardized influenza serological assays. We describe a multiplex assay for the study of neutralizing antibodies that are directed against both influenza H5 and H7 HA. This assay permits the measurement of neutralizing antibody responses against two antigenically distinct HAs in the same serum/plasma sample thus increasing the amount and quality of serological data that can be acquired from valuable sera. Sera obtained from chickens vaccinated with a monovalent H5N2 vaccine, chickens vaccinated with a bivalent H7N1/H5N9 vaccine, or turkeys naturally infected with an H7N3 virus were evaluated in this assay and the results correlated strongly with data obtained by HI assay. We show that pseudotypes are highly stable under basic cold-chain storage conditions and following multiple rounds of freeze-thaw. We propose that this robust assay may have practical utility for in-field serosurveillance and vaccine studies in resource-limited regions worldwide.

1. Introduction

The increased number of outbreaks of H5 and H7 low pathogenicity avian influenza (LPAI) and high pathogenicity avian influenza (HPAI) viruses in poultry has major public and animal health implications and significant economic impact. The evaluation of evidence of influenza infection or vaccination efficiency in poultry species is assessed via the measurement of immunological responses against avian influenza viruses and serological assays representing an important tool for serosurveillance studies particularly in new outbreak locations. Although vaccination, combined with improved biosecurity has successfully prevented significant mortalities and production loss, on-going evolution of the virus requires the development of surveillance systems to ensure that vaccination continues to be effective [1].

Influenza A viruses infecting poultry are divided into two groups (HPAI and LPAI) on the basis of their ability to cause disease and with different human and animal health implications. Evidence [2, 3] supports the hypothesis that HPAI viruses arise as a result of mutations after the virus has been introduced from wild birds into poultry and thus, it is believed that LPAI viruses are the progenitors of the highly pathogenic variants [4]. It has been recognized as important to control not only HPAI viruses, but also LPAI strains in domestic poultry [5] despite current knowledge on the mechanisms of mutation from LPAI to HPAI being insufficient for predicting which influenza strains will mutate into HPAI variants [6, 7]. Despite significant efforts being put into the development of avian vaccines, serological surveillance represents one of the major tools for evaluating the immune state of avian populations especially for the ability of certain subtypes

to mutate (antigenic drift mechanism) due to their long-term circulation among vaccinated populations [8]. Serology represents a powerful and sensitive approach for detecting the presence of avian influenza antibodies in a population but the occurrence of antigenic drift and shift must be taken into consideration as it can render subtype-specific serologic tests (HI or neutralization assays) less sensitive for new or emerging strains of influenza [9]. Additionally, serologic cross-reactivity with antigenically distinct influenza viruses can occur as a consequence of precedent vaccination or exposure, resulting in a more complicated interpretation of the serological findings. To address these issues, new assays are warranted as summarized within Appendix A of the Consultation Summary (May 2010) of an FAO-OIE-WHO Joint Technical consultation on Avian Influenza at the Human-Animal Interface (7–9 October, 2008, Verona, Italy) which makes the recommendation to "develop and validate more sensitive and specific tests for detecting antibodies to avian influenza viruses in avian and nonavian species including humans" [10]. As substitution rates are significantly higher in influenza HA and NA genes compared with internal genes, retroviral and lentiviral pseudotypes bearing HA and NA envelope glycoproteins devolved from the rest of the virus are ideal tools to monitor the effects of viral evolution on serological outcomes as previously shown [11–14]. They can be used as sensitive, low-containment assays for measuring antibody responses against HPAI and LPAI influenza strains [15] and potentially against all different influenza subtypes [16, 17] because, upon availability of the novel viral RNA/cDNA, HA/NA genes can be sequenced, readily cloned or custom synthesized, and pseudotyped lentiviral vectors prepared for use in neutralization assays. Therefore, this assay can be continually updated to measure the efficacy of current vaccines and therapeutics as well as serosurveillance. Also, the use of lentiviral pseudotypes has shown additional advantage compared to other serological assays since this system can potentially be adapted to a "multiplex format" with beneficial repercussions when large scale serological investigations need to be undertaken. In this study, the flexibility of the influenza pseudotype system has been exploited to develop a multiplex assay to study the neutralizing antibody responses directed against HAs belonging to Influenza Group 1 (HPAI H5N1 clade 1 A/Vietnam/1194/2004 and HPAI clade 2.1.3.2 A/Indonesia/5/2005) and Group 2 (HPAI H7N1 A/chicken/Italy/13474/1999). By the incorporation of different luciferase (Renilla and firefly) reporter genes into the lentiviral genome of two separate pseudotypes, each bearing an antigenically distinct envelope glycoprotein on its surface, the presence of neutralising antibodies against two influenza HAs has been evaluated within a single serum sample in a single assay plate well. Initially, sera from chickens vaccinated with a monovalent vaccine (H5N2) or from turkeys naturally infected during an H7N3 influenza outbreak were tested by pseudotype neutralization assay (pp-NT) assay using the "monoplex" format and serological results were compared to the standard reference HI test. Subsequently, H5 and H7 influenza pseudotypes were used for the screening of a panel of sera collected from chickens vaccinated with a bivalent vaccine (H5N9/H7N1) using a multiplex format where subtype-specific antibody responses in the same serum sample directed against H5 and H7 pseudotypes were evaluated exploiting the use of two different reporters and offering a new assay format for in-field serosurveillance and vaccine studies. We have also shown that these pseudotypes are highly stable at basic cold-chain storage conditions of −20°C and +4°C and after multiple rounds of freeze-thaw making these assays potentially applicable for use in-field in endemic areas as we have described recently for rabies and lyssaviruses [18, 19].

2. Materials and Methods

2.1. Serum Samples. All avian sera were provided by the FAO, OIE, and National Reference Laboratory for Newcastle Disease and Avian influenza (Istituto Zooprofilattico Sperimentale delle Venezie) and consisted of ten sera H5 positive collected from chickens vaccinated with the inactivated H5N2 (A/chicken/Hidalgo/28159-232/1994) vaccine (no. 1–10), ten sera H7 positive (no. 11–20) collected from turkeys during an Italian outbreak caused by an LPAI H7N3 virus (A/turkey/Italy/2002), ten sera positive for both H7 and H5 collected from chickens vaccinated with an inactivated bivalent vaccine produced with the LPAI H7N1 (A/chicken/1067/1999), and H5N9 (A/chicken/Italy/22A/1998) strains. Forty negative sera were included in the study and were obtained from chickens tested AI antibody-free by enzyme-linked immunosorbent assay (ELISA) and agarose gel immunodiffusion (AGID) assay using standard protocols described previously [20, 21]. Additionally, two hyperimmune sheep sera, SH454 raised against NIBRG-14 (H5N1 HA) and 02/294 raised against A/chicken/Italy/13474/1999 (H7N1 HA), were kindly provided by NIBSC.

2.2. Inhibition of Haemagglutination (HI) Test. All avian sera employed in the study were tested by HI at FAO, OIE, and National Reference Laboratory for Newcastle Disease and Avian influenza, Istituto Zooprofilattico Sperimentale delle Venezie with different reference antigens routinely used for avian influenza surveillance in Italy, namely, H5N2 (A/turkey/Italy/1980), H7N3 (A/turkey/Italy/9289/V02), H7N1 (A/Africa starling/England/983/1979), H5N9 (A/chicken/Italy/22A/1998), and H7N1 (A/chicken/Italy/1067/1999). For the HI tests, standard protocols were used as described previously [22].

2.3. Firefly Luciferase and Renilla Luciferase H5/H7 Pseudotypes. Lentiviral vector (carrying the luciferase reporter gene, pCSFLW) pseudotyped with HA envelope glycoproteins derived from the HPAI H5N1 viruses (clade 1 A/Vietnam/1194/2004 and clade 2.1.3.2 A/Indonesia/5/2005) and the HPAI H7N1 virus (A/chicken/Italy/13474/1999) were produced as described previously [23, 24], except that the neuraminidase activity was provided by a cognate NA plasmid in lieu of exogenous bacterial NA addition. In parallel, using the same transfection protocol and the same batch of HEK 293T/17 producer cells, HPAI H7 pseudotypes (A/chicken/Italy/13474/1999) carrying the Renilla luciferase

gene (pCRLFW), were generated [24]. Using the firefly luciferase as marker for infection of HEK 293T/17 target cells, titration of H5, and H7 influenza pseudotype was carried out [23] in order to calculate the input virus dose required for the proceeding pseudotype-based neutralization assays. The titres of influenza pseudotypes were quantified by luminescence expression, expressed as relative luminescence units (RLU) measured by luminometer (GloMAX 96, Promega). Two controls were required for the titration: a negative control (cell only) and the Δ-envelope glycoprotein control (No HA and NA). In addition, for stability studies pCSLZW, expressing the lacZ gene [18], was used in conjunction with the clade 1 A/Vietnam/1194/2004 HA and exogenous bacterial NA (1 unit/mL; Sigma, UK) to produce lacZ pseudotype viruses, and infection of HEK 293T/17 cells was detected using the X-gal substrate as described by us previously [18]. Lentiviral pseudotypes bearing rabies CVS-11 [25] and HIV-1 [26] envelope glycoproteins were utilized for comparison.

2.4. Firefly Luciferase (Monoplex) pp-NT Assay. Serum samples (5 μL) were twofold serially diluted in culture medium (DMEM GlutaMAX supplemented with 15% FBS and 1% Penicillin/Streptomycin) and mixed with pseudotype virus (500,000 RLU luciferase input) at a 1 : 1 v/v ratio. After incubation at 37°C for 1 hour, 1×10^4 HEK 293T/17 cells were added to each well of a white 96-well flat-bottomed tissue culture plate. 48 hours later, pseudotype transduction titres obtained at each of a range of dilution points were expressed as RLU/mL, and an arithmetic mean was calculated. For each serum sample, RLUs were normalized and compared with the signal detected in the absence of pseudotype virus (equivalent to 100% neutralization) and the signal of the negative control (equivalent to 0% neutralization). The 50% inhibitory doses (IC_{50}) were determined as the reciprocal of serum dilution resulting in a 50% reduction of a single round of infection (reporter gene mediated signal).

2.5. Firefly and Renilla (Multiplex) pp-NT Assay. To allow detection of neutralizing antibody responses against two different influenza viruses (H5 and H7) in the same well of a 96-well flat-bottomed tissue culture plate, fixed amounts (corresponding to 500,000 RLUs estimated by prior pseudotype titration) of both influenza pseudotypes (one containing the firefly reporter gene and the other the Renilla reporter gene) were added to each well in which twofold serially diluted serum samples (5 μL) were dispensed together with cell culture medium (DMEM GlutaMAX supplemented with 15% FBS and 1% Penicillin/Streptomycin). After 48 hours, the neutralizing antibody responses against each subtype were detected by using the Dual-Glo reagent (Promega) which differentiates between the two reporter genes as detailed in the manufacturer's instructions, so that neutralizing antibody titre for each influenza pseudotype could be recorded for each serum sample concurrently.

2.6. Data Analysis and Sequence Analysis. Data analyses were undertaken using Excel and GraphPad Prism (Version 6). Antibody titres observed for H5 (A/Vietnam/1194/2004 and A/Indonesia/5/2005) and H7 (A/chicken/Italy/14374/1999) influenza pseudotypes when used in the monoplex and multiplex assays were expressed as geometric mean titer (GMT). Firstly, the IC_{50} values were calculated, as described above, and the serum dilution resulting in 50% neutralizing activity reduction for each serum sample (tested in duplicate) was transformed to logarithmic scale. Subsequently, the geometric mean of duplicate observations was calculated. Statistical analyses for all the data and correlation coefficients (Pearson's correlation analysis) were performed using GraphPad Prism. The radial tree and HA amino acid identity grid were constructed with MATLAB (MathWorks).

3. Results

3.1. Construction of H5 and H7 Lentiviral Pseudotypes. We have constructed H5N1 and H7N1 pseudotypes (with A/Viet Nam/1194/2004 HA and NA, A/Indonesia/5/2005 HA and NA, and A/chicken/Italy/13474/1999 HA and NA) encoding the firefly luciferase reporter gene, and additionally, for use in a multiplex assay, an H7N1 pseudotype (with A/chicken/Italy/13474/1999 HA and NA) encoding a Renilla luciferase reporter. The phylogenetic relationship between these pseudotype serological antigens (and the other antigens utilized in this study) can be visualized on a radial tree in Figure 1. Using firefly luciferase as a marker for infection of HEK 293T/17 cells, it was shown that high titre functional pseudotypes bearing these three different envelope glycoprotein pairs were successfully produced (data not shown). Based on these virus titres, it was decided to use 500,000 RLU as the input virus dose for subsequent neutralization assays.

3.2. Stability of H5 Lentiviral Pseudotypes. The requirements and reliability of cold-chain storage in laboratories undertaking AI serology vary greatly, especially in resource limited regions of the world. Therefore, if these pseudotype-based assays are to be adopted in these regions in the future, it is assumed, primarily for cost reasons, that lacZ will be the reporter gene of choice and that these laboratories may have frequent disruptions to ideal pseudotype storage conditions (−80°C) or simply may have no access to a −80°C freezer. We therefore undertook a series of A/Viet Nam/1194/2004 HA pseudotype virus stability investigations by storage of this virus at the higher temperatures of −20°C (standard freezer), +4°C (standard fridge), and room temperature and by subjecting the pseudotype virus to multiple freeze-thaw cycles. The initial titre of the H5 lacZ pseudotype was 4.3×10^5 IFU/mL and >80% infectivity remained after five cycles of freeze-thaw (Figure 2). In parallel, pseudotypes bearing rabies CVS-11 and HIV-1 envelope glycoproteins were subjected to the same freeze-thaw regimen and were found to lose approximately 4% and 9% activity, respectively, per freeze-thaw cycle (Figure 2). In relation to temperature storage variations and their effect on pseudotype viability, Figure 3 shows that H5 A/Viet Nam/1194/2004 HA pseudotypes stored at −20°C maintained infectivity (of >80% compared to storage at −80°C) for at least 6 months, making these assays readily applicable in the vast majority of laboratories

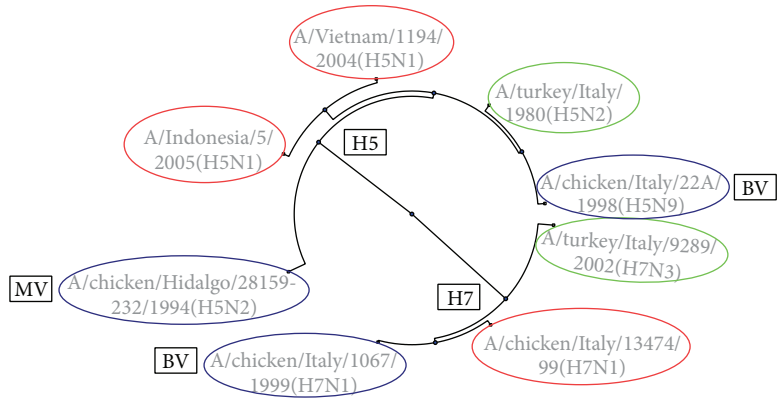

Figure 1: Radial phylogenetic tree showing the relationship between the full-length HA genes of vaccine and serological antigens (MATLAB Software). MV: monovalent vaccine strain, BV: bivalent vaccine strain. Viruses encircled in blue represent vaccine strains, in red represent pseudotype antigen strains, and in green represent HI antigen strains. The amino acid identity of these strains is shown in Figure 4.

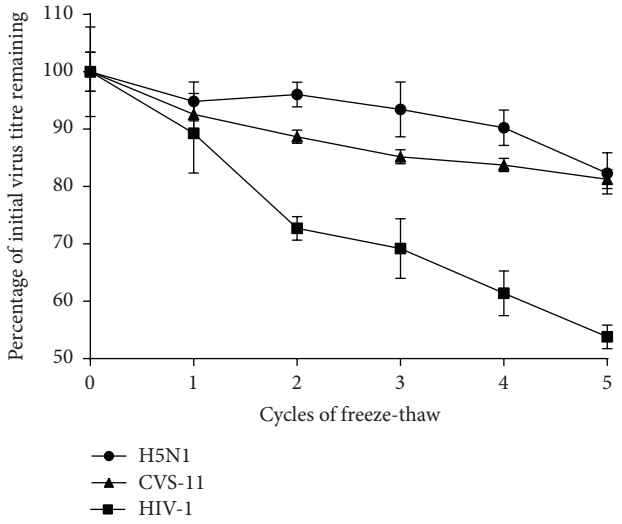

Figure 2: The influence of freeze-thaw cycles on pseudotype virus titre. The stability of H5N1 lacZ pseudotypes was evaluated by subjecting aliquots of virus to 5 cycles of freeze-thaw. Results for pseudotypes bearing the rabies virus (CVS-11) and HIV-1 envelope proteins are shown for comparison. One biological replicate of each pseudotype virus was used to generate three technical replicates. Error bars represent standard deviation.

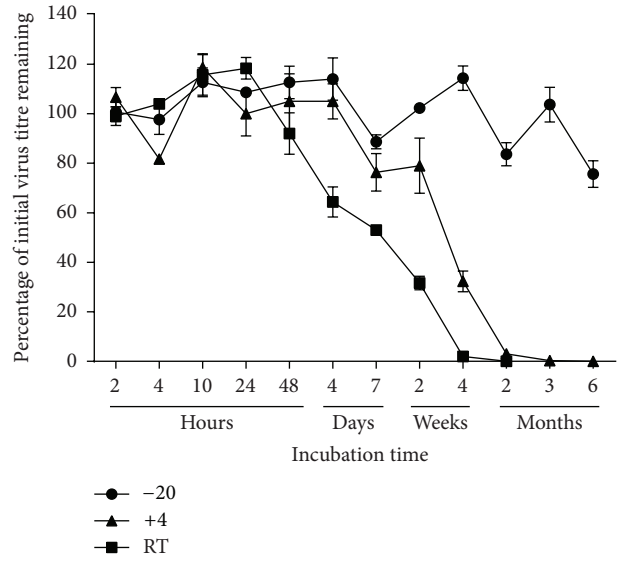

Figure 3: The influence of different storage temperatures on pseudotype virus titre. The stability of H5N1 lacZ pseudotypes was evaluated by storing aliquots of virus at different temperatures for up to 6 months. Pseudotype titres for the time course are given relative to the titre of virus stocks maintained at −80°C which is considered the optimal storage temperature for retroviral pseudotypes. One biological replicate pseudotype virus was used to generate three technical replicates. Error bars represent standard deviation.

worldwide. As also shown in Figure 3, these viruses could additionally be stored at +4°C for up to 4 weeks (with a 50% reduction in infectivity) and at room temperature (23°C) for 1 week (with a 50% reduction in infectivity).

3.3. Monoplex pp-NT Assay Using HPAI H5 and H7 Influenza Pseudotypes. Three panels of sera (H5 positive, H7 positive, and 40 negative serum samples) were initially tested using a monoplex pseudotype-based format. An initial pilot study was carried out where H5N1 hyperimmune (SH454) and H7N1 (02/294) sheep sera were tested for the ability to neutralize influenza pseudotypes bearing the HAs from H5N1 A/Vietnam/1194/2004 and H7N1 A/chicken/Italy/13474/1999.

The H5 influenza pseudotypes were neutralized by the SH454 sera (100% inhibition of pseudotype entry at 1:1280 serum dilution) but not by the 02/294 sera whilst the H7 pseudotypes were neutralized by 02/294 (with a 100% inhibition at dilution 1:1280) but not by SH454. This lack of cross-neutralizing antibody response between H5 and H7 subtypes was consistent with the different clustering, within Group 1 and Group 2, of HA-subtypes based on phylogenetic relationship analysis of influenza subtypes. According to this analysis, H5 HA belongs to Group 1 "cluster 1" (together with H1, and H2, H6) and H7 HA belongs to Group 2 "cluster 7"

Table 1: Comparison of neutralizing activity of a panel of sera collected from chickens vaccinated with an inactivated H5N2 monovalent vaccine.

Serum number	H5 positive sera (H5N2 monovalent vaccine)			
	GMT titres H5N1 A/Vietnam/1194/04	GMT titres H5N1 A/Indonesia/5/05	GMT titres H7N1 A/chicken/13474/99	HI titres H5N2 A/chicken/Italy/80
1	2560	2560	28	2048
2	2560	2560	113	2048
3	640	1280	10	512
4	1810	2560	10	2048
5	453	453	10	1024
6	1280	905	28	1024
7	226	226	10	512
8	113	160	113	128
9	640	905	28	512
10	640	453	57	512
H5N1+	1280	—	—	—
Negatives (40 tot.)	≤1 : 10	≤1 : 10	—	1 : 2

GMT titres were calculated and expressed as the reciprocal of serum dilution at which a 50% inhibition of pseudotype (IC_{50}) entry was observed.

	A/Vietnam/1194/2004(H5N1)/1-568	A/Indonesia/5/2005(H5N1)/1-568	A/chicken/Hidalgo/28159-232/1994(H5N2)/1-564	A/turkey/Italy/1980(H5N2)/1-564	A/chicken/Italy/22A/1998(H5N9)/1-564	A/chicken/Italy/13474/99(H7N1)/1-564	A/turkey/Italy/9289/2002(H7N3)/1-532	A/chicken/Italy/1067/1999(H7N1)/1-560
A/Vietnam/1194/2004(H5N1)/1-568	100%	97%	87%	91%	91%	41%	40%	42%
A/Indonesia/5/2005(H5N1)/1-568	97%	100%	86%	91%	91%	41%	39%	41%
A/chicken/Hidalgo/28159-232/1994(H5N2)/1-564	87%	86%	100%	90%	90%	41%	40%	42%
A/turkey/Italy/1980(H5N2)/1-564	91%	91%	90%	100%	95%	41%	41%	42%
A/chicken/Italy/22A/1998(H5N9)/1-564	91%	91%	90%	95%	100%	41%	40%	42%
A/chicken/Italy/13474/99(H7N1)/1-564	41%	41%	41%	41%	41%	100%	92%	98%
A/turkey/Italy/9289/2002(H7N3)/1-532	40%	39%	40%	41%	40%	92%	100%	93%
A/chicken/Italy/1067/1999(H7N1)/1-560	42%	41%	42%	42%	42%	98%	93%	100%

Figure 4: Amino acid identity grid for pseudotype, HI, and vaccine antigen strains. MV: monovalent vaccine antigen, BV: bivalent vaccine antigen, pp: pseudotype antigen, and HI: haemagglutination inhibition assay antigen.

(together with H10 and H15) [27]. Subsequently, a panel of ten sera collected from chickens vaccinated with a monovalent inactivated vaccine produced with an H5N2 strain (A/chicken/Hidalgo/232/1994) were run in order to more comprehensively evaluate the utility of this assay in an avian serological setting (tested using H5N1 A/Vietnam/1194/2004, H5N1 A/Indonesia/5/2005, and H7N1 A/chicken/13474/1999 pseudotyped particles). Serological profiles obtained by pp-NT assays were also compared with those obtained by HI tests. All ten sera positive by HI test using an H5N2 A/chicken/Italy/1080 reference antigen (with titres ranging from 1 : 128 to 1 : 2048) were also confirmed positive by pp-NT assay using H5N1 A/Vietnam/1194/2004 (GMT range 1 : 113 to 1 : 2560) and H5N1 A/Indonesia/5/2005 (GMT range 1 : 60 to 1 : 2560) (Table 1). When this panel was tested against H7 HPAI pseudotypes, six of ten sera were also found positive with GMT ranging from 1 : 28 to 1 : 113 (Table 1).

As shown in Figure 5(a), titres obtained via HI correlated strongly with titres obtained using HPAI H5 pseudotypes belonging to two different clades: clade 1 A/Vietnam/1194/2004 ($r = 0.87$, $P < 0.0001$) and clade 2.1.3.2 A/Indonesia/5/2005 ($r = 0.87$, $P < 0.0002$) despite the fact the HAs used in the two serological assays were not optimally matched. The percentage amino acid identities between the pseudotype antigens, the HI antigen, and the vaccine antigen are shown in Figure 4.

It was also observed (Figure 5(b)) that all ten sera neutralized both H5 HPAI pseudotyped viruses but with a different magnitude; it was found that the absolute titers (expressed as mean ± SD) of H5 A/Vietnam/1194/2004 (222.96) were significantly lower than those obtained for the same panel tested by A/Indonesia/5/2005 (1206.2) as confirmed by P value <0.0001 and $r = 0.93$ when analyzed using Student's t-Test (paired data set) (Figure 5).

Table 2: Comparison of neutralizing activity of panel of sera collected from naturally infected turkeys with H7N3 A/turkey/Italy/2002.

Serum number	H7 positive sera (H7N3 positive, naturally infected)		
	GMT titres H7N1 A/chicken/13474/99	GMT titres H5N1 A/Vietnam/1194/04	HI titres A/ty/Italy/9289/V02 H7N3
11	320	10	64
12	320	10	32
13	2560	10	128
14	28	10	64
15	2560	10	128
16	80	10	32
17	226	10	32
18	640	28	32
19	640	10	128
20	1280	10	64
H7N1+	1 : 1280	≤1 : 10	—
Negatives (40 tot.)	≤1 : 10	—	1 : 2

GMT titres were calculated and expressed as the reciprocal of the serum dilution at which 50% inhibition of pseudotype (IC_{50}) entry was observed.

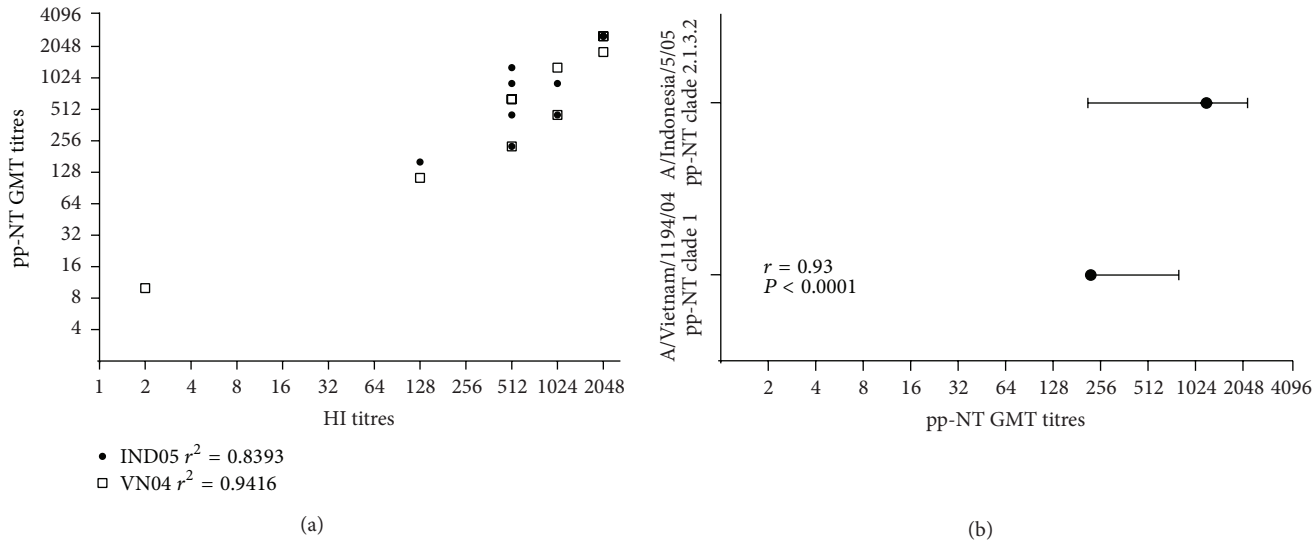

Figure 5: (a) Comparison of pp-NT with HI titers. Scatterplots showing the correlation of antibody logarithmic titers measured by pp-NT versus HI. For the pp-NT assay, HPAI H5 from A/Vietnam/1194/04 VN04 and A/Indonesia/5/05 (IND05) were tested. Pearson's correlation analysis was carried out and the coefficient of determination (r^2) for each strain reported on the graph. (b) Paired t-test performed by using GraphPad.

Next, a panel of ten sera obtained from turkeys naturally infected with an H7N3 strain with titres ranging from low (1 : 32) to high (1 : 128) as tested by HI (HI reference antigen: H7N3 A/turkey/Italy/9289/V02) were subsequently tested using H7 pseudotypes A/chicken/Italy/13474/1999 and H5 A/Vietnam/1194/2004. All ten sera (no. 11 to no. 20) were found positive when tested against H7 pseudotypes with GMT range from 1 : 28 to 1 > 2560 while only one serum sample was positive (GMT of 1 : 28) against H5 A/Vietnam/1194/2004 (Table 2).

As previously shown for the panel of H5 positive sera, a comparative serological approach was undertaken in order to assess whether the results obtained with the pseudotype neutralization assay reflected those obtained with HI test using a regression analysis on paired datasets (generated from all 51 samples comprising also the negative sera) and the Pearson's correlation test. The results of this analysis revealed a highly statistically significant correlation ($P < 0.001$) between antibody titers obtained with both assays. The correlation coefficient between pp-NT and HI for the panel of H7 positive was 0.72 (Figure 6).

3.4. Multiplex Assay by Using HPAI H5 and H7 Influenza Pseudotypes Expressing Firefly and Renilla Luciferase Reporter Gene. For a panel of sera collected from chickens vaccinated with an inactivated bivalent vaccine (BV) produced with the avian influenza vaccine strains H7N1 (A/chicken/Italy/

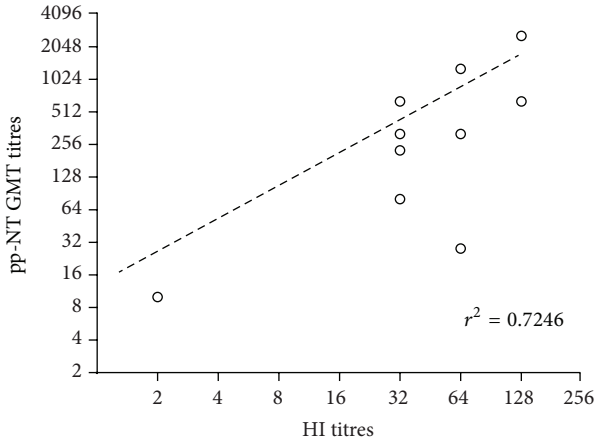

Figure 6: Comparison of pp-NT with HI titers. Scatterplots showing the correlation of antibody logarithmic titers measured by pp-NT (using HPAI H7 A/chicken/Italy/13474/99 pp) versus HI (HI antigen: H7N3 A/ty/Italy/9289/V02). The total number of sera was 51. Graph shows the linear regression fitted to the data using GraphPad.

1067/1999, LPAI) and H5N9 (A/chicken/Italy/22A/1998, LPAI), monoplex (as described before) and multiplex (by using firefly/Renilla) pp-NT assays were undertaken against H5 A/Vietnam/1194/2004 and H7 A/chicken/Italy/13474/1999 pseudotypes. As shown in Table 3, 9/10 sera were confirmed positive when tested by the monoplex assay against H5 A/Vietnam/1194/2004 with titres (expressed as GMT) ranging between 1 : 28 to 1 : 905; only one serum sample (n. H5+7s3), positive by HI (1 : 64), was found negative by pp-NT (GMT = 10). Neutralizing antibody responses against H5 A/Indonesia/5/2005 were also found positive (10/10 sera) with GMTs ranging between 1 : 28 and 1 : 905 and showing, as seen for the panel of H5 positive sera (Table 1), overall higher GMTs compared to H5N1 pseudotypes belonging to clade 1. Also for H7N1 pseudotypes the GMTs mirrored those obtained with H5N1 pseudotypes (GMTs from 1 : 40 to 1 : 453) with one serum sample (n. H5+7s3) negative (GMT = 10) and two sera at the proposed positive threshold of 1 : 40.

Subsequently, neutralizing antibody titres, obtained when standard and dual H5/H7 assays were undertaken (GMT reported in Table 3), revealed a strong correlation between the results of the standard and dual pp-NT assays (0.86; $P < 0.0001$; Pearson's correlation; Figure 7).

Neutralizing antibody titres obtained by monoplex assay (using H5 A/Vietnam/1194/2004 carrying the firefly luciferase gene) mirrored those obtained when the same panel of sera were tested against H5 A/Vietnam/1194/2004 (firefly gene) mixed with H7 A/chicken/13474/1999 carrying the Renilla gene ($r = 0.85$, $P = 0.001$). Similar results were observed for antibody responses against H7 A/chicken/13474/1999 tested in monoplex and multiplex assays ($r = 0.91$, $P = 0.0002$) (Figure 7). The magnitude of neutralizing antibody responses observed by pp-NT assays reflected those obtained by the standard HI test although the HI test had to be performed for evaluating HA-mediated antibody responses versus both influenza antigens H5N9 (A/chicken/Italy/22A/1998) and H7N1 (A/chicken/Italy/1067/1999) (Table 3).

4. Discussion and Conclusion

Since 1997, H5 and H7 outbreaks in domestic poultry have been increasing in frequency and it is likely to be due to a complex set of factors such as improved diagnostic tools, climate fluctuations, and changes in trade flows of poultry products [28]. One logical step to understand and limit the possible spread of avian influenza viruses to humans and to control the circulation amongst avian species is the monitoring of AI virus exposure in poultry initially via identification of active infections. However, due to the ability of influenza viruses to circumvent immunity acquired through infection or vaccination by progressive antigenic drift, serological surveillance of avian samples is also particularly important [29, 30]. Serological techniques play a key role in various aspects of influenza surveillance, vaccine development, and evaluation and they can be used to assess the presence of antibodies to past infections and responses to a circulating influenza strain or vaccine components [31]. From a veterinary point of view, serological and virological surveillances are necessary not only as monitoring systems for AI viruses circulating among poultry species but also as a prevention and control tool for those strains with possible pandemic potential [32, 33].

Recent studies have provided the impetus that the future of avian serology, rather than moving towards a single assay approach, is the implementation of a strategy that involves conventional and novel technologies to be used in conjunction with validated and standard tests. Comparative serology aims to achieve a more holistic view of the serological response and newer assays like the pseudotype-based neutralization assay presented in this study are key [14, 23]. We have shown previously that retroviral pseudotypes (MLV) based on A/Viet Nam/1194/2004 can be used to measure antibody responses in chickens immunized with H5N1, H5N2, H5N3, H5N7, and H5N9 avian viruses [11]. In this current study, lentiviral pseudotypes have been employed to form the basis for the development of a multiplex reporter (firefly luciferase and Renilla luciferase) neutralization assay for H5 and H7 subtype viruses. This pseudotype system allows the measurement of neutralizing antibody responses against two antigenically distinct AI HA envelope glycoproteins in the same avian serum sample. The individual components employed for the construction of the pseudotypes used for this multiplex assay have been chosen from a set of interchangeable plasmids which we have available for assay development. These are retroviral and lentiviral plasmids coding for the *gag-pol* core structural proteins, HA and NA expression plasmids, and retroviral vectors incorporating the reporter gene. Firefly and Renilla luciferases were employed in this study, but potentially a wide range of reporter genes can be used in these assays (green fluorescence protein (GFP), red (RFP)/yellow (YFP), secreted embryonic alkaline phosphatase (SEAP), and lac-Z) [18, 19, 34, 35]. In order for pseudotype assays to have wide applicability and deployment

Table 3: Evaluation of antibody responses in sera collected from chickens vaccinated with a bivalent vaccine (H5/H7) by using the monoplex and multiplex assay formats.

Serum number	HI H7N1	H7 pp-NT monoplex A/ck/Italy/13474/99	H7 pp-NT multiplex (with H5 VN04)	Serum number	HI H5N9	H5 pp-NT monoplex VN04	H5 pp-NT monoplex H5 IND05	H5 pp-NT multiplex (with H7)
H5+7s1	32	40	40	H5+7s1	32	57	80	40
H5+7s2	16	57	80	H5+7s2	32	57	80	28
H5+7s3	32	10	10	H5+7s3	64	10	28	10
H5+7s4	16	40	40	H5+7s4	16	28	40	28
H5+7s5	32	453	320	H5+7s5	64	905	453	453
H5+7s6	32	226	226	H5+7s6	32	160	160	226
H5+7s7	16	113	113	H5+7s7	32	226	320	160
H5+7s8	16	57	57	H5+7s8	64	57	80	57
H5+7s9	16	80	113	H5+7s9	32	453	905	320
H5+7s10	32	226	320	H5+7s10	32	905	905	1280

Values are reported as geometric mean titres. Left side: values for H7 A/chicken/Italy/13474/99 tested in monoplex and multiplex are reported. Right side: values for H5 A/Vietnam/1194/04 tested in monoplex and multiplex are reported and the panel of sera was additionally tested against A/Indonesia/5/05.

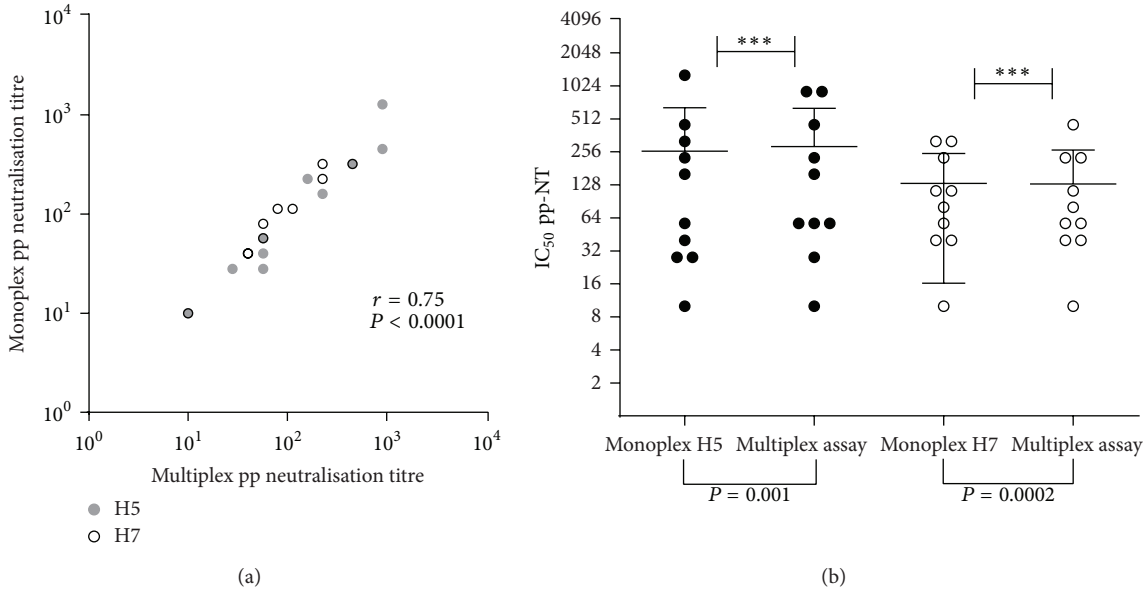

Figure 7: (a) Correlation of monoplex versus multiplex IC_{50} pseudotype neutralization titres for sera collected from chickens vaccinated with an inactivated bivalent vaccine produced with the AI strains H7N1 (A/ck/Italy/1067/99, LPAI) and H5N9 (A/ck/Italy/22A/98, LPAI). Antibody titres between monoplex and multiplex assays correlate when tested with both H5 and H7 pseudotypes. Neutralizing titres against H5 A/Vietnam/1194/2004 (grey dots) and H7 A/chicken/Italy/13474/1999 (empty circles) were determined in separate wells (single) or in the same well (multiplex). Correlation coefficient and P values were calculated using Pearson's correlation. Plot drawn with GraphPad. (b) IC_{50} values for each sera tested by monoplex and multiplex pp-NT assays using H5 A/Vietnam/1194/04 (firefly luciferase gene) and H7 A/chicken/Itlay/13474/1999 (carrying firefly luciferase and Renilla luciferase gene) were calculated and plotted (the wide horizontal bar represents the means of IC_{50} titres). Results were subsequently analyzed by performing Student's t-test on the paired dataset.

potential within different laboratories worldwide, the availability of different reporter systems is highly desirable. The HIV-based GFP reporter plasmid (pCSGW), which we have described previously in the context of pseudotype-based neutralization assays [23], has been modified by PCR subcloning to express alternative reporter genes. These are firefly luciferase (pCSFLW), Renilla luciferase (pCSRLW), and *lac*-Z (pCSLZW) [19, 24]. Of the three types, the luciferase reporter based assays are the most sensitive and reproducible and also the simplest to use in terms of hands-on time and downstream data analysis. This was the reason they were chosen for the serological assays described in this current study. However, due to the relatively high cost of the necessary reagents (luciferase assay) and necessity for specialized equipment (luminometer), luciferase assays may have limited applicability for laboratories in resource poor regions. GFP based assays do not require any supplementary reagents but do necessitate specialized equipment (fluorescent microscope

or 96-well plate flow cytometry facility). The cost of a basic fluorescent microscope however is now under $5000 making this technology applicable for middle to low-resource lab deployment. The lac-Z based assays are the most cost effective as the necessary reagents are cheaply available and specialized equipment is unnecessary making them ideal for deployment in resource poor areas where serosurveillance in domestic poultry is likely to be carried out. In addition to firefly/Renilla (used in this study), it is technically feasible to multiplex pairs of pseudotype viruses carrying GFP/RFP and lacZ/SEAP combinations for low-resource laboratory use. Lac-Z was thus chosen as the reporter gene of choice for the "in-field" applicability studies involving the freeze-thawing and storing of pseudotype viruses outside of a −80°C facility as was used recently for similar studies with lyssavirus pseudotypes [18]. Our results showed them to be highly suitable for such use as they were stable over time at different storage temperatures and when subjected to multiple cycles of freeze-thaw. Interestingly the pseudotype virus bearing the HIV envelope glycoprotein was significantly more sensitive to the freeze-thaw procedure than the viruses bearing influenza or rabies virus glycoproteins. This is most likely due to the fact that HIV glycoprotein is relatively unstable when frozen. Additionally, with the multiplex assay using the dual reporter gene system, the interassay variability is likely to be reduced since only a single serum dilution series needs to be performed. The same preparation of target cells is used for two viruses and the antibody response to the H5 subtype virus may serve as an internal "serocontrol" for the antibody response to the H7 subtypes and vice versa as two separate luciferase reporters were employed.

Results collected from pp-NT assays were statistically significant when performed in monoplex and multiplex from both H5 ($P = 0.001$) and H7 ($P = 0.0002$) influenza strains with results from the monoplex mirroring those obtained with the multiplex assay (Figure 7). This system could be subsequently refined with the possibility of increasing the multiplexing capability (use of more reporter systems by detecting luminescence and fluorescence signals, e.g., GFP/RFP with firefly/Renilla luciferase) and it could readily be adapted to high-throughput if large serum panels are used. There are also beneficial economic implications to the use of this assay since the antibody responses against two viruses do not require high-containment facilities and relatively fewer reagents than HI and MN tests. The pp-NT assay described here is both "serum sparing" and "antigen sparing" as only ≤5 μL and, especially for certain HPAI strains, less than 10 μL (corresponding to a pseudotype input of 10e6 RLU) pseudotypes per 96-well plate is required. It is possible with the multiplex pp-NT assay to measure neutralizing antibody responses against large panels of H5/H7 influenza viruses and drift variants faster and more accurately than laborious wild-type virus microneutralization, thus providing comprehensive data on antigenic evolution of avian influenza viruses.

Moreover, the major limitations to the use of HI assay are that it is not practical for general influenza A screening with significant level of intralaboratory variability as demonstrated in human serology [36]. It requires a greater amount of sera and the occurrence of cross-reactivity between subtypes needs to be taken into account. On the contrary, pseudotype particles have been shown to be particularly sensitive with the potential to detect antibody responses and variations within influenza subclades and also showing statically significant correlation when compared to the HI test (Table 1) (Figures 5 and 6) [23]. Recent studies have raised the possibility that the lower incorporation of HA spikes into lentiviral pseudotypes, compared to the wild virus, makes pseudotypes more sensitive by allowing the binding of antibodies to the antigenic sites on the HA head and also on the HA stalk [37, 38]. Based on the data obtained in this study, future refinement of this assay is warranted and it contributes towards the recommendations for the development of new assays as outlined in the FAO-OIE-WHO Joint Technical Consultation document [10]. In addition, this study provides the basis for future composite studies where collaborating laboratories can be involved to determine whether the level of intra- and interlaboratory variability in pp-NT assay is lower than that found with HI or indeed MN enabling the pp-NT assay to become accepted for large scale testing, not only in the context of avian and human influenza surveillance but also for integrated surveillance of other "neglected" influenza strains (circulating in horses, pigs, seals, and dogs for e.g.) [39].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Eleonora Molesti and Edward Wright contributed equally to the work.

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Review Article

Targeting TLR2 for Vaccine Development

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Novel and more effective immunization strategies against many animal diseases may profit from the current knowledge on the modulation of specific immunity through stimulation of innate immune receptors. Toll-like receptor (TLR)2-targeting formulations, such as synthetic lipopeptides and antigens expressed in fusion with lipoproteins, have been shown to have built-in adjuvant properties and to be effective at inducing cellular and humoral immune mechanisms in different animal species. However, contradictory data has arisen concerning the profile of the immune response elicited. The benefits of targeting TLR2 for vaccine development are thus still debatable and more studies are needed to rationally explore its characteristics. Here, we resume the main features of TLR2 and TLR2-induced immune responses, focusing on what has been reported for veterinary animals.

1. Introduction

The innate immune system senses microorganisms through germ-line encoded receptors, the pattern recognition receptors (PRRs), which include the membrane associated toll-like receptors (TLRs) [1]. Based on the knowledge that stimulation of PRRs by pathogen-associated molecular patterns (PAMPs) has a determinant role in shaping the profile of the subsequent adaptive immune response [2, 3], the conjugation of antigens with PRR ligands has been extensively explored in the last decades for the development of improved vaccines [4–7]. For that purpose, much attention has been paid to PRR ligands inducing strong polarized Th1 and cytotoxic T lymphocyte (CTL) responses, for example, ligands for TLR3, TLR7/8, or TLR9, since these are immune mechanisms poorly induced by vaccination with nonlive, inactivated or subunitary, vaccines. Activation through TLR2 is not recognized as a strong polarizing stimulus, resulting in Th responses with variable characteristics. However, TLR2 offers unique properties to be explored in vaccine development. The possibility to covalently attach TLR2 ligands to antigens, the enhancement of direct- and cross-presentation of antigens coupled to TLR2-targeting lipid moieties, the capacity to induce balanced Th responses and even regulatory mechanisms, and the mucosal imprinting properties of TLR2 stimulation are characteristics that have potential to help solving actual vaccine challenges. Here, we will review the present knowledge on the modulation of the immune response by immunogenic formulations targeting TLR2 and discuss its potential for the development of immunization strategies in the veterinary field.

2. TLR2

2.1. The Receptor. TLRs are transmembrane type I glycoproteins with a structure composed by three domains. The N-terminal extracellular domain, which is involved in the recognition of their ligands, consists of leucine-rich repeats (LRR) with the conserved motif "LxxLxLxxN" with around 20 to 30 amino acids. This domain is followed by a transmembrane region then extended intracellularly by a cytoplasmic toll/IL-1 receptor (TIR) domain, needed for signal transduction [1, 8, 9].

Phylogenetically, TLR2 belongs to a TLR family that includes TLR1, TLR6, TLR10, TLR14, and possibly the avian TLR15 [10]. TLR2 is located at the surface of the cell and,

upon binding of its ligands, dimerises with TLRs of the same family (see below). In consequence, the juxtaposition of the cytoplasmic TIR domains recruits the signaling adaptors MyD88 and TIRAP, initiating a signaling pathway that leads to activation of NF-κB transcription factor and MAPK which activate the AP-1 transcription factor [8, 11, 12].

Like other TLRs, TLR2 evolved under strong selection pressure, being preserved in all the vertebrate species tested so far [10, 13]. Sequence information on TLR2 is available for the generality of domestic animals, including cattle, sheep, goat, buffalo, horse, pig, chicken, dog, and cat [14–18].

Species-specific variations in TLR2 have been reported, mainly at the extracellular domains, possibly reflecting adaptation to different microbial environments [19]. Peculiarities in chicken TLRs include the existence of two types of TLR2 (TLR2a and TLR2b) and of TLR1 (TLR1La and TLR1Lb), originated by gene duplication, and the absence of TLR6 [13]. TLR15, apparently unique to avian species, is phylogenetically related to the TLR2 family [13].

2.2. Cells and Tissue Expression. TLR2 expression has been reported in antigen presenting cells (APCs), namely, macrophages, monocytes, and dendritic cells (DCs), including CD8α^+, CD8α^-, and plasmacytoid DCs in the mouse and interstitial and Langerhans DCs but not plasmacytoid DCs in humans [20]. TLR2 immunomodulatory influence can also be exerted directly on B cells, CD4$^+$ and CD8$^+$ T cells, T_{reg} cells, $\gamma\delta$ T cells, natural killer (NK) cells, neutrophils, basophils, and some epithelial cells [21, 22].

Tissue and cell distribution of TLR2 expression in domestic animals follows in general terms what has been described for mice and humans (for comprehensive reviews, see [14, 15]). Most of the information available, summarized in Table 1, has been obtained by reverse transcription (RT)-PCR and data on distribution and levels of the protein itself is sparse due to the lack of characterized specific antibodies for domestic animals. However, in the last few years, efforts have been made to fill this gap and anti-TLR2 antibodies have been used to assess TLR2 expression in different species, namely, bovine and ovine [23], porcine [17, 24, 25], chicken [26], and dogs [27].

In the bovine, differences in TLR2 expression on monocytes, macrophages, and DCs were found by RT-PCR. Monocytes and monocyte-derived macrophages have shown a higher signal, alveolar macrophages and bone marrow-derived DCs an intermediate signal and monocyte-derived DCs, as well as CD172a$^+$ and CD172a$^-$ DC subsets of afferent lymph, have shown weaker signals [28]. These differences were later confirmed by flow cytometry using anti-TLR2 antibodies [23]. Expression of TLR2 on ovine and bovine peripheral blood mononuclear cells (PBMCs) was detected only in CD14$^+$ monocytes [23]. No differences were observed comparing different sheep breeds [23]. Das et al. [29] analysed TLR2 sequences from nilgai, buffalo, sheep, and goat and, interestingly, found that nilgai immune cells and tissues express more TLR2 transcripts than buffalo.

Studying the expression of TLR2 in gut-associated lymphoid tissues from adult swine, Tohno et al. [30] showed that TLR2 mRNA was preferentially expressed in the mesenteric lymph nodes and Peyer's patches in levels higher than that of spleen, and Western blotting confirmed the high TLR2 expression in these structures. Beside immune cells, like T and B cells, TLR2 expression was also detected in membranous cells (M cells). Its detection in the apical membrane of the pocket-like M cells suggests a possible role in ligand-specific transcytosis and transport in these cells.

After raising a panel of monoclonal antibodies for porcine TLR2, Alvarez et al. [24] could demonstrate TLR2 expression in monocytes, macrophages, and granulocytes but not on peripheral blood lymphocytes. TLR2 expression was also detected in nonimmune cells lining body entry sites like tracheobronchial and intestinal epithelial cells, bile ducts in the liver, renal tubules, and basal layer of the epidermis [24]. Expression of TLR2 and TLR6 was demonstrated in porcine alveolar macrophages by Western blot and using antibodies against these receptors, their relevance in the sensing of *Mycoplasma hyopneumoniae* was shown [17]. Also in the horse, TLR2 expression was detected by RT-PCR in alveolar macrophages [31], as well as in respiratory epithelia [32] and PBMCs [33].

Expression of chicken TLR2a and TLR2b was detected in high levels by Western blot in heart, liver, gizzard, and muscle [26] and was also identified by RT-PCR in heterophils, monocytes, macrophages, and B and T cells [34, 35].

Ishii et al. [18] studied the mRNA expression of canine TLR2 in different dog tissues and found it in blood mononuclear cells, lymph node, lung, liver, spleen, bladder, pancreas, small intestine, large intestine, and skin. Bazzocchi et al. [27] found that TLR2 mRNA is constitutively expressed in canine blood neutrophils and, by flow cytometry, it was detected on the blood neutrophils, monocytes, and, at lower levels, lymphocytes.

For the cat, TLR2 expression was reported in lymphoid tissues (spleen and thymus), in lymphocytes (CD4$^+$ and CD8$^+$ T cells and, in higher levels, CD21$^+$ B cells) [36], in bone marrow-derived DCs [37], and in the oral mucosa [38].

2.3. The Natural TLR2 Ligands. TLR2 is usually described as the TLR recognizing the largest range of ligands. These include components from bacterial cell walls such as lipoproteins, peptidoglycan (PGN), lipoteichoic acid (LTA), lipopolysaccharides (LPS) from some bacterial species (e.g., *Porphyromonas gingivalis*), porins from *Neisseria*, lipoarabinomannan from mycobacteria, and zymosan and phospholipomannan from yeast cell walls, among others [6, 8, 39]. The recognition of such a variety of ligands is attributed to the formation of heterodimeric structures with other membrane molecules, like TLR1, TLR6, CD36, CD180/RP105, or dectin-1 [8]. This is however a controversial issue, since some authors argue that bacterial lipoproteins are the only ligands recognized by TLR2 at physiological concentrations [40]. In addition, TLR2 stimulation by most of other ligands was attributed to contamination with lipoproteins [40–42].

Lipoproteins are membrane structural components of bacteria with diverse molecular structure but with a common lipidic modification at an N-terminal cysteine [43, 44].

Table 1: (a) TLR2 expression; (b) specificities reported for veterinary species.

(a)

Human and mouse	Reference
Antigen presenting cells: macrophages, monocytes, and DCs ($CD8\alpha^+$, $CD8\alpha^-$, and plasmacytoid DCs in mouse; interstitial and Langerhans DCs but not plasmacytoid DCs in humans)	[20]
Lymphocytes: B cells, $CD4^+$, and $CD8^+$ T cells, Treg cells, $\gamma\delta$ T cells, and NK cells	[21, 22]
Granulocytes: neutrophils, basophils	
Some epithelial cells	

(b)

Species	Cells and tissues reported to express TLR2	Method[a]	Information on the level of expression	Reference
Bovine	Monocytes	RT-PCR/FC	Strong	[23, 28]
	Monocyte derived-macrophages	RT-PCR/FC		
	Alveolar macrophages	RT-PCR/FC	Intermediate	
	Monocyte derived-DCs	RT-PCR/FC		
	$CD172^+$ DCs	RT-PCR/FC	Weak	
	$CD172^-$ DCs	RT-PCR/FC		
	$CD21^+$ B cells	RT-PCR	No signal	[28]
Ovine	$CD14^+$ monocytes from PBMCs	FC		[23]
Nilgai and Buffalo	PBMCs, monocytes, DCs, testes, skin	RT-PCR	Higher in Nilgai than Buffalo	[29]
Buffalo	Kidney, endometrium, bone marrow, trachea	RT-PCR	Higher in endometrium and bone marrow	
Swine	Mesenteric lymph nodes and Peyer's patches	RT-PCR, IHC, FC	Higher than in spleen by RT-PCR	[30]
	Heart, thymus, lung, kidney, skeletal muscle, small intestine	RT-PCR	Lower than in spleen	
	M cells	IHC, FC		
	T and B cells	FC	Higher in T cells than in B cells	
	Monocytes, macrophage, and granulocytes, but not on peripheral blood lymphocytes	FC		[24]
	Epithelial cells lining body entries (Lung, jejunum, kidney, liver)	IHC		
	Alveolar Macrophages	WB		[17]
Equine	PBMCs	RT-PCR		[33]
	Alveolar macrophages	RT-PCR		[31]
	Respiratory epithelial tissues	RT-PCR		[32]
Chicken	Heart, liver, gizzard, muscle	RT-PCR, WB	Strong	[26]
	Spleen, caecal tonsil, bursa, liver	RT-PCR	Strong	[34]
	Heterophils, monocytes, macrophages, B and T cells	RT-PCR		[34, 35]
Canine	Blood mononuclear cells, lymph node, lung, liver, spleen, bladder, pancreas, small intestine, large intestine, and skin	RT-PCR		[18]
	Blood neutrophils	RT-PCR		[27]
	Blood neutrophils, monocytes	FC	Higher levels	
	Lymphocytes	FC	Lower levels	
Feline	Spleen, thymus	RT-PCR		[36]
	$CD4^+$ T cells, $CD8^+$ T cells, $CD21^+$ B cells	RT-PCR	Higher in B cells than in T cells	
	BM-DCs	RT-PCR		[37]
	Palatoglossal mucosa	RT-PCR		[38]

[a]RT-PCR: reverse transcription-PCR; FC: flow cytometry; IHC: immunohistochemistry; WB: Western blot.

In the diacylated specimens, that is, lipidated with two fatty acid residues, the modification consists in a di-O-acylated-S-(2,3-dihydroxypropyl) cysteine. The triacylated specimens have a third fatty acid, bound through an amide link to the same N-terminal cysteine. Examples of diacylated forms are the M161Ag lipoprotein of *Mycoplasma fermentans* from which the macrophage-activating lipopeptide (MALP)-2 is derived [45, 46], the LP44 lipoprotein from *Mycoplasma salivarium* from which the fibroblast-stimulating lipopeptide (FSL)-1 is derived [47], and the synthetic lipopeptide di-palmitoyl-S-glyceryl cysteine (Pam_2C) SK_4. The Braun's lipoprotein from *Escherichia coli* is the prototype of the outer membrane triacylated lipoproteins from Gram-negative bacteria and some synthetic lipopeptides used as TLR2 stimulators, for example, tri-palmitoyl-S-glyceryl cysteine (Pam_3C) SK_4, have a lipid modification analogue to this lipoprotein [48–50]. Other examples of triacylated lipoproteins are OspA from *Borrelia burgdorferi* [51] and the 19 kDa lipoprotein from *Mycobacterium tuberculosis* [52, 53]. The capacity to stimulate through TLR2 of both diacylated and triacylated lipoproteins is conferred by the lipidic N-terminal moiety [54, 55]. The initial studies pointed that diacylated lipoproteins are signalized through TLR2/6 heterodimers, while the triacylated molecules do so through TLR2/1 heterodimers [52, 56–58]. However, later studies suggested that lipopeptide activation through TLR2 may occur independently of TLR1 and TLR6 [59].

In 2007, Jin and collaborators determined by crystallography the structure of the complex TLR1-TLR2-lipopeptide, allowing a structural comprehension of the heterodimerisation induced by the ligand [60]. The binding of the triacylated lipopeptide (Pam_3CSK_4) induces the formation of an "m" shaped heterodimer of the TLR1 and TLR2 ectodomains. This dimerisation occurs by the insertion of the two ester-bound fatty acids in a pocket in TLR2 and the insertion of the amide-linked fatty acid in a hydrophobic channel in TLR1 [60]. The role played by the three lipid chains thus explains the incapacity of the diacylated peptide Pam_2CSK_4 to dimerise TLR2 and TLR1. On the other hand, Kang et al. [61] showed that TLR2/6 heterodimer has a reduced affinity for triacylated lipopeptides because TLR6 lacks a proper binding site for the amide-bound lipid chain. They also showed that, in the TLR2-TLR6-diacylated lipopeptide complex, the increased hydrophobic area found in the interface of the two dimerised receptors appears to compensate the absence of interaction between a lipid chain and TLR6. For TLR2 ligands without patent hydrophobic regions for TLR2 binding, such as PGN and zymosan, a structural support for the receptor dimerisation is lacking [61]. TLR10 is nonfunctional in the mouse, but in the human, it was shown to form dimers with TLR2, recognizing triacylated lipopeptides and other microbial components [62]. However, this receptor fails to activate typical TLR-induced signaling and its role remains elusive.

In general terms, TLR2 ligands are the same across different vertebrate species; however, some species specificities have been reported. For example, by cotransfection of bovine TLR1 and TLR2 in HEK293 cells, Farhat et al. [63] showed that the ester-bound acid chains of triacylated lipopeptides need to have at least 12 carbon atoms to activate the bovine heterodimer, contrasting with the murine heterodimer that could already be activated by lipopeptides with fatty acids of only 6 carbon atoms. Willcocks et al. [64] showed that, for some TLR2 ligands, bovine primary macrophages or cells transfected with bovine TLRs respond at lower levels than humans.

Irvine et al. [65] cloned equine TLR2, TLR1, and TLR6 and addressed their responses to classical TLR2 ligands. Functionality of TLR2/1 and TLR2/6 heterodimers was demonstrated, with LTA inducing responses similar to those observed with human heterodimers. Pam_2CSK_4 activation of TLR2/6 was identical for receptors of both species, while, in opposition to what is observed in human, Pam_3CSK_4 was less potent than Pam_2CSK_4 in activating the equine TLR2/1 heterodimer.

Different studies addressed the ligand recognition by both types of chicken TLR2 and TLR1 [26, 66, 67]. Together, these studies show that the avian TLR2a and TLR2b form heterodimers with the avian TLR1La and TLR1Lb, allowing the recognition of the same range of ligands that bind the mammalian TLR2 heterodimers, including Pam_3CSK_4, MALP-2, FSL-1, and PGN.

3. Immunogenic Formulations Targeting TLR2

As occurred with other TLR ligands, the use of lipoproteins and lipopeptides of bacterial origin as adjuvant molecules is well prior to the knowledge of their receptors and mode of action. Soon after the pioneer studies describing and characterizing a lipoprotein present in the cell wall of *E. coli*, published by the groups of Braun and Inouye around 1970 [50, 68–75], it was demonstrated that this lipoprotein, then called Braun's lipoprotein, presented mitogenic properties in mouse B cells [76]. The study of the fragments obtained by hydrolysis of the native lipoprotein enabled Bessler et al. [77] to attribute its mitogenic capacity to the N-terminal triacylated moiety and this was confirmed soon later through the chemical synthesis of lipopeptides with structure analog to the lipid moiety of that region [49]. Following these works, the adjuvant properties of the lipid moiety were tested *in vivo*. The mice inoculation with synthetic lipopeptides covalently bound to a nonimmunogenic peptide of the epidermal growth factor receptor led to the induction of specific antibodies two weeks later after one single administration [78] and the inoculation of guinea pigs with synthetic peptides from the foot-and-mouth disease virus (FMDV) conjugated with the Pam_3CSS lipopeptide resulted in the induction of neutralizing antibodies and protection against viral infection [79]. At the end of the 1980s, it was reported that the lipopeptides stimulated *in vitro* not only lymphocytes but also human monocytes and mouse macrophages [80] and Deres et al. [81] demonstrated the possibility of inducing *in vivo* CTL responses, restricted to MHC class I, by the inoculation of mice with synthetic lipopeptides conjugated with epitopes from the influenza virus nucleoprotein. Based on these works, as well as in earlier demonstrations of the immunomodulatory effect of protein lipidation [82–85], many studies using synthetic lipopeptides

in different disease and immunization models were carried out.

However, it was only at the end of the 1990s that, shortly after the publication describing the cloning and characterization of the human receptor homolog to the *Drosophila* toll [86], different studies reported that TLR2 is a receptor for bacterial lipoproteins [48, 54, 87, 88]. This definitely contributed to understand the adjuvant properties of lipopeptides and lipoproteins and drove a renewed interest in their use as adjuvants. Meanwhile, many other molecules have been claimed to bind TLR2, including nonlipidated molecules, and, although their interaction with the receptor is not entirely elucidated, some have also been used experimentally as adjuvants (e.g., [89, 90]). Among the strategies proposed to explore TLR2 stimulation for the modulation of immune responses, the different systems allowing covalently link lipid moieties to proteic antigens present the most promising applications in vaccinology.

3.1. Recombinant Bacterial Lipoproteins Expressed in Fusion with Heterologous Antigens. The first plasmidic vectors for the expression of proteins in fusion with bacterial lipoproteins in *E. coli* aiming at vaccine development were reported in the 1990s. Some of these works had the single objective of exhibiting antigens at the surface of the host bacteria [91, 92], but others were developed also with the purpose of making use of the adjuvant properties of the lipid moiety for the induction of immune responses against heterologous antigens [93, 94]. These vectors consist in partial or complete sequences derived from bacterial lipoprotein genes followed downstream by coding sequences for the heterologous antigens. Among the lipoproteins used as partners in these chimeric structures or used as sources of the lipidation signals are the colicin E2 lysis lipoprotein from an *E. coli* colicinogenic plasmid [94], OprI lipoprotein from *Pseudomonas aeruginosa* [93], Braun's lipoprotein from *E. coli* [95, 96], 26 kDa lipoprotein (Rv1411) from *Mycobacterium tuberculosis* [97], Wza lipoprotein from *Vibrio anguillarum* [98], Ag473 lipoprotein from *Neisseria meningitidis* [99], and OMP19 lipoprotein from *Brucella abortus* [100]. Apart from the Rv1411 lipoprotein from *Mycobacterium tuberculosis*, all the mentioned lipoproteins are originally from Gram-negative bacteria where they are found anchored to the outer membrane. These molecules are first expressed in the cytoplasm as prolipoproteins with an N-terminal signal peptide and are then translocated by the Sec translocon across the inner membrane to the periplasmic side where the processing takes place [101]. The initial lipidation step consists in the binding of a diacylglycerol group through a thioether linkage to the cysteine residue located at the N-terminus of the mature sequence, followed by the cleavage of the signal peptide. The third acyl chain is then attached to the amine group of the same cysteine residue through an amide linkage. Mature lipoproteins are finally transported and anchored on the outer membrane by the Lol System [101, 102]. Due to this maturation process, triacylated forms of the recombinant lipoproteins are only present in the outer membrane of the expression hosts and purification strategies were developed to purify these fully mature forms from the outer membrane [103]. In the cases in which lipoproteins are purified from whole bacterial cell lysates, immature forms, including diacylated lipoproteins, are also present in the final formulations [104, 105].

In some of these cloning and expression systems, multiple cloning sites were included downstream of the lipoprotein gene offering a flexible platform for the cloning of heterologous antigens and were even proposed for shotgun cloning viral genomes and screening for T cell antigens [106]. In some cases, C-terminal hexahistidine tails were also added to enable the purification of the fusion proteins by metal affinity chromatography. When the lipid moieties were characterized, palmitic acid was the predominant fatty acid found, although other fatty acids, including unsaturated, were also present [103, 107].

It is worth mentioning here that there are also examples of vaccine formulations using lipoproteins as homologue antigens, extracted from their native hosts or produced in other expression hosts (e.g., [108, 109]). The classical example is OspA from *Borrelia burgdorferi* for vaccination against Lyme disease [110].

3.2. Synthetic Lipopeptides. The chemical synthesis of peptides linked to lipid moieties is another widely used strategy to produce self-adjuvant formulations. Epitopes extended by Pam_3C or Pam_2C mimic tri- and diacylated bacterial lipid moieties, but many different variations to this structure have also been developed. These include single-chain palmitoyl-peptides and the more complex lipid core peptide (LCP) and multiple antigen lipophilic adjuvant carrier (MALAC) systems. The covalent attachment of TLR2 agonists to intact proteins has also been reported [111]. Their description and the relations between their structural characteristics and activity have been extensively discussed in detailed reviews (see, e.g., [112–114]) and thus we will not focus on that. However, it is important to stress that differences in the lipid moiety structure, such as the length of the fatty acids and chirality of the glyceryl modification, affect the TLR2-activating properties and this may reflect on the immune response elicited.

Another important point to consider is that some of these synthetic ligands have peptidic and lipid structures very different from the typical bacterial TLR2 ligands and, in certain cases, the dependency on TLR2 activation for their immunomodulatory properties remains to be elucidated. However, for monoacetylated lipopeptides and some other lipoamino acid based lipopeptides the activation through TLR2 is documented [115–117].

4. Immunomodulation by Formulations Targeting TLR2

A rational use of adjuvants in the development of better subunit vaccines relies on the understanding of how stimuli exerted at vaccination are translated in specific immune mechanisms, including their magnitude, profile, persistence, and localization. The innate activation through PRRs plays a central role in this shaping of the adaptive immunity and here

we resume what has been reported, mainly based on mouse and human studies, concerning TLR2 and TLR2-targeting immunogenic formulations (Table 2).

4.1. Modulation of APC Migration and Antigen Internalization. TLR activation has been implicated in the several steps that culminate in the development of specific immune responses, including APC migration. Recently, addressing different adjuvants on influenza subunit vaccines, a role for TLR2 activation in leukocyte migration to inflammation foci was suggested [118]. Pam_3CSK_4 was shown to be more efficient than CpG and resiquimod, respectively, TLR9 and TLR7/8 ligands, at inducing an early recruitment of $CD11b^+$ blood cells, mainly neutrophils, at the injection site and this observation correlated with the higher capacity of Pam_3CSK_4 to enhance antibody responses against influenza antigens [118]. TLR ligands were shown to transiently reduce the motility of DCs at the inflammatory sites, allowing for an extended contact between DCs and the antigen at the site of inflammation [119, 120]. However, the DC activation through TLRs downregulates receptors for inflammatory chemokines and upregulates CCR7 promoting their subsequent migration through lymphatic vessels and localization in the T cell areas of the regional lymph nodes [4, 121–123], and this was reported for TLR2 agonists as well [124, 125].

Internalization of pathogens is also regulated by TLR activation at the inflammatory sites. An initial transitory increasing in the internalization of antigens in response to TLR ligands occurs and is followed by the characteristic reduction in the endocytic capacity of mature DCs, which is congruent with a phenotype specialized in processing and presentation of antigens [126–129].

The above-mentioned transitory reduction in DC motility and the enhancement of antigen internalization was observed upon stimulation of different TLRs including TLR2 [120, 129]. Also demonstrating a TLR2 role in antigen internalization, Schjetne et al. [130] showed that targeting TLR2 with an anti-TLR2 monoclonal antibody leads to the internalization of the ligand into endosomes and its presentation under MHC class II.

4.2. Antigen Processing and Presentation in the Context of MHC Class II. Although still controversial [131–133], growing evidences support a determinant role of TLR activation in the control of phagosome maturation, with consequent impact on the regulation of antigenic presentation by APCs. Studies developed by the groups of Blander and of Medzhitov sustain the existence of two modes of phagosome maturation, one constitutive and the other inducible and controlled by TLR signalling [134–136]. According to these studies, the phagocytosis of bacteria leads to a phagolysosomal fusion rate superior to that observed in phagocytosis of apoptotic cells. This difference is only observed in the presence of TLR signalling and involves activation of MAPK p38 through MyD88 activation. Notably, this control of maturation occurs autonomously in the phagosome; that is, the inducible mode is only observed at the phagosomes containing TLR ligands. In addition, TLR activation has been shown to control autonomously the MHC class II loading in the phagosome [136] and to stabilize MHC class II at the cell surface [137]. This autonomous control of phagosome maturation and MHC class II loading may contribute to explain the enhancement of specific immune responses when the antigen is covalently linked to the TLR ligands or is incorporated in the same physical particle [138–141]. The TLR2-targeting formulations in which the antigen is chemically linked to lipid moieties or expressed as fusion with a bacterial lipoprotein ensure TLR activation inside the same phagosomes that contain the antigen and thus are particular interesting tools to modulate antigen processing and presentation. In fact, clearly increased antibody and cellular responses were observed *in vivo* when the antigens were covalently linked to lipopeptides or lipoproteins in comparison to admixed formulations [140, 142, 143].

For the development of an effector response by $CD4^+$ T cells, the recognition of a peptide in the context of MHC class II must be accompanied by the engagement of costimulatory molecules expressed at the DC surface, like CD80, CD86, and CD40. In the absence of costimulation, T cells are instructed towards a regulatory or anergic phenotype, resulting in tolerance to the presented antigen [144]. The upregulation of these molecules and of MHC class II at the surface of DCs is generally induced through TLR activation [121]. TLR2 is not an exception. In several studies, the activation of APCs with synthetic lipopeptides and recombinant lipoproteins resulted in cell maturation, with upregulation of MHC and costimulatory molecules (e.g., [99, 142, 145–152]). Moreover, the presentation of MHC class II epitopes to specific T cells by DCs has been demonstrated to be enhanced in the presence of TLR2 agonists [129, 149].

4.3. $CD4^+$ T Cell Polarization. The fate of $CD4^+$ T cells is a key issue for the type of immunity elicited upon immunization and its adequacy to the challenge is of capital importance for the success of a vaccine. In this respect, the consequence of TLR activation is not the same for the different TLR ligands and this is probably the most controversial point regarding TLR2 activation and the use of TLR2-targeting formulations in vaccination.

Some authors associate the activation through TLR2 with the induction of Th2 responses [151–155]. According to the model proposed by Dillon et al. [151], the activation via this receptor induces a high level of ERK1/2 signalling resulting in the stabilization of the transcription factor c-Fos suppressing IL-12(p70) production and promoting IL-10 secretion, thus favouring Th2 type responses. Moreover, Gautier et al. [156] attribute the secretion of IL-12(p70) by DCs, essential for Th1 polarization, to an autocrine-paracrine loop of type I interferon (IFN) initiated in response to TLR activation. Stimulation via TLR2 induces the activation of a MyD88-dependent signalling pathway, with activation of NF-κB and the MAPK pathway, resulting, among other effects, in the production of proinflammatory cytokines but not of type I IFN. This would justify the incapacity to polarize responses towards Th1 type via TLR2.

However, other authors pointed TLR2 stimulation or the use of adjuvants composed of TLR2 ligands as an efficient

Table 2: Immunomodulation by TLR2 ligands in mouse and human models.

TLR2 ligand[a]	Species and cells/observations[b]	Reference
Modulation of APC migration and antigen internalization		
Recruitment of leukocytes		
Pam_3CSK_4	**Mouse**/Recruitment of $CD11b^+$ blood cells at injection site, mainly neutrophils	[118]
Transient reduction of DC motility at inflammatory sites		
Pam_3CSK	**Mouse BM-DCs, spl. DCs**/Podosome disassembly, increasing in cell spreading, switch from podosomes to focal contacts	[120]
Increase in DC antigen internalization		
Pam_3CSK_4; Pam_3CSK	**Mouse BM-DCs, spl. DCs**/Transiently increased pinocytosis	[120, 129]
Mouse anti-human TLR2 mAb	**Human Mo-DCs**/Internalization of an anti-TLR2 monoclonal antibody into endosomes	[130]
Pam_3CSK_4	**Mouse BM-DCs, D1 cells**/TLR2-independent; clathrin- or caveolin dependent; covalent link-dependent	[140]
Promotion of DC migration to regional lymph nodes		
Pam_3CSK_4	**Mouse BM-DCs, BM-Mf**/↘inflammatory chemokine receptors, ↗CCR7	[125]
Antigen processing and presentation in the context of MHC class II		
Enhanced presentation on MHC class II		
Pam_3CSK_4	**Mouse BM-DCs, spl. DCs**/When antigen is coadministered with TLR2 ligand but not sequentially	[129]
MALP-2	**Mouse BM-DCs**/When DCs are loaded with MHC class II Ova peptide in the presence of MALP-2	[149]
Upregulation of costimulatory molecules and MHC class II		
rLipo-D1E3; OprI BLP; PGN, LTA; MALP-2	**Mouse BM-DCs**	[99, 147–149]
[Th]-K(P_2CSS)-[B]	**D1 cells**	[142]
Pam_3CSK_4; 19-kDa and Tp47 LPs	**Human PBMC-DCs**	[145]
[Th]-K(P_2CSS)-[Tc]	**D1 cells**	[146]
Pam_3C	**Human Mo-DCs**	[130]
BPPcysMPEG	**Mouse**/*In vivo*, in $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC subsets	[150]
P. gingivalis LPS	**Mouse**/*In vivo*, in $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC subsets	[152]
Pam_3CSK_4	**Mouse**/*In vivo*, in $CD11c^+CD11b^+$ and $CD11c^+CD11b^-$ splenic DC subsets	[151]

Table 2: Continued.

TLR2 ligand[a]	Species and cells/observations[b]	Reference
$CD4^+$ T cell polarization		
Induction of Th2 responses		
Pam_3CSK_4	**Human Mo-DCs; Mouse/**↗ERK1/2 signalling, stabilization of c-Fos, ↘IL-12p70, ↗ of IL-10	[151, 153]
FSL-1	**Mouse/**Higher IgG1, lower IgG2a; ↗IL-10 but not IL-12p70 by splenocytes; ↗MAPK and c-Fos in splenocytes	[154]
P. gingivalis LPS	**Mouse/**↗IL-13, IL-5, IL-10 but not IFN-γ by specific $CD4^+$ T cells; no IL-12p70 by $CD8\alpha^+$ DCs	[152]
Pam_3CSK_4	**Mouse/**↗IL-13, IL-1β, GM-CSF, B7RP-1, but low IL-12, IFN-α, IL-18, IL-27 by BM-DCs. *In vivo* ↗IgE and IgG1 but not IgG2a; ↗IL-13 and IL-5 but not IFN-γ by specific $CD4^+$ T cells after restimulation or i.n. challenge	[155]
PGN, Pam_3C, and zymosan	**Human Mo-DCs/**No IL-12p70 while inducing IL-12p40. Related with the incapacity to induce type I IFN	[156]
Induction of Th1 responses		
K(Pam)-[Th] versus K(Chol)-[Th]	**Mouse/**Pam-LP induced higher IFN-γ and IL-2 and lower IL-4 by HSV1-specific $CD4^+$ T cells and higher IgG2a/IgG1 ratio	[117]
OprI-COOHgp63 BLP	**Mouse/**↗IgG2a; Leishmaniosis protection correlated with IFN-γ production	[143]
[Th]-K(P2CSS)-[Tc]	**Human Mo-DCs/**Production of IL-12p70 ≥ than that induced by LPS	[157]
Pam_2CSK_4 and Pam_3CSK_4	**Human/**↗IFN-γ in CB-PBMCs co-cultured with allogeneic DCs	[158]
Pam_3CSK_4	**Mouse/**↗IgG2a, ↘IgG1 and no IgE after oral immunization with gliadin	[159]
Pam_3C, 19-kDa, and Tp47 LPs	**Human PBMCs and Mo/**↗IFN-γ but not IL-4 in PBMCs; anti-IL-12 antibody ↘T cell proliferation; ↗IL-12p40 in Mo	[160]
PGN, Pam_3C, Pam_3CSK_4	**Mouse splenocytes/**↗IL-12p70 and IFN-γ (but less than LPS)	[161]
rlipo-E7m	**Mouse/**↗IL-12 in BM-DCs; Higher IFN-γ and lower IL-5 by restimulated splenocytes	[162]
19- and 38-kD BLPs	**Human Mo; THP-1 cells/**↗IL-12p40 in THP-1 cells; 19-kD lipoprotein ↗IL-12p70 in Mo	[87]
19 kDa BLP; Tp47, OspA, and 19 kDa LPs; Pam_3CSK_4	**Human Mo-DCs and Mo/**↗IL-12p40 and IL-10 in Mo; ↗IL-12p40 and IL-12p70 in Mo-DCs (IL-12p70 higher for the lipoprotein than for the lipopeptides)	[163]
Pam_3CSK_4 and MALP-2	**Mouse Th1 cells/**Direct stimulation ↗IFN-γ, cellular survival and proliferation in the absence of TCR stimulation; not observed for ligands of other TLRs	[164]
Induction of Th17 responses		
PGN, Pam_3CSK_4, MALP-2	**Human Mo-LC/**MALP-2 and PGN ↗IL-6, IL-1β, and IL-23 by Mo-LC; ↗IL-17 by allogeneic $CD4^+$ T cells cocultured with $CD1^+$ Mo-LC stimulated with PGN or Pam_3CSK_4	[165]
Induction of Th1/Treg differentiation and inhibition of Th2 responses		
OprI BLP	**Mouse/**↘airway eosinophilia, IL-4 and IL-13 after i.n. coadministration of OprI with Ova allergen	[147]
LP40	**Human PBMCs and T cells; Mouse *in vivo* models./**↗IL-10, IFN-γ and IL-12 by human PBMCs, both directly and on antigen restimulation; ↘allergy in different models (↘Th2 cells, IgE, and eosinophilia)	[166]
Pam_3CSK_4	**Mouse/**↗IL-12p35 and IL-10 in BM-DCs and oral myeloid DCs; treated DCs ↗IFN-γ and IL-10 by naïve $CD4^+$ T cells; Sublingual administration with antigen in Ova-sensitized mice ↘airway hyperresponsiveness and Ova-specific IL-5 and IL-10 in cervical lymph nodes	[167]
Regulatory role		
Zymosan	**Human Mo-DCs and Mouse spl. DCs, *in vitro*; Mouse, *in vivo*/**↗high IL-10, low IL-6 and IL-12p70 by DCs; *In vivo*: low costimulatory molecules on splenic DCs or proinflammatory cytokines, induction of specific T cells secreting IL-10 but little Th1 or Th2 cytokines, and unresponsiveness to challenge with the antigen plus adjuvant (IFA)	[168]
Zymosan	**Mouse spl. DCs/**↗Aldh1 and IL-10; leads to metabolize vitamin A and stimulate Treg cells	[169]
Zymosan	**Mouse/**T cells from treated mice showed reduced ability to induce diabetes in NOD-Scid mice	[170]
FSL-1	**Mouse/**↗Treg cells in the draining lymph node and ↗ the growth of tumor. Anti-CD25 antibody abrogated the protumor activity of FSL-1	[171]
Pam_2 lipopeptides	**Mouse/**↗Treg cells in a TLR2- and IL-10- dependent manner	[172]
Antiregulatory role		
Pam_3CSK_4	**Mouse/**Direct activation of Tregs, with TCR and IL-2 stimulation, induced proliferation and temporary loss of suppressive properties, which are restored after removing the stimulus	[173, 174]
Pam_3CSK_4	**Mouse/**↘suppressive function of Treg cells; ↗tumor-specific CTL activity	[175]
Pam_3CSK_4	**Mouse/**$CD4^+$ effector T cells became resistant to Treg-mediated suppression	[176]

Table 2: Continued.

TLR2 ligand[a]	Species and cells/observations[b]	Reference
Cross-presentation and $CD8^+$ T cell cytotoxicity		
Pam_3CSK_4-[Tc]	**Mouse**/Induction of specific CTL responses higher than peptide admixed; *In vitro* cross-presentation of peptides in fusion, but not admixed, and independently of TLR2	[140]
[Th]-K(P_2CSS)-[Tc]	**HLA-A2kb transgenic Mouse; Human Mo-DCs**/Induction of specific CTL responses. Lipopeptide-pulsed human DCs activated antigen-specific IFN-γ production by autologous $CD8^+$ T cells	[157]
[Th]-K(P_2CSS)-[Tc] versus Pam_2CSS-[Th]-[Tc]	**Mouse**/Branched lipopeptide more potent in the primary response; both induced CTLs and conferred long-term protection against Ova-expressing tumor cells	[177]
[Th]-K(Pam or Chol)-[Tc]	**Mouse**/Induction of CTL responses by i.n. route and enhanced protection against influenza challenge	[178]
Pam_3CSS-[Tc]	**Mouse**/Specific CTL activity induced *in vivo* against influenza virus	[81]
Hda-[Tc]	**Mouse**/Specific CTL activity induced *in vivo* against HIV-1 virus	[179]
K(Pam)-[Tc]	**Human Mo-DCs**/Lipopeptides but not the peptide were endocytosed; ↗IFN-γ of matched specific $CD8^+$ T-cells	[180]
Pam_3CSK_4-[Tc]	**Mouse**/Induced *in vivo* tetramer positive and IFN-γ producing $CD8^+$ T cells	[181]
MALP-2	**Mouse BM-DCs**/↗immunoproteasome proteins LMP2, LMP7, and MECL; ↗proteolytic activity	[149]
$(K(Pam))_{1,2,or\ 3}$-[Th]-[Tc]	**Mouse**/↗specific antiviral CTL responses irrespective of the number of lipid moieties	[182]
BPPcysMPEG	**Mouse**/↗specific CTL response in a TLR2 and $CD4^+$ Th dependent manner, but independent of IFN-α. Direct link of the antigen to BPP ↗CTL	[150]
FSL-1	**Mouse**/Immunization of FSL-1 and tumor-associated antigens ↗specific CTLs	[171]
rlipo-E7m	**Mouse**/↗CTL responses and therapeutic and prophylactic protection against tumor challenge	[162]
Induction of NK cells activity		
Stimulation of NK cell activity		
Pam_2C lipopeptides	**Mouse**/Variations in the activation capacity among lipopeptides with different peptide sequences	[183]
Pam_2CSK_4 versus MALP-2	**Mouse**/MALP-2 much less effective in inducing NK cell activity	[184]
Induction of ADCC by NK		
FSL-1	**Mouse**/Coadministration with tumor associated antigens ↗antitumor ADCC by NK cells	[171]

Table 2: Continued.

TLR2 ligand[a]	Species and cells/observations[b]	Reference
Induction of antibody responses		
Induction of antibody secreting cells (ASC) differentiation		
Natterins	**Mouse**/Generation of long-lived ASCs	[185]
Pam_2CSK_4; Pam_3CSK_4	**Mouse**/Together with CD40 signal, ↗differentiation of B cells into ASCs	[186]
Increasing of antibody responses		
Fusion lipoproteins	**Mouse**	[99, 143]
Synthetic lipopeptides	**Mouse**	[117, 146, 154, 155]
Induction of mucosal responses		
Immunization via mucosal surfaces		
$((Pam)K)_3$-[Th]-[Tc]	**Mouse**/Intravaginally delivery ↗HSV-2-specific memory CTLs locally and in the spleen. Significantly weaker response observed in $TLR2^{-/-}$ mice	[187]
MAP-Pam_3C	**Mouse**/↗specific IgA in mucosal secretions and IgG in the serum after oral immunization. Intragastric delivery ↗systemic T cell stimulation and specific CTL activity	[188]
LT-IIa-B5	**Mouse**/i.n. immunization recruited DCs to the NALT, ↗antigen uptake, CCR7 and migration to draining lymph node. TLR2-dependent ↗ of specific $CD4^+$ T cell proliferation, salivary IgA, and serum IgG	[124]
Pam_3CSK_4	**Mouse**/i.n. immunization enhanced serum antibody responses and protection against influenza	[189]
[Th]-K(P_2CSS)-[Tc]	**Mouse**/i.n. immunization induced $CD8^+$ CTL responses in the lung and systemic after influenza challenge	[146]
Mucosal imprinting of specific immunity		
Pam_3CSK_4	**Mouse**/Pretreatment of extraintestinal DCs ↗retinal dehydrogenases and confer the capacity to induce gut-homing lymphocytes	[190]

[a] rLipo-D1E3 and rlipo-E7m: Domain 1 of the Ag473 lipoprotein from *Neisseria meningitidis* fused to a sequence of dengue virus envelope protein (E3) or inactive E7 oncoprotein of human papillomavirus (E7m); OprI or OprI-COOHgp63 BLPs: outer membrane lipoprotein I from *Pseudomonas aeruginosa*, alone or fused with a truncated 32-kDa version, the major cell surface glycoprotein gp63 of *Leishmania major*; [Th]: $CD4^+$ T cell epitope; [Tc]: $CD8^+$ T cell epitope; [B]: B cell epitope; K: Lysine with a palmitic (Pam), diacylated (P2CSS), or cholesterol (Chol) moiety attached to ε-amino group; 19-kDa, Tp47, and OspA LP: synthetic tripalmitoyl lipopeptides based on the sequences of 19-kDa lipoprotein from *Mycobacterium tuberculosis*, 47-kDa lipoprotein from *Treponema pallidum*, and OspA lipoprotein from *Borrelia burgdorferi*; 19-kDa and 38-kD BLP: 19-kDa and 38-kD lipoproteins from *Mycobacterium tuberculosis*; LP40: synthetic lipopeptide CGP 40774; Hda: α-aminohexadecanoic acid; BPPcysMPEG: pegylated synthetic derivative of MALP-2; MAP: multiple antigen peptide; LT-IIa-B5: pentameric B subunit of type IIa enterotoxin of *Escherichia coli*.

[b] ↗: enhancement; ↘: decrease; BM-DCs: bone marrow-derived DCs; spl. DCs: splenic DCs; Mo-DCs: monocyte-derived dendritic cells; Mo: monocytes; Mo-LC: monocyte-derived Langerhan-like cells; D1 cells: a line of immature DCs derived from spleen cells; PBMC-DCs: PBMC-derived DCs; CB-PBMCs: cord blood-derived PBMCs; THP-1 cells: human monocytic cell line; i.n.: intranasal.

strategy to induce Th1 responses through APC activation [87, 117, 143, 157–163]. Moreover, according to Imanishi et al. [164], the stimulation of mouse Th1 cells by TLR2 ligands directly induces IFN-γ production, as well as cellular survival and proliferation in the absence of TCR stimulation. The same is not observed for ligands of other TLR receptors, suggesting an important role of TLR2 activation in the promotion and maintenance of Th1 responses.

In other experimental conditions, the induction of Th17 polarization by IL-1β, TGF-β, and IL-23 produced by human Langerhans cells stimulated via TLR2 was reported [165]. Synthetic lipopeptides or bacterial lipoproteins have also been shown to induce Th1/T_{reg} differentiation and inhibition of Th2 responses, suggesting a potential application in the treatment of asthmatic diseases [147, 166, 167]. In fact, a regulatory role has been attributed to TLR2 in several studies and its targeting for the induction of tolerogenic responses has also been proposed [168–172, 191]. However, experimental evidences support that the direct activation of T_{regs} by the TLR2/1 ligand Pam_3CSK_4, together with stimulation through TCR in the presence of IL-2, induces proliferation and temporary loss of suppressive properties, which is restored after removing the stimulus [173, 174]. Furthermore, the same TLR2 ligand was reported to exert antitumor effects, either through the reduction of the suppressive function of T_{regs} [175] or by the enhancement of the resistance of T effector cells to T_{reg} suppression [176].

The observations supporting divergent TLR2 polarizing properties were obtained from distinct models, using various TLR2 ligands and looking at different levels of the immune response, from the molecular signaling level up to *in vivo* context. As underlined by Mele and Madrenas [192] and supported by other studies [171], it is clear that TLR2 plays both proinflammatory and regulatory roles and that through TLR2 activation, alone or in combination with other stimuli, both effector and regulatory immune mechanisms can be elicited *in vivo* depending on not yet completely clarified factors. To focus on understanding these discrepancies should be a priority for rationally exploring TLR2 stimulation in vaccine development.

4.4. Cross-Presentation and $CD8^+$ T Cell Cytotoxicity. TLR activation has been also implicated in the induction of cross-presentation of antigens by DCs as consequence of enhanced antigen internalization and delivery to the cytosol as well as increase in TAP and proteasome activity [128, 129]. A MyD88-dependent cross-presentation mechanism that requires the dislocation of TAP to the early endosomes was also reported [193], suggesting a spatial separation between endogenous MHC class I-restricted antigen presentation and cross-presentation of exogenous antigens, the latter being biased toward antigens containing PAMPs. Cross-presentation and *in vivo* induction of CTL by TLR activation are usually attributed to TLR ligands high inducers of type I IFN [126, 194, 195], which is not the case of TLR2 ligands. However, different studies demonstrate the induction of these mechanisms by antigens conjugated with TLR2 ligands [140, 146, 157, 177, 178]. In fact, this has been from the beginning one of the most appealing characteristics of these immunogenic formulations [81, 196]. Initially, it was suggested that this capacity could be due to the access of lipidated peptides to the cytoplasm of APC, facilitated by the interaction of the membrane lipids with those of the lipoprotein, with consequent entering in the MHC class I processing pathway [179]. Another explanation resided in the physical properties conferred to the formulations by the lipids, for example, the possible formation of micellar structures and the consequent processing by APCs identical to that observed with particulate antigens [180]. Although these mechanisms are fundamentally of physical nature and TLR-independent, the activation of these receptors also plays an important role in the induction of CTL responses. In fact, Khan et al. [181] showed that *S* and *R* glycerol configurations of Pam_3CSK_4 are equally internalized by DCs but diverge at their capacity to induce cytokines and maturation markers on these cells, as well as on the induction of CTL responses *in vivo*. This suggests a determinant role of TLR2 activation on promoting the CTL immune mechanisms. Additionally, it was demonstrated that DC stimulation with the TLR2 ligand MALP-2 induces the expression of proteins of the immunoproteasome LMP2, LMP7, and MECL and an increasing in the proteolytic activity and thus the antigenic processing, suggesting that lipopeptides may indirectly increase the responses restricted to MHC class I [149].

Monoacylated lipopeptides, lipidated through the covalent binding of palmitic acid to the lateral chain of lysine, induce $CD8^+$ T cell responses [180, 182] and, although monoacylation do not correspond to the native structure of bacterial lipoproteins, Zhu et al. [117] found that these molecules enhance internalization and DC maturation through TLR2. Zhang et al. [187] also demonstrated the induction of herpes simplex virus (HSV)-2-specific memory $CD8^+$ CTL both locally and systemically after intravaginal immunization with a peptide extended by a lipid moiety with three palmitic acids and showed that this response was significantly lower in $TLR2^{-/-}$ and $MyD88^{-/-}$ mice. The inoculation of ovalbumin (Ova) peptides together with BPP, a synthetic derivative of the MALP-2, induced CTL responses which were much higher when the peptides were directly linked to the TLR2 ligand comparing to admixed antigens and adjuvants [150].

4.5. Induction of NK Cells Activity. TLR2-dependent NK cell activation was demonstrated to play a role in the immune response against different virus and bacteria [197–201] suggesting the possibility to explore TLR2 ligands as inducers of NK cell activity in therapeutic or prophylactic immunomodulation. The activation of NK cells by different TLR2 agonists has been demonstrated, although the requirement for accessory cells, namely DCs, and accessory cytokines is still debatable [202–204]. Also, variations in the capacity to stimulate NK cells were found among different TLR2 ligands. For example, the activation of NK cells by MALP-2 through stimulation of TLR2 on bone marrow-derived DCs is much less effective than Pam_2CSK_4 [184] and the peptide primary sequence in synthetic Pam_2C lipopeptides has been demonstrated to influence the capacity for

NK activation, both directly through TLR2 on NK cells and via DCs [183].

In vivo, the subcutaneous injection of Pam_2CSK_4 around NK sensitive B16D8 tumor cells led to tumor retardation and this activity was abrogated by injection of asialoGM-1 antibodies, while this antitumor activity was not observed for MALP-2 ligand which was less effective in inducing NK activity [184]. However, in another study, the administration of diacylated lipopeptides (MALP-2 and different Pam_2 peptides) in mice induced IL-10 and T_{reg} cells that prevented effective antitumor therapeutic responses [172]. The authors also reported the production of IL-10 by NK cells stimulated by the TLR2 agonists *in vitro*. In an antitumor prophylactic immunization, Kiura et al. [154] reported that coadminisration of tumor associated antigens with the diacylated lipopeptide FSL-1 could induce antitumor antibody dependent cellular cytotoxicity (ADCC) by NK cells.

The role of TLR-mediated activation of NK cells in immune responses against infectious and tumor disease is now emerging [205]. TLR2-mediated activation of NK activity for the shaping of the adaptive response, as well as the activation of NK activity in the context of a recall response, will certainly be an interesting research field for the development of future prophylactic vaccines.

4.6. Induction of Antibody Responses. Antibody titres, affinity, avidity, and neutralizing capacity are for many diseases the best correlative of protection and to elicit a proper and long-lasting antibody response is thus frequently a desirable achievement in vaccination.

The role of TLR activation in antibody responses and its longevity is a theme of actual research. Pasare and Medzhitov [206] showed that the generation of T-dependent antigen-specific antibody responses requires activation of TLRs in B cells. Kasturi et al. [207] have shown that administration of Ova in synthetic nanoparticles together with the TLR4 ligand monophosphoryl lipid A (MPL) and, simultaneously, with the TLR7 ligand R837 leads to synergistic increases in the anti-Ova antibody response and provides a sustained memory for 1.5 years. This was dependent on DCs, Th cells, and the direct TLR stimulation on B cells.

The specific role of TLR2 in the development of antibody responses is now emerging. TLR2 has been recently demonstrated to be involved in the generation and longevity of antibody secreting cells (ASC) [185] and the addition of CD40 signalling to TLR1/2 and TLR2/6 agonists have been shown to stimulate differentiation of B cells into ASC [186]. Moreover, lipidated formulations targeting TLR2 are widely described as good inducers of antibody responses (e.g., [99, 117, 143, 146, 154, 155, 159]).

4.7. Mucosal Immune Responses. Many of the most relevant diseases of humans and veterinary animals are caused by infectious and parasitic agents entering the target hosts through mucosae. Immunization via different mucosal surfaces using TLR2-targeting formulations has been demonstrated to induce strong immune responses, including mucosal IgA and serum IgG as well as local and systemic $CD8^+$ CTL [124, 146, 187–189]. Efficient induction of mucosal immune responses is not usually achieved by parenteral administration of vaccines, requiring the presentation of antigens directly at the mucosal surfaces. However, TLR2/1 signals, but not signals from other TLRs, have been shown to be capable of educating extraintestinal DCs with gut-specific imprinting properties [190]. Considering that an immune response elicited apart from the strongly regulatory mucosal environment may be more freely modulated, this capacity of localizing immune responses at the mucosal level through nonmucosal immunization may open the possibility to better tailor the adaptive mucosal immunity. For this purpose, TLR2-targeting formulations are particularly interesting tools.

5. Immunomodulation through TLR2 for Veterinary Vaccines

For the veterinary species, the modulatory effect of TLR2 activation on immune response is much less characterized than in humans or in the mouse model. However, *in vitro* activation of APCs or PBMCs by TLR2 ligands was demonstrated for different species as it was the adjuvanticity of TLR2-targeting formulations when inoculated *in vivo*. Evidences on the capacity to elicit CTL responses were also obtained but information on the immune response profiles is sparse. Here, we compile some of the most relevant studies available regarding immunomodulation through TLR2 in veterinary species.

5.1. Ruminants. In ovine, stimulation of bone marrow-derived DCs by LTA resulted in upregulation of MHC class II in the $CD11b^{dull}$ subset, which acquired strong capacity of stimulating $CD4^+$ T cells in allogenic assays [208]. In bovine, Pam_3CSK_4 stimulation of monocyte-derived DC also induced DC maturation, with upregulation of MHC class I, MHC class II, CD40, CD80, CD86, and CD1b molecules, and lead to the production of IL-12 and TNF-α. In addition, stimulated DCs promoted IFN-γ secretion when cocultured with allogeneic PBMCs [209]. In contrast, the stimulation of macrophages lead to the downregulation of MHC expression and to an almost null effect on IL-12 and TNF-α production and on IFN-γ secretion in mixed leukocyte reaction [209]. However, Franchini et al. [210] reported that bovine macrophages produce nitric oxide (NO) and TNF-α in response to TLR2 activation and that this production is strongly increased by costimulation with IFN-γ.

Nelson et al. [211] have shown the possibility to induce active vitamin D_3 (1α,25-dihydroxyvitamin D3) in bovine monocytes through TLR4 and TLR2 activation. Notably, in the mouse, the induction of vitamin D3 in APCs was shown to be related with the imprinting of skin-tropism by APCs in T cells [212]. How this could be exploited for the induction of skin-tropic responses in farm animals remains to be studied. More recently, it was reported that bovine $\gamma\delta$ T cells directly respond to TLR2 ligands with increased proliferation and cytokine production in a TCR-independent manner [213].

Investigating the potential of *Mycobacterium bovis* antigens to stimulate delayed-type hypersensitivity (DTH) response in cattle, Whelan et al. [214] have shown that the combination of Pam_3CSK_4 lipopeptide with the ESAT-6 antigen allowed the induction of DTH in experimentally infected calves which did not occur when the antigen was used alone. *In vitro*, the authors showed the induction of TNF-α production by bovine DCs stimulated by Pam_3CSK_4 and pointed this induction of proinflammatory cytokines as a possible explanation for what was observed *in vivo*.

Aiming at optimizing the protective efficacy of *Mycobacterium bovis* BCG, Wedlock et al. [215] tested combinations of BCG with culture filtrate proteins formulated with a depot adjuvant and mixed with different stimulatory molecules: MPL, a synthetic mycobacterial phosphatidylinositol mannoside-2 (PIM2) and Pam_3CSK_4. Evaluating different pathological and microbiological disease parameters, such as the proportion of animals with tuberculous lesions in the lungs and lymph nodes and the number of *M. bovis* culture-positive lymph nodes, the inclusion of Pam_3CSK_4 in the vaccine formulation was shown to induce the best protection.

The potential to use lipopeptides for vaccination against foot-and-mouth disease was tested using seven peptides containing FMDV-specific B-cell linear epitopes from structural and nonstructural proteins, synthesized with a Pam_3C moiety, and delivered intramuscularly emulsified with Montanide ISA 9 [216]. Twenty days after a single immunization, the animals were challenged and four of the seven immunized animals were protected. No correlation was found between protection and antibody titre or virus-specific proliferation but all protected animals showed a strong T-cell response against at least one of the peptides used for immunization.

Also in cattle, lipopeptides with a palmitic acid coupled to the NH_2-terminal amino acid and delivered in Freund's adjuvant were used to boost an anti-*Neospora caninum* SRS2 (NcSRS2) DNA immunization [217]. Lipopeptide boosting induced strong immune response, characterized by increased NcSRS2-specific lymphocyte proliferation, IFN-γ-secreting cells, and levels of specific IgG1 and IgG2a antibodies. Regarding these parameters, this immunization strategy reproduced the immune response observed against *N. caninum* infection in cattle.

5.2. Horse. Stimulation of equine monocytes with Pam_3CSK_4 induced the production of TNF-α, IL-6, IL-1β, and IL-10 [218], and in a whole blood assay TNF-α, IL-6, and IL-1β were also induced by PGN and LTA [219]. Using equine infectious anemia virus (EIAV) CTL epitopes synthesized on multiple antigenic peptide (MAP) system linked to Pam_3C, Ridgely and McGuire [220] demonstrated the capacity to stimulate CTL activity *in vitro* on PBMCs obtained from horses of different ELA-A haplotypes chronically infected. The stimulated cells were able to specifically lyse EIAV-infected target cells. In addition, immunization of horses with a Pam_3C-MAP-CTL epitope induced transitory peptide and virus-specific CTL and, although it neither prevented infection nor affected viral load, it induced a protective effect against development of clinical disease following virus challenge, in which vaccinated horses showed less severe fever and thrombocytopenia and did not develop anemia during the first 2 months after infection [221]. In another approach for anti-EIAV immunization with lipopeptides, Fraser et al. [222] inoculated horses with a pool of peptides containing Th and CTL epitopes extended by a palmitic acid molecule to each of the free NH_2 groups. The immunized horses showed significant postimmunization proliferative responses to Th peptides but no evident CTL response. After challenge, the immunized group also had a significant increase in the proliferative response to the Th peptides and PBMCs from four of five immunized horses showed CTL activity when stimulated 2 weeks later. Nonetheless, no significant protection was observed considering level and course of viral load, the platelet counts, or fever.

5.3. Swine. Immunizing pigs against mouse IgG, an increase in the anti-mouse IgG titres was observed by targeting the antibody to TLR2 [223]. In this work, it was also shown that the *in vitro* proliferative response of PBMCs obtained from pigs immunized with mouse IgG was enhanced when restimulation was performed using an anti-TLR2 mouse monoclonal antibody comparing with restimulation with an isotype-matched control.

Using outer membrane preparations from bacteria expressing African swine fever virus (ASFV) antigens in fusion with the OprI lipoprotein, the entering of the antigens in the class I pathway of antigen presentation and the possibility to identify ASFV epitopes specifically recognised by porcine CTL [196] as well as to stimulate specific CTL activity *in vitro* [106] were demonstrated. In a different study [224], with the purpose to test OprI as an adjuvant for a subunit vaccine against classical swine fever (CSF), it was shown that this lipoprotein activated porcine monocyte-derived DCs, upregulating CD80/86 and MHC class II expression, as well as proinflammatory cytokines. The antigenic restimulation of lymphocytes obtained from CSFV-immune pigs cocultured with autologous monocyte-derived dendritic cells was also enhanced by OprI, as measured by proliferation and IFN-γ production. *In vivo*, a subunit vaccine adjuvanted with OprI induced partial protection against CSFV infection but less effective than a water-oil-water adjuvanted vaccine tested in parallel.

5.4. Chicken. Stimulation of chicken splenocytes with Pam_3CSK_4 upregulated not only Th1-associated cytokines IFN-γ and IL-12 but also the Th2-associated cytokine IL-4 [225]. The direct stimulation of chicken $CD4^+$ T cells by Pam_3CSK_4 also significantly upregulated IFN-γ but not IL-4, IL-13, and IL-10 [226]. In a study comparing the effect of three different TLR2 ligands on chicken splenocytes, the results observed suggest different kinetics in the production of proinflammatory cytokines. Pam_3CSK_4 induced high IL-1β response, while FSL-1 induced an early and prolonged expression of IL-8. The three TLR2 ligands, Pam_3CSK_4, FSL-1, and lipomannan, induced a mixed Th profile with upregulation of IFN-γ, IL-12, IL-4, and IL-13 [227]. Stimulating chicken monocytes, He et al. [35] demonstrated

the induction of iNOS mRNA and of NO production by LTA but not by Pam_3CSK_4. Erhard et al. [228] tested the adjuvant effect of Pam_3CSK_4 and Pam_3CS linked to Th epitopes when administered together with different antigens. Although varying with the antigen, the antibody responses were enhanced and in certain cases the combination of more than one adjuvant induced even better responses. Testing two lipoproteins from *Pasteurella multocida* as vaccine antigens, Wu et al. [109] report high protection rates in chicken immunized with *E. coli* expressed lipoprotein E after inoculation in double emulsion adjuvant. OprI lipoprotein was shown to bind *in vitro* and *in vivo* to epithelial cells of the trachea and the small intestine of chickens suggesting its potential use as a carrier for antigen delivery at mucosal surfaces [229].

Based on previous demonstration of the immunostimulatory properties of protozoan HSP70 through TLR2 and TLR4, Zhang et al. [90] investigated if *Eimeria tenella* HSP70 could enhance the immunity elicited by *E. tenella* antigen microneme protein 2 (EtMIC2) against avian coccidiosis. EtHSP70 induced the production of IL-12 and IFN-γ in chicken embryo fibroblasts and when inoculated *in vivo* together with EtMIC2 resulted in increased body weight gains, decreased oocyst shedding, and increased antibody responses. Levels of IL-12, IFN-γ, and IL-17 were also higher compared with the inoculation of the antigen alone. Chickens immunized with EtHSP70 alone also revealed a protective effect against *E. tenella* infection.

6. Concluding Remarks

Vaccination in veterinary animals is a cost-effective strategy to promote animal health and may have an important impact on public health by contributing in reducing the use of antibiotics and controlling zoonotic diseases. The development of new vaccines largely relies on the understanding of how activation of innate immunity through PRRs shapes the subsequent adaptive immune response. The possibility of enhancing antigen presentation by covalently linking TLR2 ligands to the antigen and the particular TLR2 properties at influencing the type and localisation of specific immunity are interesting features that can help at solving some of the present vaccine challenges. However, considering the inconsistencies in results regarding the profile of immune responses, it is of major relevance to address how the specific immune mechanisms elicited upon immunization targeting TLR2 are affected by different factors, such as type of ligand, route of administration, doses, and synergies with other innate stimuli. To extend these studies to the field of veterinary vaccinology further implies to address species-specificities. Clarifying these aspects will allow us in the future to make the innate stimulus adequate for a particular challenge in a given species.

Abbreviations

ADCC: Antibody dependent cellular cytotoxicity
ASC: Antibody secreting cells
APC: Antigen presenting cell
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
EIAV: Equine infectious anemia virus
FMDV: Foot-and-mouth disease virus
LPS: Lipopolysaccharide
LTA: Lipoteichoic acid
MPL: Monophosphoryl lipid A
NO: Nitric oxide
NK: Natural killer
OprI: Outer membrane lipoprotein I
Ova: Ovalbumin
Pam_2C: Di-palmitoyl-S-glyceryl cysteine
Pam_3C: Tri-palmitoyl-S-glyceryl cysteine
PNG: Peptidoglycan
PRR: Pattern recognition receptor
TLR: Toll-like receptor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Generation of Recombinant Porcine Parvovirus Virus-Like Particles in *Saccharomyces cerevisiae* and Development of Virus-Specific Monoclonal Antibodies

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Porcine parvovirus (PPV) is a widespread infectious virus that causes serious reproductive diseases of swine and death of piglets. The gene coding for the major capsid protein VP2 of PPV was amplified using viral nucleic acid extract from swine serum and inserted into yeast *Saccharomyces cerevisiae* expression plasmid. Recombinant PPV VP2 protein was efficiently expressed in yeast and purified using density gradient centrifugation. Electron microscopy analysis of purified PPV VP2 protein revealed the self-assembly of virus-like particles (VLPs). Nine monoclonal antibodies (MAbs) against the recombinant PPV VP2 protein were generated. The specificity of the newly generated MAbs was proven by immunofluorescence analysis of PPV-infected cells. Indirect IgG ELISA based on the recombinant VLPs for detection of PPV-specific antibodies in swine sera was developed and evaluated. The sensitivity and specificity of the new assay were found to be 93.4% and 97.4%, respectively. In conclusion, yeast *S. cerevisiae* represents a promising expression system for generating recombinant PPV VP2 protein VLPs of diagnostic relevance.

1. Introduction

Porcine parvovirus (PPV), first isolated from sows in Germany [1], has been found to occur worldwide [2–4]. PPV is the major causative agent in a syndrome or reproductive failure in swine. This syndrome is characterized by stillbirth, mummified fetuses, early embryonic and fetal death, delayed return to estrus, and infertility (abbreviated as *SMEDI*) [5, 6]. PPV is also shown to be an agent able to increase the effects of porcine circovirus type 2 infection in the clinical course of postweaning multisystemic wasting syndrome [7], which is a significant disease in global swine production [8].

Five different groups of porcine parvoviruses (PPV) have been identified: classic PPV (PPV1), PPV2, PPV3 (known as porcine PARV4, hokovirus, or partetravirus), and PPV4 and porcine bocaviruses, which all have substantial genetic divergence [9–12]. Recently, a new parvovirus provisionally proposed to be named as PPV5 was discovered in the United States [13].

Classic PPV has one serotype subdivided into four clinical genotypes (biotypes) according to their pathogenicity. The NADL-8 strain can cause viremia and crosses the placenta to infect fetuses, leading to fetus death [14]. In contrast, nonpathogenic NADL-2 strain is currently widely used as an attenuated vaccine and causes only limited viremia without crossing the placental barrier in experimental infections [15]. The other two groups are the Kresse and IAF-A83 strains, which are associated with dermatitis and enteric diseases, respectively [16].

PPV is a small, nonenveloped virus, assigned to the genus *Parvovirus*. This group of viruses also infects rodents and carnivores and belongs to the Parvoviridae subfamily within the Parvoviridae family [9]. PPV has a negative, single-stranded DNA of about 5 kb with distinct hairpin termini. The genome contains two major open reading frames (ORFs), each located in the same frame of the complementary strand. ORF1 encodes three nonstructural proteins and the structural proteins VP1, VP2, and VP3 are encoded in ORF2. VP1 and VP2 are translated from differently spliced RNAs, whereas VP3 is formed by proteolytic cleavage of VP2 [17, 18].

VP2 consists of an eight-stranded antiparallel β-barrel motif with 4 large loops between β-strands. These loops are shown to possess many B-cell epitopes and can tolerate insertions [19, 20] that make PPV VP2 a potential antigen carrier and play a key role in PPV diagnosis and immune prophylaxis [21]. The structure of PPV capsid composed of baculovirus system generated recombinant VP2 is available in 3.5 Å resolution (PDB Accession Number 1K3V) [22].

Immunogenic major capsid protein VP2 of PPV has been synthesized in several expression systems including bacteria [21, 23]. PPV VP2 protein expressed using the baculovirus expression vector system was shown to assemble into virus-like particles (VLPs) similar in size and morphology to the original virions. Such VLPs were shown to induce antibodies in immunized pigs [24] and guinea pigs [25]. VLPs generated in baculovirus system exhibit positive immunoreactivity for PPV and are used in most commercial ELISA tests [26]. Most recently, immunogenic PPV VP2 protein was synthesized in yeast *Pichia pastoris* [27]

The formation of recombinant antigenic human parvovirus capsid protein VLPs in *S. cerevisiae* has been recently demonstrated [28, 29]. Regarding costs, yield, and ease of handling, VLP production in yeast represents an alternative to the recombinant baculovirus expression system, which is so far the dominating source of VP2-derived VLPs of parvoviruses [27, 28].

In the current study, we have generated the PPV VP2 protein as VLPs in *S. cerevisiae* expression system, demonstated their structural and antigenic similarity with viral capsids and developed a new indirect IgG ELISA based on the use of PPV VP2-derived VLPs. Moreover, we have developed a panel of PPV VP2 protein-specific monoclonal antibodies and demonstrated their reactivity with PPV-infected cells.

2. Materials and Methods

2.1. Serum Samples. One hundred and eighty-three swine serum samples from farms in Lithuania ($n = 160$), Romania ($n = 14$), and Ukraine ($n = 13$) were collected in years 2008–2010 and used in this study. Samples were stored at −70°C prior to testing.

2.2. Viral DNA Isolation. Viral nucleic acids (NAs) were extracted from porcine kidney cell culture PK-15 (ATCC CCL-33) infected with porcine parvovirus strain NADL-2. NAs were extracted using commercial QIAamp UltraSens Virus kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's manual and stored at −70°C until use.

2.3. Cloning and Characterization of PPV VP2 Gene. The PPV VP2 gene was amplified using High Fidelity Enzyme Mix (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania) directly from extracted NAs using the following pair of primers (IDT, Munich, Germany):

PPV-vp2-F 5′-TCTACTAGTACAATGAGTGAAA-ATGTGGAACAA-3′

PPV-vp2-R 5′-GAGACTAGTCTAGTATAATTTT-CTTGGTATAAGT-3′

The primers used for amplification incorporated *Bcu*I site (underlined) for subcloning into the yeast vector pFX7. The thermal cycle conditions were the following: initial denaturation for 3 min at 95°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and then the final elongation at 72°C for 10 min. The PCR amplification product was digested with *Bcu*I and inserted into *Xba*I-linearized and dephosphorylated yeast expression plasmid pFX7 under control of yeast GAL1-10 promoter [30] and confirmed by PCR and subsequent DNA sequence analysis. The nucleotide sequence of the amplified PPV VP2 was compared with those in GenBank using the Basic Local Alignment Search Tool (BLAST). All DNA manipulations were performed according to standard procedures [31] using enzymes and kits from Fermentas/Thermo Fisher Scientific. Recombinant constructs were screened in *Escherichia coli* DH5α F′.

2.4. Strains, Media, Yeast Transformation, Cultivation, and Protein Purification. Recombinant construct containing PPV VP2 sequence was screened in *E. coli* DH5αF′ cells. *Saccharomyces cerevisiae* haploid strain AH22 *MATa* (*leu2 his4 pep4*) was used for the expression of PPV VP2 protein. Selection of yeast transformants resistant to formaldehyde was carried out on the YEPD (1% yeast extract, 2% peptone, 2% dextrose, Difco, Sparks, MD, USA) agar supplemented with 5 mM formaldehyde. *S. cerevisiae* transformants were grown in YEPD medium supplemented with 5 mM formaldehyde or in YEPG induction medium (1% yeast extract, 2% peptone, 2% galactose, Difco). Cultivation of transformed yeast cells, expression and purification of PPV VP2 was performed as previously described [32, 33]. After purification, the total protein concentration was determined by the Bradford assay (Roth, Karlsruhe, Germany) with bovine serum albumin (BSA) used as a standard.

2.5. SDS-PAGE and Western Blotting Analysis. The samples were boiled in a reducing sample buffer and separated in gel electrophoresis in SDS-Tris-glycine buffer. Proteins were visualized by staining with Coomassie Brilliant Blue (Sigma-Aldrich Co., St. Louis, MO, USA). For Western blotting, proteins were electrotransferred to Immobilon P membrane (Millipore, Bedford, MA, USA) as described by Sambrook and Russell [31]. The membranes were blocked with 5% milk in phosphate buffered saline (PBS) for 2 h. The blocking

solution was removed and the blots were incubated with the MAbs against PPV VP2 protein (undiluted hybridoma supernatants). Secondary antibodies conjugated to horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA, USA) were used for detection of specific antibody binding. The blots were stained with 3,3′,5,5′-tetramethylbenzidine (TMB) ready-to-use chromogenic substrate (Clinical Science Products Inc., Mansfield, MA, USA).

2.6. Electron Microscopy. After purification by CsCl ultracentrifugation, suspension of the recombinant PPV VP2 protein was placed on 400-mesh carbon coated copper grids (Agar Scientific, Stansted, UK). The protein samples were stained with 2% aqueous uranyl acetate solution (Reachim, Moscow, Russia) and examined with a Morgagni-268 electron microscope (FEI, Eindhoven, The Netherlands).

2.7. Characterization of Serum Samples by Commercial Test. Porcine serum samples were assayed for the presence of anti-PPV antibodies using a commercial INGEZIM PPV compact kit (Ingenasa, Madrid, Spain). This is an enzymatic assay based on the blocking ELISA technique which uses MAb specific for porcine parvovirus VP2 protein, and baculovirus expression systems generated recombinant capsid of VP2. The sera were tested according to the recommendations of the manufacturer. Two blocking percentage (BP) values were used for result interpretation: samples with BP higher than 30% were considered as positive and samples with BP lower than 25% were considered as negative. Samples with BP between both values were considered as doubtful.

2.8. Indirect ELISA. Polystyrene microtiter plates (Nerbe plus, Winsen/Luhe, Germany) were coated with 50 ng per well of recombinant PPV VP2 protein, diluted in 100 μL of 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBST (phosphate buffered saline with 0.05% (v/v) Tween 20, (Bio-Rad, Richmond, CA, USA)) and then blocked by the addition of 150 μL of blocking buffer per well (1x Roti-Block, Carl Roth GmbH & Co.) and incubation at room temperature for 1 hour. After blocking, the plates were washed three times with PBST and 100 μL aliquots of serum specimens, diluted 1 : 400 in PBST with 1% BSA, were added to the wells. Antigen concentration and serum dilution level for this assay were determined by titration to reach optimal conditions for sensitivity and specificity (data not shown). After 2 h of incubation at 37°C, the plates were rinsed three times with PBST. HRP-conjugated rabbit anti-pig IgG (Sigma-Aldrich Biosciences, Seattle, USA) diluted 1 : 30 000 in PBST, containing 1% BSA, were added to the wells in 100 μL aliquots and incubated for 1 h at 37°C. The plates were washed as described above. Binding of specific antibodies was visualized by the addition of 100 μL/well of TMB substrate (Clinical Science Products Inc., Mansfield, MA, USA). After 10 min of incubation at the room temperature, the reaction was stopped by adding 100 μL/well of 10% sulphuric acid and the optical density (OD) was measured at 450 nm (reference filter 620 nm).

2.9. Production of Monoclonal Antibodies. MAbs to recombinant PPV VP2 were produced essentially as described by Kohler and Milstein [34]. Eight-week-old female BALB/c mice (obtained from a breeding colony at the Center for Innovative Medicine, Vilnius, Lithuania) were immunized at days 0, 28, and 56 by a subcutaneous injection of 50 μg of recombinant PPV VP2 protein. For an initial immunization, the antigen was emulsified in complete Freund's adjuvant (Sigma-Aldrich). Subsequent immunizations were performed without an adjuvant, with the antigen dissolved in PBS. Three days after the final injection, mouse spleen cells were fused with Sp2/0-Ag 14 mouse myeloma cells using polyethylene glycol 1500 (PEG/DMSO solution, Hybri-Max, Sigma-Aldrich). Hybrid cells were selected in growth medium supplemented with hypoxanthine, aminopterin, and thymidine (50×HAT media supplement, Sigma-Aldrich). Samples of supernatant from wells with viable clones were screened by an indirect ELISA (as described above) using goat anti-mouse IgG (Bio-Rad) diluted 1 : 5000 to detect specific antibodies to PPV VP2. Hybridomas secreting specific antibodies to PPV VP2 protein were subcloned twice by a limiting dilution assay. Hybridoma cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) containing 15% fetal calf serum (Biochrom) and antibiotics. Antibodies in hybridoma culture supernatants were isotyped using the mouse monoclonal antibody isotyping kit (Pierce, Thermo Scientific) in accordance with the manufacturer's protocol.

2.10. Immunofluorescence Assay. The reactivity of the MAbs with PPV-infected cells was analyzed by immunofluorescence assay (IFA) using porcine parvovirus FA substrate slides (VMRD, Inc., Pullman, USA) containing fixed swine testicle cells infected and noninfected with porcine parvovirus strain KY-11. The slides were treated according to the manufacturer's protocol, incubated with undiluted hybridoma supernatants and developed with fluorescein isothiocyanate- (FITC-) labelled secondary antibody (BD Biosciences, Franklin Lakes, NJ, USA). The immunostained slides were observed by fluorescent microscope Olympus IX-70 (Olympus, Tokyo, Japan).

3. Results and Discussion

3.1. Expression and Purification of PPV VP2 VLPs in Yeast. The gene encoding PPV VP2 was derived from the nucleic acids extract obtained from PPV-infected cell culture. The 1700 bp sequence amplified by PCR was sequenced and confirmed to be identical to VP2 gene from porcine parvovirus strain NADL-2 (GenBank Entry Number NC001718). The gene was cloned into *S. cerevisiae* expression vector pFX7 under the galactose-inducible promoter. A SDS-PAGE analysis of the lysate of induced yeast biomass revealed a major protein band of approximately 64 kDa (Figure 1, lane 2). No additional protein bands were observed in crude lysates of *S. cerevisiae* harboring empty yeast vector pFX7 (Figure 1, lane 1). After centrifugation of lysates through 30% sucrose cushion, the pellets harboring recombinant proteins were

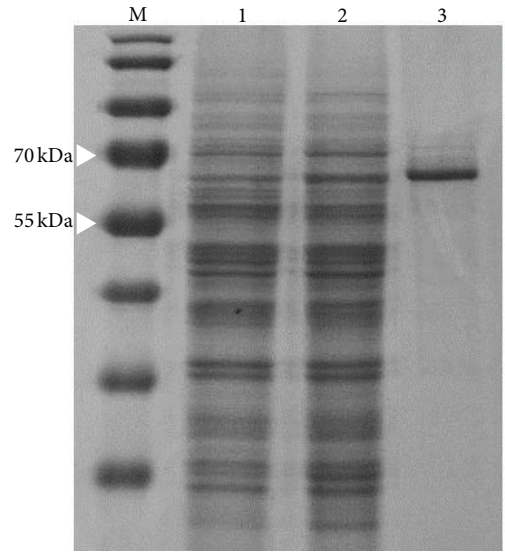
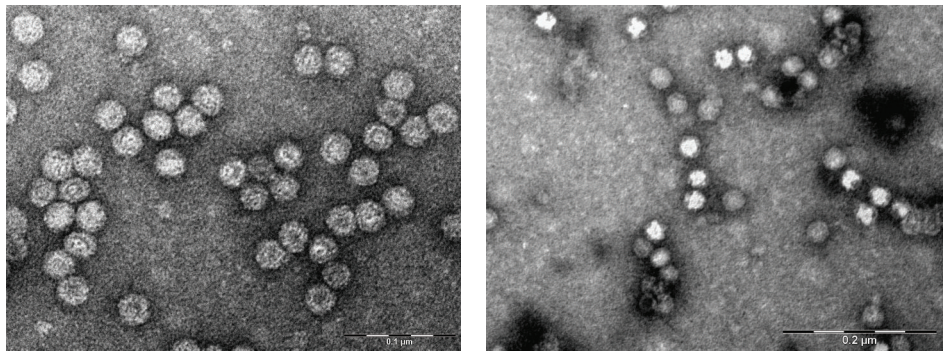


Figure 1: Analysis of *S. cerevisiae* cell lysates and purified PPV VP2 protein by SDS-PAGE. Lysates of *S. cerevisiae* harboring plasmids pFX7 (lane 1) and pFX7-PPV VP2 (lane 2) as well as CsCl-gradient purified PPV VP2 protein (lane 3) were separated in a 12% SDS-PAGE gel and stained with Coomassie Brilliant blue. PageRuler Prestained Protein Ladder (Fermentas/Thermo Fisher Scientific) was used as molecular mass standard in lane M.



(a) (b)

Figure 2: Electron micrograph of recombinant PPV VP2 VLPs in CsCl fraction ((a), scale bar = 100 nm) and VLPs resolubilized after lyophilisation ((b), scale bar = 200 nm).

subjected to CsCl-gradient centrifugation. CsCl gradients revealed recombinant PPV VP2 protein (Figure 1, lane 3) in fractions with buoyant density of 1.286–1.308 g/mL.

In several preparative procedures, the yield of purified recombinant PPV VP2 protein was found to be 8-9 mg per liter of induced yeast culture. There was no significant yield difference using fresh or frozen biomass. After CsCl gradient purification, the recombinant VP2 protein was dialyzed against PBS and stored at −20°C in PBS containing 50% glycerol.

Formation of VLPs by PPV VP2 protein was confirmed by negative staining electron microscopy. Typical icosahedral structures of parvoviruses with a diameter of approximately 25–30 nm were observed indicating that PPV VP2 protein is self-assembled to VLPs (Figure 2(a)). VLPs of PPV produced in *S. cerevisiae* expression system were similar to those previously generated in insect cells [35] or native PPV particles [17]. Treatment with 25 mM EDTA or 10 mM EGTA did not cause the dissociation of recombinant VLPs indicating that the assembled structures do not require divalent ions (data not shown). The recombinant PPV VP2-derived VLPs were found to be stable when lyophilized and stored at −20°C longer than a year and VLPs remained intact when resolubilized in PBS as no pentamers or disrupted particles were observed by electron microscopy (Figure 2(b)). Moreover, the ELISA results using freshly prepared and resolubilized PPV VP2 antigen were fully concordant (data not shown). The stability of VLPs is crucial to ensure their successful transportation and possible application in the point-of-care tests.

VP2 protein is a major immunogen of most parvoviruses [36, 37]. Therefore, it is successfully employed for the serodiagnostics as well as epidemiological studies of PPV infection [24]. Moreover, VP2 protein is the major agent for developing

Table 1: Summary of the concordance of results obtained with the newly developed indirect IgG ELISA and with the commercial INGEZIM test.

ELISA test with recombinant antigen		INGEZIM PPV compact			Total
		Positive	Negative	Doubtful	
Indirect IgG	Positive	128	1	0	129
ELISA test	Negative	9	38	11	58
	Total	137	39	11	187

Table 2: MAb isotypes and specificity.

MAb clone	MAb isotype	Indirect ELISA results using yeast *S. cerevisiae* generated antigens		
		Porcine parvovirus VP2	Hantaan (Fojnica) nucleocapsid (N) protein	Tioman nucleocapsid (N) protein
1F8	IgG1	+	–	–
4F11	IgG1	+	–	–
6D1	IgG1	+	–	–
10A7	IgG1	+	–	–
16A1	IgG2a	+	–	–
16G11	IgG1	+	–	–
22G2	IgG2a	+	–	–
23A7	IgG2a	+	–	–
25C5	IgG1	+	–	–

+, OD in ELISA ≥ 1.0;–, no reactivity.

vaccines [38]. To meet the need for stable recombinant VLPs of PPV, several expression systems were tested as an alternative to baculovirus expression system that is a major source of the antigen for the market [26]. *E. coli* [23], *Lactobacillus casei* [21], and recently yeast *Pichia pastoris* [27] were reported to have been successfully used for producing PPV VP2 protein, but VLP formation in these expression systems has not been confirmed. To our knowledge, our study provides the first evidence of stable recombinant PPV VP2 VLPs not produced in baculovirus expression system.

S. cerevisiae expression system has been shown to be efficient in producing antigenic VLPs of diagnostic relevance of human parvoviruses [28, 29] and porcine circovirus [39]. Therefore, it represents a potential system to meet the need for VLP-forming antigens to detect and differentiate a number of newly discovered porcine.

3.2. Indirect IgG PPV ELISA. PPV VP2 protein-derived VLPs generated in *S. cerevisiae* were used to develop an indirect ELISA for the detection of PPV-specific IgG antibodies in swine serum specimens. In order to test the antigenic properties of yeast-derived VLPs, 187 serum samples were tested using INGEZIM PPV compact test as a gold standard and further retested with the newly developed Indirect IgG PPV ELISA. Both assays were performed in parallel for every serum sample to determine the sensitivity and specificity of the new Indirect IgG PPV ELISA. The cut-off value for the new assay was calculated as the mean OD value of the 39 negative sera (identified with the commercial kit) plus 2 standard deviations ($\overline{x}$ + 2SD) resulting in 95% confidence. The mean OD value and SD were 0.150 and 0.090, respectively. Therefore, sera with OD values above 0.330 were considered positive (n = 129) and those with OD value below this cut-off were assessed as negative (n = 58) in the newly developed Indirect IgG PPV ELISA.

Thirty-eight out of the 39 sera tested as negative with a commercial kit were assessed as negative by the Indirect IgG PPV ELISA. Nine out of 137 positive and all 11 doubtful serum samples by INGEZIM assay showed the OD value below the cut-off in the Indirect IgG PPV ELISA and were considered as negative (Table 1). Thus, the calculated specificity and sensitivity for the new Indirect IgG PPV ELISA were 97.4% (38/39) and 93.4% (128/137), respectively. All 9 false-negative samples of the new assay were weak positive in INGEZIM kit showing blocking percentage in the 33–45% range. All samples above BP equal to 30% were considered positive in this commercial kit. The only false-positive sample in the Indirect IgG PPV ELISA showed OD = 0.354 that is just above the cut-off OD of 0.330. To obtain more precise estimation of the sensitivity and specificity of the new assay, additional evaluation with more serum samples and alternative assays must be done in the future. Alternatively, the precision of the test can be improved using other formats of ELISA. In summary, results of the current study are promising to the use of PPV VP2 antigen synthesized in yeast *S. cerevisiae* in diagnostic kits.

3.3. Generation of Monoclonal Antibodies and Their Characterization. Purified recombinant PPV VP2 protein was used to immunize mice and generate PPV VP2-specific MAbs.

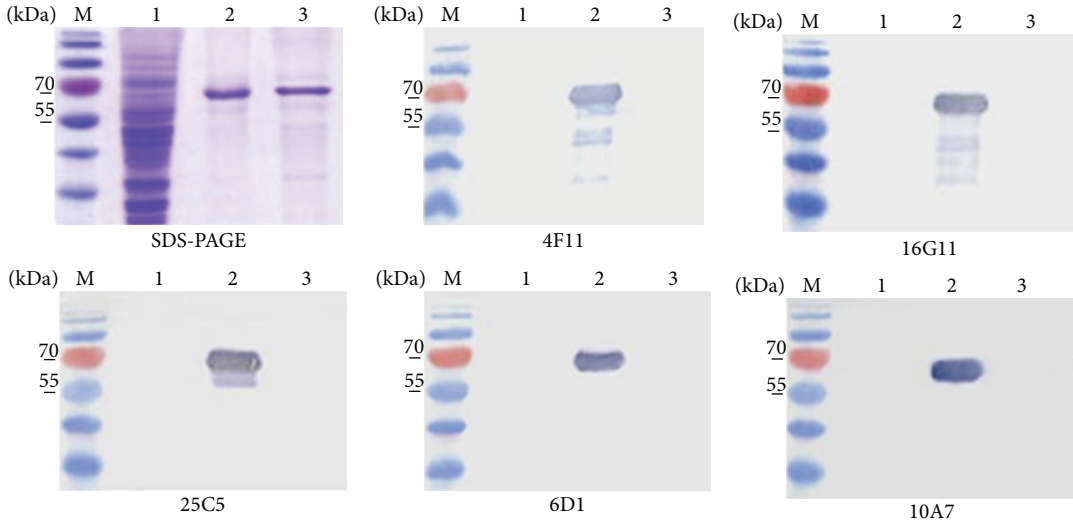


Figure 3: Western Blot analysis of MAbs raised against PPV VP2. Lanes 1: lysates of *S. cerevisiae* harboring plasmids pFX7; lanes 2 and 3: yeast synthesized recombinant PPV and human bocavirus 1 VP2 proteins, respectively. PageRuler Prestained Protein Ladder (Fermentas/Thermo Fisher Scientific) was used as molecular mass standard in lanes M. MAb number used in each WB is indicated below the corresponding blot picture. Only blots for linear-epitope recognizing MAbs are provided.

After screening and cloning of positive hybridoma clones, 9 stable hybridoma cell lines producing IgG antibodies were derived. Six MAbs produced by hybridoma clones were of IgG1 subtype and the remaining three were found to be of IgG2a subtype. All MAbs reacted specifically with recombinant PPV VP2 protein in ELISA and did not react with other yeast-expressed proteins used as a negative control (Table 2).

To characterize the nature of the epitopes recognized by the MAbs, their reactivity in Western blotting was analyzed. The MAbs 4F11, 16G11, 25C5, 6D1, and 10A7 recognized SDS-denatured PPV VP2 protein in Western blotting assay (Figure 3, lanes 2). This result indicates that these MAbs recognize SDS-denatured epitopes of the PPV VP2 protein. The other four MAbs (clones 1F8, 16A1, 22G2, and 23A7) did not recognize SDS-denatured PPV VP2 protein in Western blotting assay (data not shown), suggesting that these MAbs recognize conformation-dependent epitopes.

The specificities of the MAbs were further analyzed by IFA to verify the ability of the MAbs to recognize native virion. For this purpose, commercial porcine parvovirus control slides containing virus-infected and noninfected fixed cells were used. None of the MAbs reacted with noninfected cells, which confirms the specificity of the assay (Figure 4, negative control). Both groups of MAbs recognizing linear or conformational epitopes reacted with infected cells; however, only the latter ones produced images with sharp nucleus-shaped patterns. In contrast, the MAbs recognizing linear epitopes produced signal outside the nuclei but in lesser intensity (Figure 4). This difference could be explained by the possibility that PPV VLPs finish their assembly in the nucleus forming conformational epitopes. Taking into consideration trimer translocation model for other parvoviruses [40], conformational epitopes might be available only in intact capsid but not in trimmers or pentamers formed by VP2 protein. This possibility emphasizes the importance of properly assembled VLPs to elicit strong immune responses when using recombinant antigens as potential vaccines. Further epitope mapping needs to be done to answer if linear epitopes remain accessible on the intact VLP surface or are hidden within the structure. However, our generated MAbs represent an attractive tool for studying intracellular PPV infection and capsid formation process.

The PPV VP2-derived VLPs generated in *S. cerevisiae* have not been yet tested for a possible use as a vaccine in pigs; however, considering results on the antigenic structure and the immunogenicity in mice described in this study, this is an attractive alternative to the currently used recombinant PPV vaccines. In previous studies, PPV VP2-derived VLPs have been shown to be effective epitope carriers to elicit a strong immune response in mice [41, 42]. Furthermore, yeast expression system does not require additional contaminant elimination procedure as described for baculovirus expression system [35] for such recombinant subunit vaccine preparation. Therefore, PPV VP2-derived VLPs generated in yeast *S. cerevisiae* are a promising platform for new PPV vaccine development.

4. Conclusions

In this study, we have demonstrated that yeast *S. cerevisiae* is a suitable host for the production of recombinant PPV VP2 protein as stable immunogenic VLPs. The recombinant yeast-derived PPV VP2 protein can be employed in an indirect ELISA for detection of PPV-specific IgG antibodies in swine sera with high specificity and sensitivity. The MAbs

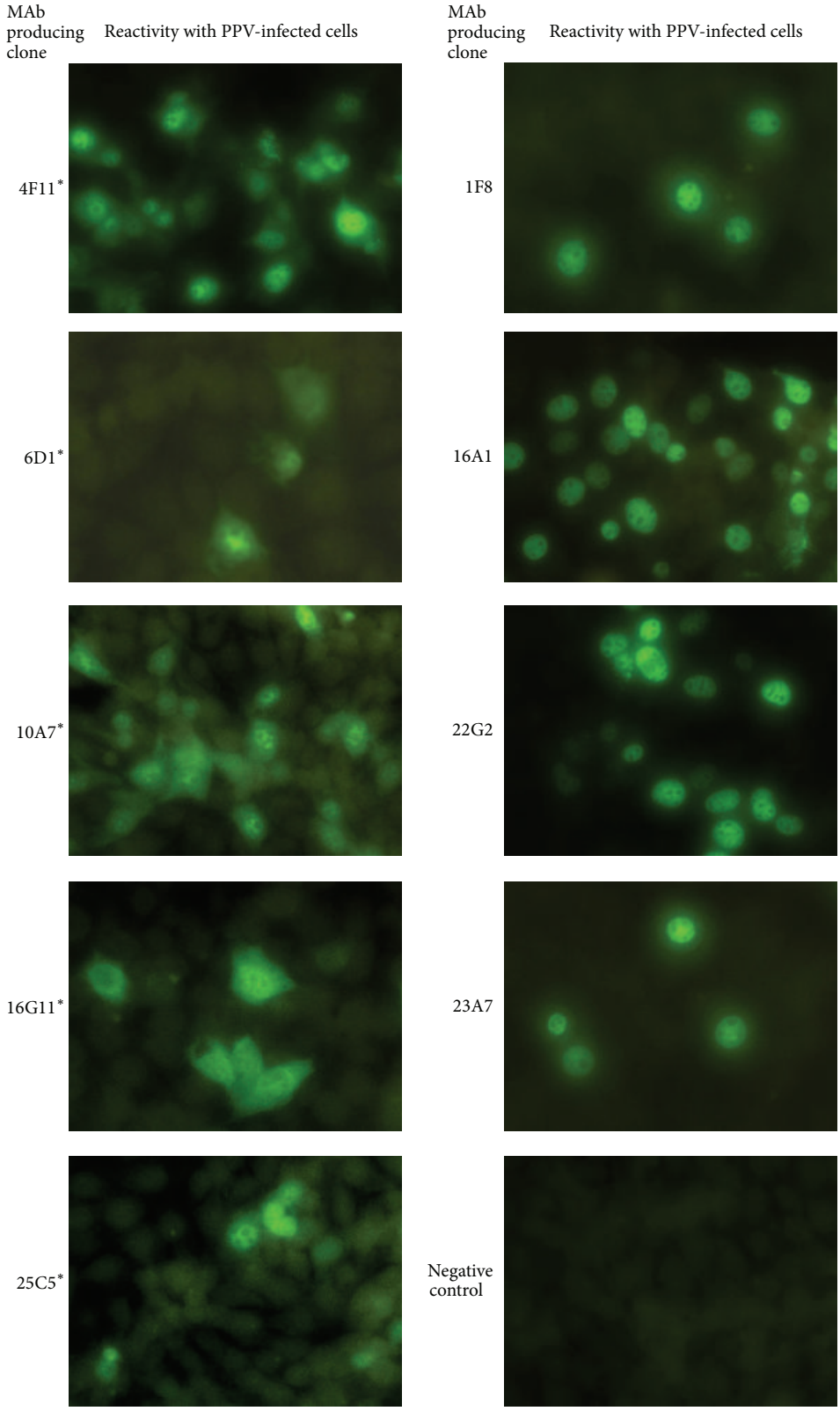


Figure 4: Fluorescence microphotographs showing the reactivity of 9 MAbs raised against yeast-derived PPV VP2 protein with PPV-infected cells on commercial slides (VMRD, Inc.). The codes of the MAbs are indicated on the left side of each picture. The MAbs recognizing linear PPV VP2 epitopes are indicated with an asterisk. As a negative control, negative control serum included in the kit is used.

raised against yeast-derived PPV VP2 VLPs recognize virus-infected cells and differentiate conformational and linear epitopes of PPV VP2 protein.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Simultaneous Detection and Differentiation of Highly Virulent and Classical Chinese-Type Isolation of PRRSV by Real-Time RT-PCR

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Porcine reproductive and respiratory syndrome (PRRS) is a leading disease in pig industry worldwide and can result in serious economic losses each year. The PRRS epidemic situation in China has been very complicated since the unprecedented large-scale highly pathogenic PRRS (HP-PRRS) outbreaks in 2006. And now the HP-PRRS virus (HP-PRRSV) and classical North American type PRRSV strains have coexisted in China. Rapid differential detection of the two strains of PRRSV is very important for effective PRRS control. The real-time RT-PCR for simultaneous detection and differentiation of HP-PRRSV and PRRSV by using both SYBR Green and TaqMan probes was developed and validated. Both assays can be used for rapid detection and strain-specific identification of HP-PRRSV and PRRSV. However, the TaqMan probe method had the highest detection rate whereas the conventional RT-PCR was the lowest. The real-time RT-PCR developed based on SYBR Green and TaqMan probe could be used for simultaneous detection and differentiation of HP-PRRSV and PRRSV in China, which will benefit much the PRRS control and research.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is widely accepted as being one of the most economically important diseases affecting swine industry [1]. In 2006 there was an unparalleled large-scale outbreak of the so-called high fever disease in most provinces of China that affected more than 2,000,000 pigs, leading to concerns within the global swine industry and in relation to public health [2–4]. In March 2007 the disease was identified in the Hai Duong province of Vietnam and it spread countrywide affecting more than 65,000 pigs [5, 6]. The outbreaks caused extensive concern worldwide [7]. Studies demonstrated that highly virulent porcine reproductive and respiratory syndrome virus (HP-PRRSV) was the major causative pathogen of the so-called high fever disease [2]. Genetic analysis indicated that the HP-PRRSVs isolated from China and Vietnam shared a discontinuous deletion of 30 aa in nonstructural protein 2 (NSP2), as compared with the North American type PRRSV strains (NA PRRSV) [2, 5, 8]. Since 2006, the HP-PRRSV and classical North American type PRRSV strains coexist in China. Now PRRS epidemic situation is very complicated in China, of which the predominant form is the HP-PRRSV. Rapid differential detection of the two strains of PRRSV is very important for effective PRRS control. Therefore, it is imperative to develop an assay for simultaneous detection and strain identification of HP-PRRSV and PRRSV.

The current immunoassay, such as immunohistochemistry and serological methods, cannot differentiate between the two strains of PRRSV. Conventional RT-PCR is time-consuming, lowly sensitive, and also prone to contamination. The development of real-time RT-PCR technology offers the opportunity for more rapid, sensitive, and specific detection of virus. The current two major genotypes, the European (EU) and the North American (US) strains, have been rapidly identified by SYBR Green-based or TaqMan probe-based real-time RT-PCR assay [9–11]. A specific TaqMan probe real-time RT-PCR has been developed for assaying the HP-PRRSV

Table 1: Primers and probes used in the real-time RT-PCR and conventional RT-PCR assays.

Primers and probes		Sequences (5′-3′)	Products (bp)	
			HP-PRRSV	PRRSV
Conventional RT-PCR	NSP2-F	AACACCCAGGCGACTTCA	787	874
	NSP2-R	GCATGTCAACCCTATCCCAC		
Real-time RT-PCR	NSP2-qF	GTGGGTCGGCACCAGTT	85	172
	NSP2-qR	GACGCAGACAAATCCAGAGG		
Probes	Pb-H	FAM-CGCGTAGAACTGTGACAACAACGCTGA-TAMRA [12]		
	Pb-N	HEX-AAAATTGGCTCACTCAAGGGCGTCA-TAMRA		
	Pb-all	FAM-CACAGTTCTACGCGGTGCAGG-TAMRA		

[12], but it is not able to differentially detect the HP-PRRSV and PRRSV.

In this research, the real-time RT-PCR for simultaneous detection and differentiation of HP-PRRSV and PRRSV by using both SYBR Green and TaqMan probe was developed and validated. These two methods provided alternative diagnostic assays in diverse PRRSV epidemiological circumstances.

2. Materials and Methods

2.1. Virus Strains and Clinical Samples. HP-PRRSV (GD and XH) and PRRSV (CH-1a) virus strains were kindly supplied by Dr. Guihong Zhang (South China Agricultural University, China). PRRSV (CC), PRV, FPV, and FCV were kindly provided by Laboratory Animal Center in Jilin University, China. 39 and 477 serum samples were obtained from 6 pig farms in South China in 2008 and 2011, respectively. 15 sera as described previously were from pigs experimentally infected with HP-PRRSV and PRRSV [13]. The viral RNA of the virus-infected cell culture and serum was extracted by using QIAamp Viral RNA Mini Kit according to the manufacturer's instruction (Qiagen). First-strand cDNA was synthesized using the extracted total RNA and AMV Reverse Transcriptase from Reverse Transcription System of Promega according to the manufacturer's instruction (Promega).

2.2. PCR Primers and Probes. The difference of genome sequence between the HP-PRRSV and PRRSV was the 87-base deletion in the fixed site in NSP2 gene [2, 12]. After aligning 20 HP-PRRSV and PRRSV strains isolated from China and the US strain (VR-2332) sequences obtained from the NCBI database, the NSP2 region was selected to design an assay for discriminating between HP-PRRSV and PRRSV strains. The differential detection based on real-time RT-PCR using SYBR Green I and TaqMan probes was performed employing the same primer pair (Table 1). Real-time RT-PCR for PRRSV detection based on dual-colour TaqMan probes was performed using strain-specific probes including a Pb-H (only detecting HP-PRRSV strain) [12], Pb-N (only detecting PRRSV strain), and Pb-all (simultaneously detecting both HP-PRRSV and PRRSV strains) (Table 1).

SYBR Green I real-time PCR was carried out using SYBR Premix Ex Taq (TaKaRa) and the LightCycler 480 Real-Time PCR System (Roche Applied Science). Amplification was performed in a 10 μL reaction mixture containing 5.0 μL SYBR Premix Ex Taq (2×), 0.2 μL of each forward (NSP2-qF) and reverse (NSP2-qR) primer (10μM), 1.5 μL cDNA or plasmid DNA, and 3.1 μL H2O. The amplification conditions were 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 40 s. Fluorescent signal was detected for each cycle at the end of the 60°C extension step. For each assay, a standard curve was generated with 10-fold serially diluted plasmid standards of 10^2–10^6 copies/μL. Meanwhile positive and negative reference samples were detected along with unknown samples. After 40 amplification cycles, melting curve analysis was carried out with the conditions of 95°C for 1s and 60°C for 15 s and then increased to 95°C while continuously collecting the fluorescent signal. The melting temperature (Tm) of each strain was analyzed to verify the PRRSV type.

The 10 μL duplex TaqMan probe real-time PCR reaction mixtures contained 5.0 μL Premix Ex Taq (2×) (TaKaRa), 0.2 μL of each forward (NSP2-qF) and reverse (NSP2-qR) primer (10 μM), 0.2 μL of each probe (Pb-H and Pb-N or Pb-all and Pb-N, 10 μM), 1.5 μL cDNA or plasmid DNA, and 2.7 μL H_2O. The amplification conditions were 95°C for 10 s, followed by 45 cycles of 95°C for 5 s and 60°C for 40 s. For each assay, a standard curve was generated with 10-fold serially diluted plasmid standards of 10^1–10^6 copies/μL. The FAM (6-carboxyfluorescein) and HEX (hexachloro-6-carboxyfluorescein) signals were detected for each cycle at the end of the 60°C extension step.

2.3. Conventional RT-PCR and Preparation of Standard Plasmid DNA. The conventional RT-PCR was performed by using the NSP2-F and NSP2-R primers described in Table 1. 10 μL reaction mixture contains 0.5 μL cDNA, 5.0 μL 2 × PCR reaction mix, 0.4 μL NSP2-F (10 μM) primer, 0.4 μL NSP2-R (10 μM) primer, 0.1 μL Taq DNA polymerase (2.5 U/μL), and 3.6 μL H_2O. The negative controls included the reagents without cDNA template. The reaction mixtures were performed at the amplification condition: 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension step of 5 min at 72°C. The PCR products were detected by 1.5% agarose gel electrophoresis in 1 × TAE. Then the PCR products were cloned into the plasmid pMD20-T (TaKaRa) and propagated in competent *Escherichia coli* DH5α cells according to the manufacturer's instructions. Plasmid DNA was purified using the E.Z.N.A. Plasmid Mini

Table 2: Intra- and interassay reproducibility of real-time PCR.

Concentration of standard plasmid (copies/μL)	n	Intra-assay (Cp)			Interassay (Cp)		
		Mean	SD	CV (%)	Mean	SD	CV (%)
HP-PRRSV (SYBR)							
10^6	3	14.96	0.02	0.13	14.82	0.29	1.96
10^4	3	21.78	0.04	0.18	21.49	0.57	2.65
10^2	3	28.5	0.16	0.56	28.48	0.27	0.95
PRRSV (SYBR)							
10^6	3	15.47	0.02	0.13	15.75	0.31	1.97
10^4	3	22.55	0.05	0.22	22.71	0.19	0.84
10^2	3	29.6	0.01	0.03	29.96	0.34	1.13
HP-PRRSV (FAM)							
10^6	3	15.9	0.02	0.13	15.98	0.06	0.38
10^4	3	22.51	0.01	0.04	22.63	0.15	0.66
10^2	3	29.71	0.02	0.07	29.59	0.17	0.57
PRRSV (HEX)							
10^6	3	16.59	0.15	0.90	16.47	0.04	0.24
10^4	3	23.41	0.09	0.38	23.44	0.37	1.58
10^2	3	29.75	0.04	0.13	29.56	0.28	0.95

Kit I (Omega) and quantified by measuring OD_{260} using spectrophotometer ND-1000 (Wilmington, USA).

3. Results and Discussion

3.1. SYBR Green I Real-Time PCR. 10-fold serial plasmid dilutions were tested and used to construct the standard curve. The generated standard curve covered a linear range of 3.93×10^2 to 3.93×10^6 copies/μL for HP-PRRSV and 8.56×10^2 to 8.56×10^6 copies/μL for PRRSV. Both standard curves had a slope of −3.410 to −3.443 and an efficiency of 1.964 to 1.952, which indicate a high PCR efficiency of the experiment (Figures 2(a) and 2(b)). The amplification with primers NSP2-qF and NSP2-qR yielded 85 bp and 172 bp amplified product within NSP2 of both HP-PRRSV (GD) and PRRSV (CH-1a), respectively (Figure 1), which was sufficient to discriminate between melting peaks of the two PRRSV strains. The mean and standard deviation of Tm of HP-PRRSV and PRRSV were 85.17 ± 0.12°C and 87.27 ± 0.07°C, respectively (Figure 3(b)).

3.2. TaqMan Probe Real-Time PCR. The generated standard curve covered a linear range of 3.93×10^1 to 3.93×10^6 copies/μL for HP-PRRSV and 8.56×10^1 to 8.56×10^6 copies/μL for PRRSV. Both standard curves had a slope of −3.256 to −3.400 and an efficiency of 2.028 to 1.968, which indicate a high PCR efficiency of the experiment (Figures 2(c) and 2(d)). Two TaqMan probes specific to HP-PRRSV and PRRSV strains combined in duplex real-time PCR system can specifically detect the two PRRSV strains. When the two TaqMan probes of Pb-H (FAM) and Pb-N (HEX) were combined in a duplex real-time PCR system, only the FAM fluorescent signal could be observed in the template of GD HP-PRRSV strain, and only the HEX fluorescent signal could be observed in the template of CH-1a PRRSV strain (Figure 4). However, when Pb-N (HEX) and Pb-all (FAM) were combined in a duplex real-time PCR system, only HEX fluorescent signal could be observed when the template was CH-1a PRRSV strain, and FAM fluorescent signal could be observed when the templates were GD and CH-1a strains (Figure 4).

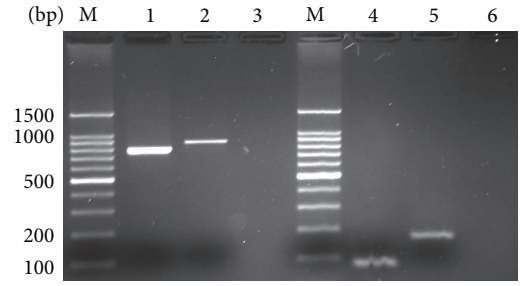

Figure 1: Conventional PCR results of PRRSV NSP2 gene. M: 100 bp marker; 1 and 4: HP-PRRSV (GD) strain; 2 and 5: PRRSV (CH-1a) strain; 3 and 6: negative.

3.3. Validation of Real-Time PCR Assay. Specificity of real-time PCR using SYBR Green I and TaqMan probe was determined by analyzing nucleic acid extracts of other viruses (PRV, FPV, and FCV), host cells (Marc145, PK15), and H_2O. The results of the specificity test of the two methods showed that there were no cross-amplifications from other viruses or host cells (Figures 3(a) and 4), which confirmed that the primers and probes used in this study were highly specific for both HP-PRRSV and PRRSV.

Table 3: Detection results of samples by conventional and real-time PCR.

Samples	Number	Methods					
		Conventional PCR		SYBR Green I		TaqMan probe	
		HP-PRRSV	PRRSV	HP-PRRSV	PRRSV	HP-PRRSV	PRRSV
Reference strains	4	2	2	2	2	2	2
Serum 1	15	6	4	6	5	6	6
Serum 2	39	0	5	0	6	0	8
Serum 3	477	2	0	19	0	34	7

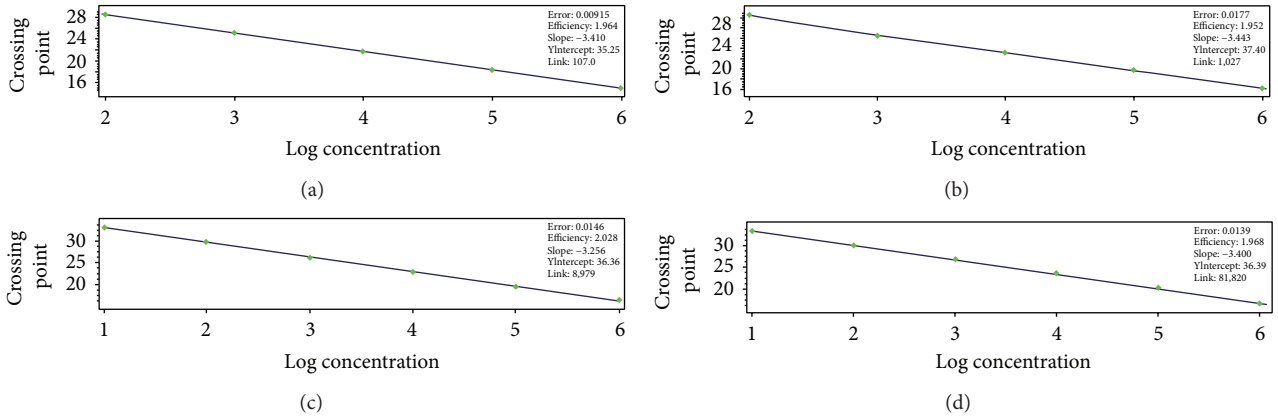

Figure 2: Standard curves were generated based on Cp values of 10-fold dilutions of plasmid DNA. Regression lines between the Cp (C_T) values and the input concentrations of HP-PRRSV (a) and PRRSV (b) plasmid DNA in real-time RT-PCR detected using SYBR Green I and HP-PRRSV (c) and PRRSV (d) using TaqMan probe, respectively.

10-fold serially diluted plasmid standards of HP-PRRSV (pMD20-GD) and PRRSV (pMD20-CH1a) were used as templates for sensitivity tests in both conventional PCR and real-time PCR using SYBR Green I and TaqMan probe. The results showed that real-time PCR using both SYBR Green I (Figure 6) and TaqMan probe (Figure 5) can be used to detect concentrations at least 10^0 copies/μL of plasmid standards whereas the sensitivity of conventional PCR was only 10^3 copies/μL.

The intra- and interassay reproducibility were evaluated using three replicates of 10^6, 10^4, and 10^2 copies/μL plasmid standards of both pMD20-GD and pMD20-CH1a. Mean and coefficient of variation (CV) for the C_T value were calculated. The results showed that neither the CVs of intra-assay nor the CVs of interassay were more than 5% (Table 2), indicating the reproducibility of the two assays.

Our results showed that real-time PCR using both SYBR Green I and TaqMan probe could be used to simultaneously detect and differentiate HP-PRRSV and PRRSV in China. But the TaqMan probe method had the highest detection rate, whereas the conventional RT-PCR was the lowest. The SYBR Green I real-time PCR assay is timesaving, easy to handle, and highly sensitive. Yang et al. detected the PRRSV and CSFV RNA by SYBR Green I-based quantitative PCR and found that both sensitivity and specificity were equal or superior to conventional RT-PCR [14]. Although Tian et al. developed a rapid SYBR one step real-time RT-PCR for detection of PRRSV [15], it could not be used for simultaneous detection and differentiation of HP-PRRSV and classical North American type PRRSV (PRRSV). Kleiboeker et al. developed dual labeled probes quantitative PCR, which could simultaneously detect NA- and EU-PRRSV [16]. However, this assay could not simultaneously detect and differentiate between both HP-PRRSV and classical North American type PRRSV (PRRSV) strains in China. The TaqMan probe method provided more accurate results than SYBR Green I with melting curve analysis. SYBR Green I real-time PCR assay was simpler, rapider, and lower in cost than TaqMan probe method. In addition to the high specificity, sensitivity, and reproducibility, the real-time PCR assay based on both SYBR Green I and TaqMan probe established by us could recognize coinfection of HP-PRRSV and PRRSV. Because the two types of PRRSV isolates coexist in Chinese swine herds, recombination could occur. Therefore, the results provided alternative diagnostic assays in diverse PRRSV epidemiological circumstances.

3.4. Testing of Clinical Samples. To compare and evaluate the developed real-time RT-PCR and conventional RT-PCR, 2 reference strains of H-PRRSV (GD and XH) and N-PRRSV (CH-1a and CC) and 15, 39, and 477 serum samples were tested. The results were shown in Table 3. The results of 4 reference strains for real-time PCR assays were consistent with that of conventional RT-PCR method. The results of

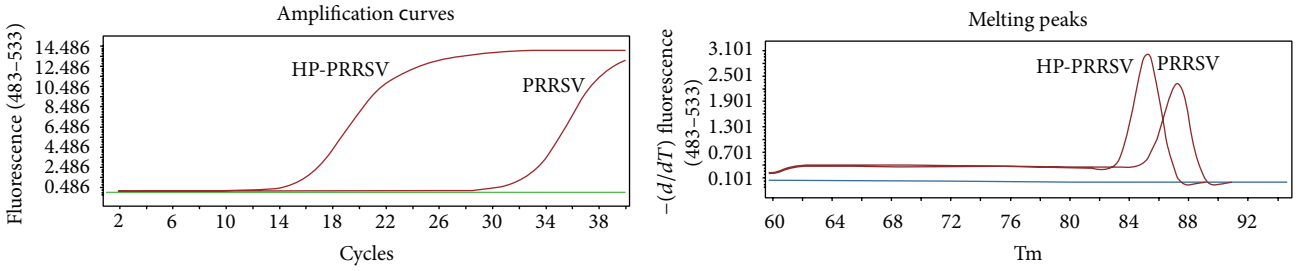

Figure 3: Specific amplification curves and melting curve analysis by SYBR Green I real-time PCR. (a) Specific amplification curves. Fluorescent curves were observed when HP-PRRSV (GD) and PRRSV (CH-1a) were used as templates; no fluorescent signals were observed when the templates were other viruses and host cells. (b) Melting curves. Tm of HP-PRRSV = 85.17±0.12°C; Tm of PRRSV = 87.27±0.07°C.

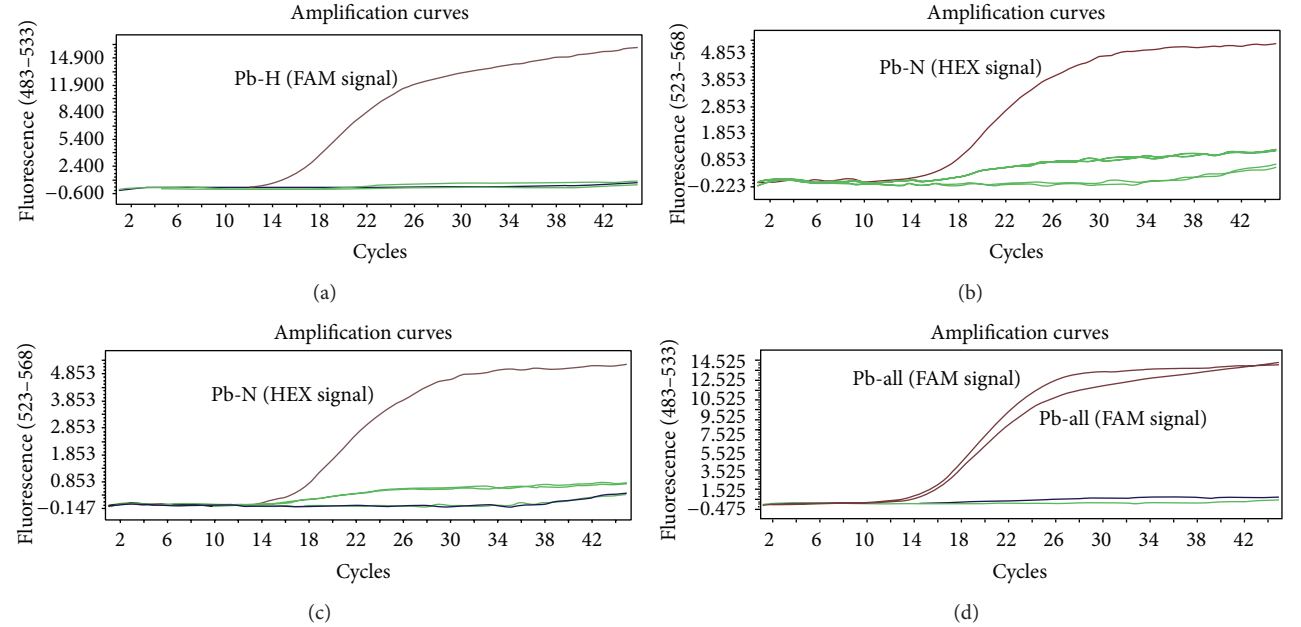

Figure 4: Specific amplification curves by duplex TaqMan probe real-time PCR. When Pb-H (FAM) and Pb-N (HEX) probes were combined in a duplex real-time PCR system, only the FAM fluorescent signal could be observed when the template was GD HP-PRRSV strain, no FAM signal was detected when the templates were CH-1a PRRSV strain and other viruses (a), and vice versa, only the HEX signal could be collected when the template was CH-1a PRRSV strain (b). When Pb-N (HEX) and Pb-all (FAM) were combined in a duplex real-time PCR system, the Pb-N (HEX signal) probe could only detect PRRSV strain (c), whereas Pb-all (FAM signal) probe could detect both HP-PRRSV and PRRSV strains (d).

531 serum samples showed that the TaqMan probe real-time PCR had the highest detection rate, whereas the conventional RT-PCR had the lowest detection rate. To evaluate comprehensively the practicality of this assay, clinical samples that span a broader geographical origin should be tested in the future.

4. Conclusions

The real-time RT-PCR for simultaneous detection and differentiation of HP-PRRSV and PRRSV by using both SYBR Green and TaqMan probes was developed and validated. Both assays can be used for rapid detection and strain-specific identification of HP-PRRSV and PRRSV. A total of 535 samples were tested by real-time PCR and conventional RT-PCR. The results of 4 reference strains for real-time PCR assays were consistent with that of conventional PCR method. The results of 531 serum samples showed that the TaqMan probe method had the highest detection rate whereas the conventional RT-PCR was the lowest. The real-time PCR developed based on SYBR Green and TaqMan probe could be used for simultaneous detection and differentiation of HP-PRRSV and PRRSV in China, which provided two alternative diagnostic assays in diverse PRRSV epidemiological circumstances.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

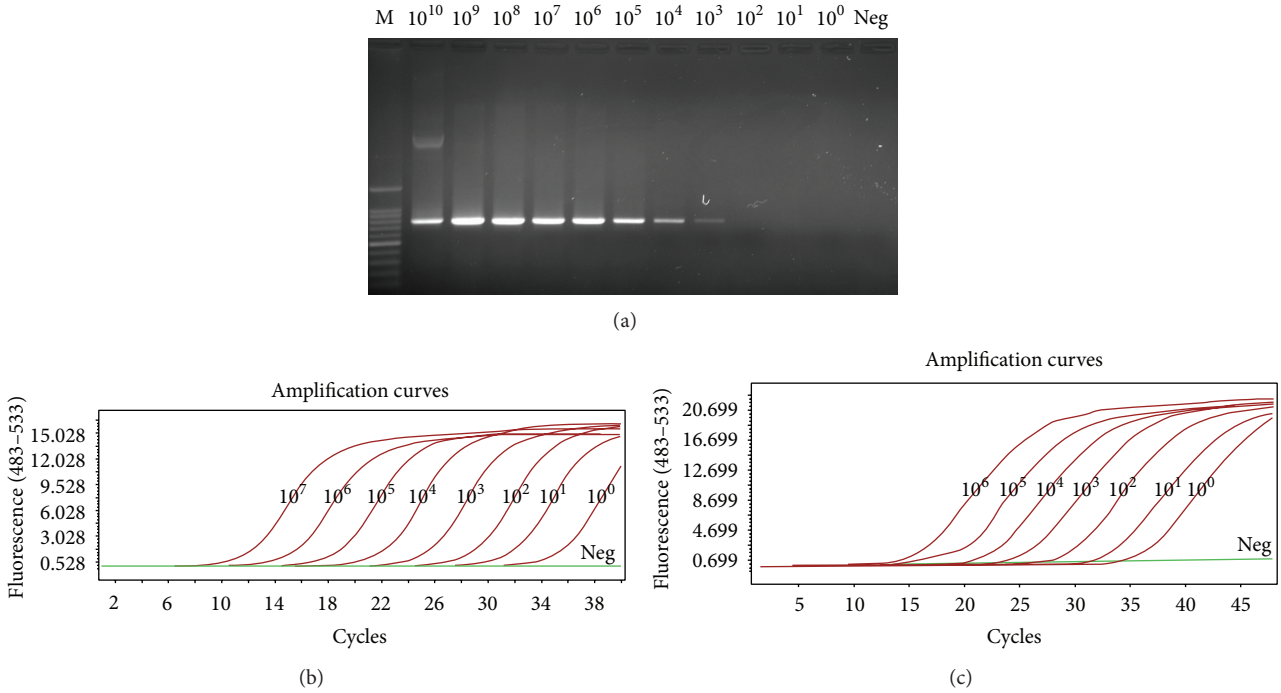

FIGURE 5: Comparison of sensitivity for HP-PRRSV detection by conventional RT-PCR and real-time PCR. Samples were 10-fold serially diluted plasmid standards of HP-PRRSV. M: 100 bp marker; Neg: negative control.

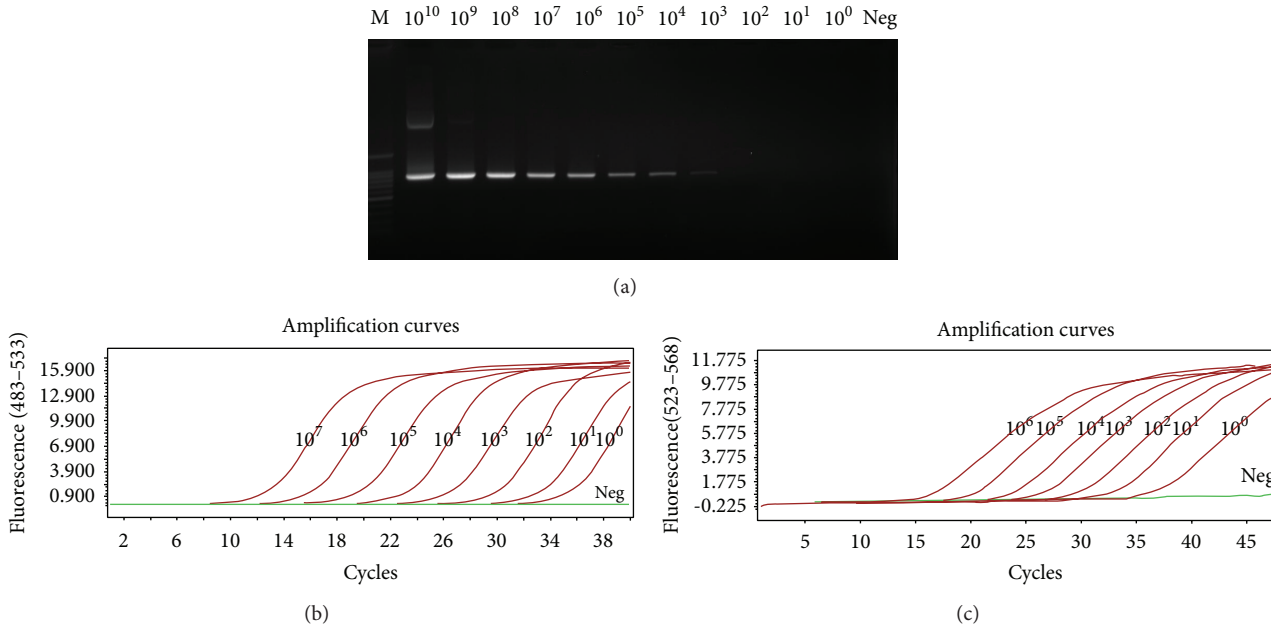

FIGURE 6: Comparison of sensitivity for PRRSV detection by conventional RT-PCR and real-time PCR. Samples were 10-fold serially diluted plasmid standards of PRRSV. M: 100 bp marker; Neg: negative control.

Acknowledgments

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Research Article

Attenuation and Immunogenicity of a Live High Pathogenic PRRSV Vaccine Candidate with a 32-Amino Acid Deletion in the nsp2 Protein

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A porcine reproductive and respiratory syndrome virus (PRRSV) QY1 was serially passed on Marc-145 cells. Virulence of different intermediate derivatives of QY1 (P5, P60, P80, and P100) were determined. The study found that QY1 had been gradually attenuated during the in vitro process. Pathogenicity study showed that pigs inoculated with QY1 P100 and P80 did not develop any significant PRRS clinic symptoms. However, mild-to-moderate clinical signs and acute HP-PRRSV symptoms of infection were observed in pigs inoculated with QY1 P60 and P5, respectively. Furthermore, we determined the whole genome sequences of these four intermediate viruses. The results showed that after 100 passages, compared to QY1 P5, a total of 32 amino acid mutations were found. Moreover, there were one nucleotide deletion and a unique 34-amino acid deletion found at 5′UTR and in nsp2 gene during the attenuation process, respectively. Such deletions were genetically stable in vivo. Following PRRSV experimental challenge, pigs inoculated with a single dose of QY1 P100 developed no significant clinic symptoms and well tolerated lethal challenge, while QY1 P80 group still developed mild fever in the clinic trial after challenge. Thus, we concluded that QY1 P100 was a promising and highly attenuated PRRSV vaccine candidate.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a severe viral disease in pigs, characterized by reproductive failure in sows and respiratory problems in pigs. Since its emergence in the US in the 1980s, PRRS was found worldwide and had caused great financial losses to the swine industry in the world [1–4]. PRRS is caused by PRRS virus (PRRSV), which belongs to order Nidovirales, family Arteriviridae, genus *Arterivirus* [5]. PRRSV is susceptible to genetic diversity. On the basis of phylogenetic analysis of PRRSV isolates, the virus can be divided into two genotypes: type I (Europe-like) typified by LV and type II (NA-like) typified by VR-2332 [6, 7]. Within the type II PRRSV, it is overall divided into 9 monophyletic lineages [8]. Both type I [9] and type II PRRSV were reported in China. It was noticed that the variant PRRSV which was also named highly pathogenic PRRSV (HP-PRRSV), emerged in 2006, had affected more than 200 millions pigs, and had caused huge economic losses to Chinese swine industry [3].

PRRSV has a positive-sense RNA genome of approximately 15.1–15.5 kb. The genome contains at least 10 open reading frames (ORFs) [10]. The ORF1a and ORF1b are located downstream of 5′ untranslated region (UTR) and occupy around two-thirds of the genome and yield at least 14 smaller viral nonstructural proteins (NSPs, Nsp1α, Nsp1β, and Nsp2-12) related to the viral transcription and replication. ORF2a, ORF2b, and ORFs 3–7, located upstream of the

3′UTR, encode the minor structural proteins (GP2, E, GP3, and GP4) and major structural proteins (GP5, M, and N), respectively [11–15]. An additional novel structural protein encoded by ORF5a was identified to be important for PRRSV replication [10, 16].

Live attenuated virus vaccine is considered to be the most economic method to achieve immunization [17]. Several different live-attenuated vaccines had been developed to control this disease and these vaccination strategies had greatly reduced the economic loss caused by PRRS [18–21]. Live-attenuated vaccines were somehow an effective way of inducing immunity and protecting herds from losses associated with infections by highly virulent strains of PRRSV [22]. All of the commercially available live-attenuated vaccines were derived from field wild-strains after sequential passages of the virus on cell line; it was shown that both structural and nonstructural viral proteins and perhaps the interaction of different proteins led to PRRSV virus attenuation in vivo [18, 19, 23–25] and overattenuation of HP-PRRSV would result in insufficient immunogenicity, which had been reported [26]. Thus, a satisfactory balance between attenuation and good immunogenicity for PRRSV live vaccine requires a better understanding on the PRRSV attenuation. In the present study, we described a PRRSV isolate QY1 which was serially passed on Marc-145 cells; genome sequences and the level of attenuation of different derivative of QY1 were determined as well. Additional studies were carried out to evaluate the immunogenicity of its attenuated phenotype for a better understanding of the possible relationship between genetic mutations and PRRSV attenuation.

2. Materials and Methods

2.1. Virus Culture. PRRSV strain QY1 was isolated in June 2007. Marc-145 cell culture was applied to virus propagation, and the inoculated monolayers of Marc-145 cells were placed at 37°C, 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (Gibco) when 70%–80% cytopathic effects (CPE) was visible. The cell culture supernatants were harvested by freezing and thawing for 3 times, which were diluted as the ratio of 1 : 50 or 1 : 100 to start the next passage. Virus purification and titer assessment were carried out every 10 passages.

2.2. Virus RNA Extraction and Genome Sequencing. Total viral RNAs were extracted from different QY1 virus passages using TRIzoL Reagent (Invitrogen, USA). Complete genomic sequences of P5, P60, P80, and P100 were determined, and the ORF5 gene was determined at every 10-passage interval as a viral mutation insight. Total RNA was dissolved in nuclease-free water and stored at −70°C for further use. The reverse transcription (RT) and the polymerase chain reaction (PCR) were conducted using PrimeScript One Step RT-PCR kit (TaKaRa, Japan). A 3′-full RACE Kit (TaKaRa, Japan) was employed to amplify the 3′UTR according to instructions of the manufacturer. 17 primers pairs were designed to amply the full length of viral-genome based on sequence of the VR-2332 [27]. PCR products were purified from agarose gel by using an E.Z.N.A. Gel Extraction Kit (OMEGA, USA) according to the manufacture's recommendation. The PCR products were cloned into pMD19-T vector (TaKaRa, Japan) and sent to Shanghai Sango Biotech, China, for sequencing. A quality sequence represented at least threefold genome coverage and the sequence results were analyzed using DNAStar lasergene version 7.2.

2.3. Pathogenicity Study Design and Experimental Challenge. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Committee of Animal Experiments of South China Agricultural University (approval ID 201004152). All efforts were made to minimise suffering.

A total of fifty 35-day-old PRRSV-free piglets were obtained from a farm that was negative for PRRSV and PCV2 infections. The piglets were randomly divided into five groups with 10 animals in each group. Each group of piglets was housed separate from other groups in biological safety level 2 (BSL2) facilities provided by Guangdong Dahuanong Animal Health Products Co., Ltd. At 6 weeks of age, piglets in groups 1, 2, 3, and 4 were inoculated intramuscularly with 2×10^5 tissue culture infective does (TCID50) QY1 virus of P5, P60, P80, and P100, respectively. Group 5 was intramuscularly injected with Dulbecco's Modified Eagle's Medium (DMEM), which was used as the negative control. Then piglets were monitored daily for clinical signs, including anorexia, lethargy, diarrhea, dyspnoea, and body temperature. Animals were weighed at 0, 7, 14, 21, and 28 days after inoculation (dpi). Serum samples were collected on 0, 3, 7, 10, 14, 21, and 28 dpi. Five pigs from each group were randomly selected and were necropsied on 14 dpi, followed by lungs examination for gross and microscopic changes.

The remaining pigs ($n = 5$) of groups 3, 4, and 5 were intramuscularly injected with 2×10^5 of the P5 virus on 28 dpi for experimental challenge, respectively. Animals were monitored daily for the presence of clinical signs of anorexia, lethargy, diarrhea, and dyspnoea and body temperature. Blood samples were collected on 28, 35, and 42 dpi. All pigs were necropsied on the 14th day after challenge, and gross pathological lung lesions were evaluated. Lung tissues were collected for histological examination as well.

2.4. Serology. Serum samples collected on 0, 7, 14, and 21 dpi were used for PRRSV specific antibody responses using a commercial ELISA kit 2XR (IDEXX Laboratories Inc., Westbrook, ME) according to the manufacturer's instructions. Samples with sample-to-positive (S/P) ratios ≥0.4 were considered positive for antibodies against PRRSV. In addition, serum samples from 21, 28, 35, and 42 dpi were used for virus neutralization assays as described by Plagemann et al. [28].

2.5. Viremia. Virus isolation and viral titration assay in serum were conducted. Briefly, 50 μL of serum was added on a monolayer of Marc-145 cells in 1 well of a 24-well plate for virus isolation. Each well was examined for cytopathic effect and assessed as positive or negative daily for one week

of culture. Titration was performed by preparing 10-fold dilutions of each positive sample and adding 50 μL of each dilution to 4 wells of a monolayer of Marc-145 cells in a 96-well plate. The 50% tissue culture infective dose ($TCID_{50}$) per mL was calculated according to the method of Reed and Muench.

2.6. Histological Examination. Samples of lung (two sections from the cranial lobe and one from each of middle, accessory, and caudal lung lobes) were collected and fixed in 10% neutral buffered formalin and routinely processed for histopathological examination. The microscopic sections were examined in blind fashion and assigned a score for severity of interstitial pneumonia (0 to 4) as previously described [29].

2.7. Statistical Analysis. Statistical analysis of antibody and virus titers was performed using SPASS 7.0 software. One-way analysis of variance (ANOVA) was used to evaluate the differences among the geometric mean antibody and virus titers. The level of statistically significant difference was set as $P < 0.05$.

3. Result

3.1. Clinical Signs and Weight Gain. The negative-control group showed no clinical symptoms and was PRRSV free until the end of the pathogenicity study (up to 28 dpi). All piglets infected with QY1 P5 virus developed typical clinical symptoms of HP-PRRSV including high fever, anorexia, depression, lethargy, dyspnea, and skin cyanosis and 2/10 piglets in this group died on 9 dpi and 11 dpi, respectively. Other pigs of this group began to decrease in severity on 12 dpi except that two pigs still showed severe weakness and moribund condition and were euthanized on 14 dpi. Febrile response was shown in Figure 1. All pigs inoculated with P5 exhibited high fever (≥40.5°C) on 4 dpi, which lasted for 6 days. Pigs inoculated with QY1 P60 exhibited mild to moderate clinical symptoms, such as anorexia, depression, and lethargy and four pigs in this group showed moderate dyspnea and high fever but the average body temperature of this group was below 40.5°C. The P60 group exhibited clinical symptoms beginning on 3 or 4 dpi, which reached peak on 10 dpi and resolved from 14 to 21 dpi. 4/10 pigs infected with QY1 P80 exhibited moderate fever (40°C–40.5°C) on 5 dpi, which lasted for 5 days. Inoculation with P80 clearly induced elevated temperature following inoculation, suggesting that there was residual virulence in the viruses. In contrast, animals inoculated with QY1 P100 did not show any significant clinical symptoms throughout the experiment. Rectal temperature of pigs infected with QY1 P100 was within normal range; the performance on weight gaining was shown in Figure 2. No significant difference in weight gaining was observed among P80, P100, and control groups. The least weight gain was found among pigs infected with the virulent virus QY1 P5 followed by the moderate virulent virus QY1 P60.

At 28 dpi, pigs inoculated with either QY1 P80 ($n = 5$) or P100 ($n = 5$) were challenged with QY1 P5. Pigs in these two groups did not show high fever or clinical symptoms after challenge exposure, which indicated that these pigs were completely protected following inoculation with either QY1 P80 or P100. After challenge exposure, all pigs in group 5 developed acute clinical symptoms of HP-PRRSV, including anorexia, lethargy, diarrhea, dyspnoea, and persistent high body temperature. Most importantly, 2/5 pigs in this group died on 9th and 10th day after challenge, respectively.

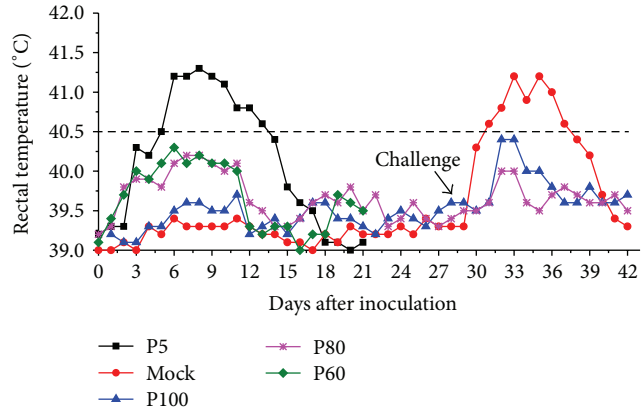

Figure 1: Mean rectal temperature following inoculation with different QY1 passage virus. 28 days after inoculation, five pigs in P80, P100, and mock groups were challenged with QY1 P5.

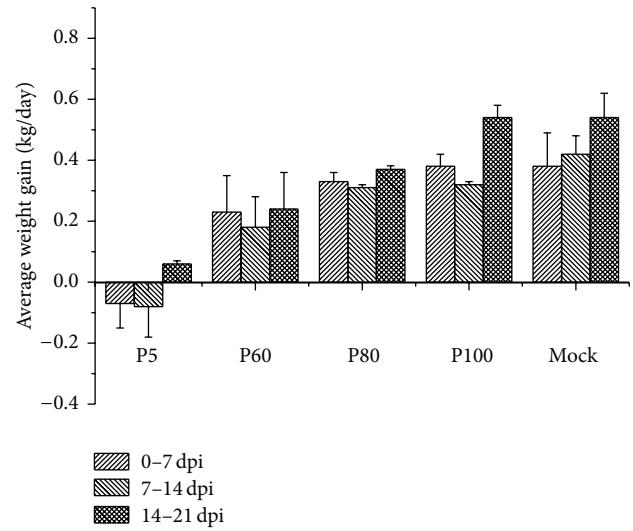

Figure 2: Average weight gains after PRRSV inoculate (0–21 dpi). Data were express as mean ± S.D. of the numbers of pigs alive at the time of the measurement.

3.2. Antibody Response. All pigs were negative for PRRSV antibody test at the time of inoculation (Figure 3). The serum of negative control pigs showed no responses for PRRSV antibodies throughout the course of the pathogenicity study. On 7 dpi, 7/10, 8/10, and 7/10 pigs were seroconverted in P5, P60, and P80 inoculation groups, respectively. P60 group showed a significant high S/P ratio ($P < 0.05$) than the other

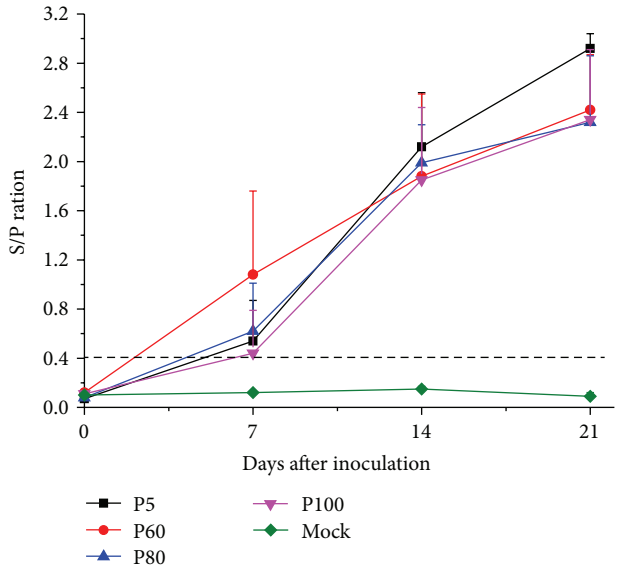

Figure 3: Mean anti-PRRSV antibody levels measured by ELISA. Samples were considered positive for antibody to PRRSV if the sample-to-positive (S/P) ratio was equal to or greater than 0.4. The geometric mean data with standard deviations (error bars) were shown.

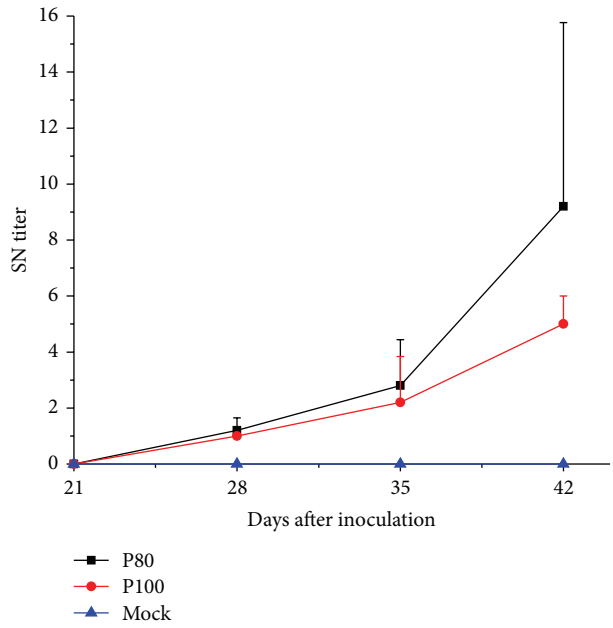

Figure 4: Virus neutralizing titers. Serum collected at −7, 0, 7, and 14 days after challenge were examined for neutralization with PRRSV. The data were expressed as the geometric mean ± S.D. of 5 pigs in each group. The serum was considered neutralizing at fourfold or higher dilution in positive response.

groups. 5/10 pigs in P100 inoculation group were seroconverted by 7 dpi. Consequently, all pigs were seroconverted to anti-PRRSV on 14 dpi. The virulent virus P5 inoculation group displayed a higher antibody titer compared to the other groups at 14 and 21 dpi ($P < 0.05$), respectively, while P60, P80, and P100 infectious group induced similar levels of anti-PRRSV antibody responses in pigs at these time points. Serum samples collected on 21, 28, 35, and 42 dpi were used for virus neutralizing assays. The results showed that PRRSV-specific neutralizing antibodies were absent before challenge and not detectable until 7th day after challenge. QY1 P80 group developed a better virus neutralizing reaction, demonstrating a higher level of serum neutralizing antibody against QY1 P5 at both days 35 and 42 than QY1 P100 inoculated group (Figure 4).

3.3. Viremia. Serum samples were analyzed by titration. The virulent virus P5 showed the highest viral titer at all collection time points, of which in the attenuation phenotype P100 was the lowest. After reaching the peak value on 7 dpi, titers of each group began to decline. There is less than one log decrease in growth before 14 days between the passage levels. One pig of QY1 P100 group maintained high levels of viremia whereas other pigs did not on 14 dpi, which caused a large variation at this time point (Figure 5). On 21 dpi, all pigs infected with P80 and P100 were PRRSV negative confirmed by both RT-PCR detection and viral isolation (Table 1).

3.4. Gross Lesions and Microscopic Lesions. Gross lung lesions scores were shown in Figure 6(a). At necropsy, pigs infected with PRRSV induce similar lesions but different in severity among groups. All pigs inoculated with virulent P5 virus showed obvious lung lesions, which affected the cranial, middle, and accessory, characterized by the lung's failure to collapse and the parenchyma being firm and rubbery. Furthermore, the average lung lesion score in this group was significantly higher ($P < 0.01$) than other PRRSV infected groups. 2/5 pigs in P60 inoculated group displayed moderate lesions and the other pigs in this group developed mild lung lesions. Mild lesions were also observed in P80 inoculated group, whose average lung lesions were similar for all the five pigs in this group. Gross lung lesions above 10% in P60 and P80 groups indicated residual pathogenicity in these passage levels. No obvious lung lesions were observed in the P100 and negative control groups. The varying degree of severity of microscopic lung lesions among each group was consistent with the display of gross lung lesions. Virulent P5 virus infected groups developed acute PRRSV infection signs, which were characterized by collapsed alveoli with an infiltration of macrophages and an accumulation of immature lymphocytes in the interstitium (Figure 7). Scores of microscopic lung lesions of pig inoculated with virulent P5 virus were significantly higher ($P < 0.01$) than that of P80, P100, and mock groups (Figure 6(b)). Lung lesions in P60 and P80 infected group were moderate on 14 dpi while P100 inoculated group was minimal or absent at this time point. Pigs inoculated with P80 or P100 virus were sacrificial on 14 day post-challenge no obvious pathological damage was found, indicating that pigs could tolerate lethal challenge.

3.5. Sequence Analysis. The full-length nucleotide sequences of QY1 P5 strain and its derivative passages P60, P80, and

Table 1: Development of viremia in inoculation pigs.

Groups	Days after inoculation						Days after challenge		
	0	3	7	10	14	21	0	7	14
P5	0/10	10/10	10/10	10/10	7/10	3/5	—	—	—
P60	0/10	10/10	10/10	10/10	6/10	3/5	—	—	—
P80	0/10	10/10	10/10	10/10	8/10	0/5	0/5	0/5	0/5
P100	0/10	6/10	7/10	6/10	4/10	0/5	0/5	1/5	0/5
MOCK	0/10	0/10	0/10	0/10	0/10	0/5	0/5	5/5	5/5

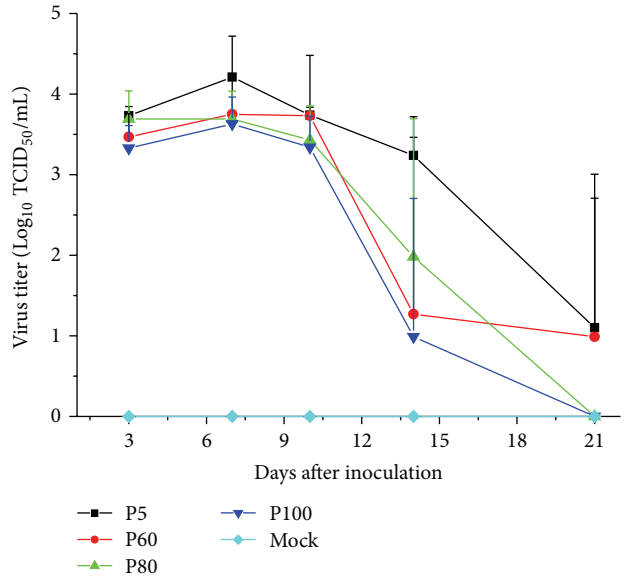

Figure 5: Level of viral titers on Marc-145 cells in the serum at each collection time point. The data were expressed as the geometric mean ± S.D. from 5 pigs in each group. No PRRSV was detected from any of the samples collected from pigs in the mock infected group.

P100 were determined. The sequence data showed that the genomic sequence of QY1 P5 was 15,357 nucleotides (nts) in length, including a 189-nt 5′UTR, a 14,981-nt coding region comprising 10 ORFs, and a 187-nt 3′UTR with 37-nt ploy (A) tail. Comparative genomics analysis revealed that QY1 P5 shared a high identity with the HP-PRRSV strains including JXA1, HuN4, SY0608, and WHU1, which was 98.6%–99.6% homology at the nucleotide level. The unique molecular feature, discontinuous 30-amino acid deletion in Nsp2, was observed which was known to be one of the characteristics of the epidemic strains isolated in China in 2006 [2, 14]. The QY1 P5 showed 89.6% nucleotide identity with VR-2332 strain, but only 61.3% sequence identity with LV strain, revealing that QY1 P5 belongs to the HP-PRRSV. Sequence identities between the parental strain P5 and its three derivatives (P60, P80, and P100) were ranging from 99.6% to 99.8% at the nucleotide level. Compared with the parental strain, the numbers of nucleotide substitutions that occurred in P60, P80, and P100 were 29, 40, and 48, respectively. Taken together, all these implied that virus mutations increased over time, although some of these mutations were synonymous or were unable to pass from one generation to the next. In addition, two deletions appeared during the in vitro passage, with one nucleotide acid deletion occurring in the 5′UTR, besides the other novel noncontinuous 34-amino acid deletion located in nsp2 between positions 464 and 498. No insertion was observed during the series of passages. The RT-PCR and sequence analysis of viral RNA isolated from serum sample ($n = 5$) collected from P100 inoculated group 14 days after challenge confirmed the unique deletions were genetically stable in in vivo study.

3.6. Amino Acid Mutations during the Attenuation Process. Compared to the QY1 parental strain P5, a total of 47 altered nucleotides resulting 34 amino acid mutations were found in QY1 P100 genome which were located in ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, and ORF5. Among the 34 amino acid mutations, 20 of 34 occurred in nsps and 14 mutations were found in the structural proteins. No mutation was found in the M and N protein-encoding region. Compared to the genome sequence of QY1 P5, P60 with 11 amino acid mutations was observed in P60, while 14 mutations occurred from P60 to P80 and 8 mutations were found from P81 to P100. Detailed analysis of the nucleotide mutations and corresponding amino acid changes was shown in Table 2. Mutations that occurred in the viral attenuation process would somehow be associated with viral attenuation. Compared to the other test virus, we proposed that the following virulence-associated amino acids were related to the PRRSV attenuation (Table 3). Additionally, a unique 34 aa deletion (464–498, corresponding to VR-2332) was identified in the nsp2. Compared to the deletion location found in TJM, a commercial attenuated-live vaccine strain with continuous deletion of 120 aa (628–747, corresponding to VR-2332) in the nsp2 protein [24], the deletion found in the QY1 P100 located upstream of nsp2.

4. Discussion

Mutations associated with PRRSV attenuation had been revealed in several studies; the investigators suggested that multiplate regions would relate to the attenuation of PRRSV [18, 24, 30–33]. In this study, a highly pathogenic PRRSV strain named QY1 P5 was passaged in vitro, and mutations occurred during this process, including 34 amino acid changes, 1 nucleotide deletion, and 34 amino acid deletions in the QY1 P100. Mutations were found in the genome except for nsp4, nsp6, nsp12, ORF6, and ORF7. Animal experiment

Table 2: Nucleotide and amino acids changes among P5, 60, 80, and 100 of PRRSV strain GDQY1.

ORFs	Encoding protein (aa length[a])	nt position[b]	P5	P60	P80	P100	aa position[c]	P5	P60	P80	P100
ORF1a	Nsp1α (180)	333	T	C	C	C	48	G	G	G	G
		677	A	C	C	C	163	H	P	P	P
	Nsp1β (203)	986	C	C	T	T	266	P	P	L	L
	Nsp2 (950)	1586	A	G	A	G	466	D	G	D	G
		1813	A	A	G	G	542	K	K	E	E
		2000	A	A	A	G	604	Q	Q	Q	R
		2018	T	T	T	G	610	I	I	I	T
		2527	A	A	G	G	780	K	K	E	E
		2560	A	A	A	G	791	M	M	M	V
		2729–2830			—[d]	—	846–880			—	—
		2986	A	G	G	G	933	E	G	G	G
		3178	G	A	A	A	997	D	N	N	N
		4220	G	G	A	A	1344	R	R	K	K
	Nsp3 (446)	4447	C	C	C	T	1419	C	C	C	C
		5120	C	C	C	T	1644	A	A	A	V
	Nsp5 (170)	6076	A	G	G	G	1963	E	K	K	K
		6156	C	T	T	T	1989	V	V	V	V
		6157	A	C	C	C	1990	K	Q	Q	Q
		6177	T	C	C	C	1996	F	F	F	F
		6248	G	G	A	A	2053	L	L	L	L
		6357	C	T	T	T	2056	A	A	A	A
	Nsp7 (259)	7134	A	A	T	T	2315	K	K	N	N
ORF1b	Nsp9 (643)	8139	G	G	G	T	178	A	A	A	S
		8310	G	G	A	A	235	E	E	K	K
		8322	T	T	A	A	239	L	L	I	I
		8427	C	T	T	T	274	P	S	S	S
		8269	A	A	G	G	554	I	I	M	M
	Nsp10 (441)	10895	C	C	T	T	408	F	F	F	F
	Nsp11 (223)	11321	G	G	G	A	109	K	K	K	K
OR2a	GP2 (257)	12012	G	A	A	A	10	L	L	L	L
		12131	A	A	T	T	50	Y	Y	F	F
		12334	A	A	G	G	118	I	I	V	V
		12377	G	G	A	A	132	S	S	N	N
		12592	C	C	A	A	204	H	H	N	N
ORF2b	E (73)	12012	G	A	A	A	9	D	N	N	N
		12131	A	A	T	T	48	L	L	F	F
ORF3	GP3 (255)	12840	C	T	T	T	79	H	Y	Y	Y
		13032	T	C	C	C	143	F	L	L	L
		13275	T	T	T	C	223	H	H	H	H
		13277	G	G	A	A	224	Q	Q	Q	Q
ORF4	GP4 (179)	13275	T	T	T	C	42	S	S	S	P
		13277	G	G	A	A	43	D	D	N	N
		13561	C	C	T	T	137	H	H	H	H
ORF5	GP5 (200)	13853	G	G	G	T	52	G	G	G	V
		13864	T	T	T	C	56	L	L	L	P
		13936	G	T	G	T	80	G	V	G	V

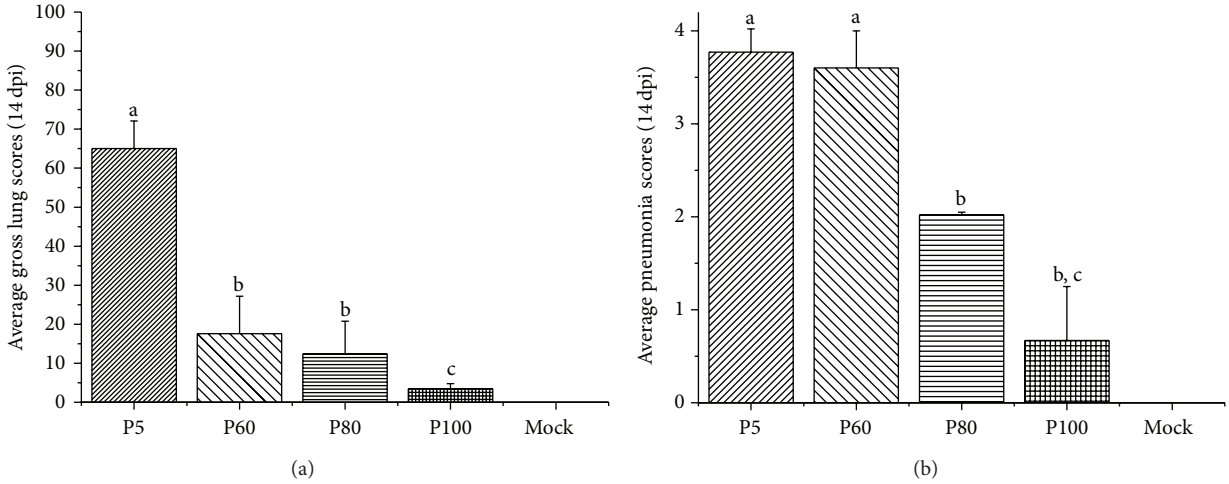

Figure 6: Average gross lung scores (a) and pneumonia scores (b) were recorded. The data were expressed as the mean ± S.D. from 5 pigs in each group. Different letter superscripts denote significant differences at $P < 0.05$.

Table 2: Continued.

ORFs	Encoding protein (aa length[a])	nt position[b]	P5	P60	P80	P100	aa position[c]	P5	P60	P80	P100
		14033	C	T	T	T	112	Y	Y	Y	Y
		14284	A	G	G	G	196	Q	R	R	R

[a]Amino acids length of each NSP.
[b]Nucleotide position mapped in the viral genome.
[c]Amino acids position mapped in each nonstructural or structural protein.
[d]Dash stands for deletions in those positions.

revealed that QY1 P5 had been gradually attenuated in vitro. Pigs inoculated with QY1 P100 did not show any clinical signs, while signs of fever were observed in P80 infection pigs (4/10) during 5 to 10 dpi. Pigs inoculated with P60 showed mild-to-moderate clinical signs and QY1 P5 infected pigs developed acute HP-PRRSV infection symptoms, including high fever and severe respiratory disease. The virus attenuation was further supported by pathological examination, which revealed that pigs inoculated with the latter passage virus exhibited attenuated phenotype compared to animals inoculated with early passage virus.

The QY1 P5 strain had the typical genomic character of the HP-PRRSV strains and exhibited 98.6%–99.6% nucleotide identity with the Chinese HP-PRRSV strains. The present study clearly demonstrated that PRRSV strain QY1 P5 is a highly pathogenic virus. QY1 P5 caused typical clinical signs of "swine high-fever syndrome"; two pigs inoculated with P5 died. Animals infected with QY1 P5 reach over 41°C on 6 dpi and lasted for 5 days, and the virus replicated in swine to a high virus level in serum. Conversely, no pigs infected with the other intermediate viruses reach a temperature of 40.5°C and lower virus level in serum was observed in these groups compared to the P5 infected group. These results indicated that virulence of the QY1 P5 strain was attenuated remarkably after serial in vitro passage.

The 5′ and 3′UTR of PRRSV were shown to be important in the viral replication and transcription of PRRSV [34]. Studies of poliovirus vaccine identified mutations in the 5′-leader as important determinants for the attenuation of the vaccines [35]. However, no studies had showed that the mutations found in the 5′UTR were associated with PRRSV attenuation. 5′UTR was highly conserved during the PRRSV attenuating process [30]. Compared to VR-2332 strain, most of the HP-PRRSV isolates exhibited only one nucleotide deletion at position 119 within 5′UTR. In the present study, there was an additional guanine deletion that occurred at position 120 of 5′UTR during the QY1 attenuation process (at the early passage of 19); however, viral derivatives QY1 P60 which contained the continuous two nucleotide deletions in 5′UTR still showed mild-to-moderate virulence. Additionally, similar deletion had also been found in field strains BJPG and GX1002 with unknown virulence. The deletions found in 5′UTR were interesting; however, the relative contribution of such deletion to the attenuation of QY1 remains undefined and needs to be addressed in future studies.

Nsp2 gene deletions in both PRRSV genotypes were naturally found in the field [27, 36–38]. It had been demonstrated that as many as 403 amino acids within the nsp2 hypervariable region were dispensable for viral viability in vitro [39] and the selected nsp2 gene deletion mutations could delay seroconversion and growth attenuation in swine [40]. In this study, a unique 34 aa deletion (464–498, corresponding to VR-2332) was identified in this region. As a result, it was showed that the mutants with the new deletion in nsp2 showed higher titers than the original virus when cultured

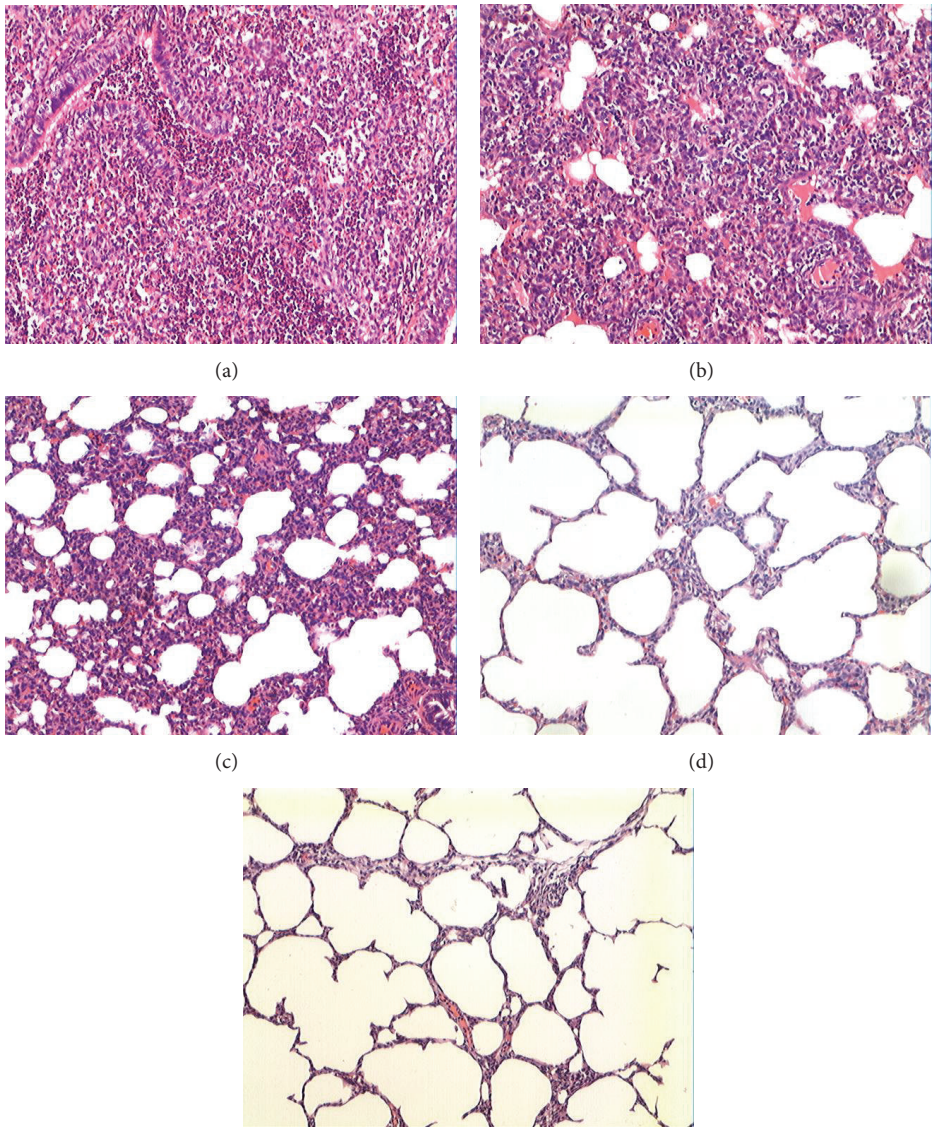

Figure 7: Histologic analysis of lungs from inoculated and control pigs. 14 days after inoculation, five pigs from three groups inoculated with P5 (a), P60 (b), P80 (c), and P100 (d) and the control group (e) were examined for histopathology.

Table 3: Nine unique amino acids positions that varied in PRRSV attenuation process.

VR-2332/RespPRRSV-MLV	JXA1/JXA1R	HuN4/HuN4-F112	TJ/TJM	QY1/QY100
GP2 (L10/F10)	GP2 (L10/F10)			GP2 (L10/F10)*
	GP2 (Y50/F50)	GP2 (Y50/S50)		GP2 (Y50/F50)
		GP2 (I118/V118)		GP2 (I118/V118)
E (D9/Y9)	E (D9/N9)	E (D9/N9)		E (D9/N9)
	E (L48/F48)	E (L48/F48)		E (L48/F48)
	GP3 (H79/N79)		GP3 (F143/L143)	GP3 (H79/N79)
	GP4 (D43/G43)	GP4 (D43/N43)	GP4 (D43/G43)	GP4 (D43/N43)
			GP5 (G80/V80)	GP5 (G80/V80)
		GP5 (Q196/R196)	GP5 (Q196/R196)	GP5 (Q196/R196)

*Synonymous mutation.

in Marc-145 cells (about 2 log10 of TCID50/mL). However, both mutants QY1 P80 and P100 growth attenuated in pigs and exhibited lower level of viral replication in vivo than their parental strains. It was known that a continuous deletion of 120 aa (628–747, corresponding to VR-2332) in the nsp2 protein was found in a commercial attenuated-live vaccine strain TJM [24]. Compared to the deletion location found in TJM, the deletion found in the QY1 P100 located upstream of nsp2 and such deletion found in QY1 P100 was similar to a HP-PRRSV isolate GDQY2 that we reported previously, which possesses a 35 aa deletion at amino acid positions 470–505 corresponding to VR-2332 [27]. Large spontaneous deletion in the nsp2 gene during in vitro cell culture passage has been reported previously, but such a deletion was found to have no effect on PRRSV virulence [41]. Thus, deletions found in the nsp2 of QY1 P100 were probably not the major factor for PRRSV virulence decrease. However, as the HP-PRRSV with a unique discontinuous deletion of 30 amino acids was the dominant strain of PRRSV in mainland China, the attenuation phenotypes of QY1 P100 with a new deletion in nsp2 would serve as a satisfied marker attenuated live-vaccine candidate.

Serial passage is a method that has been commonly applied for PRRSV MLV vaccines development, yet the mechanisms involved in PRRSV attenuation are largely unknown. It had been shown that major virulence determinants are located in NSP3-8 and ORF5, while the other NSPs and ORF2 were also involved in virulence determinants [42]. Based on our findings, the results revealed that determination sites of viral attenuation were variable and multiple, and incidental mutations always occurred in the nonstructural gene while the structural gene, especially the envelope-associated protein encoding gene, had unique amino acids changes among the attenuated PRRSV strains. $G^{12012} \rightarrow A^{12012}$ change resulted in a synonymous mutation at L^{10} located within the predicted signal sequence of GP2, which was detected during sequential passages of DGQY1, and this nucleotide mutation also altered the amino acids mutation $D^{9} \rightarrow N^{9}$ in E protein. Since parallel mutation in this position was found in other PRRSV viral attenuating process, it is possible that mutation achieved at this position was responsible for the viral attenuation. Another residue change ($L^{48} \rightarrow F^{48}$) located in ORF2 was also found in two commercial MLV vaccines JXA1R and HUN4-F112, which seemed to be a unique molecular signature among the HP-PRRSV attenuated strains. It was shown that GP3, as a structural protein [43] presenting on virion envelope, was potentially associated with viral attenuation [30]. In this study, mutations (aa, $H^{79} \rightarrow Y^{79}$, $F^{143} \rightarrow L^{143}$) located in the antigenic region of GP3 were detected, and this mutation could influence the characteristics of antigenic sites of GP3. Besides, a varying mutation amino acid position ($D^{43} \rightarrow N^{43}$) that was found in GP4 led to an additional potential glycosylation. Thus, GP4 of QY1-P100 was likely to have five N-glycosylation sites at positions N37, N43, N84, N120, and N130, respectively. GP4 was the key glycoprotein of PRRSV that was responsible for formation of the multiprotein complex, and the amount of glycan moieties could affect the viral attachment with cell receptor CD163 [44]. However, whether N43 glycan moieties could promote the viral propagation on Marc-145 cell or affect the virulence needs further investigations. An interesting observation was a mutation at position 196 of ORF5 (aa $Q^{196} \rightarrow R^{196}$), and this change appeared in GP5 of HuN4 and TJ attenuation phenotypes as well. As was reported by Tian, the GP5Q^{196}WGRL/P^{200} epitope was immunodominant and highly conservative in all type II PRRSVs and the 53D8 MAb could not recognize this mutated R^{196}WGRL/P^{200} by IFA or IPMA [19].

The application of attenuated live vaccine such as Resp-PRRS MLV, JXA1-R, HuN4-F112, and TJM had greatly reduced the production loss caused by PRRS in China [3, 20, 30]. These live attenuated vaccines had been developed by serial passage on cell culture. In our study, QY1 P100 conferred very mild reactogenicity and high degree of immunogenicity, and pigs could tolerate lethal challenge of virulent virus, indicating that QY1 P100 was safe and satisfactorily immunogenic against HP-PRRSV. However, the protection of QY1 P100 under challenge by heterologous PRRSV strains was undefined. Such questions will be addressed experimentally in the future.

In conclusion, the data obtained from this study suggested that HP-PRRSV isolate QY1 was gradually attenuated during the in vitro passage. The virulence of PRRSV was determined by multiple factors in both structural and nonstructural genes. The results presented in the study signified some important clues for the attenuated PRRSV phenotype. The attenuated PRRSV phenotype QY1 P100 was proved to be immunogenic and protective in pigs, which could serve as a desired candidate for the attenuated live vaccine against HP-PRRSV under further evaluation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

An Overview of Live Attenuated Recombinant Pseudorabies Viruses for Use as Novel Vaccines

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Pseudorabies virus (PRV) is a double-stranded, DNA-based swine virus with a genome approximating 150 kb in size. PRV has many nonessential genes which can be replaced with genes encoding heterologous antigens but without deleterious effects on virus propagation. Recombinant PRVs expressing both native and foreign antigens are able to stimulate immune responses. In this paper, we review the current status of live attenuated recombinant PRVs and live PRV-based vector vaccines with potential for controlling viral infections in animals.

1. Introduction

1.1. Background Information on PRV. Pseudorabies virus (PRV) is a member of the family Herpesviridae, subfamily Alphaherpesvirinae [1] and the causative agent of pseudorabies (PR) or Aujezsky's disease. Infections with PRV result in nervous disorders, respiratory distress, weight loss, young piglet death, and abortion [2]. The virus has a double-stranded linear DNA genome 1.43×10^5 kb in length [3] and contains a unique long region (UL), a unique short region (US), a terminal repeat sequence (TRS), and internal repeat sequences (IRS) [4].

To date, at least 11 different glycoproteins of PRV (gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, or gN) have been identified and the genes that encode these proteins have been sequenced. The essential glycoproteins of PRV include gB gD, gH, gL, and gK; the others are considered nonessential [5, 6]. There are several nonstructural proteins of PRV such as thymus kinase (TK) and protein kinase (PK), which are associated with virulence [6, 7]; however, this subset of genes can be replaced by heterologous genes without affecting infectivity or virus propagation provided the essential genes remain intact. A schematic drawing regarding common sites for gene insertion in the PRV genome is shown in Figure 1.

The efficacy of multivalent PRV vaccines has been investigated. Herein, we review research progress using attenuated recombinant PRVs (rPRVs) as vaccine candidates with application for advancing the development of rPRV vector vaccines.

1.2. Introduction to Live Attenuated PRV Vaccines. Recombinant viruses represent a particularly promising avenue of vaccine research both for improving existing vaccines and for developing new ones [8, 9]. In principle, the design of PRV vector vaccines is predicated upon the genome of live PRV being used to insert and express genes encoding protective antigens from other pathogens including viruses, bacteria, and parasites [10]. The expressed foreign antigens can be used subsequently to stimulate relevant immune responses [11]. The existence of numerous nonessential genes in the large PRV genome permits the simultaneous insertion of multiple foreign genes in the hope of vaccinating against several diseases at the same time [12].

PRV Bartha-K61 is a common parental strain of rPRV. It is an attenuated PRV which has been passaged repeatedly in pig kidney cells, chicken eggs, and chicken embryo cells [13]. In this strain, the complete gE and part of gI genes have been

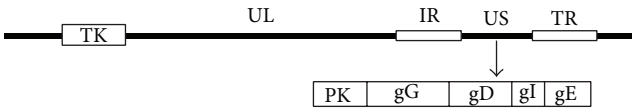

Figure 1: Common sites in the PRV genome for inserting exogenous genes. The genes encoding TK, PK, gG, gD, gI, and gE are the most common sites for inserting exogenous sequences. The TK gene is located within the unique long region (UL), and the PK, gG, gD, gI and gE genes are located within the unique short region (US). IR = internal repeat sequences; TR = terminal repeat sequence. The drawing is not to scale.

deleted [14]. Nonetheless, this construct has met with good success in developing multivalent vaccines to control various infectious diseases [4, 10, 15].

The common strategy for using rPRV involves constructing a transfer vector harboring a portion of the PRV genome. This vector is transfected into susceptible cells along with the native PRV, and then the cells are screened for the presence of the recombinant. In addition to a portion of the PRV genome, the transfer vector also contains a promoter, the foreign genes of interest, and a reporter gene. PRV sequences should appear at the start and end of the vector to permit homologous recombination between the arms of the vector and virus genomes. One study demonstrated that the human cytomegalovirus (CMV) promoter is more efficient than the PRV promotor in directing viral gene synthesis [4]. As such, the immediate early gene promoter of CMV has become the most common promoter used in these constructs. It can also be used for identification of rPRVs. In addition to conventional approaches to generating recombinants, viral genomes can be cloned into bacterial artificial chromosome (BAC) vectors. The use of herpesvirus BACs for generating site-directed and transposon mutagenic recombinants has been reviewed [16].

2. The Efficacy of Live Attenuated PRV Vaccines

To date, most of the foreign genes that have been inserted into the PRV genome encode key antigens derived from animal viruses. A summary of constructs developed to date is provided in Table 1 which includes parental PRV strains, foreign genes, and insertion sites. The examples which follow provide a more in-depth discussion of successes using this technology.

2.1. PRRSV/PRV Recombinant Virus Vaccines. Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive strand RNA virus which is a member of the familyArteriviridae [17]. It causes tremendous economic losses worldwide and is among the most important diseases in countries where swine are intensively raised [18–20]. The genome of PRRSV is 15 kb in length and contains nine open reading frames (ORFs) designated ORF1a, ORF1b, ORF2a, ORF2b,ORF3, ORF4, ORF5, ORF6, and ORF7 [21–23].

A decade ago, an attenuated rPRV, rPRV-GP5, was developed that expresses the GP5 envelope protein of PRRSV; the GP5 protein is encoded by ORF5. The rPRV-GP5 was able to confer significant protection against clinical symptoms and reduce pathogenic lesions caused by PRRSV challenge in vaccinated pigs. Pigs immunized either with rPRV or with PRRSV inactivated vaccine remained clinically healthy before and after challenge. Following immunization, only a short period (3 days) of mild fever ($\leq 41^{\circ}$C), gradually improving lung and kidney lesions, and short-term viremia (2 and 3 weeks, resp.) resulted; however, no anti-PRRSV antibody was detected before challenge [24]. In order to improve the protective efficacy of rPRV-GP5, a modified GP5 gene (GP5 m) was synthesized wherein a Pan DR T-helper cell epitope (PADRE) sequence was inserted between the N-terminus and the neutralizing GP5 epitope. The new rPRV-GP5 m elicited a higher level of PRRSV-specific neutralizing antibodies and cellular immune responses than the rPRV-GP5 [25].

Recently, this group generated another construct named rPRV-GP5 m-M that expresses two major membrane-associated proteins (GP5 and M) of PRRSV within the same vector [11]. Mice immunized with rPRV-GP5 m-M developed PRV-specific humoral immune responses and provided complete protection against a lethal PRV challenge. At the same time, high levels of PRRSV-specific neutralizing antibodies and lymphocyte proliferation responses were observed in the immunized mice. Once proof of principle was demonstrated in mice, studies advanced to piglets. When compared to the commercially available PRRSV killed vaccine, rPRV-GP5 m-M immunized animals generated higher PRRSV-specific neutralizing antibodies and higher lymphocyte proliferation responses resulting in better protection against PRRSV. These data indicate that PRV is an excellent vector for developing virus-based vaccines against PRV and PRRSV.

2.2. PCV2/PRV Recombinant Virus Vaccine. Porcine circovirus type 2 (PCV2) is the primary cause of postweaning multisystemic wasting syndrome (PMWS), which is a worldwide disease that debilitates pigs with lymphadenopathy and interstitial pneumonia [26, 27]. PCV2 is a single-stranded circular DNA virus and a member of the family Circoviridae [28]. PCV2 has three major ORFs; ORF1 encodes two replication-related proteins, Rep and Rep', which are essential for viral DNA replication [29]; ORF2 encodes the major capsid protein of PCV2; and ORF3 encodes the nonstructural ORF3 protein [30]. A rPRV expressing a fusion protein of ORF1 and 2 was constructed and its immunogenicity tested in mice and pigs [10]. The rPRV-PCV2 elicited strong anti-PRV and anti-PCV2 antibodies in BALB/c mice wherein rPRV-PCV2 protected mice against a lethal challenge with a virulent PRV. In pigs, rPRV-PCV2 elicited significant immune responses against PRV and PCV2.

A second rPRV was constructed expressing only the ORF2 gene that was also used to immunize piglets. Results showed that the rPRV-ORF2 elicited significant humoral

immune responses to both PRV and PCV2 wherein PCV2-specific lymphocyte proliferation responses could be detected by 49 days after immunization [31]. The rPRV-ORF2 was better at eliciting protective immune responses in piglets than rPRV expressing both ORF1 and 2. These findings demonstrate that rPRV-PCV2 may be a suitable bivalent vaccine against PRV and PCV2 and that multiplicity is not always the optimal approach to vaccine development.

2.3. FMDV/PRV Recombinant Virus Vaccine. Foot-and-mouth disease virus (FMDV) is highly contagious and affects all cloven-hoofed domestic animals including cattle, sheep, goats, pigs, and buffalo [32]. It is a positive single-stranded RNA virus approximately 8.5kb in length and belongs to the family Picornaviridae. The FMDV capsid precursor P1-2A is cleaved and released from the polyprotein by L protease and processed by viral protease 3C to form four structural proteins, VP1, VP2, VP3, and VP4 [33]. FMDV has seven serotypes, A, O, C, Asia1, SAT1, SAT2, and SAT3, each of which contains multiple subtypes [34–37].

Using PRV as a vector, the VP1 gene was fused to the PRV genome. The immunogenicity of the recombinant product was tested in 15 FMDV seronegative white pigs. Although the antibody levels were lower than those induced by commercially available FMDV vaccines and protection against virulent FMDV was not observed, the rPRV-VP1 construct still alleviated clinical symptoms in infected pigs [38]. To improve the immune response, another rPRV was generated that expresses P1-2A of FMDV; its protective effects were evaluated in white pigs [39]. In contrast to the earlier version, these pigs exhibited high levels of neutralizing antibodies to both FMDV and PRV and further showed strong CTL responses against FMDV antigen activation. Following challenge, replication of FMDV was significantly lower in pigs vaccinated with the new rPRV construct when compared to the commercially available vaccine.

Recently, another rPRV which coexpresses P1-2A and the viral protease 3C was developed and tested in piglets [40]. These results showed that rPRV-P12A3C induced a high level of neutralizing antibodies and FMDV-specific lymphocyte proliferative responses. Relative to the inactivated FMDV vaccine which provided 100% protection, the rPRV-P12A3C induced only 60% protection in challenged piglets but was able to reduce pathogenic lesions. These findings suggest that rPRV-P12A3C was better at protecting piglets than the previous constructs and support further development of vaccines against both FMDV and PRV. Work must now focus on targeting other serotypes of FMDV with the hope of finding one vaccine with good efficacy against all or most serotypes.

2.4. PPV/PRV Recombinant Virus Vaccine. Porcine parvovirus (PPV) is an important cause of reproductive failure in swine. It is characterized by fetal death, mummification, stillbirth, and prolonged farrowing intervals [41]. PPV is a single-strand DNA virus, which is a member of the familyParvoviridae. Its genome is 5kb is size and contains two large ORFs; the left ORF encodes the nonstructural protein NS1 and the right ORF encodes three capsid proteins [42]. One of the three capsid proteins, VP2, can self-assemble into virus-like particles (VLPs) that are immunologically indistinguishable from inactivated whole-virus vaccines [43].

A rPRV was constructed to express the VP2 gene of PPV [44]. Piglets vaccinated with rPRV-VP2 elicited PRV- and PPV-specific humoral immune responses and generated complete protection against a lethal dose of PRV. This finding lends further support to the development of bivalent vaccines and in particular, against PRV and PPV.

2.5. FMDV/PPV/PRV Recombinant Virus Vaccine. A rPRV coexpressing P1-2A of FMDV and VP2 of PPV was constructed and used to vaccinate BALB/c mice [12]. Both total antibody and neutralizing antibody levels to PRV were equivalent to the commercially available PRV vaccine. Protection to FMDV or PPV was >60% when compared to inactivated vaccines. Neutralizing antibody titers induced by the rPRV construct against FMDV or PPV were 50% of the level induced by their respective inactivated vaccines.

Unlike previous constructs, this vaccine candidate demonstrated the feasibility of using rPRV to develop trivalent vaccines, in particular against PRV, FMDV, and PPV. Future work should be performed in swine to test the utility of such vaccine in the natural host for these viruses.

2.6. CSFV/PRV Recombinant Virus Vaccine. Classical swine fever virus (CSFV) is a significant impediment to global trade in swine products and results in considerable financial loss [45]. CSFV is an enveloped, positive, single-stranded RNA virus which belongs to the genus *Pestivirus* of the family Flaviviridiae [46, 47]. Its genome, which is 12.3 kb long, encodes a single glycoprotein [48], glycoprotein El (later called E2), which is highly immunogenic and capable of inducing protective immune responses [49, 50].

A bivalent rPRV was synthesized that was gD/gE negative and that expressed glycoprotein E2. Vaccination of piglets exhibited strong protection against both Aujeszky's disease and CSFV [51] supporting the use of rPRV-based bivalent vaccines against CSFV.

2.7. SIV/PRV Recombinant Virus Vaccine. Swine influenza virus (SIV) is a type A virus, which is enveloped and consists of negative single-stranded RNA. It is a member of the family Orthomyxoviridaeand its genome encodes 10 viral proteins. RNA segment 4 contains the gene encoding the large hemagglutinin (HA) glycoprotein which is the major surface glycoprotein. It is also a major immunogen which induces subtype-specific protective cellular and humoral immune responses in animals [52, 53]. Segment 5 encodes the nucleoprotein (NP) gene [54]

A rPRV expressing the HA gene of serotype H3N2 subtype SIV (A/Swine/Inner Mongolia/547/2001) was constructed [55] and its immunogenicity was tested in mice. Upon challenge, no virus could be isolated from the vaccinated mice; however, mild pathological lesions were observed in the lungs. At the same time, the rPRV-HA construct protected mice from challenge using a heterologous virulent

SIV (A/Swine/Heilongjiang/74/2000) as well. The rPRV-HA vaccine represents a candidate vaccine against SIV. Recently, Klingbeil et al. [56] used BAC technology to generate a HA-based vaccine derived from the swine H1N1 virus cloned into PrV. The resulting virus showed little difference from the parental strain. Pigs given a single injection of the vaccine produced high levels of antibody directed at the H1N1-derived HA protein and were protected from clinical signs of infection when challenged.

2.8. Other rPRV Vaccines. PRV has a wide range of hosts including swine, sheep, cattle, and dogs [3]. As such, the PRV vector has been used to develop recombinant vaccines in other hosts and in systems unrelated to viral protection, that is, protozoan parasites.

2.8.1. Toxoplasma gondii/PRV Recombinants. A rPRV was constructed expressing SAG1 from the protozoan parasite, *T. gondii* [57]. The SAG1 protein domain belongs to a group of glycosylphosphatidylinositol (GPI)-linked proteins with SAG1 related sequences that can be found on the surface of the parasite. The protective character of the rPRV-SAG1 construct was tested in BALB/c mice. All mice vaccinated with the rPRV-SAG1 developed high levels of specific antibodies against *T. gondii* lysate antigen (TLA) and neutralizing antibodies. In addition, they observed an increase in the splenocyte proliferative response, IFN-γ and IL-2 and strong cytotoxic T lymphocyte responses. When the mice were challenged with the highly virulent RH strain of *T. gondii*, the rPRV-SAG1 construct induced partial protection (60%). This is likely related to the significantly complex life cycle of protozoan parasites and the stage specificity of SAG1 expression.

In order to improve the protective response, two additional rPRVs expressing SAG1 or the micronemal protein MIC3 (rPRV-MIC3) were developed and used to immunize BALB/c mice separately and simultaneously [58]. All mice vaccinated with the rPRVs induced high levels of antibodies to *T. gondii* lysate antigen, splenocyte proliferation, IFN-γ, and IL-2. Further experiments indicated that rPRVs stimulated humoral and cellular immune responses in vivo. The vaccinated mice survived a lethal challenge with *T. gondii* RH strain; however, protection was not complete.

These results support previous studies showing the utility of expressing *T. gondii* protective antigens in PRV as a novel approach for developing vaccine candidates against pseudorabies and toxoplasmosis; however additional research is needed to increase the survivability of host animals to parasite challenge. One approach is to make a multivalent vaccine that targets more than one stage of infection or to test other parasite antigens. Unlike viruses, parasites are far more complex both biologically and genetically which complicates the approach to recombinant vaccine development.

2.8.2. Schistosoma japonicum/PRV Recombinants. Three rPRVs expressing *S. japonicum* glutathione S-transferase (Sj26GST), fatty acid binding protein (SjFABP), or both were constructed and named rPRV/Sj26GST, rPRV/SjFABP, and rPRV/Sj26GST-SjFABP, respectively [59]. Their abilities to protect mice and sheep against *S. japonicum* challenge were evaluated. The results showed that all rPRVs induced specific antibody responses against total worm extracts, increased splenocyte proliferation, and elevated IFN-γ and IL-2 levels in the immunized mice. However, better immune stimulation was observed in animals given rPRV/Sj26GST-SjFABP than in those given either rPRV/Sj26GST or rPRV/SjFABP. Further, in all immunized sheep, the treatment was deemed safe and the worm and egg burdens were demonstrably reduced following challenge.

These results indicated that the multivalent rPRV-based vaccines for *S. japonicum* can produce significant protection and are capable of preventing infection from protozoan parasites. However, less than 100% protection, which is very common among putative parasite vaccines, has hindered acceptance and further development.

2.8.3. JEV/PRV Recombinants. A rPRV expressing the NS1 protein of Japanese encephalitis virus (JEV) was constructed [60]. Both BALB/c mice and pigs were immunized. A test using 10^6 pfu, in mice, piglets, and pregnant sows indicated a good safety profile for the rPRV. Animals given the rPRV-NS1 virus developed JEV-specific humoral and cellular immune responses and protected the animals from a lethal challenge with the virulent PRV Ea strain. These experiments provided evidence that the rPRV may serve as a candidate for generating a novel vaccine that can be used for controlling pseudorabies and Japanese encephalitis.

2.8.4. Rabies Virus/PRV Recombinants. A rPRV expressing the rabies virus glycoprotein was constructed [61]. This recombinant virus was deemed safe for dogs by oral and intramuscular inoculation routes and induced protective immune responses against both rabies and pseudorabies. Neutralizing antibody titers against rabies and pseudorabies were demonstrably elevated by 5 weeks after vaccination and remained as such for at least 6 months. This experiment indicates that constructs designed herein survived well in the host such that the immune profile of vaccinated animals was long-lived.

3. Other Virus Vectors

Clearly, there are other viral genomes that can serve as vaccine vectors such as adenovirus, poxvirus, and baculovirus. These have all been tested as delivery vehicles for exogenous antigens that had been previously expressed in PRV vectors. Adenoviruses are currently one of the most applied systems for gene delivery. As vectors, they have a high capacity for the insertion of foreign genes (5–36 kb), are able to transduce a broad range of cell types [62], and are commercially available in kit form for subsequent genetic modification.

Poxviruses are the largest known group of animal DNA viruses. They have been extensively used as expression vectors for vaccination, expression of large foreign genes, and induction of cellular and humoral immune responses [63]. Among the more common poxviruses are modified vaccinia

Table 1: General information of recombinant PRVs.

Insertion sites in PRV genome	Parental PRV strains	Foreign Genes [references]
TK gene	Bartha-K61 strain	GP5 of PRRSV [24]
TK gene	Bartha-K61 strain	The HA gene of H3N2 subtype SIV [55]
Between PK and gG gene	Bartha-K61 strain	The major immunodominant surface antigen 1 (TgSAG1) of the protozoan parasite, *T. gondii* [57]
Between PK and gG gene	Bartha-K61 strain	The glycoprotein of rabies virus [61]
Between PK and gG gene	Bartha-K61 strain	The glutathione S-transferase (Sj26GST) and the fatty acid binding protein (SjFABP) of *Schistosoma japonicum* [59]
gG gene	TK-/gG-/LacZ + strain	The VP1 gene of FMDV [38]
gG gene	TK-/gG-/LacZ + strain	The capsid precursor encoding regions of [39]
gG gene	TK-/gG-/EGFP + strain	The main surface antigen 1 (SAG1) and the micronemal protein MIC3 of the protozoan parasite, *T. Gondii* [58]
gG gene	TK-/gG-/LacZ + strain	The NS1 gene of Japanese encephalitis virus [60]
gD gene	gE-/gD-strain	The envelope glycoprotein E2 of CSFV [51]
gI gene	TK-/gE-/gI-strain	The VP2 gene [44]
Between gE and gI gene	TK-/gE-/gI-/LacZ + strain	Two major membrane-associated proteins (GP5 and/or M) (GP5 contains a native GP5 and a modified GP5) of PRRSV [11]
Between gE and gI gene	TK-/gE-/LacZ + strain	ORF1 and partial ORF2 gene/ ORF2 gene of PCV2 [10, 31]
Between gE and gI gene	TK-/gE-/gI-strain	The capsid precursor polypeptide P12A and nonstructural protein 3C of FMDV [40]
Between gE and gI gene	TK-/gE-/LacZ + strain	The protein precursor P1-2A of FMDV and VP2 protein of PPV [12]
gE gene	TK-/gE-/LacZ + strain	The modified GP5 [25]

virus Ankara (MVA), fowlpox virus, and orf virus. MVA has been a smallpox vaccine for many years and more recently it has been used as a viral vector for preventing both cancer and infectious diseases [64]. Other examples include the use of a canarypox-based recombinant containing the PrM and E genes of the West Nile Virus (WNV) to induce protection in cats and dogs. This study resulted in the expression of the WNV genes and the induction of protective immunity [65]. The orf virus has been used to generate protective immunity against CSV using the E2 gene [66] and pseudorabies in pigs [67]. Inasmuch as the orf virus rarely causes system infections and has a narrow infection host range; it is a logical choice for developing multivalent viral vaccines.

Baculovirus is an excellent tool to overexpress recombinant proteins in insect cells. Its host specificity was originally thought to be restricted to cells derived from arthropods; however, recent studies have shown that baculoviruses carrying mammalian cell-active promoters are capable of transferring and expressing foreign genes in a variety of mammalian cell types as well as in animal models [68]. Baculovirus systems have been very popular because like adenoviruses, they also are available commercially and in kit form for easy genetic modification.

Above-mentioned viruses have also been used to develop recombinant live viruses bearing components of PRRSV, PCV2, FMDV, CSFV, and SIV. A comparison of key immunological efficacies among these many virus vectors is shown in Table 2.

4. Concluding Remarks

Past successes of rPRV as a vector for expressing exogenous antigens has resulted in new rPRVs being constructed that are less pathogenic. There are many advantages of rPRV. First, live attenuated PRV has a large genome wherein half of the genome is considered nonessential thus permitting modification without affecting key characters such as infectivity. Although some of these genes are associated with virulence, their deletion and replacement by foreign genes has no adverse effects on the propagation of PRV [31]. Representative information regarding common insertion sites in parental viruses is summarized in Table 1. The benefits of viral vectors are that they not only express their own protective antigens, but any inserted exogenous genes as well. Inasmuch as they use host machinery to replicate and express proteins, the resultant exogenous gene products have a higher probability of being correctly modified or folded posttranslationally, something which is lacking in bacterial systems. As such, products derived from rPRVs are more likely to mimic native

Table 2: Comparisons of immunological efficacies among different virus vectors.

Viruses and vectors	Inserted gene	Host	Neutralizing Ab	Other responses	Reference
PRRSV					
Canine adenovirus type 2	GP5 and M	Mouse	Appeared at 14 days post immunization (dpi) peaked at 42 dpi maximum titer = 16	Anti-PRRSV Ab appeared at 14 dpi; CTL appeared at 28 dpi	[7]
Adenovirus	GP5 and M	Mouse	Appeared at 14 dpi peaked at 56 dpi, maximum titer = 102	Specific lymphocyte proliferation responses appeared at 28 dpi; CTL appeared at 28 dpi	[69]
MVA (Poxvirus)	GP5 and M	Mouse	Appeared at 14 dpi peaked at 70 dpi maximum titer = 8.12	High IFN-γ (72.6 pg/mL)	[60]
Baculovirus	GP5 and M	Mouse	Appeared at 21 dpi peaked at 42 dpi maximum titer = 8	High IFN-γ (147.84 pg/mL)	[52]
PRV	GP5 and M	Mouse	Appeared at 42 dpi peaked at 70 dpi maximum titers = 21.3		[11]
	GP5m and M	Piglets	Appeared at 42 dpi peaked at 84 dpi maximum titer = 160	Anti-PRRSV Ab appeared at 28 dpi	
PCV2					
Adenovirus	ORF2	Piglets	Titers = 1 : 36 (27 dpi) and 1 : 48 (37 dpi)	Specific Ab appeared at 10 dpi; protection = 60%	[55]
Baculovirus	ORF2	Mouse	Appeared at 21 dpi peaked at 42 dpi maximum titer = 16	Specific Ab appeared at 21 dpi; high IFN-γ (286 pg/mL)	[70]
PRV	ORF2	Piglets	Appeared at 21 dpi PCV2 Ab not detected	Specific Ab appeared at 21 dpi; PCV2-specific lymphocyte proliferation appeared at 49 dpi (low)	[71]
FMDV					
Adenovirus	whole capsid and non-structural protein 3C	Piglets	non-detected	Protection = 75% low FMDV Ab	[72]
Fowlpox virus (Poxvirus)	whole capsid and non-structural protein 3C	Mouse		Specific Ab appeared at 10 dpi	
		Piglets	Peaked at 30 dpi decreased by 49 dpi	Specific Ab appeared at 10 dpi; protection = 75%	[21]
Pseudotype baculovirus	whole capsid and non-structural protein 3C	Mouse	Titer = 13 (21 dpi) Titer = 35 (49 dpi)	High IFN-γ (1917 pg/mL)	[73]
PRV	whole capsid and non-structural protein 3C	Piglets	Appeared at 21 dpi (variable)	Virus-specific lymphocyte and non-proliferative responses higher than recombinant; protection = 60%	[63]
CSFV					
Adenoviruses	E2 glycoprotein	Rabbits	Titer = 13.8 (21 dpi) Titer = 218.8 (35 dpi)		[43]
		Piglets	Antibody level was at 90% inhibition rate	Protection = 40%	
Orf virus (Poxvirus)	E2 glycoprotein	Piglets	Appeared at 21 dpi Titer = 37 (49 dpi)	Protection = 100%	[74]
PRV	E2 glycoprotein	Piglets	Appeared at 42 dpi Titer = 37	Protection = 100%	[6]

Table 2: Continued.

Viruses and vectors	Inserted gene	Host	Neutralizing Ab	Other responses	Reference
SIV					
Adenoviruses	HA gene of type H3N2	Mouse	Appeared at 14 dpi	HA inhibiting (HI) Ab appeared at 14 dpi peaked at 35 dpi Titer = 8; Maximum titer = 32 Protection = 83.3%	[31]
PRV	HA gene of type H3N2	Mouse		HI Ab appeared at 21 dpi Peaked at 42 dpi Titer = 2 Maximum titer = 4 Protection = 80%.	[9]

immunogens and correctly induce humoral and/or cellular responses in immunized animals. As shown above, one can target multiple diseases within a single vector construct. Second, there is minimal risk using PRV gene-deletion vaccines. PRV vaccine strains have been used for decades and exhibit high safety and efficacy profiles in vivo. Third, PRV has a broad host range including pigs, cattle, goats, and dogs among others. This makes it possible to target animal diseases in multiple hosts without resorting to multiple vector constructs to express the antigens. Fourth, native PRV induces cellular immunity and causes latent infection. Therefore, rPRVs can be maintained for long periods in a given host thereby providing constant stimulation of the protective immune responses. Finally, PRV can be propagated in various cell lines including SPF chicken embryo fibroblast cells. This permits simplifying virus production and keeping manufacturing costs under control.

Other points to consider when developing PRV-based vector vaccines are that this vector system requires a strong promoter to maintain high and stable expression levels. Also, selection of nonessential genes in the PRV genome to be replaced with the foreign genes of interest can affect optimizing the immune response. Given competing interests between vector-derived and exogenous protein-derived immune responses, recombinant constructs should be characterized with respect to optimal inoculation dosage. For development of effective rPRV vaccines, the pathogenic features, protective mechanisms, and the epidemiology of diseases must be taken into account in all future work.

Many of the rPRV vaccine candidates that have been reported here either have not been further pursued or are not yet commercially available. In general, there are factors that complicate advancing these products to the marketplace. First, optimizing viral infection and replication are required to produce efficient and safe vaccines suitable for release into the environment. To this end, identifying more appropriate nonessential regions within the virus is needed to enhance expression of exogenous genes particularly when multivalent rPRVs are being developed. This is not a trivial task in view of the large genome size of PRV and the interplay between essential regions and exogenous genes that can affect viral virulence and replication. Second, modifications to the parent PRV in generating a rPRV are often required to eliminate or replace existing marker genes or important regulatory elements to make the construct more suitable for clinical application. Third, plans are needed to transition between available vaccinations and those derived from rPRVs. Concurrent or overlapping vaccinations of the two will have a significant and deleterious impact on the efficacy and propagation of subsequent rPRV-based immunizations. Finally, many of the studies using rPRVs have not been advanced to the natural host, that is, swine. Problems with the high cost of clinical trials, manufacturing sufficient amounts to advance these studies and releasing biologicals into the environment are often limiting factors. Yet these studies are necessary to get a more comprehensive picture of the immunogenicity of the expressed genes, the persistence of the viral infection, and longevity of the stimulation in the natural host and to study the potential for tumorigenesis when using uniquely modified rPRV-based vectors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Effect of TLR9 Agonist CpG Oligodeoxynucleotides on the Intestinal Immune Response of Cobia (*Rachycentron canadum*)

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Cytosine-guanine oligodeoxynucleotide (CpG ODN) motifs of bacterial DNA are recognized through toll-like receptor 9 (TLR9) and are potent activators of innate immunity. However, the interaction between TLR9 and CpG ODN in aquatic species has not been well characterized. Hence, cobia TLR9 isoform B (RCTLR9B) was cloned and its expression and induction in intestine were investigated. RCTLR9B cDNA consists of 3113bp encoding 1009 amino acids containing three regions, leucine rich repeats, transmembrane domain, and toll/interleukin-1 receptor (TIR) domain. Intraperitoneal injection of CpG ODN 2395 upregulated RCTLR9 A and B and MyD88 and also induced the expressions of Mx, chemokine CC, and interleukin IL-1β. Cobia intraperitoneally injected with CpG ODN 1668 and 2395 had increased survival rates after challenge with *Photobacterium damselae* subsp. *piscicida*. In addition, formulation of CpG ODN with formalin-killed bacteria (FKB) and aluminum hydroxide gel significantly increased expressions of RCTLR9 A (50 folds) and B (30 folds) isoforms at 10 dpi (CpG ODN 1668) and MyD88 (21 folds) at 6 dpv (CpG ODN 2395). Subsequently, IL-1β increased at 6 dpv in 1668 group. No histopathological damage and inflammatory responses were observed in the injected cobia. Altogether, these results facilitate CpG ODNs as an adjuvant to increase bacterial disease resistance and efficacy of vaccines in cobia.

1. Introduction

Teleost innate immunity plays an important role in the initial protection against invading pathogens [1]. The gastrointestinal (GI) tract is generally recognized as an organ crucial not only to the digestion/absorption of nutrients, but also to the immunity [2]. The GI tract serves as an important barrier and protects the fish from feed-borne pathogens [3–5]. The posterior segment of fish intestine is immunologically active and is armored with various immune cell types, including B cells, macrophages, granulocytes, and T cells, that play vital roles in local immune responses during the course of immunization and inflammation [6–8]. In addition, the fish intestinal epithelial cells are constantly exposed to pathogens and are involved in the innate immunity of GI tract. These cells recognize pathogen-associated molecular patterns (PAMPs) through the toll-like receptors (TLRs) and induce immune responses in the intestinal lumen [9].

TLRs are transmembrane proteins recognizing conserved pathogenic structures and activating immune effector molecules [10] to form a linkage between innate and adaptive immunity [11]. The main immune functions of TLRs are (1) inducing the expressions of pro/anti-inflammatory, cyto- and chemokine that link to the adaptive immune

system; (2) initiating antimicrobial effects or pathways; and (3) maintaining commensal and mucosal homeostasis [12]. The role that toll-like receptor 9 (TLR9) plays in the innate immune responses to bacterial and synthetic DNA containing unmethylated CpG motifs has been well characterized [13]. The human TLR9 gene can splice into different isoforms during transcription generating 5 TLR9 isoforms (TLR9A, B, C, D, and E). These TLR9 isoforms are differentially expressed in various immune organs and cells, such as spleen, peripheral blood mononuclear cells (PBMC), and lymph nodes, which may attribute to differential profiles of proinflammatory cytokines and cytotoxic T-lymphocyte differentiation [14–16]. In teleost, only sea bream and croaker TLR9 B isoforms have been identified, but the functions of TLR9 B isoforms remain unclear [10, 17].

Cytosine phosphate-guanine (CpG) oligodeoxynucleotides (ODNs) are DNA fragments with a high frequency of CpG motifs simulating the immunostimulatory activity of bacterial DNA [18, 19]. CpG ODNs, unlike most conventional adjuvants, are able to stimulate both humoral and cell-mediated immune responses in immunized animals [20]. The interaction of TLR9 with CpG motifs initiates a cascade of events resulting in the secretion of T helper (Th) 1-type cytokines and chemokines. Productions of the chemokine and interferon-gamma-inducible protein-10 (IP-10) are early indicators of Th1 type immune responses [21, 22]. In mammals, intraperitoneal administration of CpG ODNs is effective in stimulating the expressions of intestinal TLR9 and chemokines due to the lower interleukin (IL)-10 level in human neonates and pig intestine [23, 24]. In teleost, administration of CpG ODNs has been found to increase TLR9 expression in Atlantic salmon [25], sea bream [26], and turbot [27]. However, to our best knowledge, the immune responses involved in the gut immunity in response to intraperitoneal stimulation of CpG ODNs in cobia have not been investigated.

The cobia industry has been suffering from various infectious diseases associated with *Photobacterium damselae* (*P. damselae*) subsp. *piscicida* [28–31] that has a high binding and invading capacity to the epithelial cells of fish intestine [32, 33]. Hence, the immunostimulatory effects of CpG ODNs might be able to enhance the intestinal immunity of cobia and increase the resistance to infections caused by *P. damselae* subsp. *piscicida*. The objectives of this study are to (1) clone RCTLR9B; (2) analyze the expressions of RCTLR9A RCTLR9B, MyD88, Mx, IgM, chemokine CC, and IL-1β in response to the stimulation of CpG ODNs; (3) evaluate the protection efficiency of intraperitoneal administration of CpG ODNs against *P. damselae* subsp. *piscicida* infections; and (4) assess the CpG adjuvanticity by intraperitoneal injection of CpG ODNs formulated with formalin-killed bacteria (FKB) and aluminum hydroxide gel (alum).

2. Materials and Methods

2.1. Cloning of Cobia TLR9 Isoform B

2.1.1. Partial Cloning of Cobia TLR9 Isoform B. Partial sequence of cobia TLR9B was obtained using the primers (TLR9F1 and TLR9R1) which were designed based on TLR9 sequences of other fish species (*Acanthopagrus berda* number EU256332, *Dentex tumifrons* number EU256335, *Larimichthys crocea* A number EU655704, *Larimichthys crocea* B number EU655705, *Sparus aurata* A number AY751797, and *Sparus aurata* B number AY751796) using Vector-NTI (Invitrogen, Carlsbad, CA, USA) [34]. PCR was carried out using cobia cDNA from the liver as a template under the following condition: one cycle at 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were sequenced and used for designing real-time PCR primers.

2.1.2. Rapid Amplification of cDNA Ends (RACE). To obtain the full-length cDNA sequence of RCTLR9 isoform B (RCTLR9B), 5′ and 3′ RACE were implemented using the SMARTer RACE cDNA amplification kit (Clontech, USA). The RCTLR9 gene primers, TLR9B-RacF1 and TLR9B-RacR1, were designed from the partial RCTLR9B cDNA sequence (JX073035.1). Touchdown-PCR was carried out for 3′ and 5′ RACE using TLR9B-Rac F1/UPM and TLR9B-RacR1/UPM primer sets (Table 1), under the following conditions: one cycle of initial denaturation at 94°C for 2 min followed by 5 cycles of 94°C/30 s, and 72°C/3 min; next 5 cycles 94°C/30 s, 70°C/30 s, and 72°C/3 min; next 25 cycles 94°C/30 s, 68°C/30 s, and 72°C/3 min; and one cycle at 72°C/5 min. Nested PCR for 5′ and 3′ RACE was performed using TLR9B-NGSP1/NUP for 5′ and TLR9B-NGSP2/NUP for 3′ nested primer sets (Table 1). For nested PCR, 1 μL of primary RACE-PCR product was used as a template with the following conditions: initial one cycle of 94°C/2 min and then 30 cycles of 94°C/30 s, 68°C/30 s, and 72°C/3 min followed by one cycle of 72°C/5 min. The PCR products were sequenced and analyzed using Vector-NTI program and BLASTx was performed using NCBI website (http://www.ncbi.nlm.nih.gov/blast/) on the GenBank database.

2.1.3. Analysis of TLR9 cDNA Sequences. The full-length cDNAs of RCTLR9B were assembled using vector NTI program. The deduced amino acid sequence, molecular weight (kDa), pI, and protein analysis were conducted by the ExPASy proteomic tool (http://www.expasy.org/tools/). The protein domains were predicted using SMART software (http://smart.embl-heidelberg.de/). Homologous sequences were searched using BLAST program available at the NCBI website with default settings on the GenBank database. The multiple sequence alignment was created using the CLUSTALW (http://www.ebi.ac.uk/clustalw2/).

2.2. CpG ODNs. CpG ODNs were purchased from Bioneer (Korea). The ODNs were phosphorothioate modified

Table 1: Primer name, sequence, product size, and target gene details used in this study.

(a) Primers used for cloning of TLR9 isoform B

Primer name	Primer sequence (5′-3′)	Application
TLR9F1[a]	ATCTCAGCCACAATCAGATC	Partial cloning
TLR9R2[a]	AGTTTGGGAAACATCTCATC	
TLR9B1 RacF[b]	CTGTGAATACCCTGAGTCTCAACAGGG	RACE
TLR9B RacR[b]	GTGATGCTGTGAATCAGTACCAGCC	
Universal primer mix (UPM)	Long 0.2 μM CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT Short 0.4 μM-CTAATACGACTCACTATAGGGC	
Nested universal primer mix (NUP)	AAGCAGTGGTATCAACGCAGAGT	
TLR9B1-NGSP1	GCCTTTATGTCCTGCCCAAAGCACC	
TLR9B-NGSP2	CTTCCTCGTCTGTTCCATCTTGGCTGT	

(b) Primers used for gene expression studies

Primer name	Primer sequence (5′-3′)	Product size (bp)	Target gene	Gene bank accession number	Application
B3-5[c]	ACAGACTGTTCCTCCTCCCC	532	3′UTR	HM754627	cDNA quality
B3-2[c]	GAAACCTCCAACAGCGGG				
RAF	AAGGACCTGTACGCCAACA	330	β-Actin-3	HM754627	
RB1	TGGCGTCTCGCATCGTTT				
TLR9A-RC F	TCTGTTCCATCTTGGCTGTG	160	TLR9A	KC180322	qRT-PCR
TLR9A-RC R	CTGGTTTCTGGTGTCAAACA				
TLR9B-RC F	GCCTTCCTCGTCTGTTCCAT	178	TLR9B	JX073035.1	
TLR9B-RC R	ACAGCCTGGTTTCTGGTGTC				
MyD88-F1Q	GAGGTGTAAGAGGATGGTGGT	183	MyD88	KF018033.1*	
MyD88-R2Q	GGTGGGGAATGGCTTTGTCAT				
RCIL-1βF1	CAGGCAGAACAACCACTGAC	170	IL-1β	AY641829	
RCIL-1βR2	TTCCAAGTCCAGTCCTTTGG				
RCMxF1	TGGACATAGCAACCACAGAG	157	Mx	AY834185	
RCMxR2	TTCTTCAGGTGGATGACCTC				
RCIgM-F1	AGACAGCCTGCAGGGAAAAG	182	IgM	JX025102	
RCIgM-R2	TGTTCCTTTCCCCCAGTAGT				
CC1eF	ATTACAATAAGAACCCTGTGC	185	CC	JF975593	
CC1eR	TCTTTCCTGGGATGGATTTG				
β-Actin RC-F	ACAGACTGTTCCTCCTCCCC	160	β-Actin-3	HM754627	
β-Actin RC-R	AAAATCCTGAGTCAAGCGCC				

[a]Partial sequence primers.
[b]Touchdown primers for 5′ and 3′ designed from TLR9 isoform B partial sequence.
[c]Primers designed from 3′UTR of cobia β-actin-3.
*Partial sequence cloned in this study.

throughout the sequence. Sequences of CpG ODNs are as follows:

B-Class CpG 1668 T∗**C**∗**G**∗T∗**C**∗**G**∗T∗T∗T∗T∗G ∗T∗**C**∗**G**∗T∗T∗T∗T∗G∗C∗T∗G,

C-Class CpG 2395 T∗**C**∗**G**∗T∗**C**∗**G**∗T∗T∗T∗T∗**C** ∗**G**∗G∗**C**∗**G**∗**C**∗**G**∗**C**∗**G**∗C∗**C**∗**G** and 2137 (control CpG with phosphorothioate modified inverted),

T∗**G**∗**C**∗T∗**G**∗**C**∗T∗T∗T∗T∗G∗T∗**G**∗**C**∗T∗T∗T∗ T∗G∗T∗**G**∗**C**∗T∗T (phosphorothioate modifications are marked with ∗ and CG and GCs are in bold).

The CpG ODNs were suspended in phosphate buffer saline (PBS, pH 7.2) and stored at −20°C until used.

2.3. Experimental Design and Fish Sampling. Cobia fish (approximately 20 g body weight) were procured from a local farm and they were maintained in recirculatory aerated tanks for acclimatization up to one week. During acclimatization, they were fed with a commercial diet and proper water quality (water level 200 L, temperature 28°C, and salinity 30 ppt) was maintained. Fish were randomly divided into four groups and each tank was stocked with ten fish (25 g). Fish were intraperitoneally (i.p.) injected with 0.5 mL PBS for

the control group and 0.5 mL PBS containing 10 μg unmethylated CpG ODN 1668, 2395, and 2137, respectively. At each time interval (1, 3, 6, and 10 days after stimulation), two fish were sampled and the posterior part of the intestine was hygienically dissected and immersed in PBS (pH 7.2). After the mucus and digested feed inside the intestine were completely removed, the samples were immediately immersed in TRIzol reagent (Invitrogen, USA). Total RNA isolation of the intestine was carried out according to manufacturer's instruction with minor modifications. The RNA pellet was dissolved in Rnase free water (Qiagen) and preserved in −80°C until used.

2.4. Protection Efficiency of Intraperitoneal Administration of CpG ODNs against Bacterial Challenge in Cobia. Cobia fish (20–25 g) were divided into five groups ($n = 10$ each) and acclimatized for three days. During acclimatization, fish were fed with a commercial feed daily. After three days, cobia were injected intraperitoneally (i.p) with 0.5 mL of PBS and 0.5 mL PBS containing 10 μg CpG ODNs 1668, 2395, and 2137. After 48 h, cobia were challenged intraperitoneally (i.p) with live *P. damselae* subsp. *piscicida* bacteria (LD_{50}—3.25×10^6 CFU/mL). Thereafter, the water quality was properly maintained by vigorous aeration and by monitoring constant water temperature and salinity (28°C and 30 ppt). The behavior and mortality after challenge were recorded daily in individual group.

2.5. The Adjuvanticity of CpG ODNs

2.5.1. Preparation of Bacterial Antigens. Bacteria were prepared from the frozen stock using the following conditions: *P. damselae* subsp. *piscicida* (BCRC 9714) was cultured in 5 mL brain heart infusion (BHI) broth containing 2% NaCl at 28°C/overnight and then 1 mL broth of the stock was transferred to 100 mL broth and grown until the O.D reaches 1.0 (600 nm). The bacteria were harvested by centrifugation (6000 ×g) at 4°C for 5 min. The pellet was washed twice in PBS (pH 7.2) and the bacterial suspension was inactivated by adding formalin to a final concentration of 3% and incubated overnight at 4°C. The inactivated bacterial solution was centrifuged at 6000 ×g for 10 min and thoroughly washed 3 times to remove formalin residues. The inactivation of FKB was confirmed by plating 100 μL of solution on BHI + 2% NaCl plates and incubated at 28°C overnight.

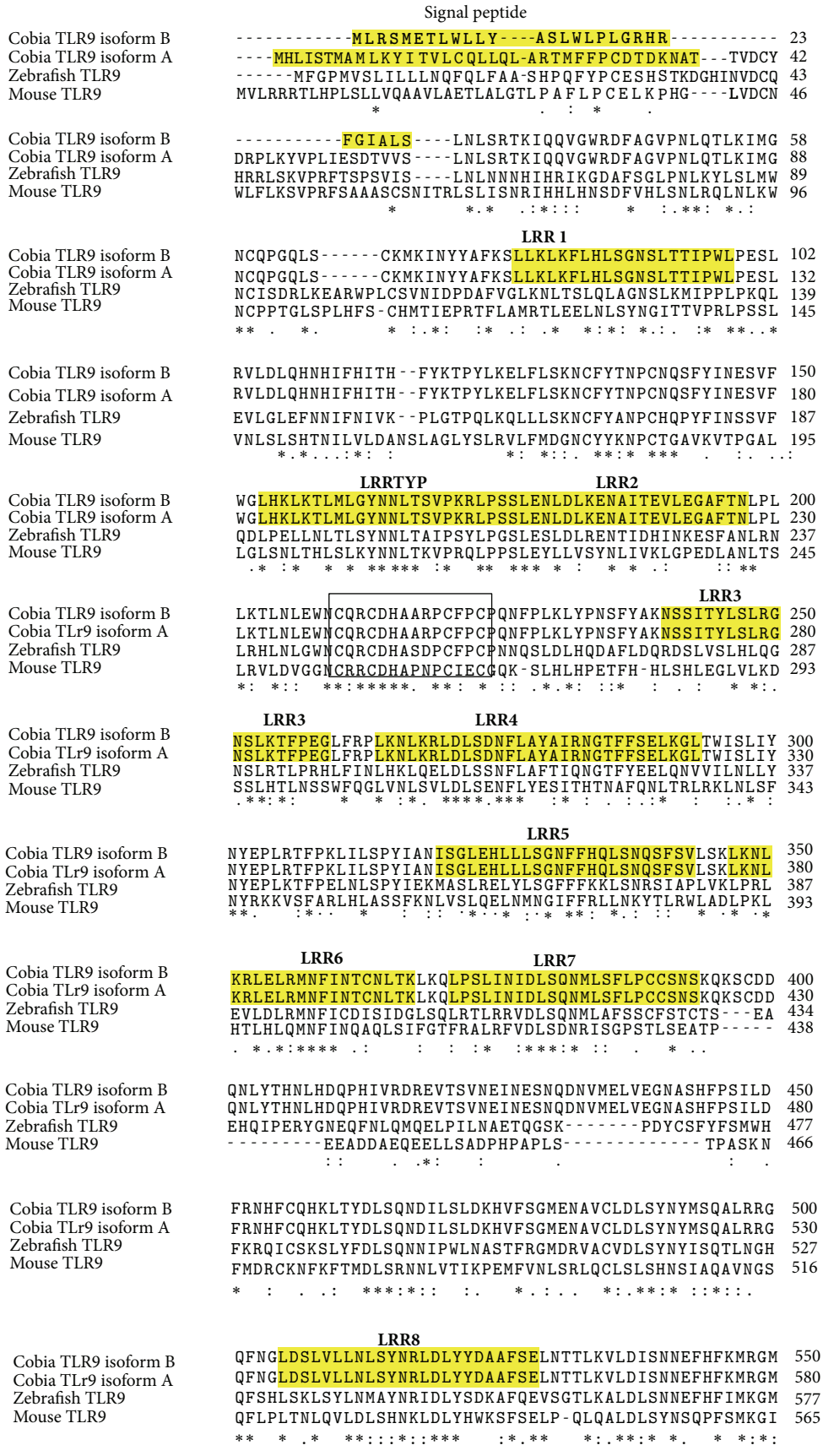
2.5.2. Formulation of CpG ODNs with FKB and Alum. Each dose (100 μL) of vaccine contained 10 μg of CpG ODNs (1668, 2395, or the control 2137) in 10 μL PBS formulated with 45 μL of 2% alum (Alhydrogel, Invivogen) and 45 μL of FKB. Formulations were mixed at room temperature on a shaker at approximately 30 rpm for 30 min and stored at 4°C until used.

2.5.3. Immunization and Sampling. All cobia were procured from a local fish farm in Pingtung, Taiwan, and were acclimatized as previously described. After acclimatization, twenty fish per tank were stocked into four different groups. The first group was immunized intraperitoneally (i.p.) with 100 μL PBS. The second group was immunized with 100 μL/fish vaccine containing 10 μg/10 μL of 1668 CpG ODNs plus 45 μL FKB. The third (FKB + alum + CpG ODN 2395) and fourth groups (FKB + alum + 2137) were immunized with 45 μL of 2% alum designated as FKB + alum + CpG ODN 1668. All formulations were injected in a total volume of 100 μL/fish. After injection, the behavior of fish was observed daily and the optimal conditions were maintained. For gene expression studies, the posterior intestine was sampled for total RNA isolation at 1, 3, 6, and 10 days after injection (dpi) as described in Section 2.3.

2.5.4. Histopathology. Two fish from each group were sacrificed at 3, 6, and 10 dpi, respectively, for histopathological examination. Tissue samples from the posterior part of the intestine were fixed in 10% neutral buffered formalin for standard procedures of light microscopy. Samples were processed, paraffin-embedded (Tissue-Tek TEC), and cut into 4-5 μm sections using standard microtome (Leica RM2235) before being stained with hematoxylin and eosin (Tissue-Tek DRS). Stained slides were examined (Olympus) for signs of inflammation, dilation of lamina propria, and necrosis of epithelial cells using a double blind design. Slides were recoded to avoid observation bias before they were sent to the pathologist for further examination.

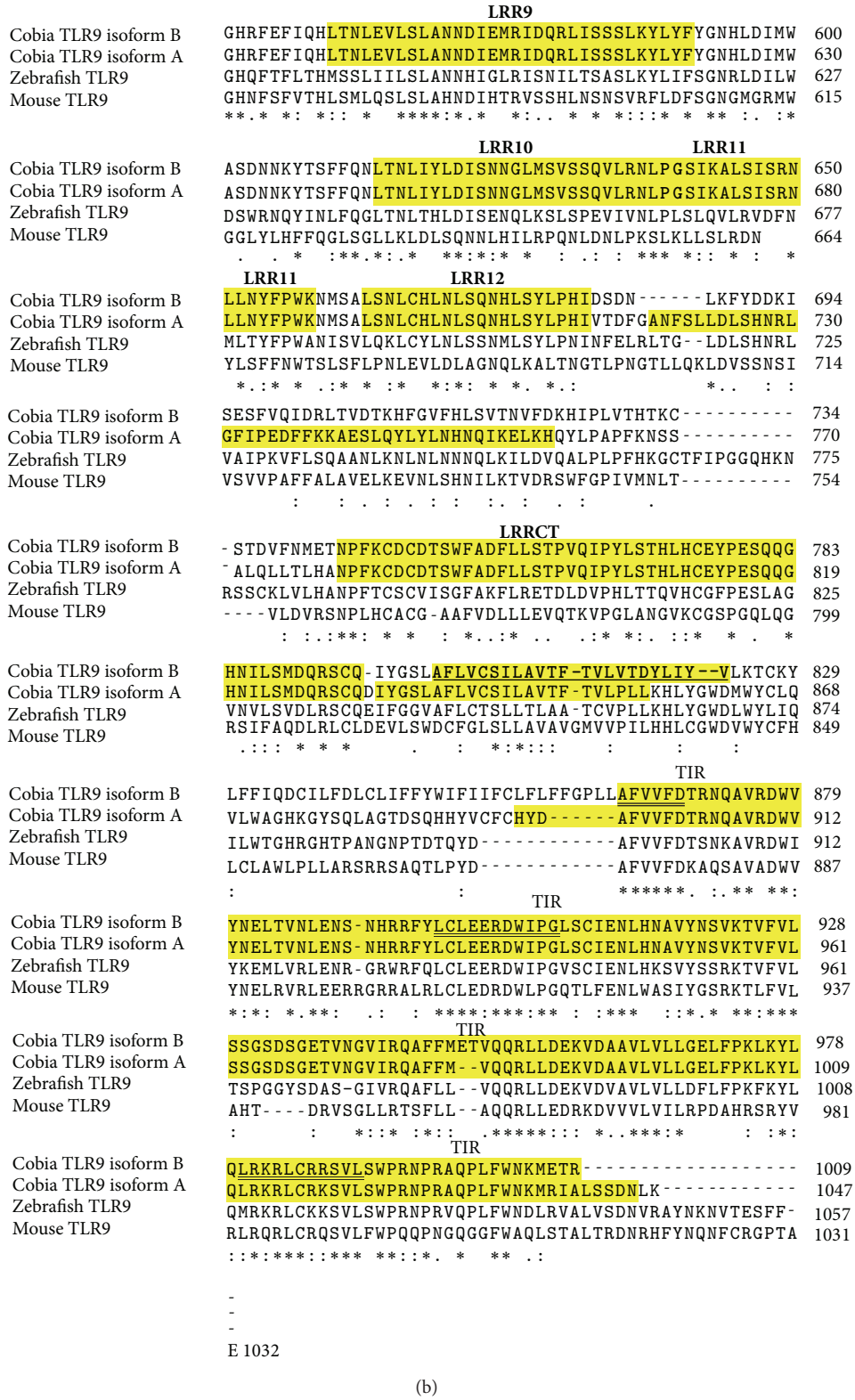
The sign of inflammation was scored from 0 to 3 (0 = normal, 1 = mild, 2 = moderate, and 3 = severe) [35]. Dilation of lamina propria, necrosis of epithelial cells, atrophy, deposits, serosa necrosis, submucosal necrosis, hypertrophy, and hyperplasia were also evaluated based on the histopathological changes as (−) no histopathology, (+) mild histopathology, (++) moderate histopathology, and (+++) severe histopathology.

2.6. cDNA Synthesis and Quantitative Gene Expression. The quality of total RNA was analyzed by a spectrophotometer using 260/280 nm UV. First strand cDNA was synthesized by reverse transcriptase on 2 μg of total RNA using MULV reverse transcriptase enzyme (Lucigen, USA) with an oligo-dT primer. All the cDNA samples were stored at −20°C until used. The genomic contamination and quality of cDNA synthesis were determined by using PCR-amplification by designing primers on intron-exon flanking region and 3′UTR region of cobia β-actin3 (HM754627). The expression levels of TLR9 A and B (KC180322 and JX073035), MyD88 (KF018033), IL-1β (AY641829), Mx (AY834185), IgM (JX025102), and CC-chemokine (JF975593) were analyzed by gene specific primers (Table 1). Real-time PCR was carried out in an ABI 7500 real-time detection system (Applied Biosystems, USA). The amplification was performed in a total volume of 10 μL, containing 5 μL of SYBR green I real-time PCR master Mix (Kapa Biosystems), 1 μL of cDNA, 0.2 μL of each primer, and 3.6 μL of DEPC water. The real-time PCR program was 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. Dissociation and melting curve of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. After amplification, data acquisition



(a)

Figure 1: Continued.



(b)

Figure 1: Amino acid sequence alignment of cobia (*Rachycentron canadum*) TLR9 isoform B deduced protein to cobia TLR9 (*Rachycentron canadum*, AGD79973.2), zebrafish TLR9 (*Danio rerio*, NP_001124066), and mouse TLR9 (*Mus musculus*, NP_112455.2). **LRR**, **LRRTYP** , and **LRRCT** domains are indicated with bold letters shaded with yellow accent; transmembrane domains are bold and underlined. CXXC motifs are indicated by black box; highly conserved motifs in TIR domains are double underlined. - - - - Sequence gaps, ∗ identical residues,**:** conserved substitution, and**.** semiconserved substitution.

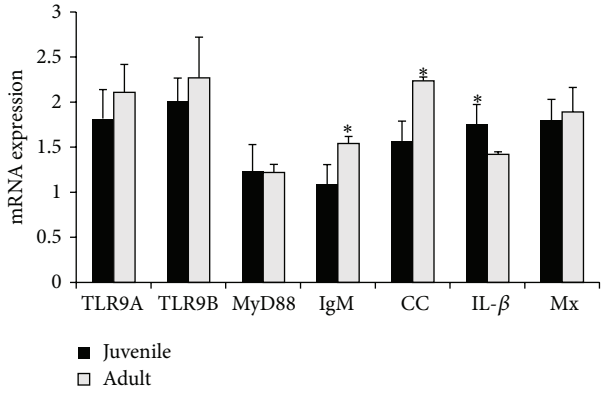


Figure 2: Quantitative analyses of toll-like receptor 9 and immune-related gene expression in intestine of juvenile and adult. The relative expression variance is showed as ratio (the amount of gene mRNA expression normalized to the corresponding beta actin values). Data are shown as mean ± SD ($n = 2$). The significant difference is indicted with asterisk (∗) in the figure.

and analysis were conducted using the sequence detection software (SDS version 2.1. Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was chosen as the calculation method [36]. The difference between the cycle threshold (Ct) value of the target gene and the reference gene (β-actin) called ΔCT was calculated. ΔΔCT = (ΔCT of target gene or PBS-injected group for the target gene at each time point) − (ΔCT of the initial control).

2.7. Statistical Analysis. Data were analyzed using the SPSS16 (SPSS Inc., Chicago IL). Data distribution was determined using descriptive statistics. Differences in the means among the treatments were analyzed using ANOVA and compared using post hoc multiple comparison using Duncan's multiple range test. A P value of <0.05 was considered significant.

3. Results

3.1. Cloning of Cobia TLR9 Isoform B. The full length of RCTLR9B (KF963251) was 3113 bp encoding 1009 amino acid residues. The RCTLR9B protein is homologous to *Rachycentron canadum* isoform A (AGD79973), *Larimichthys crocea* isoform A (ACF60624), *Epinephelus coioides* isoform A (ACV04893), and *Dentex tumifrons* (ABY79218) at 89%, 66%, 65%, and 64%, respectively. A signal peptide located at the 1–29 position of amino acid sequence was identified. In addition, 12 leucine repeats (LRR), LRRTYP (typical), LRRCT (LRR C-terminal), and a 244 amino acid Toll-interleukin-1 receptor (TIR) domain in RCTLR9B were observed. The CXXC motif involved in ligand binding was identified on the LRR motif region at 209–223 amino acid segment containing two conserved motifs separated by six amino acid residues (Figure 1, indicted by a box). Three subsections of TIR domain known to be important in signaling and receptor localization were conserved in RCTLR9A, zebrafish, and mouse TLR9 (Figure 1).

3.2. Immune Genes Expression in Juvenile and Adult Intestine. The mRNA expressions of TLR9 isoform A and B, MyD88, IL-1β, IgM, chemokine, and Mx were examined in juvenile and adult cobia (Figure 2). The expressions of IgM and chemokine genes in the adult are significantly ($P < 0.05$) higher than those in the juvenile. The level of IL-1β was higher in the juvenile when compared to that in the adult. The expressions of TLR9 A and B isoforms, MyD88, and Mx were not significantly different between the juvenile and the adult.

3.3. The Expression of Immune Genes in the Intestine after the Stimulation of CpG ODNs

3.3.1. TLR 9 A. After CpG ODN 1668 injection, RCTLR9A transcript increased significantly ($P < 0.05$) after 3 dpi, decreased to the lowest level at 6 dpi, and subsequently increased at 10 dpi (Figure 3(a)). While after CpG ODN 2395 injection, the expression bottomed after 3 dpi and sharply peaked at 6 dpi. Among different ODNs, CpG ODN 2395 resulted in the highest fold change of RCTLR9A mRNA in comparison with those of CpG ODN 1668 and CpG ODN 2137.

3.3.2. TLR 9 B. No significant difference of expression was shown in CpG ODN 1668 ODN treated group at 1 dpi (Figure 3(b)). The expression peaked at 6 dpi and later reduced to the lowest. However, the level of RCTLR9B with CpG ODN 2395 stimulation was found to be significantly higher at 6 dpi.

3.3.3. MyD88 Expression. After injection with CpG ODN 1668, the level of MyD88 transcript increased after 1 dpi and peaked at 3 dpi and then reduced to the lowest at 6 dpi and slightly increased at 10 dpi. Significant differences were found at 3 and 6 dpi (Figure 3(c)). The level of MyD88 transcript after injection with CpG ODN 2395 continually increased after 3 dpi, peaked at 6 dpi, and then sharply bottomed at 10 dpi.

3.3.4. Mx Expression. CpG ODNs 1668 being a class B did not induce high fold change of Mx expression in intestine (Figure 4(a)). However, stimulation with CpG ODNs 2395 has induced significant high expression at 1 dpi and at 3 dpi the expression decreased and later it increased significantly higher at 6 dpi and reduced to the lowest at 10 dpi.

3.3.5. IgM Expression. After CpG ODN 1668 stimulation, IgM transcription peaked at 1 dpi and then decreased at 3 dpi and reached the lowest level at 10 dpi (Figure 4(b)), while the level of IgM transcript with CpG ODN 2395 stimulation increased after 1 dpi and reached the highest level at 3 dpi and sharply reduced at 6 dpi.

3.3.6. Chemokine CC Expression. CpG ODN 1668 has resulted significantly in the increase of chemokine CC and peaked at 3 dpi and then the expression decreased at 6 dpi

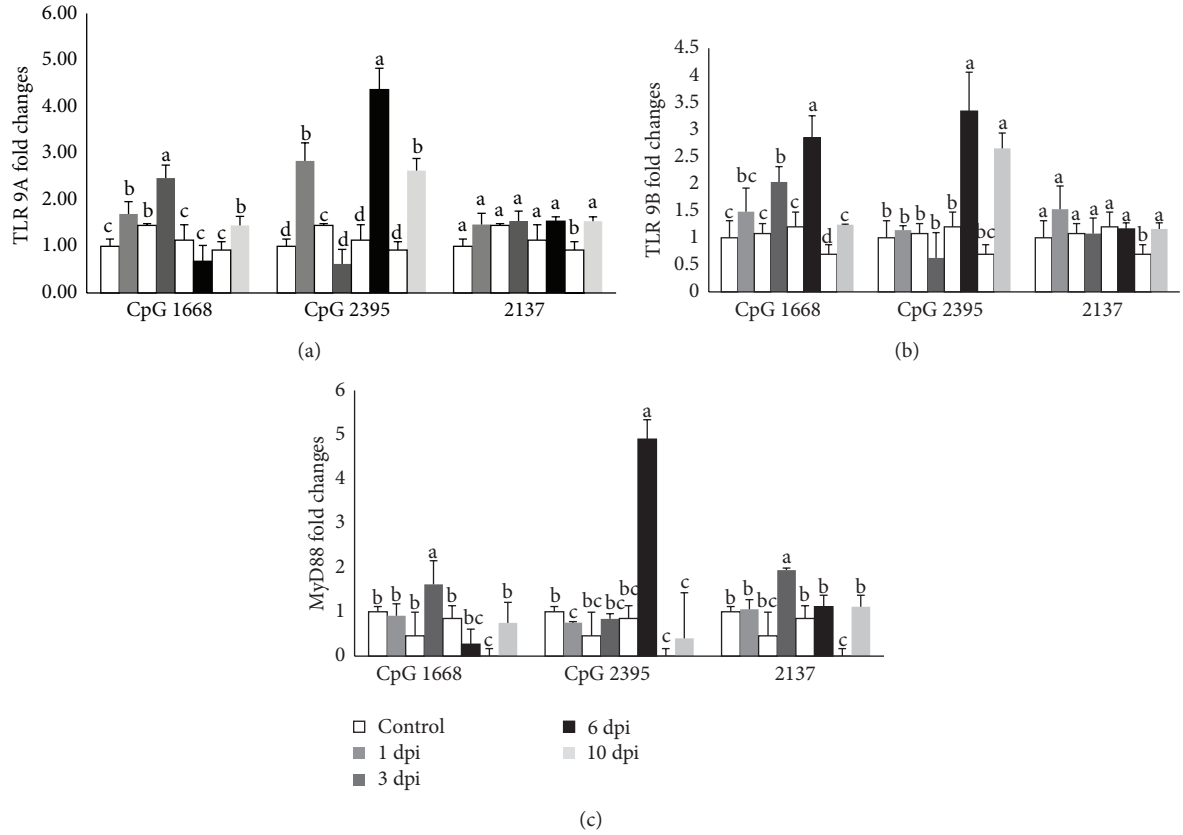


Figure 3: The relative mRNA expressions of TLR 9A (a); TLR 9B (b); and MyD88 (c) in cobia intestine after stimulating with different CpG ODNs (1668, 2395) and ODN 2137 as control measured by quantitative real-tie PCR at 1, 3, 6, and 10 days after stimulation. The expression values represented as "fold change" were compared to the noninjected control samples and β-actin was used as a reference gene. The results are presented as the mean ± SD ($n = 2$) and mean values with different alphabetical letters are significantly different ($P \leq 0.05$).

after injection (Figure 4(c)). The level of chemokine transcript after injection with CpG ODN 2395 bottomed at 3 dpi and reached the highest level at 6 dpi and then dropped at 10 dpi.

3.3.7. IL-1β Expression. Expression profile of IL-1β in intestine with CpG ODNs 1668 resulted in constant increase after 3 dpi and peaked at 6 dpi; later the expression decreased at 10 dpi (Figure 4(d)). However for CpG ODN 2395 stimulation showed the expression bottomed after 3 dpi and peaked significantly higher at 6 dpi and decreased to the lowest at 10 dpi.

3.4. Protection Efficiency of CpG ODNs in Cobia against Bacterial Challenge. Experiment was conducted to determine whether CpG ODN injected cobia can be protected against bacterial challenge. Results revealed that CpG ODN 1668 and 2395 can protect cobia when challenged with *Photobacterium damselae* subsp. *piscicida* (Figure 5). Fish injected with PBs alone and injected with CpG ODN 2137 began to die at 3 days and 5 days after challenge. Fish injected with PBS showed 90% mortality within 10 days. Among the CpG ODN injected groups, the highest mortality was observed in CpG ODN 2137. The highest survival rate was obtained from CpG ODN 1668 (90%) and followed by CpG ODN 2395 (70%).

3.5. The Adjuvanticity of CpG ODNs

3.5.1. TLR 9 Isoform A and B Expressions. Both RCTLR9 A and B expressions increased at 1 dpi (~9 and 8 folds) and decreased at 3 dpi after i.p injection of FKB + alum + CpG 1668 (Figures 6(a) and 6(b)). Thereafter, both isoforms A and B increased at 6 dpi (~38 and 42 folds). Expression of isoform A increased at 10 dpi (~51 folds), in contrast, isoform B decreased (~33 folds) during the same time. Expressions of RCTLR 9A and B after being administrated with FKB + alum + CpG 2395 increased significantly until at 6 dpi (~27 and 26 folds) and then decreased at 10 dpi. In control treatment with FKB + alum + 2137, expression of RCTLR9B increased significantly (~5 folds) until at 6 dpi and then decreased at 10 dpi.

3.5.2. MyD88 Expression. Figure 6(c) showed that after administration the expression of Myd88 was fluctuated both in FKB + alum + CpG 1668 and in FKB + alum + CpG 2395. The expression of MyD88 decreased from 1 dpi to 3 dpi and increased significantly at 6 dpi in both CpG-ODN 1668

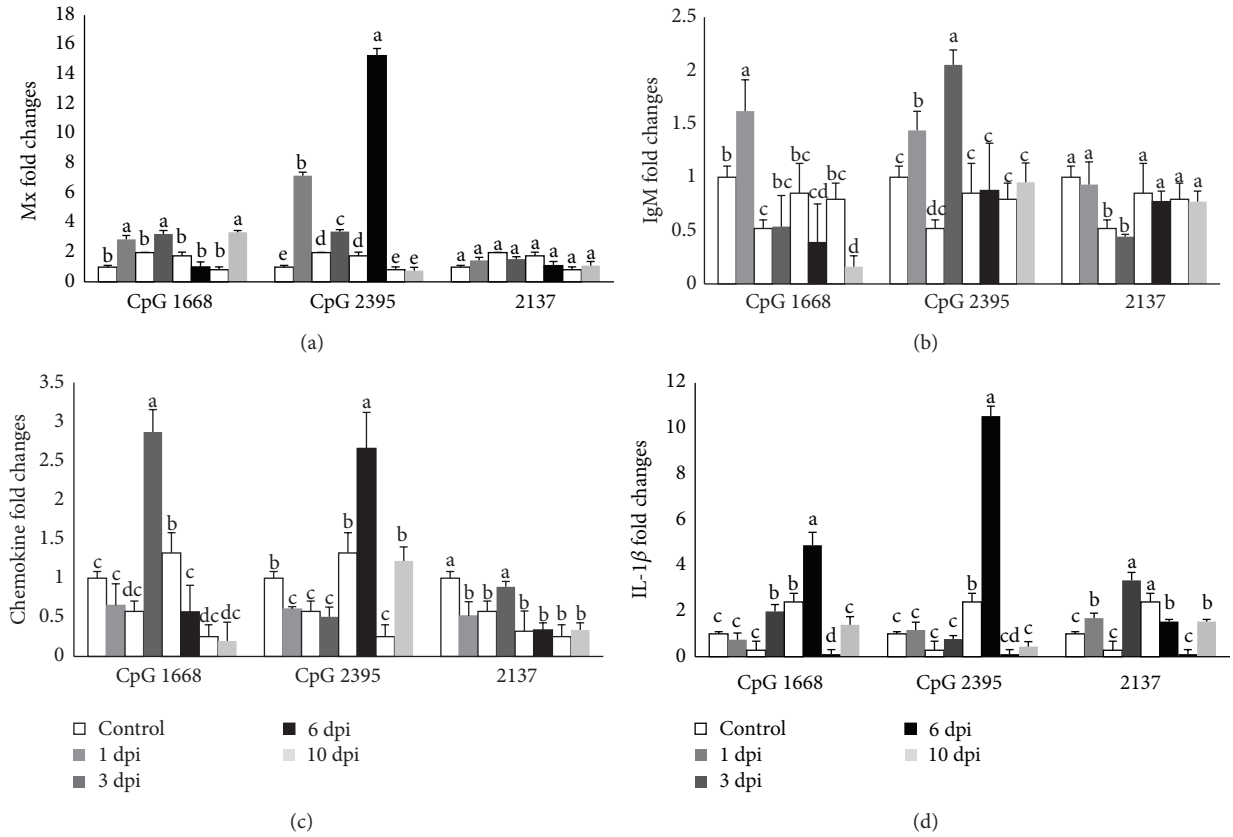


Figure 4: The relative mRNA expressions of Mx (a); IgM (b); CC (c); and interleukin 1-β (d) in cobia intestine after stimulating with different CpG ODNs (1668, 2395) and ODN 2137 as control measured by quantitative real-tie PCR at 1, 3, 6, and 10 days after stimulation. The expression values are represented as "fold change" and compared to the noninjected control samples and β-actin was used as a reference gene. The results are presented as the mean ± SD ($n = 2$) and mean values with different letters are significantly different ($P \leq 0.05$).

and CpG-ODN 2395 (~10 and 22 folds) but then decreased and reached the lowest level (~1 fold) at 10 dpi. In control group FKB + alum + 2137, profile of MyD88 showed fluctuated expression. The expression decreased at 3 dpi, increased at 6 dpi, and bottomed at 10 dpi. FKB + alum + CpG 2395 group showed the highest fold changes compared with the other treatment.

3.5.3. IL-1β Expression. IL-1β expressed low in all three treatments (FKB + alum + CpG 1668; FKB + alum + CpG 2395; and FKB + alum + 2137 treatments) in terms of fold changes. The expression profile of IL-1β in both FKB + alum + CpG 1668 and FKB + alum + CpG 2395 became significantly low after 3 dpi. The expression profile of the control group was higher than that of the treatment group in most of observation time (Figure 6(d)). Only at 6 dpi, treatment group showed an increase in expression. In control 2137 group, the expression profile of IL- 1β showed fluctuation of no significant difference.

3.6. Histological Observation. Normal gross morphology on the intestinal wall of cobia was examined using light microscopy (LM) after hematoxylin and eosin (H and E) staining in all groups. No inflammatory cells were found in the lamina propria of the cobia intestine. Additionally, fish from the control group showed an intestinal epithelium consisting of a single layer of columnar epithelial cells with abundant goblet cells joined by apparently intact junctional complexes (Figures 7 and 8). No cell debris was observed in the gut lumen. A continuous mucus layer was evident over the apical surface of the cells. No signs of damage, edema, or inflammation were observed.

4. Discussion

RCTLR9B has a transmembrane domain, indicating its localization at the endosomal membrane. The CLUSTALW alignment of cobia (RCTLR9B and RCTLR9A), zebrafish, and mouse TLR9 showed high homology according to the amino acid sequence (Figure 1). Motifs in the cDNA sequence have been proposed to bind to the PAMPs of TLR9 [37]. Our results demonstrated that RCTLR9B has CXXC motifs between 209 and 223 amino acids, suggesting its binding nature to the ligand. In human, five isoforms of TLR9 have been reported (TLR9A–E) [14]. Differential localizations of these TLR9 isoforms in various cells types raise the question of functional significance of computational modeling,

structure, and biological relevance a specific isoform may play during inflammation. TLR9 A, C, D, and E are confirmed to be predicted to be located on the ER, but TLR9 B, on the other hand, is located on mitochondria [14]. In teleost, yet only two TLR9 isoforms have been identified in sea bream [10] and croaker [17]. However, the cellular localization of these isoforms in teleost has not been studied.

Both RCTLR9A and RCTLR9B were expressed at a similar level in healthy juvenile and adult cobia. No significant differences in expressions were found between RCTLR9A and RCTLRB or between the juvenile and the adult. This is in agreement with a previous study, in which both TRL9 isoforms were found in the intestine of healthy *Sparus aurata* [10]. However, in juvenile croaker the expression of TLR9B was significantly higher than that of TLR9A [17]. In addition, the expressions of MyD88 and Mx were not significantly different between the juvenile and the adult. Similar levels of expressions were observed in the intestine of healthy rohu [38]. The mRNA expressions of IgM and chemokine CC were significantly higher in adult cobia when compared to juvenile cobia. The basal expressions of CXC and chemokine CC were detected in pig jejunum, caecum, and colon before the treatment of CpG ODNs [24]. The higher expressions of IgM and chemokine CC in adults may indicate the more activated immunity due to the increased exposure to pathogens.

Since the GI tract is the major portal of entry for pathogens [32, 33], it would be meaningful to investigate the expression of genes related to immunity in response to the administration of CpG ODNs in the cobia intestine. The cobia intestinal epithelial cell line is not available; we therefore intraperitoneally injected various CpG ODNs to the fish and acquired intestinal tissues at different time points. Results demonstrated that intraperitoneal stimulation of CpG ODNs had an influence on the expression of genes related to innate immunity in the cobia intestine. The expression of immunity-related genes in the intestine was found to be dependent on the type of CpG ODNs and the time, namely, day after injection (dpi). Fish injected with CpG ODN 2395 had significantly increased expressions of both RCTLR9A and RCTLR9B at 6 dpi, which was reflected on the significantly upregulated expression of MyD88 in comparison with that of fish injected with CpG ODN 1668. A human epithelial cell line, HT-29, was found to spontaneously express TLR9 mRNA and protein, and stimulation with CpG ODNs and bacterial DNA induced the expression of proinflammatory cytokine, including IL-1β and IL-8 [39, 40]. In the present study, the expression of IL-1β remained unchanged at 1 and 3 dpi, peaked at 6 dpi, and returned to the baseline at 10 dpi. The delayed IL-1β response may be due to the following reasons: (1) the activation of TLR9 by CpG ODNs does not always lead to immediate upregulation of an inflammatory cytokine and (2) the presence of TLR9 receptor-ligand interactions leads to intestinal homeostasis which results in the delayed response of inflammatory cytokines [41]. Further investigations would be required to elucidate the relationship between TLR9 and elicited inflammatory responses after the stimulation of CpG ODNs in the cobia intestine.

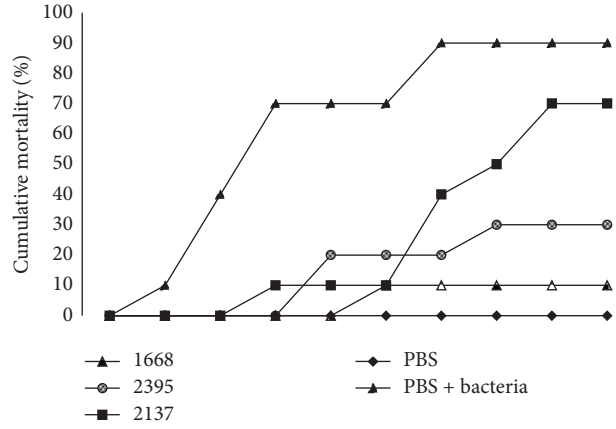


Figure 5: Cumulative percent mortalities of fish injected intraperitoneally (i.p) with 100 μL PBS alone, PBS alone with bacterial challenge, CpG ODN 1668, 2395, and control ODN 2137. After 48 h after CpG ODN injection, cobia was i.p. injected with 50 μL of 3.25×10^6 bacteria (*Photobacterium damselae* subsp. *piscicida*). The group injected with ODN 2395 and 1668 displayed lower mortalities than any other group, $n = 10$ fish per group.

In response to the stimulation of CpG ODNs, the activated T helper cells draft immunocompetent cells into the intestinal mucosa and influence the chemokines secreted by immune cells, such as macrophages, activated NK cells, and T cells, which determine the subsequent Th1/Th2 immune responses [42, 43]. Intraperitoneal injection of CpG ODN 1668 and 2395 significantly increased the mRNA expression of chemokine CC in the cobia intestine at 3 dpi and 6 dpi, respectively, which might be due to the engagement of immune cells with Th1 type property to the intestinal mucosa after the injection of CpG ODNs. In the piglets administered with CpG ODN D19, the percentage of macrophages and dendritic cells, as well as the expression of chemokine CC, in the intestinal tissue was significantly elevated [24].

CpG ODN 1668 is recognized as a potential immunostimulant for Atlantic salmon, common carp, and Japanese flounder [44–46]. In this study, a CpG ODN other than CpG ODN 1668 and CpG ODN 2395 (a class C CpG ODN) also showed protective effects and significantly increased the survival rate of cobia challenged with live *P. damsela*e subsp. *piscicida*. Similar results found that olive flounder injected with CpG ODN 1668 and 2395 had the highest survival rate against *M. avidus* and *E. tarda* infection, respectively [47, 48]. In the mouse model, administration of CpG ODNs alone has been demonstrated to increase the resistance to listeriosis [49] and *Helicobacter pylori* infection [50]. Oral administration of CpG ODNs protected (90%) newborn mice from *Cryptosporidium parvum* enteric infection [23, 51]. It is difficult to accurately analyze the linkage between the cytokine level and survival rate in aquatic species because only very limited information is available in the literatures. In the present study, the expressions of chemokine CC and IL-1β were significantly increased in cobia injected with CpG ODNs. Whether the significantly increased survival rate in cobia injected with CpG ODN 1668 and 2395 was due to upregulated expression of cytokines needs to be confirmed in

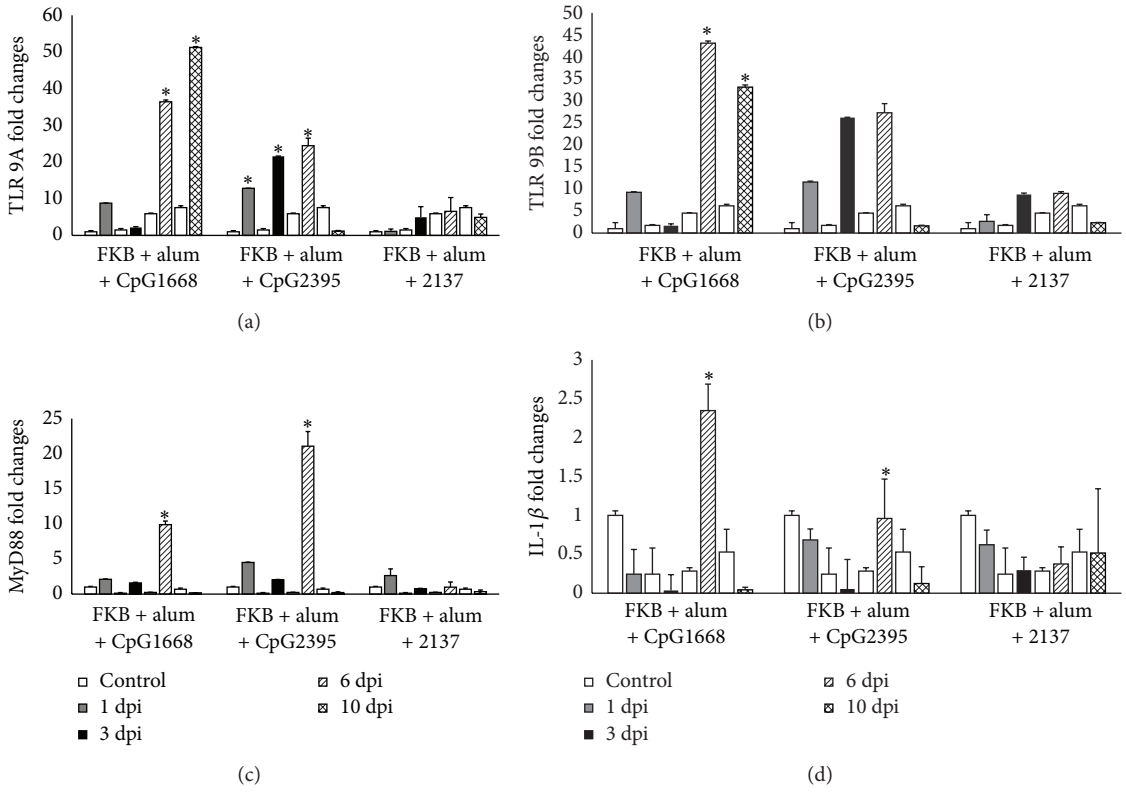


Figure 6: The relative mRNA expressions of (a) TLR9 A, (b) TLR9 B, (c) MyD88, and (d) IL-1β in the posterior cobia intestine stimulated with formalin-killed vaccine mixed with CpG and alum and PBS-injected and measured by quantitative real-time PCR. The sampling was carried out at 1, 3, 6, and 10 days after stimulation and β-actin was used as a reference gene. The results are presented as the mean ± SD ($n = 2$) and mean values with asterisk (∗) are significantly different ($P \leq 0.05$).

the future study. However, it has been reported that excessive activation of the immune system with overwhelming production of proinflammatory cytokines can be harmful leading to microcirculatory dysfunction, tissue damage, shock, or even death in severe cases [52]. Moreover, different types of CpG ODNs can elicit different immune responses, for example, the profile of upregulated genes. CpG ODN 2395, being a C-class CpG ODNs, is intermediate between A-class and B-class CpG ODNs in terms of the immunostimulatory activity [53]. The presence of CpG motifs at the 5′-end of C-Class ODN is necessary for inducing strong interferon (IFN)-α production [54]. In this region of the CpG ODNs, the required sequence appeared to be similar to that of the B-class CpG ODNs, where the presence of a 5′-TCG is very critical to the immunostimulatory effects [55].

The effect of CpG ODNs on the adaptive immunity of cobia was also explored in the present study. Formulation of CpG ODNs as the adjuvant to enhance the immunogenicity of vaccines has been extensively studied in mice, rabbits, and cattle [56–59]. The combination of FKB, alum, and CpG ODNs (either 1668 or 2395) significantly enhanced the expressions of RCTLR9A and RCTLR9B when compared to the control ODN 2137. Presumably, CpG ODNs initiated the signal transduction in endosomes where TLR9 is located [60–62]. It is noteworthy that the increased expression of TLR9 (~50 folds) in cobia injected with CpG ODN 1688 formulated with alum and FKB was dramatically higher than that of cobia injected with CpG ODN 1688 (~3 folds) alone. This indicated that alum and bacterial antigen (FKB) stimulated the immune responses to upregulate the expression of TLR9, which may facilitate the uptake of the CpG ODNs. CpG ODNs and alum worked synergistically to enhance immune-potentiating and antigen-sparing effects of a vaccine against swine influenza virus [63], which was not observed when CpG ODNs or alum was used alone. The increased expressions (~5 folds) of RCTLR9A and RCTLR9B in cobia injected with the control ODN 213 formulated with alum and FKB may be attributed to the immunostimulatory effects of alum and FKB. In addition, ODNs on a phosphorothioate backbone without CpG motifs have been shown to nonspecifically stimulate TLR9-dependent or -independent activation [64, 65]. The increased expression of MyD88 at 6 dpi implied that the Myd88-dependent signaling pathway of TLR9 was activated in response to CpG ODNs, because this was not seen in cobia injected with the control ODN 2137. The expression of IL-1β, a proinflammatory cytokine, was significantly upregulated at 6 dpi in cobia injected with the CpG ODNs, but not the control ODN, with alum and FKB. In a vaccination study on Japanese flounders, the transcription of IL-1β was significantly increased in both vaccinated and nonvaccinated groups at 1 dpi indicating that the handling and injecting procedure during vaccination may induce inflammatory

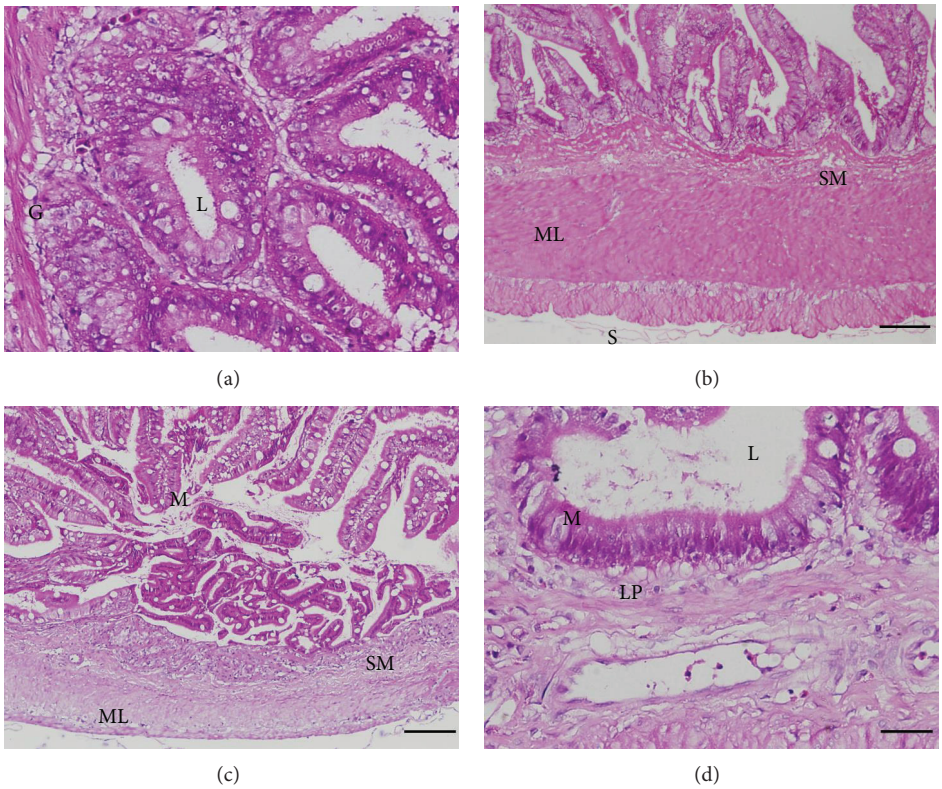


Figure 7: Histopathological observations in posterior part of cobia intestine injected with control PBS (i) and stimulation of cobia mixed with FKB, alum and 2137 (ii), 2395 (iii), and 1668 (iv) at 6 days after stimulation. Letters in the figure are denoted as M: mucosa epithelium, L: lumen, ML: muscular layer, SM: submucosa, LP: lamina propria, S: serosa, and G: goblet cells.

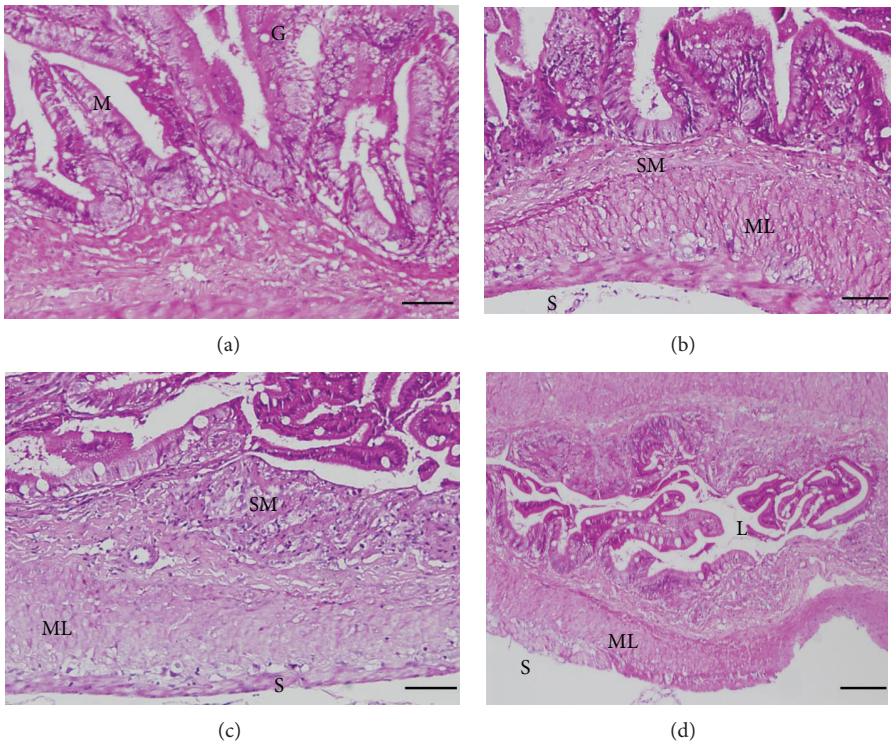


Figure 8: Histopathological observations in posterior part of cobia intestine injected with control PBS (i) and stimulation of cobia mixed with FKB, alum and 2137 (ii), 2395 (iii), and 1668 (iv) at 10 days after stimulation. Letters in the figure are denoted as M: mucosa epithelium, L: lumen, ML: muscular layer, SM: submucosa, LP: lamina propria, S: serosa, and G: goblet cells.

responses temporally [66]. Although IL-1β has been indicated to upregulate during the course of infections in many mammals, the expression of IL-1β in the posterior distal intestine remained unchanged in rainbow trout infected with *A. salmonicida* [67]. In addition, the expression of IL-1β may be affected by the amount of antigens present in the posterior intestine of Atlantic cod [68].

Fish vaccines formulated with alum salts are generally accepted due to their safety and satisfactory immunostimulatory effects [69]. In olive flounders, the dosage at 500 μg alum alone per fish only induced very mild inflammation without abnormal histopathological changes [70]. When the dosage was increased to 1600 μg per fish, severe inflammation and mortality were observed. In the present study, only 45 μg alum was used in our vaccines. Moreover, addition of 10 μg CpG ODNs to the vaccine formulation did not cause undesired side effects, such as lamina propria dilation and epithelial cell necrosis, at the site of injection as indicated by the histological observation.

5. Conclusion

The cobia TLR9B gene was cloned and ligand binding region CXXC motifs were found on the cobia TLR9B protein. Since TLR9 is the cellular receptor for CpG ODNs, the immunostimulatory effects of CpG ODNs in the intestine were investigated by intraperitoneal injection of various CpG ODNs to cobia fish. Results revealed that the expressions of RCTLR9 and proinflammatory chemokine genes were upregulated and were dependent on the type of CpG ODNs used. The CpG ODNs injected cobia also had significantly increased survival rates after challenge with live *P. damsela*e subsp. *Piscicida*. Finally, the adjuvanticity of CpG ODNs was examined by formulating CpG ODNs as the adjuvant in a vaccine for cobia. The expressions of cobia TLR9, MyD88, and IL-1β were significantly elevated in cobia injected with CpG ODNs formulated with alum and FKB. No signs of tissue damage and overwhelming inflammatory responses were observed at the site of injection. Application of CpG ODNs may be used to increase the disease resistance and efficacy of vaccines in cobia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Generation of Recombinant Schmallenberg Virus Nucleocapsid Protein in Yeast and Development of Virus-Specific Monoclonal Antibodies

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Schmallenberg virus (SBV), discovered in continental Europe in late 2011, causes mild clinical signs in adult ruminants, including diarrhoea and reduced milk yield. However, fetal infection can lead to severe malformation in newborn offspring. To develop improved reagents for SBV serology, a high-level yeast expression system was employed to produce recombinant SBV nucleocapsid (N) protein. Recombinant SBV N protein was investigated as an antigen in SBV-specific IgG enzyme immunoassay and used for generation of monoclonal antibodies (MAbs). Yeast-expressed SBV N protein was reactive with anti-SBV IgG-positive cow serum specimens collected from different farms of Lithuania. After immunization of mice with recombinant SBV N protein, four MAbs were generated. The MAbs raised against recombinant SBV N protein reacted with native viral nucleocapsids in SBV-infected BHK cells by immunofluorescence assay. The reactivity of recombinant N protein with SBV-positive cow serum specimens and the ability of the MAbs to recognize virus-infected cells confirm the antigenic similarity between yeast-expressed SBV N protein and native viral nucleocapsids. Our study demonstrates that yeast expression system is suitable for high-level production of recombinant SBV N protein and provides the first evidence on the presence of SBV-specific antibodies in cow serum specimens collected in Lithuania.

1. Introduction

In 2011, an unidentified disease in cattle was first reported in Germany in a farm near the town of Schmallenberg [1]. Metagenomic analysis identified a novel *Orthobunyavirus*, which subsequently was isolated from blood specimens of infected animals. This new virus was called Schmallenberg virus (SBV) after the place of origin of the collected samples. Clinical symptoms of diseased cows include fever, reduced milk yield, and diarrhoea. Also, SBV infection has been implicated in many cases of severely malformed bovine and ovine offspring [2–7]. The inoculation of 9-month-old calves with blood of cattle that were RT-qPCR positive for SBV caused fever and mucous diarrhoea, providing experimental evidence that SBV might be responsible for the clinical signs observed [1]. Analysis of viral genomic sequences has led to the classification of SBV in the Bunyaviridae family and the *Orthobunyavirus* genus. Recent analysis revealed that SBV is most related to Douglas and Sathuperi virus belonging to the Simbu serogroup of *Orthobunyavirus* genus [8]. The majority of bunyaviruses are transmitted by arthropod vectors. Epidemiological data existing so far are in accordance with the hypothesis that SBV is transmitted by biting midges (*Culicoides* spp.). Recently, some studies have

reported the presence of the SBV genome in different species of *Culicoides* collected in different countries of Europe. It has been reported that some *Culicoides* species are present inside farm buildings during the winter and are able to complete their life cycle in animal enclosures. It is possible that SBV is able to persist from year to year in the vector population despite winter temperatures as described in reviews [6, 7]. The qRT-PCR is the primary diagnostic assay used by laboratories in affected countries [1]. This assay has limitations in detecting infected individuals based on blood samples, as it only detects viral RNA when the animal is viraemic [9]. Furthermore, the virus can be isolated on insect and hamster cell lines. For the detection of SBV-specific antibodies, indirect immunofluorescence tests, microneutralization tests, and commercial SBV-based indirect ELISA have been used [9–12].

The genetic structure of SBV is typical for Bunyaviridae, containing a tripartite RNA genome of negative polarity. The genome of SBV contains three segments of single-stranded negative-sense RNA called the large (L), medium (M), and small (S) segments. The L segment encodes the RNA-dependent RNA polymerase; M segment encodes surface glycoproteins Gn and Gc and nonstructural protein NSm. The S segment encodes nucleocapsid protein N and nonstructural protein NSs [13]. The S segment of SBV was shown to share 96.7% nucleotide sequence similarity with S segment of Shamonda virus. Comparably, the similarity between SBV and Sathuperi virus S segment nucleotide sequence is 94% [14].

The N protein of bunyaviruses is the most abundant viral antigen present in the virion and in the infected cells, thus making it an excellent target for serology [15–17]. Recombinant N proteins of different hantaviruses, generated in *Escherichia coli,* insect cells, or yeast have been widely used for serological diagnosis of hantavirus-specific antibodies in human sera and oral fluid [18–22].

The synthesis of N proteins of different European, Asian, and American hantaviruses in yeast expression system has been shown to result in large yields. The proteins were highly pure after nickel chelation purification and during stable long-term storage. Moreover, the recombinant hantavirus N proteins were strongly immunogenic in rabbits and mice. The yeast-expressed N proteins of different hantaviruses have been employed to develop highly sensitive and specific ELISAs and immunoblot tests [17–22]. Initial studies based on *E. coli* expression systems for hantavirus diagnostics have demonstrated lower specificities of these tests due to *E. coli* contaminants remaining in recombinant protein preparation [23, 24]. These problems were eliminated using yeast expression system [17–22].

Epidemiologic situation in regard to SBV infection may differ greatly from country to country and warrants further study. Indeed, to determine the true occurrence and prevalence of the SBV infection, fast, convenient, and cheap diagnostic tests are needed. In the current study, we have generated the N protein of SBV in yeast expression system, demonstrated its antigenic similarity with viral N protein, and developed N protein-specific MAbs reactive with SBV in infected cells.

2. Materials and Methods

2.1. Strains, Media, Yeast Transformation, and Cultivation. Recombinant construct containing SBV N gene sequence was amplified in *E. coli* DH5αF′ cells. *Saccharomyces cerevisiae* AH22-214 *MATa* (*leu2 his4 pep4*) was used for expression of SBV N protein. Selection of yeast transformants resistant to formaldehyde was carried out on the YEPD (1% yeast extract, 2% peptone, and 2% dextrose) agar supplemented with 5 mM formaldehyde. *S. cerevisiae* transformants were grown in YEPD medium supplemented with 5 mM formaldehyde or in YEPG induction medium (1% yeast extract, 2% peptone, and 2,5% galactose) as described previously [25].

2.2. Cloning of SBV N Protein-Encoding Sequences into Yeast Vectors and Purification of Recombinant N Protein from Transformed Yeast. All DNA manipulations were performed according to standard procedures [26]. Enzymes, molecular mass standards, and kits for DNA manipulations were purchased from Thermo Fisher Scientific Baltics (Vilnius, Lithuania). SBV N gene was chemically synthesized by GenScript USA Inc. (Piscataway, NJ, USA) according to the published sequence GenBank accession number HE649914.1 [1]. The XmaJI sites compatible with XbaI site for cloning into yeast expression vectors were inserted into the ends of SBV N gene during gene synthesis. For the generation of N-terminally hexahistidine-tagged SBV N protein, the gene was cloned into XbaI site of the *S. cerevisiae* expression vector FX7-6-His under control of galactose inducible *S. cerevisiae GAL10* promoter described previously [17]. The resulting plasmid pFX7-SBV-6-HisN was used for transformation of yeast *S. cerevisiae* AH22-214, as described previously [25]. The primary structure of the cloned gene was confirmed by sequencing.

Cultivation of transformed yeast cells as well as the expression and purification of recombinant proteins was performed as previously described [17, 25]. Briefly, 100 mL of YEPD growth medium (yeast extract 1%, peptone 2%, and glucose 2%) supplemented with 5 mM formaldehyde was inoculated with the transformed yeast cells and grown with shaking at 30°C for 24 h. After addition of 100 mL of YEPG induction medium (yeast extract 1%, peptone 2%, and galactose 5% supplemented with 5 mM formaldehyde), the yeast cells were grown for additional 17 h. The cells were harvested, washed with distilled water, and frozen at −20°C until further use. Thawed cells were resuspended in 8 mL of disruption buffer (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.5% glycerol, 1% Tween-20, 10 mM imidazole, 2 mM PMSF, and pH 8.0) and 8 g of glass beads (0.5 mm diameter, Sigma-Aldrich Co., St. Louis, MO, USA) was added. Cells were disrupted by vortexing at 4°C for 5 min. The cell debris was sedimented by centrifuging the obtained yeast lysates at 3000 ×g for 5 min. Insoluble proteins were spun down by centrifugation at 10.000 ×g for 10 min at 4°C. The supernatant was mixed with 2 mL of Ni-NTA resin equilibrated in disruption buffer and binding was performed by shaking for 1 h at room temperature (RT). N protein purification was performed on a polypropylene column, according to the manufacturer's recommendations for denaturing purification

of insoluble proteins (Qiagen, Hilden, Germany). The main portion of the protein was eluted in buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, and pH 4.5). To ensure the purity of the eluted recombinant N protein SDS-PAGE, Coomassie Brilliant Blue staining and western blot were performed. Eluted protein was dialysed against sodium acetate buffer (50 mM sodium acetate, 100 mM sodium chloride, and pH 5.0) and stored at −20°C with 40% of glycerol. Yeast transformant containing the plasmid pFX7-6-His without any insert was used as a negative control.

2.3. SDS-PAGE and Western Blot Analysis. Protein samples were boiled in a reducing sample buffer and separated in a SDS-Tris-glycine buffer through polyacrylamide gel electrophoresis (PAGE). Proteins were visualized by staining with Coomassie Brilliant Blue (Sigma-Aldrich Co.). For western blot, purified proteins were electrotransferred to Roti-PVDF membrane (Carl Roth GmbH & Co., Karlsruhe, Germany). The membrane was blocked with RotiBlock (Carl-Roth GmbH & Co.) blocking solution for 2 h at RT and rinsed in PBS with 0.1% Tween-20 (PBST). The membrane was then incubated for 1 h at RT with primary antibodies at working dilution in PBST with 10% RotiBlock and subsequently incubated with goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad, Hercules, CA, USA) 1 : 4000 diluted in PBST with 10% RotiBlock. The enzymatic reaction was developed using 4-chloro-1-naphthol and H_2O_2 (Fluka, Milwaukee, WI, USA). For the analysis of MAb specificity, undiluted hybridoma supernatants were used. To check the purity of recombinant N protein, MAb against 6-His-tag epitope (Thermo Scientific, Rockford, IL, USA) was used as a primary antibody.

2.4. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) for Investigation of SBV N Protein-Specific Mouse Antibodies. Micro test plates (Nerbe Plus GmbH, Winsen/Luhe, Germany) were coated with 100 μL/well of SBV N protein dissolved in the coating buffer (0.05 M sodium carbonate, pH 9.5) to a concentration of 5 μg/mL. The plates were incubated overnight at 4°C. The coated plates were blocked with 250 μL/well of PBS with 2% BSA for 1 h at RT. Then plates were rinsed twice with PBST. Mouse antiserum samples, hybridoma growth medium, or polyclonal antibodies were diluted in PBST, added to the wells (100 μL/well), and incubated for 1 h at RT. The plates were then incubated for 1 h with goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad) 1 : 5000 diluted in PBST. The enzymatic reaction was visualized by the addition of 100 μL of "NeA-Blue" TMB solution (Clinical Science Products Inc., Mansfield, MA, USA) to each well. The reaction was stopped by adding 50 μL/well of 1 M H_2SO_4 solution. The optical density (OD) was measured at 450 nm (reference filter 620 nm) in a microplate reader (Sunrise Tecan, Männedorf, Switzerland). The apparent dissociation constants (K_d) of MAbs were determined by an indirect ELISA. The K_d values were calculated from 4 parallel ELISA titration curves fitting logistic model obtained by incubating plate-coated SBV N protein with increasing amounts of MAbs ranging from 1.9×10^{-13} M to 3.3×10^{-8} M. The SD was determined from these 4 calculated K_d values. The K_d for each MAb was defined as the concentration (M) of the MAb that gives one-half of the maximum OD_{450} value.

2.5. Indirect IgG ELISA for Detection of SBV N Protein-Specific Antibodies in Cow Serum. Cow blood samples were collected in May-June 2013 from cow farms located in different places of Lithuania. The sera were tested for antibodies against SBV using a commercially available ELISA kit (ID Screen Schmallenberg virus Indirect, IDvet, Grabels, France) [11] before testing them with recombinant SBV N protein. In the current study, 102 serum samples were used from this collection for the evaluation of yeast-derived recombinant SBV N protein as an antigen for ELISA. Micro test plates (Nerbe Plus GmbH) were coated with 400 ng per well of recombinant SBV N protein in 100 μL of 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBST and then blocked by the addition of 150 μL of blocking reagent per well (1x Roti-Block, Carl Roth GmbH & Co.). The plates were incubated at RT for 1 hour. After blocking, the plates were washed three times with PBST and 100 μL aliquots of serum specimens, 1 : 200 diluted in PBST buffer with 10% RotiBlock (Carl Roth GmbH & Co.), was added to the wells. Plates were incubated for 1 h at 37°C and washed five times with PBST. 100 μL aliquots of rabbit antibovine IgG (Sigma-Aldrich Co.) conjugated to HRP, 1 : 10.000 (v/v) diluted in PBS with 5% RotiBlock, was added to each well and the plates were incubated for 1 h at 37°C. After washing five times with PBST, 100 μL of TMB substrate (Clinical Science Products) was added to each well and the enzyme reaction was stopped with an equal volume of 1 M H_2SO_4 solution. The optical density at 450 nm was determined for each sample using an ELISA plate reader (Sunrise Tecan).

2.6. Mass Spectrometric Analysis of Recombinant Proteins. Mass spectrometric (MS) analysis of recombinant SBV N protein was carried out according to Hellman et al. [27]. Proteins were identified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems/MDS SCIEX 4800 MALDI TOF/TOF, Framingham, MA, USA). Peptide mass spectra were acquired in reflector positive ion mode with a m/z range of 800–4000 Da. Four hundred laser shots were summarized for each sample with a mass accuracy of ±50 ppm. MS/MS spectra for dominating peptides were acquired in positive mode with the ion collision energy set to 1 keV. Five hundred laser shots were accumulated for each spectrum with a mass accuracy of ±0.1 Da. The proteins were identified in the TrEMBL database (3-23-10 release) using the Mascot algorithm.

2.7. Production of Monoclonal Antibodies. MAbs to recombinant SBV N protein were produced essentially as described by Kohler and Milstein [28]. Eight-week-old female BALB/c mice (obtained from a breeding colony at the Center for Innovative Medicine, Vilnius, Lithuania) were immunized at days

0, 28, and 56 by a subcutaneous injection of 50 μg of recombinant SBV N protein. For a primary immunization, the antigen was emulsified in complete Freund's adjuvant (Sigma-Aldrich Co.). The second and the third immunisations were performed with the antigen dissolved in PBS. Antiserum samples were collected on the 14th day after the first, second, and third immunizations and tested by an indirect ELISA for the presence of IgG antibodies specific to SBV N protein. Three days after the final injection, mouse spleen cells were fused with Sp2/0-Ag 14 mouse myeloma cells using polyethylene glycol 1500 (PEG/DMSO solution, HybriMax, Sigma-Aldrich Co.). Hybrid cells were selected in growth medium supplemented with hypoxanthine, aminopterin, and thymidine (50x HAT media supplement, Sigma-Aldrich Co.). Samples of supernatant from wells with viable clones were screened by an indirect ELISA as described above. Hybridomas secreting specific antibodies to SBV N protein were subcloned twice by a limiting dilution assay. Hybridoma cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) containing 15% fetal calf serum (Biochrom) and antibiotics. Antibodies in hybridoma culture supernatants were isotyped using the BD Pharmingen Mouse Immunoglobulin Isotyping ELISA Kit (BD Bioscience, San Diego, CA, USA) in accordance with the manufacturer's protocol. All procedures involving experimental mice were performed under controlled laboratory conditions in strict accordance with the Lithuanian and European legislation.

2.8. Immunofluorescence Assay. The immunofluorescence test was performed using SBV-infected BHK cells, clone BRS5 (L194, Collection of Cell Lines in Veterinary Medicine, Greifswald-Insel Riems) as antigen matrix in accordance with the procedure described previously [11]. Briefly, a cell suspension was seeded, incubated for 24 h at 37°C, and subsequently infected with SBV strain BH80/11. Forty-eight hours after infection the medium was removed and the cells were fixed using heat treatment (2 h at 80°C). Both, infected and uninfected cells were incubated with each MAb (1 : 10 diluted hybridoma supernatants) for 1 h at RT. After washing with Tris-buffered saline with 0.1% Tween-20 (TBST), a fluorescein isothiocyanate (FITC-) conjugated goat anti-mouse IgG (Sigma-Aldrich Co.) was added and incubated for 1 h at RT. Thereafter, the cells were washed, embedded with Dabco fluorescence conservation buffer (Sigma-Aldrich Co.), and analyzed using an inverted fluorescence microscope (Nikon Eclipse Ti-U, Nikon Instruments Inc., Melville, NY, USA).

3. Results and Discussion

3.1. Synthesis of SBV N Protein in Yeast Saccharomyces cerevisiae. To express the N protein of SBV virus, we exploited yeast vector system previously used for the high-level expression of hantaviruses N proteins [17]. Expression efficiency of recombinant SBV N protein in yeast was proven both by electrophoresis and immunoblotting. SDS-PAGE analysis of crude lysates of *S. cerevisiae* harbouring pFX7-SBV– expression vector revealed the presence of an additional protein band after induction with galactose. This band of approximately 26 kDa was present in the lysates of yeast transformed with pFX7-SBV-6-His-N (Figure 1(a), lane 3). Meanwhile, no additional band of the corresponding molecular size was observed in crude lysates of *S. cerevisiae* harbouring pFX7-6-His vector used as a negative control (Figure 1(a), lane 1). As evaluated by SDS-PAGE, the expression level of SBV N protein was approximately 2% of the total cellular protein. After cell lysis, the main quantity of SBV N protein was found in the insoluble fraction (data not shown). The yield of the His-tagged N protein after nickel-chelate chromatography was about 3.0–3.5 mg/g wet weight of yeast. Recombinant SBV N protein was soluble in 50 mM sodium acetate buffer (pH 5.0) containing 100 mM sodium chloride. The eluted N protein was visible as a single protein band in Coomassie Brilliant Blue-Stained SDS-PAGE gels (Figure 1(a)). In order to confirm the sequence identity of the full-length recombinant SBV N protein and determine its molecular weight, enzymatic digests were performed using trypsin, chymotrypsin, and endopeptidase-AspN to generate internal peptides for detailed mass spectrometry (MS) analysis. Peptide analysis confirmed the primary structure of recombinant SBV N protein predicted from DNA sequence (data not shown). Also, MS analysis revealed that the molecular weight of SBV N protein is 26 kDa, which is in line with theoretical calculated molecular weight of the protein. Western blot analysis of purified protein with the MAb against 6-His-tag epitope confirmed its identity (Figure 1(b), lane 3).

These data confirmed that we have constructed an efficient recombinant yeast expression system and obtained high-level expression of SBV N protein in yeast *S. cerevisiae*.

3.2. The Reactivity of Yeast-Derived SBV N Protein with SBV IgG-Positive Cow Sera. The reactivity of yeast-expressed SBV N protein with cow serum IgG induced by a natural SBV infection was analysed by an indirect IgG SBV ELISA using a panel of 102 cow serum specimens found to be either positive or negative for SBV-specific IgG antibodies using a commercial diagnostic kit (ID Screen Schmallenberg virus Indirect, IDvet). In the commercial kit, SBV N protein expressed in *E. coli* is used [11]. To define the positive/negative threshold of the newly developed indirect IgG SBV ELISA, 11 serum samples previously determined as SBV IgG-negative by commercial ID Screen test were used. The OD values of ELISA were corrected for nonspecific reactivity and reported as sample-to-positive (S/P) values (S/P = (OD sample/OD positive control (from the commercial test kit))$*$100) according to Breard et al. [11]. To calculate the cut-off value, the reactivities of positive and negative reference serum samples from the ID screen test kit with the recombinant SBV N protein were analysed. The cut-off value was calculated as the mean of S/P values of negative samples plus 2 SD (22 + 10) with 95% confidence. Serum sample was considered positive when its S/P value was greater than 32. The tests were run in duplicate. The correlation coefficient and the standard error were 0.92 and 0.1, respectively, between separate runs. To prove the specificity of the newly developed indirect IgG SBV ELISA, yeast-expressed hantavirus Andes N protein as

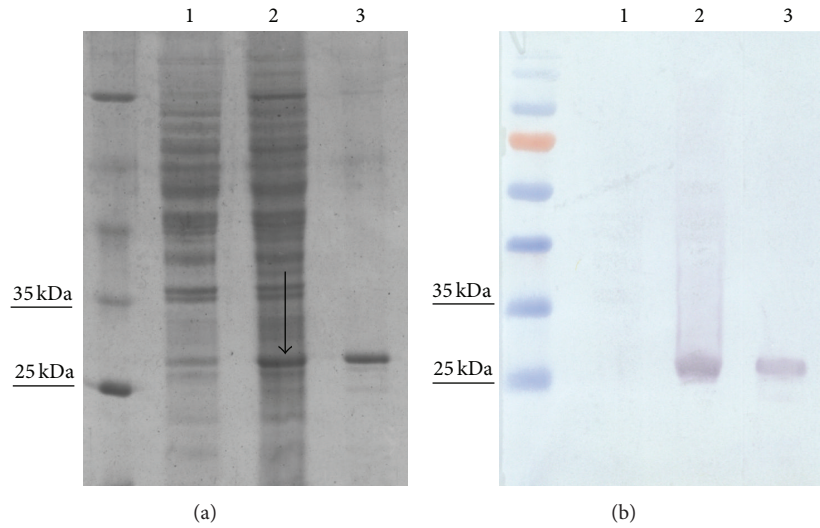

Figure 1: Analysis of yeast cell lysates and purified SBV N protein by SDS-PAGE (a) and western blot (b). Purified SBV N protein (lane 3) or 20 μg of yeast lysates (lanes 1 and 2) was separated in a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. PageRuler Unstained Protein Ladder (Thermo Fisher Scientific Baltics) was used. Lane 1, lysate of mock-transformed *S. cerevisiae* [pFX7-6-His]; lane 2, lysate of *S. cerevisiae* transformed with a plasmid [pFX7-6-His-N] encoding SBV N protein (the arrow indicates SBV N protein band); lane 3, Ni-chelate resin-purified SBV N protein. Western blotting was performed using the MAb against 6-His-tag epitope (b) (Thermo Scientific). PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Baltics) was used.

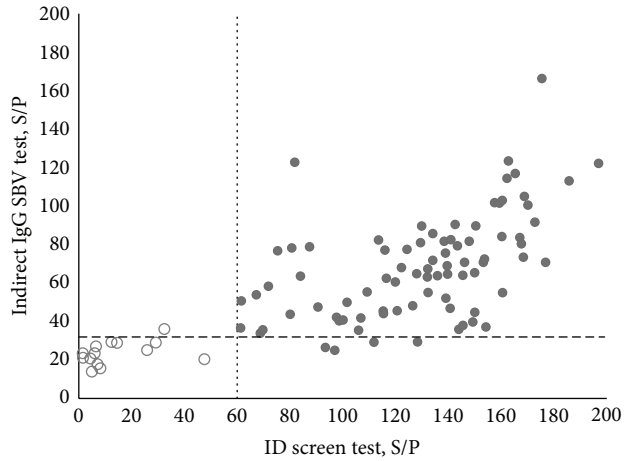

Figure 2: Antibody responses of individual cow serum specimens defined by the newly developed indirect IgG SBV ELISA based on yeast-expressed SBV N protein in comparison to the commercial ID screen test. The S/P ratios of reactivity were plotted. Grey markers represent positive serum samples and white represent negative serum samples obtained by commercial ID screen test. The dashed line represents the cut-off value of the newly developed indirect IgG SBV ELISA. The dotted line represents the cut-off value of the commercial ID screen test.

a negative control antigen was used [29]. No reactivity of cow serum specimens (OD at 450 nm was 0.15 or S/P = 11) with the recombinant hantavirus N protein was detected (data not shown).

Seventy-eight serum specimens out of 82 samples positive by the commercial test were positive and 4 were negative by the newly developed indirect IgG SBV ELISA (Figure 2). Therefore, the sensitivity of the indirect IgG SBV ELISA was calculated to be 95% (78/82 × 100). Fourteen serum samples out of 15 negative specimens by the commercial test were negative and one was positive by the indirect IgG SBV ELISA (Figure 2). Thus, the specificity of the newly developed indirect IgG SBV ELISA was 93% (14/15 × 100). Five serum specimens gave doubtful results by the commercial test. The doubtful serum specimens obtained by the commercial test were excluded from the calculation, as some authors exclude undefined or grey zone sera from the analysis [30, 31].

The high number of sera from SBV-infected cows that were found to be positive in the newly developed indirect IgG SBV ELISA indicated that yeast-expressed SBV N protein may be a suitable antigen for serological diagnosis of SBV infection in cows. The observed discrepancies with the commercial test suggest that IgG SBV ELISA should be further evaluated and optimized using more cow serum specimens collected at variable intervals of the course of the disease. Mansfield and colleagues [12] have shown that the commercial ELISA test could not recognize all SBV-positive serum samples. They conducted focus reduction neutralization assay (PRNT) that appeared to be more sensitive than the commercial ELISA. The essential difference between the PRNT assay and SBV N protein-based ELISA is that the PRNT assay allows detection of antibodies against all structural viral proteins. These results suggest that serologic ELISA tests might be improved by incorporating other viral structural proteins in the test.

This is the first report on SBV antigen expression in yeast *S. cerevisiae* and on the development of an indirect IgG ELISA test based on yeast-expressed SBV N protein. The results showed comparable agreement with commercially available test based on *E. coli*-expressed SBV N protein [11]

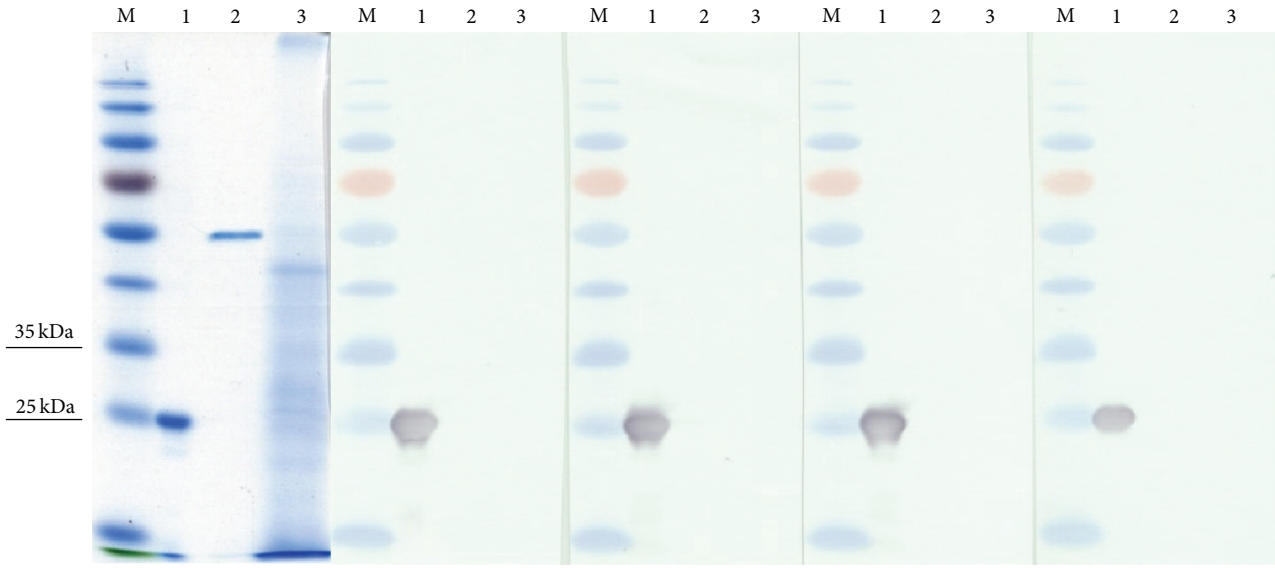

Figure 3: SDS-PAGE (a) and western blot analysis of recombinant SBV N protein with SBV N-specific MAbs ((b)–(e)). Lane M, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Baltics); lane 1, SBV N protein; lane 2, Puumala N 6 His-tagged protein; lane 3, yeast cell lysate. Undiluted hybridoma culture supernatants were used. MAbs codes are indicated at the bottom of each picture.

indicating that yeast-expressed SBV N protein could provide an alternative for analyzing SBV-specific antibodies in blood sera of SBV-infected cows.

The reactivity of yeast-expressed SBV N protein with SBV IgG-positive cow sera is in line with previous studies that demonstrated the usefulness of recombinant viral N proteins expressed in different heterologous systems for serological diagnosis of bunyaviruses infection both by western blot and ELISA [17–21]. The N protein of bunyaviruses is the most abundant viral antigen present in the virion and in the infected cells, thus making it an excellent target for serology as well as epidemiological studies of viral infections [16–18]. Recombinant N protein of hantaviruses, generated in *E. coli*, insect cells, or yeast, has been widely used for detection of hantavirus-specific antibodies in human sera and oral fluid [17–21]. SBV N protein was expressed recently in *E. coli* [11, 32] and used for the development of a commercial diagnostic kit for serologic diagnosis of SBV infection [11].

Taken together, the reactivity of SBV N protein with cow sera suggests that yeast-derived SBV N protein represents a suitable antigen for serologic detection of SBV infection and generation of virus-specific MAbs.

3.3. Generation of Monoclonal Antibodies against Recombinant SBV N Protein. Purified recombinant SBV N protein was used to immunize mice and generate SBV N protein-specific MAbs. After screening and cloning of positive hybridoma clones, four stable hybridoma cell lines producing IgG antibodies were derived: 4F3, 7B6, 8G10, and 11C10. Hybridoma clones 7B6, 8G10, and 11C10 produced MAbs of IgG1 subtype, whereas the MAb produced by clone 4F3 was of IgG2b subtype (Table 1). The apparent K_d of the MAbs ranged between 2, 47×10^{-9} and 1, 39×10^{-10} M, which indicates high-affinity binding (Table 1). All MAbs reacted specifically with recombinant SBV N protein and did not react with yeast-expressed N proteins of Puumala, Hantaan, or Dobrava-Belgrade viruses [17] used to investigate their cross-reactivity (data not shown). To characterize the nature of the epitopes recognized by the MAbs, their reactivity in western blot was analyzed. All four MAbs recognized SDS-denatured SBV N protein in western blot assay (Figures 3(b)–3(e)). This result indicates that the epitopes of the MAbs raised against yeast-derived SBV N protein are not sensitive to denaturation. Recently, it was reported that the MAb 2C8 generated against recombinant SBV N protein expressed in *E. coli* is reactive with virus-derived N protein in the lysates of SBV-infected Vero and BHK cells by western blot [32].

Table 1: Characterization of the MAbs raised against yeast-derived SBV N protein.

Clone	Subtype	K_d, M
4F3	IgG2b	$1{,}39 \times 10^{-10} \pm 2{,}9 \times 10^{-11}$
7B6	IgG1	$3{,}77 \times 10^{-10} \pm 8{,}9 \times 10^{-11}$
8G10	IgG1	$3{,}58 \times 10^{-10} \pm 9{,}3 \times 10^{-11}$
11C10	IgG1	$2{,}47 \times 10^{-9} \pm 2{,}4 \times 10^{-10}$

3.4. MAb Reactivities with SBV-Infected Cells. To prove whether the MAbs raised against recombinant SBV N protein recognize viral N protein, the reactivities of the MAbs were tested by an immunofluorescence analysis using BHK cells infected with SBV BH80/11 initially used for the isolation of SBV N protein gene. All MAbs were reactive with SBV-infected BHK cells, although the intensity of the

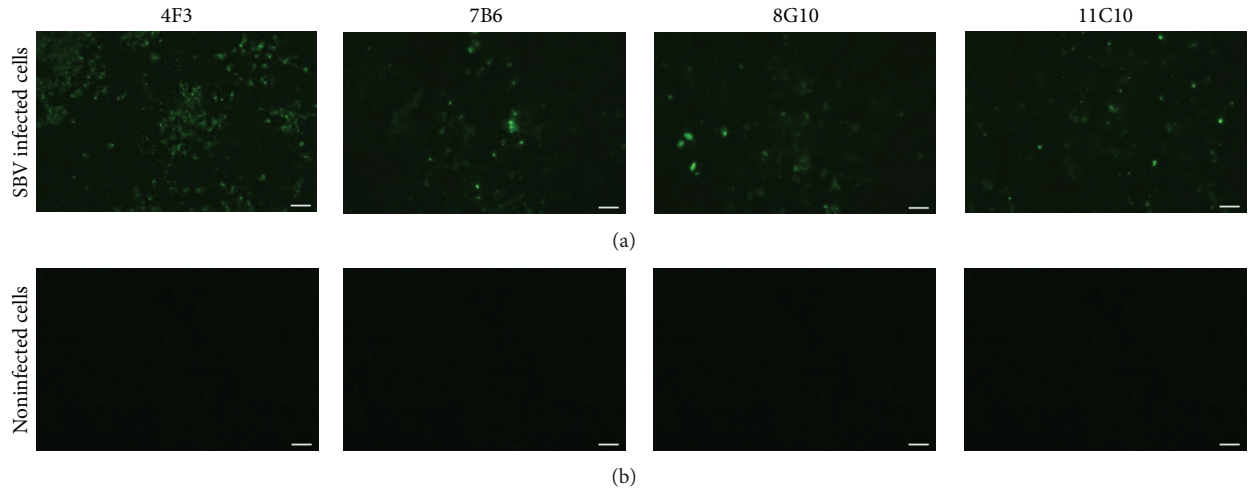

Figure 4: Fluorescence microphotographs showing the reactivity of the MAbs with BHK cells infected with SBV BH80/11 strain (a). Noninfected BHK cells were used as a negative control (b). Hybridoma culture supernatants were used at a dilution of 1 : 10. MAbs codes are indicated on the top of each picture. Scale bar: 100 μm.

immunostaining was differently dependent on MAb clone (Figure 4(a)). No immunoreactivity of the MAbs with noninfected BHK cells used as a negative control was observed (Figure 4(b)).

The immunofluorescence assay data confirm that the MAbs raised against yeast-expressed SBV N protein recognize viral nucleocapsids, which is an additional evidence on the antigenic similarity between yeast-expressed N protein and virus-derived N protein. The MAbs against SBV are of special interest, as they could be used for the development of simple and rapid laboratory diagnostic assays for direct virus detection in biological specimens.

4. Conclusions

Yeast expression system was successfully used to produce recombinant SBV N protein. Purified recombinant SBV N protein was reactive with SBV IgG-positive cow serum specimens collected in Lithuania. For the first time, the circulation of SBV virus in Lithuania was demonstrated. Immunization of mice with SBV N protein resulted in four MAbs that were reactive with SBV-infected cells. The reactivity of recombinant N protein with SBV-specific IgG in cow sera as well as the ability of the MAbs raised against recombinant SBV N protein to recognize native viral nucleocapsids confirms that yeast-expressed SBV N protein resembles native virus in regard of antigenicity and morphology. In summary, yeast-expressed SBV N protein and newly developed SBV-reactive MAbs may provide useful reagents for diagnostics and seroprevalence studies of SBV infection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Photobacteriosis: Prevention and Diagnosis

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Photobacteriosis or fish pasteurellosis is a bacterial disease affecting wild and farm fish. Its etiological agent, the gram negative bacterium *Photobacterium damselae* subsp. *piscicida*, is responsible for important economic losses in cultured fish worldwide, in particular in Mediterranean countries and Japan. Efforts have been focused on gaining a better understanding of the biology of the pathogenic microorganism and its natural hosts with the aim of developing effective vaccination strategies and diagnostic tools to control the disease. Conventional vaccinology has thus far yielded unsatisfactory results, and recombinant technology has been applied to identify new antigen candidates for the development of subunit vaccines. Furthermore, molecular methods represent an improvement over classical microbiological techniques for the identification of *P. damselae* subsp. *piscicida* and the diagnosis of the disease. The complete sequencing, annotation, and analysis of the pathogen genome will provide insights into the pathogen laying the groundwork for the development of vaccines and diagnostic methods.

1. Introduction

Photobacteriosis or fish pasteurellosis is a septicemia caused by the gram negative, halophilic bacterium *Photobacterium damselae* subsp. *piscicida*, a member of the Vibrionaceae family, that shares its species epithet with *Photobacterium damselae* subsp. *damselae* [1]. Photobacteriosis is considered one of the most dangerous bacterial diseases in aquaculture worldwide due to its wide host range, high mortality rate, and ubiquitous distribution [2]. The pathogen is able to infect a wide variety of marine fish, including the yellowtail (*Seriola quinqueradiata*) in Japan, gilthead sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), and sole (*Solea senegalensis* and *Solea solea*) in Europe, striped bass (*Morone saxatilis*), white perch (*Morone americana*), and hybrid striped bass (*Morone saxatilis* (*Morone chrysops*)) in the USA, cobia (*Rachycentron canadum*) in Taiwan, and golden pompano (*Trachinotus ovatus*) in China [3–5].

Differences in susceptibility to the disease have been described on the basis of fish age. Larvae and juveniles are more susceptible to photobacteriosis, and acute infection induces 90–100% mortality of juvenile sea bream, whereas fish over 50 g are more resistant due to more efficient phagocytosis and killing of the bacteria by neutrophils and macrophages [6]. Bacteria that reside in different tissues and inside phagocytes cause chronic and acute forms of photobacteriosis. In its acute form, multifocal necrosis is present in the liver, spleen, and kidney and bacteria accumulate freely in phagocytes, capillaries, and interstitial spaces. Chronic lesions in the internal organs are characterized by the presence of white tubercles about 0.3–0.5 mm in diameter [7].

Adherence and invasive capacities are essential in the first stage of infection [3]. *P. damselae* subsp. *piscicida* has been reported to be weakly or moderately adherent and invasive in various fish cell lines but has shown a high binding capacity to fish intestines [8]. The adherence seems to be mediated by a protein or glycoprotein receptor of the bacterial cell surface, and the internalization of the bacteria occurs through an actin microfilament-dependent mechanism [8], with cell metabolism playing an active role [9]. However, the precise nature of the mechanism responsible for adherence and interaction with host cell receptors and virulence factors contributing to the invasion of fish nonphagocytic cells is still unknown [9].

Several virulence mechanisms of *P. damselae* subsp. *piscicida* have been described. The polysaccharide capsular material plays an important role in the pathogenesis of the bacterium, conferring resistance to serum killing and increasing fish mortality [10]. Furthermore, the intracellular

survival of the pathogen is likely to confer protection against specific and nonspecific host defenses and exogenous antimicrobial agents including antibiotics [8]. Extracellular products with phospholipase, cytotoxic, and hemolytic activities may account for the damage to the infected cells, the consequent release of the microorganisms, and the invasion of adjacent cells. In particular, a key pathogenicity factor of *P. damselae* subsp. *piscicida* is an exotoxin, the plasmid-encoded apoptosis-inducing protein of 56 kDa (AIP56), abundantly secreted by virulent strains and responsible for apoptogenic activity against sea bass macrophages and neutrophils in acute fish photobacteriosis [11]. The AIP56 toxin is a zinc-metalloprotease that acts by cleaving NF-κB p-65, with the catalytic activity located in the N-terminal domain and the C-terminal domain involved in binding and internalization into the cytosol of target cells [12]. The AIP56 induces activation of caspases 8, 9, and 3, loss of mitochondrial membrane potential, release of cytochrome c into the cytosol, and overproduction of ROS, suggesting activation of both extrinsic and intrinsic apoptotic pathways [13]. Through the activation of the cell death process involving macrophages and neutrophils, the pathogen is able to subvert the immune defenses of the host and to produce infectious disease.

Another important virulence mechanism of *P. damselae* subsp. *piscicida* is the acquisition of iron from its host by using high-affinity iron-binding siderophores, low molecular weight iron-chelating molecules that interact with bacterial membrane receptors to transport iron into the bacterium [14]. Furthermore, *P. damselae* subsp. *piscicida* is able to acquire iron from hemin and hemoglobin as unique iron sources *in vitro* [14], and iron limitation results in an increased binding of hemin in virulent strains [15]. The heme uptake of the bacterium includes a TonB system to transport heme into the cytoplasm and an ATP-binding cassette (ABC) system to drive it across the cytoplasmic membrane [16, 17].

Little is known about the fish immune response to the bacterium and the factors responsible for its failure to protect against *P. damselae* subsp. *piscicida*. A transcriptomic approach has recently been applied to elucidate the early immune responses of juvenile gilthead sea bream to *P. damselae* subsp. *piscicida* infection. A rapid recognition of the pathogen is shown by the upregulation of lectins, peptides with antimicrobial activity, chemokines, and chemokine receptors, as well as protein of iron and the heme metabolism as a response against bacteria that are dependent on iron. However, this defensive reaction can be either beneficial or devastating to the host [18]. Moreover, the upregulation of genes with highly specialized suppressive functions has been observed indicating an active suppression of immunity that can be induced by the host to reduce tissue damages or by the pathogen to evade the host response [18].

2. Prevention of *P. damselae* subsp. *piscicida* Infection

Antibiotics have been the first line of defense in fish aquaculture to control photobacteriosis outbreaks, but after only a few years the pathogen acquired resistance to various antibiotics. In fact, different transferable genetic elements (R plasmids) carrying genes for resistance against kanamycin, sulphonamide, tetracycline [19–22], ampicillin [22, 23], chloramphenicol [22, 24], florfenicol [25], and erythromycin [26] have been documented in *P. damselae* subsp. *piscicida*. Differences in the geographic distribution of multidrug transferable elements have been observed among several strains collected in Japan and United States [22, 27]. Furthermore, the intracellular parasitism of *P. damselae* subsp. *piscicida* within macrophages undermines the effectiveness of chemotherapy.

Taking into account all of these issues, research has been focused on the development of effective vaccines to prevent photobacteriosis and reduce the use of antibiotics in fish farming with benefits at biological and environmental point [28]. Conventional *P. damselae* subsp. *piscicida* vaccines are based on inactivated products containing cellular (heat-o formalin killed bacteria) and soluble antigens (LPS and ribosomal formulations) for immersion and injection administration (Table 1). However, they appear to be ineffective in protecting against pasteurellosis [2, 3, 28–38]. Bacterins overexpressing a 97 kDa OMP and a 52 kDa ECP protein, involved in the internalization of the bacterium, are reported to be effective in both sea bass and yellowtail when delivered by immersion eliciting a strong antibody response in the gills and mucosae that may block pathogen entry and colonization [2]. However, the only commercially available vaccine is an ECP-enriched bacterin preparation that has been employed in several European countries with mixed results ranging from good in Spain, Turkey, and Greece to poor in Italy [28, 39]. The recommended vaccination protocol consists of two bath immersions at monthly intervals starting at the larval stage when the fish is 50 mg and an oral booster immunization when fish reaches 2 g body weight [3].

Recombinant DNA technology and biotechnological approaches have thus far been used to a very limited extent for the development of bacterial vaccines for fish and effective preventive measures against fish pasteurellosis do not yet exist. Studies on the development of subunit vaccines have recently been reported in cobia from a Taiwan *P. damselae* subsp. *piscicida* isolate [4]. Immunoproteomics, using western blotting on protein analyzed with 2DE and LC-MS/MS to isolate immune-reactive proteins, has been applied to identify *P. damselae* subsp. *piscicida* antigens that were then cloned and produced as recombinant proteins. In particular, three antigens were shown to induce a protective effect in cobia and therefore were reported as potential vaccine candidates for the development of a subunit vaccine against the pathogen. However, the protection of these vaccine candidates has not been investigated in other fish species, where *P. damselae* subsp. *piscicida* causes serious disease and high mortality, and against other *P. damselae* subsp. *piscicida* isolates [4]. Moreover, antigen combinations were studied revealing that bivalent subunit vaccines may achieve a better efficiency than monovalent or trivalent antigens [41].

In our laboratory a biotechnological approach based on the *reverse vaccinology* has been applied to design a vaccine against fish pasteurellosis [40]. New genomic sequences of *P. damselae* subsp. *piscicida* were the starting point for bioinformatic analysis aiming to identify new proteins localized

Table 1: Overview of vaccines against *Photobacterium damselae* subsp. *piscicida*.

	Type of vaccine	Type of product	Vaccination procedure	Species	References
Lipoprotein	Recombinant subunit vaccine	Experimental	Injection	Sea bass	Andreoni et al. [40]
rHSP60, rENOLASE, and rGAPDH antigens, singles or in combination	Recombinant subunit vaccine	Experimental	Injection	Cobia	Ho et al. [4] and Ho et al. [41]
Formalin-killed bacterin overexpressing a 97 kDa OMP and 52 kDa ECP	Inactivated	Licensed	Immersion	Sea bass and yellowtail	Barnes et al. [2]
Formalin-killed bacterin with Escherichia coli LPS	Inactivated	Experimental	Immersion	Sea bream	Hanif et al. [38]
Live attenuated aroA mutant	Live attenuated	Experimental	Injection or immersion	Hybrid striped bass	Thune et al. [37]
Formalin-killed bacterin, ECP, and crude capsular polysaccharide (cCPS)	Inactivated	Experimental	Injection, immersion, and oral	Sea bass	Bakopoulos et al. [36]
LPS mixed with chloroform-killed bacterin	Inactivated	Experimental	Injection	Yellowtail	Kawakami et al. [35]
ECP-enriched formalin-inactivated bacterin	Inactivated	Commercialized	Immersion	Sea bass, sea bream, and sole	Magariños et al. [39]
Live attenuated bacteria	Live attenuated	Experimental	Immersion	Yellowtail	Kusuda and Hamaguchi [34]
Ribosomal antigens	Subunit vaccine	Experimental	Injection	Yellowtail	Kusuda et al. [33]
LPS formulation	Subunit vaccine	Experimental	Immersion and spray methods	Yellowtail	Fukuda and Kusuda [32]
Heat- and formalin-killed bacterin	Inactivated	Experimental	Immersion and oral	Yellowtail	Fukuda and Kusuda [29], Hamaguchi and Kusuda [30], and Kusuda and Salati [31]

on the bacterial surface. In fact, the primary condition in selecting a bacterial protein as a vaccine candidate is its cellular localization. Cytosolic proteins are unlikely to be immunological targets, whereas surface exposed and secreted proteins are more easily accessible to the host immune system [47]. *In vitro* screening of the *in silico* selected vaccine candidates by an inhibition adherence assay revealed that immunoglobulins from mice immunized with one of the recombinant vaccine candidates were able to affect the adherence of *P. damselae* subsp. *piscicida* to fish epithelial cells. The candidate antigen, found to be involved in the adherence and internalization of *P. damselae* subsp. *piscicida* in CHSE-214 cells, was predicted *in silico* as likely lipoprotein with outer membrane localization. The N-terminal signal peptide of 20 amino acids contains the lipobox motif, 2 positively charged residues within the first 7 amino acids and a transmembrane helix of 10 residues. A database search revealed homology with hypothetical proteins and no putative conserved domain; therefore, no putative biological role could be assigned to this lipoprotein. Vaccination and challenge experiments in a laboratory trial indicated that immunization of sea bass with the recombinant antigen induced the production of specific antibodies and conferred protection against *P. damselae* subsp. *piscicida* challenge [40]. *In vivo* long persistence of lipoprotein antibodies was obtained with a single antigen administration in agreement with Ho et al. [4] who reported that multiple administrations do not increase protection in fish. The recombinant lipoprotein is potentially able to protect sea bass against *P. damselae* subsp. *piscicida* and could be an interesting candidate for the design of a recombinant vaccine against photobacteriosis. However, protection efficacy over time, increasing doses of the antigen, and its use in combination with different adjuvants must be further investigated in field experiments.

Due to the inconsistency of effective measures to prevent photobacteriosis, research has also focused on alternative methods to control the disease. Such methods include probiotics, to be applied in aquaculture to improve health, and a strain of *Pediococcus pentosaceus*, a lactic acid bacterium isolated from the intestine of adult cobia, has been investigated for its probiotic potential [48]. The acidic pH derived from metabolic acids in lactic acid bacteria culture supernatant has been shown to inhibit *P. damselae* subsp. *piscicida* growth *in vitro*. Dietary supplementation with *P. pentosaceus* in cobia enhances the growth rate and respiratory burst of peripheral blood leukocytes in fed fish. Furthermore, lactic acid bacteria feeding increased the survival rate of cobia after *P. damselae* subsp. *piscicida* immersion challenge. The mechanism affording this protection is still unclear. Although feeding with lactic acid bacteria did not increase specific

Table 2: Methods for direct identification of *Photobacterium damselae* subsp. *piscicida*.

Assay	Target	Additional culture step	Specificity	Availability on the market	References
PCR-based detection method	*16S* gene	—	*P. damselae*		Osorio et al. [42]
Multiplex PCR assay	*16S* gene *ureC* gene	—	*P. damselae* subspecies		Osorio et al. [1]
Multiplex PCR assay	*Pbp-1A* gene *ureC* gene internal amplification control	—	*P. damselae* subspecies	*Photobacterium damselae*-PCR detection Kit by Diatheva	Amagliani et al. [43]
PCR technique and plating method	*cps* gene	TCBS-1 agar	*P. damselae* subspecies		Rajan et al. [44]
Enzyme immunoassay	Polyclonal antibodies against *P. damselae* subsp. *piscicida*	—	*P. damselae* subsp. *piscicida*[a]	Aquaeia-Pp kit by BIONOR	Romalde et al. [45]
PCR-RFLP method	GenBank AY191120, AY191121 sequences	—	*P. damselae*, restriction analysis for subspecies identification		Zappulli et al. [46]

[a]Cross reactions with *P. damselae* subsp. *damselae* and *P. histaminum*.

antibody response after the immunization of cobia with inactivated *P. damselae* subsp. *piscicida* vaccine, it heightened the synergic protection against *P. damselae* subsp. *piscicida* challenge by 22% and could be administered by itself as a probiotic or with vaccination [48].

Furthermore, selective breeding for fish strains genetically resistant to photobacteriosis constitutes a potential strategy to reduce the probability of disease outbreak and avoid the dramatic consequences of high mortality in fish farms [49]. Quantitative trait loci mapping is applied to detect the regions of the host genome that are associated with resistance to the disease and marker-assisted selection is a useful approach used in several aquaculture species [50–52].

A study investigating quantitative trait loci for resistance to fish pasteurellosis in the gilthead sea bream identified two significant quantitative trait loci, one affecting late survival and another impacting overall survival, and a potential marker for disease resistance [49]. The identification of phase-specific quantitative trait loci in gilthead sea bream supports the hypothesis of a biphasic defense response with a primary infection by experimental exposure to the pathogen and a secondary infection with bacteria released from moribund and dead fish [49, 53]. Results of quantitative trait loci, mapped by identifying regions of the genome that explain complex traits such as survival, could also be used to gain a better understanding of the mechanisms of disease resistance and defense response. Further insights might also be gained through comparative mapping with other species susceptible to photobacteriosis.

3. Identification of *P. damselae* subsp. *piscicida* and Diagnosis of Infection

Rapid diagnosis of fish photobacteriosis outbreaks is essential for proper management and effective control in aquaculture. Disease diagnosis is usually made using standard microbiological methods, based on pathogen culturing and isolation steps. Biochemical and serological confirmation is also necessary to characterize the bacterium and to discriminate between the two closely related subspecies, *piscicida* and *damselae* of *P. damselae*. The miniaturized system AIP20E is usually used for a presumptive identification of the *P. damselae* subsp. *piscicida*. Although *P. damselae* generally displays a unique code of 2005004 for the *piscicida* [54] and 2015004 for the *damselae* subspecies [1], some strains exhibit aberrant reactions that can lead to misleading results [55]. Hence, differentiation of the subspecies *P. damselae* subsp. *damselae* can be achieved when three or more positive results are obtained in the lysine decarboxylase (LDC) production, motility, nitrate reduction to nitrite, gas production from glucose, thiosulfate citrate bile salts-sucrose (TCBS-1) growth, and urease tests, because all these tests yield negative results for all *P. damselae* subsp. *piscicida* strains [55, 56]. Serological methods such as agglutination or the ELISA have also been developed and commercialized [3].

To overcome the problem of time-consuming and laborious procedures, in the last few years molecular methods have been developed in order to achieve an accurate and specific identification of *P. damselae* subsp. *piscicida* and a rapid diagnosis of photobacteriosis (Table 2). The point at issue is the strong similarity at the DNA level between the two subspecies that makes it difficult to identify sequences useful for designing a subspecies-specific method [3, 42, 57]. rRNA sequences have been considered for this purpose [42], but strong similarities have been detected both in the 16S, 23S, and 5S (>99%) and the intergenic spacer regions (98–99.5%) between the two subspecies of *P. damselae*. Moreover, the mosaic-like structure of the latter makes them unsuitable for diagnostic purposes [42, 58]. Only a PCR-based method

at species level has been developed using the 16S sequences [42].

Integrated sets of methods combine the amplification of the capsular polysaccharide gene to identify the species *P. damselae* with an additional culture step on TCBS-1 agar to differentiate *P. damselae* subsp. *piscicida* from *P. damselae* subsp. *damselae* [44] or the amplification of two *P. damselae*-specific targets with restriction analysis of PCR products to obtain a unique digestion profile for *P. damselae* subsp. *piscicida* strains [46].

A multiplex PCR method based on the 16S rRNA and *ureC* genes has been proposed to discriminate between the two subspecies. The *ureC* gene is present in *P. damselae* subsp. *damselae* genome but is not found in *P. damselae* subsp. *piscicida* [1]. On the contrary, a *P. damselae* subsp. *piscicida*-specific target sequence, conserved among strains of different geographical origin but not shared by *P. damselae* subsp. *damselae*, has not yet been reported [42, 44].

An additional multiplex PCR protocol has been developed in our laboratory as a valid alternative to standard culture methods for the rapid and specific diagnosis of photobacteriosis in fish [43]. The gene coding for a penicillin binding protein 1A (GenBank accession number EU164926) was selected from a large-scale genome project as the PCR target for the identification of *P. damselae* subsp. *piscicida* because of several mismatches with the corresponding *P. damselae* subsp. *damselae* gene mainly clustered in the 3′ end of the gene. However, specificity analysis also indicated amplification of the target gene in two *P. damselae* subsp. *damselae* strains. This is due to the fact that a stronger sequence similarity to *P. damselae* subsp. *piscicida* than to other *P. damselae* subsp. *damselae* strains was found in these two *P. damselae* subsp. *damselae* strains. Hence, an additional PCR target, the *ureC* gene, lacking in the *P. damselae* subsp. *piscicida* genome, was introduced in the assay with the aim of differentiating each strain at the subspecies level together with an internal amplification control to obtain a clear distinction between truly negative and false negative results. The optimized multiplex PCR is able to correctly identify and discriminate both subspecies of *P. damselae* with a detection limit of 500 fg DNA, corresponding to 100 genomic units, twofold higher than that of immunodiagnostic systems (i.e., Bionor Aquaeia-Pp kit) [45].

4. Conclusions

Partial genome sequencing of several *P. damselae* subsp. *piscicida* strains has been previously reported [40, 59] and recently a draft of the complete genome sequence of *P. damselae* subsp. *piscicida* DI21 strain has been deposited in the public databases (GeneBank accession number PRJNA168653), but the complete gene annotation is not yet available. This information together with the comparative analysis of the genome sequence of different strains of *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* will provide further insights laying the groundwork for the development of effective vaccines and diagnostic tools for the causative agent of fish pasteurellosis.

Conflict of Interests

The authors declare no conflict of interests.

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