Chapter 1

Vaccines and Vaccination for Veterinary Viral Diseases: A General Overview

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Abstract

A high number of infectious diseases affecting livestock and companion animals are caused by pathogens of viral etiology. Ensuring the maximum standards of quality and welfare in animal production requires developing effective tools to halt and prevent the spread of those infectious diseases affecting animal husbandry. To date, one of the best strategies is to implement vaccination policies whenever possible. However many of the currently manufactured vaccines relies in classical vaccine technologies (killed or attenuated vaccines) which, under some circumstances, may not be optimal in terms of safety or adequate for widespread application in disease-free countries at risk of disease introduction. One step ahead is needed to improve and adapt vaccine manufacturing to the use of new generation vaccine technologies already tested in experimental settings. Here we present in the context of animal viral diseases of veterinary interest, an overview of some current vaccine technologies that can be approached for virus pathogens with a brief insight in the type of immunity elicited.

Key words Virus vaccines, Attenuated vaccines, Viral vectors, DNA vaccines, Subunit vaccines, Innate immunity, Adaptive immunity, Vaccine technologies

1 Viral Diseases of Animals and the Need for Vaccination

One of the biggest transformations in history occurred when mankind shifted from a hunter-gatherer to an agricultural lifestyle. During millenniums livestock and companion species (ruminants, swine, poultry, cats and dogs) were domesticated and raised first for survival (in this sense the word "livestock" is meaningful) then for profit and commerce. Since then, animal husbandry evolved as one of the most important activities for civilization and development. The importance of such activity is obvious since a proper management of land use and animal resources is always required to avoid malnutrition and famine in developing countries or in countries where intensive farming is essential for subsistence. The explosive

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growth rate of the world's human population complicates this picture (particularly in developing countries) so other sources of dietary consumption, such as farmed fish, will be more demanded in the near future. Inevitably, the intensive farming of animal species leads to the onset of diseases mainly caused by propagation of infectious pathogens, affecting animal welfare, reducing productivity, and in the worst cases, seriously undermining the economy of nations. In some cases, livestock or animal pathogens can also cause disease in humans, so means to control and eradicate them have to be implemented.

Among the plethora of infectious diseases in animals, those of viral etiology account for a high burden of cases and are among the most relevant from a veterinary perspective. In fact, approximately half of the most important animal diseases are caused by viruses, according to the OIE's classification for terrestrial and aquatic notifiable animal diseases (*see* Table 1). The listed viral diseases comprise virus from 22 different viral families and four families of virus (herpesvirus, rhabdovirus, poxvirus and paramyxovirus) concentrate a high number of diseases (Fig. 1). Several of the listed animal virus diseases can be also transmitted to humans (zoonotic diseases) either by direct contact with infected animals, infected animal tissues and fluids or by means of arthropod vectors, impacting both public health and food security. Thus, preventing transmission of infectious diseases at the animal–human interface is important for protecting the world population from both epizootics and pandemics, constituting the basis for the "One Health" concept [1, 2]. Prevention by vaccination remains as one of the most cost-effective intervention strategies against infectious diseases. For most of the listed diseases there are "licensed" or available vaccines, eventually obtained by "classical" production methodologies. An important exception is that of diseases caused by retroviruses, for which classical vaccine technologies have not been successful, and that of aquatic diseases, for which only vaccines for fish have been so far developed. In some cases the efficacy of vaccination against viral diseases of animals has been very successful, as it can be illustrated by the eradication of rinderpest [3] (probably the most deadly disease of cattle and ruminants, caused by a morbillivirus) by the use of an attenuated/avirulent strain of the causative virus. Recent evidences advice to support efforts to control emerging viral pathogens where they primarily occur, in order to avoid uncontrolled spread of deadly viruses [4]. Within this perspective, some technologies for vaccine design may constitute powerful platforms to rapidly generate new experimental vaccines based on previous knowledge about the immune responses generated in the host.

Table 1
The OIE's notifiable viral diseases and infections of terrestrial and aquatic animals

Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Bluetongue	BTV[a]	Reoviridae	Orbivirus	Live attenuated
Crimean Congo hemorrhagic fever[b]	CCHFV[a]	Bunyaviridae	Nairovirus	Not available
Equine encephalomyelitis (Eastern)[b]	EEEV[a]	Togaviridae	Alphavirus	Inactivated
Foot and mouth disease[b]	FMDV	Picornaviridae	Aphtovirus	Inactivated (BEI)
Infection with Aujeszky's disease virus (Pseudorabies)	SHV-1	Herpesviridae (α-herpesvirinae)	Suid Herpesvirus	Attenuated (deletion of glycoproteins gE, gC, gG)
Infection with rabies virus[b]	RABV	Rhabdoviridae	Lyssavirus	Inactivated/Attenuated/Recombinant poxvirus; Adenovirus
Infection with rinderpest virus	RPV	Paramyxoviridae	Morbillivirus	Attenuated
Japanese encephalitis[b]	JEV[b]	Flaviviridae	Flavivirus	Inactivated/Attenuated
Rift Valley fever[b]	RVFV[b]	Bunyaviridae	Phlebovirus	Attenuated
Vesicular stomatitis[b]	VSV[b]	Rhabdoviridae	Vesiculovirus	Inactivated/Attenuated
West Nile fever[b]	WNV[b]	Flaviviridae	Flavivirus	Inactivated/Attenuated/Recombinant canarypox/DNA vaccine (USA)
Epizootic hemorrhagic disease	EHDV[b]	Reoviridae	Orbivirus	Inactivated/Attenuated (licensed USA, Japan)
Cattle diseases				
Bovine viral diarrhea	BVDV	Flaviviridae	Pestivirus	Inactivated/Attenuated

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Table 1
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Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Enzootic bovine leukosis	BLV	Retroviridae	Lentivirus	Not available
Infectious bovine rhinotracheitis/ Infectious pustular vulvovaginitis	BoHV-1	Herpesviridae (α-herpesvirinae)	Varicellovirus	Deleted glycoprotein gE inactivated or attenuated
Lumpy skin disease	LSDV	Poxviridae	Orthopoxvirus	Attenuated
Sheep and goat diseases				
Caprine arthritis/encephalitis	CAEV	Retroviridae	Lentivirus	Not available
Infection with peste des petits ruminants virus	PPRV	Paramyxoviridae	Morbillivirus	Attenuated/Recombinant capripoxvirus
Maedi-visna	MVV	Retroviridae	Lentivirus	Not available
Sheep pox and goat pox	SPV	Poxviridae	Orthopoxvirus	Inactivated/Attenuated
Equine diseases				
Equine encephalomyelitis (Western)	WEEV[a]	Togaviridae	Alphavirus	Inactivated
Equine infectious anemia	EIAV	Retroviridae	Lentivirus	Not available
Equine influenza[b]	EIV	Orthomyxoviridae	Influenzavirus	Inactivated/Recombinant canarypox
Infection with equid herpesvirus-1	EHV-1	Herpesviridae		Inactivated/Attenuated
Infection with equine arteritis virus	EAV	Arteriviridae	Arterivirus	Inactivated/Attenuated
Venezuelan equine encephalomyelitis[b]	VEEV[a]	Togaviridae	Alphavirus	Inactivated/Attenuated

Infection with African horse sickness virus	AHSV[a]	Reoviridae	Orbivirus	Live attenuated
Swine diseases				
African swine fever	ASFV[a]	Asfiviridae	Asfivirus	Not available
Infection with classical swine fever virus	CSFV	Flaviviridae	Pestivirus	Attenuated/Subunit (E2)
Porcine reproductive and respiratory syndrome	PRRSV	Arteriviridae	Arterivirus	Live attenuated
Swine vesicular disease	SVDV	Picornaviridae	Enterovirus	Not available
Transmissible gastroenteritis	TGEV	Coronaviridae	Alphacoronavirus	Not available
Avian diseases				
Avian infectious bronchitis	IBV	Coronaviridae	Gammacoronaviridae	Inactivated/Attenuated/Inactivated multivalent
Avian infectious laryngotracheitis	ILTV	Herpesviridae (α-herpesvirinae)	Gallid herpesvirus-1	Attenuated/Recombinant herpesvirus/Recombinant fowlpox
Duck virus hepatitis	DHV-1	Picornaviridae	Avihepatovirus	Inactivated/Attenuated
Duck virus enteritis	DEV-1	Herpesviridae (α-herpesvirinae)	Anatid herpesvirus-1	Attenuated
Infection with avian influenza viruses and infection with influenza A viruses of high pathogenicity in birds other than poultry including wild birds[b]	AIV	Orthomyxoviridae	Influenzavirus A	LPAI inactivated/Recombinant fowlpox (HPAI vaccination banned or discouraged)

(continued)

Table 1
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Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
Fowl pox	FPV	Poxviridae	Avipoxvirus	Modified live attenuated
Infectious bursal disease (Gumboro disease)	IBDV	Birnaviridae	Avibirnavirus	Inactivated/Attenuated/Recombinant herpesvirus-VP2
Newcastle disease[b]	NDV	Paramyxoviridae	Avulavirus	Inactivated/Attenuated (lentogenic and mesogenic). Recombinant avian herpesvirus and avipoxvirus
Marek's disease	MDV (GaHV-2)	Herpesviridae (α-herpesvirinae)	Gallid herpevirus-1	Live attenuated
Turkey rhinotracheitis	aMPV	Paramyxoviridae	Metapneumovirus	Live attenuated/Inactivated
Lagomorph diseases				
Myxomatosis	MV	Poxviridae		Live attenuated
Rabbit hemorrhagic disease	RHDV	Caliciviridae		Recombinant poxvirus
Other infections				
Camelpox		Poxviridae		Inactivated/Attenuated
Bunyaviral infections (*Akabane, Cache Valley, Schmallenberg, and Nairobi sheep disease*)	AKAV[b] CVV[b] SBV[b] NSDV[b]	Bunyaviridae	Orthobunyavirus Orthobunyavirus Orthobunyavirus Nairovirus	Inactivated
Hendra and Nipah virus diseases[b]	HeV NiV	Paramyxoviridae	Henipaviruses	Not available

Fish diseases				
Infection with HPR-deleted or HPR0 infectious salmon anemia virus	ISAV	Orhtomyxoviridae	Isavirus	Inactivated
Infection with salmonid alphavirus	SAV	Togaviridae	Alphavirus	Inactivated
Epizootic hematopoietic necrosis	EHNV	Iridoviridae	Ranavirus	Not available
Infectious hematopoietic disease	IHNV	Rhabdoviridae	Novirhabdovirus	Inactivated/DNA
Koi herpesvirus disease	KHV	Alloherpesviridae	Cyprinivirus	Live attenuated
Red sea bream iridoviral disease	RSIDV	Iridoviridae		Formalin inactivated
Spring viraemia of carp	SVCV	Rhabdoviridae	Vesiculovirus	Not available
Viral hemorrhagic septicemia	VHSV	Rhabdoviridae	Novirhabdovirus	Not available
Mollusc diseases				
Infection with Ostreid Herpesvirus 1 microvariants	OsHV-1	Herpesviridae		Not applicable
Infection with abalone herpesvirus	AbHV	Herpesviridae (Malacoherpesviridae)		Not available
Crustacean diseases				
Infectious hypodermal and hematopoietic necrosis	IHHNV	Parvoviridae	Brevidensovirus	Not developed
Infectious myonecrosis	IMNV	Totiviridae	Totivirus	Not developed
Taura syndrome	TSV	Dicistroviridae	Aparavirus in the Family	Not developed

(continued)

Table 1
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Diseases affecting multiple species	Virus acronym	Virus family	Virus genus	Licensed vaccine type(s) available
White spot disease	WSSV	Nimaviridae	Whispovirus	Not developed
White tail disease (Infection by Macrobrachium rosenbergii nodavirus)	MrNV and XSV associate virus	Nodaviridae	Nodavirus	Not developed
Yellowhead disease	YHV	Roniviridae (O. Nidovirales)	Okavirus	Not developed
Amphibian diseases				
Infection with ranavirus	FV3	Iridoviridae	Ranavirus	Not available

[a]Arthropod-borne virus (arbovirus)

[b]Zoonotic disease

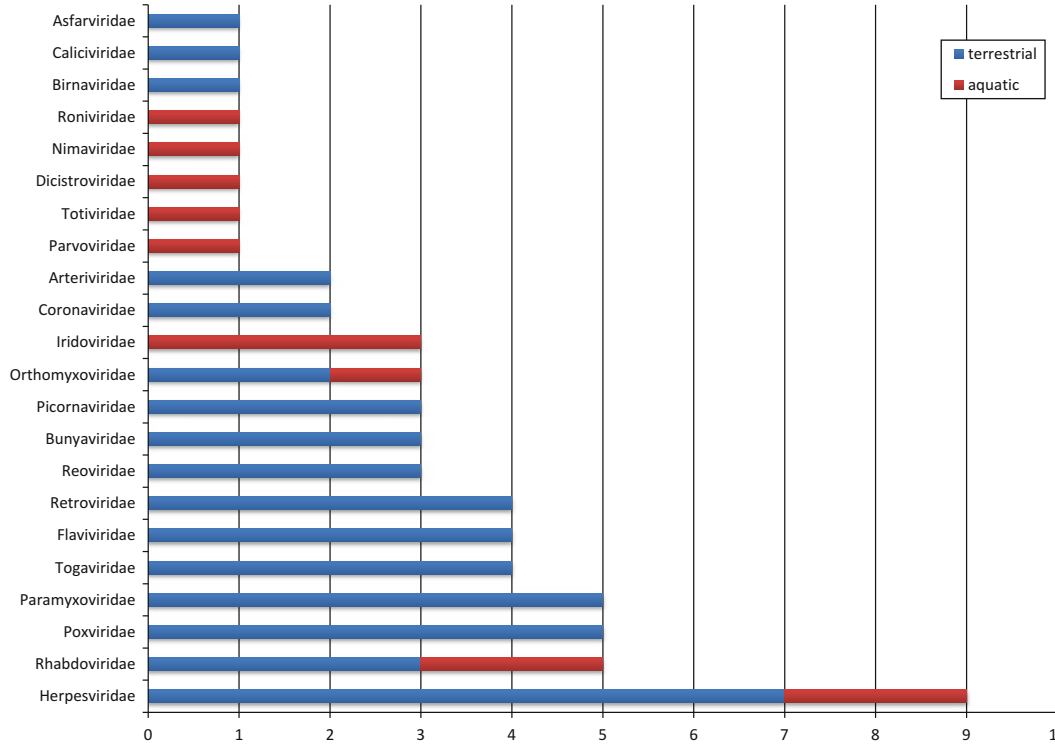

Fig. 1 Virus families including members causing notifiable animal diseases. The figure depicts the number of pathogenic members from each virus family causing important diseases in terrestrial and aquatic animals

2 Immunology Matters

The objective of vaccination is to achieve a specific stimulation of the immune system enabling the host to mount an efficient (and desirably long-lived) memory immune response that can recognize the pathogen and eventually eliminate it once is present in the organism upon infection. This can be achieved providing the appropriate antigenic stimulus (the vaccine) to activate cellular mechanisms involved in recognition of the nonself. Thus, an efficient vaccine needs to be recognized as a nonself entity and, ideally, be able to stimulate innate immune responses that further "instruct" subsequent adaptive and memory responses. The first step is carried out either by infected or by specialized phagocytic cells (antigen presenting cells or APCs, including macrophages and dendritic cells) able to present antigenic determinants to näive (B and T) lymphocytes (Fig. 2).

Though the innate immune response is broadly reactive and unspecific it strongly conditions the magnitude and the composition of the specific (adaptive or acquired) immune responses. Cellular pathogen recognition receptors (cPRRs) either membrane

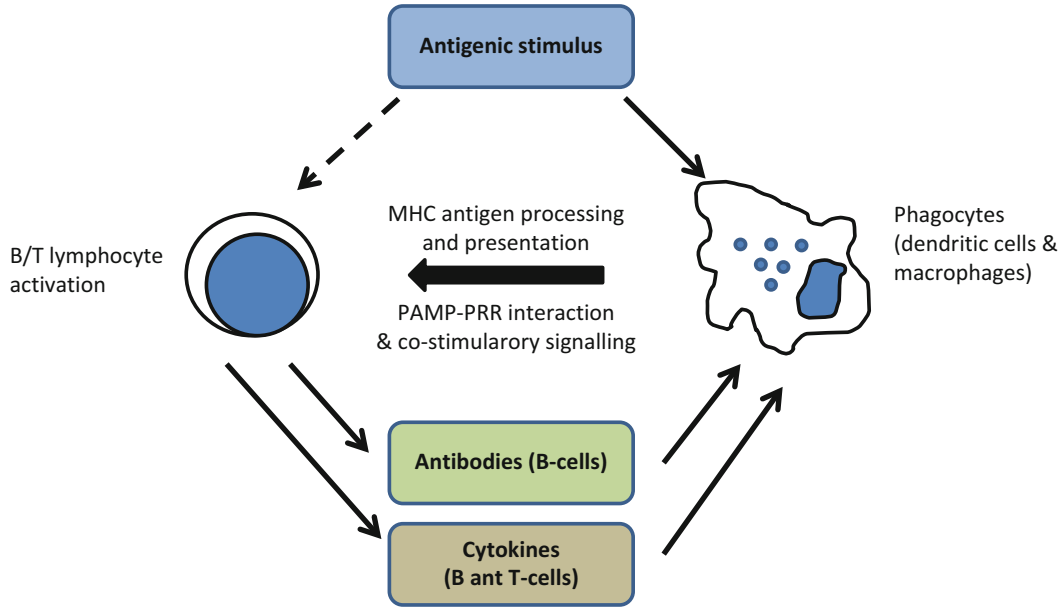

Fig. 2 The cellular cooperation in the immune response. After vaccination, specialized phagocytes present the processed antigens to näive B or T-cells that may become activated only if proper co-stimulatory signals are produced (derived from the interaction of PAMPs with cellular PRRs). Activation drives lymphocytes to secrete soluble mediators and antibodies initiating inflammatory responses (adapted from [33])

bound (Toll-like-, C-type lectin- and scavenger receptors) or cytosolic (NOD-like and RIG-like receptors) of phagocytes eventually bind to pathogen associated molecular patterns (PAMPs) carried on infecting microbes [5]. In particular, encountering of pathogenic virus ligands (such as single or double stranded RNA) to intracellular PRRs activates the phagocytic cells from a normal quiescent state by inducing NFκB-mediated gene transcription of a number of co-stimulatory molecules, proinflammatory cytokines and chemokines as well as IRF-mediated transcription of type I-interferons (IFNs) and other cytokines such as IL-1β and TNF-α [6]. Other immune cell types such as the natural killer (NK) cells express functional TLRs specifically for detecting viral PAMPs and can be also activated by type-I interferons [7]. NK cells can eliminate cells in which expression of MHC molecules is reduced upon viral infection. NK cells secrete IFN-γ which in turn can enhance the phagocytic activity of macrophages and antigen presentation by mature dendritic cells (DCs), a key player in the bridging of innate and adaptive immunity. DCs signaling to naïve lymphocytes will determine whether these cells should be eventually involved in fighting against the viral infection. This fact is exploited by those vaccines based in attenuated viruses or in replicating live virus vectors where the initiation of innate immune responses greatly aug-

ments the quality and magnitude of the adaptive response in contrast to that elicited by vaccines based on inert antigens (inactivated virus or subunit vaccines). Recently, the central role of dendritic cells or APCs in regulating the immune response has made antigen targeting to these cells a major subject for specific immune stimulation aimed to improve vaccine efficacy as well as other forms of immunotherapy [8, 9].

Upon näive lymphocyte activation by interaction with DCs the specificity of the immune response is granted and clonal expansion of B and T-cells capable of recognizing the specific antigen will take place. A pool of specific lymphocytes containing memory and effector cells will be expanded as a primary response to the vaccine stimulus. Upon infection and virus antigen encounter the secondary response will be greatly increased, potentially leading to protection and long-lived immunity by specific effector and memory cells (Fig. 3). Therefore, the two main principles to be exploited by vaccines are necessarily specificity and memory. When designing vaccines the issue of specificity is crucial for the success of a vaccine and can be approached by an adequate selection of the antigen fraction, whole antigen or antigens of choice, being able to recall

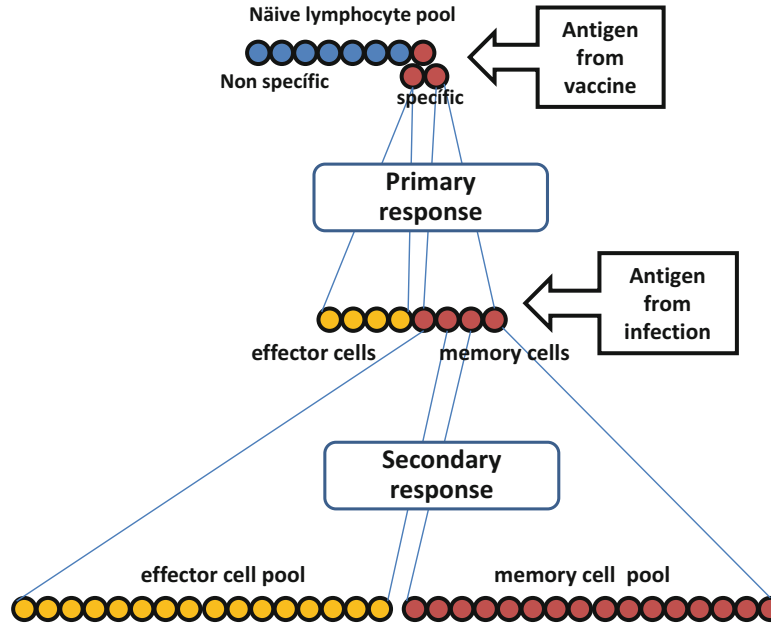

Fig. 3 Vaccination exploits the induction of specificity and immunological memory. A primary clonal expansion of lymphocytes is produced upon activation of naïve T-cells by phagocytes primed with specific vaccine antigens/stimulus. Both effector and memory cell pools are generated that upon encounter with pathogen (infection) will undergo a massive secondary expansion of both cell pools (adapted from [33])

the memory lymphocyte pools produced in the primary responses after vaccination.

Previous identification of the correlates of protective immunity upon infection is one of the logical approaches for vaccine design, for example including relevant epitopes that induce neutralizing antibodies made upon infection and/or the key T-cell epitopes responsible for helper or cytotoxic functions [10]. Ideally, this knowledge should be derived from the pathogenesis of viral infection in the target species to which the vaccine is intended for but unfortunately these type of studies are often more difficult to perform than in laboratory animals (mainly due to genetic diversity of the outbred species, the lack of reagents and markers for cell phenotype characterization, and the limitation in the number of animals used for experimentation). Nonetheless, in some cases the pathogenesis of other animal models of disease (mainly rodents), correlates well enough with that of the target species and valuable information can be obtained about the immune mechanisms of protection. After the knowledge gathered in the past decades of virus research it becomes evident that, in a general sense, for virus with less complex pathogenesis a successful immunoprophylaxis could be obtained by the generation of an immune response against surface antigens displayed on virions and/or virus infected cells. For other virus (for example poxvirus, herpesvirus, asfivirus, respiratory viruses, and lentivirus) that have developed more complex pathogenesis (i.e., induction of persistence, replication in immune privileged tissues, using immune evasion strategies, induction of harmful host immune responses) the effective vaccine should elicit, in addition to neutralizing antibodies, specific T-cell responses [11].

3 Vaccine Technologies

A first classification of vaccines has been outlined above with respect to the level of immunogenicity elicited (inactivated/killed non-infectious versus live attenuated vaccines). Therefore two broad categories of antiviral vaccines can be considered with respect to the nature of the virus used (live or death) or the relevant antigen (whole or fractionated); in fact all licensed vaccines against viral diseases available to date (both for the medical and veterinary use) could fall in either one of these categories. This dichotomy helps to categorize vaccines into four general types (Table 2).

In this categorization type I vaccines include those produced by means of inactivating methodologies while type II vaccines include all attenuated virus used as vaccines, including those generated by reverse genetics. Type III and IV vaccines include those in which only components or a fraction of the pathogen is used as the vaccine antigen. Thus, type III vaccines would include subunit vaccines, including carrier micro/nanoparticle and virus-like particles

Table 2
A proposed classification for the current vaccine technologies

VIRUS	Whole antigen	Fraction/component
Death	*Type I* (inactivated, killed)	*Type III* (subunit, VLPs, genetic DNA or RNA, killed recombinant vectors)
Live	*Type II* (modified live attenuated, reverse genetics modified)	*Type IV* (recombinant viral vectors expressing antigens)

(VLPs) vaccines produced by recombinant technologies in both prokaryotic and eukaryotic cells and inactivated recombinant vectors expressing heterologous vaccine antigens. In this category, both nucleic acid vaccines as well as peptide based vaccines could be also included. Finally, the type IV vaccines are those delivered by a live viral vector that codes and expresses particular (selected) heterologous vaccine antigens. Obviously, from all these categories further classifications can be made, depending on the formulation of the vaccine (for example, inactivated vaccines can be subdivided into those composed of whole inactivated infected cultures or purified virus fractions), and the type of adjuvant used to augment the immune responses. Attenuated vaccines could include those natural virus isolates with reduced virulence, or attenuated virus generated by serial passages, or virus rescued by means of a reverse genetics approach. Nucleic acid vaccines can be based on DNA plasmids or self-replicating RNA molecules launched by a DNA plasmid encoding a viral replicon. For each technology several methodologies for production or antigen expression can be used and further modifications and formulations applied, therefore the potential combinations that can be tested experimentally are many. The choice of one or another may depend on the experimental (preclinical) data obtained in models of infection if available. Further classification of vaccine technologies could be done on the basis of the main type of immunity provided (mucosal, systemic, humoral or cellular), preferred delivery method (oral, parenteral) or prime-boost combination (*see* Table 3).

4 Type I Vaccine Technologies

Inactivated (killed) antiviral vaccines have being used for long and are based on the disruption of the ability of a virus to replicate by generally chemical or physical methods. Among chemical methods used, formaldehyde and organic compounds such as cyclic esters (β-propiolactone) or binary ethylenimine (BEI) have been most widely used. Other cross-linking agents such as glutaraldehyde can be an option for the inactivation but its use has not been as wide as formaldehyde. Two main caveats of the use of cross-linking agents

Table 3
General features of laboratory (experimental) vaccine technologies

Type	Type of modification	Production platform	Delivery method	Adjuvants	Dosage	Immunity provided	Safety
I. Inactivated	Physical, Chemical	Eukaryotic cell culture	Parenteral	Chemical	Repeated	Humoral and Th responses	+++
II. Live Attenuated	Physical Chemical mutagens, Reverse genetics, Tissue propagation (in vitro in vivo)	Cell culture	Parenteral	none	Single Repeated	Humoral, and cellular including CTL responses	+
IIIa. Subunit & carrier technologies, glycoconjugate and peptide vaccines, microparticle and nanoparticle formulations, virus-like particles		Prokaryotic cell culture, Eukaryotic cell culture, Plant based. Chemical synthesis	Parenteral/mucosal	Chemical/ Molecular	Repeated	Humoral and Th	++++
IIIb. Nucleic acid	VpG, delivery, liposome	Prokaryotic cell culture	Parenteral	Molecular	Repeated	Humoral and cellular	+++
IV. Viral Vector based		Mammalian, Insect, Plant cell culture	Parenteral	None/molecular	Single Repeated	Humoral and	+++

for vaccine preparation can be cited; the first one is the potential for aggregation leading to disruption or modification of antigenic epitopes possibly accounting for the reduced immunogenicity of these vaccines, usually requiring two or three booster doses to maintain adequate and lasting levels of protective immunity. Another problem is the risk for incomplete inactivation leading to exacerbation of disease if the partially (or suboptimal) induced immunity cooperates with infectivity by mechanisms such as antibody dependent enhancement (ADE). In this case, monocytes or macrophages (Fc-receptors bearing cells) can be infected by virus complexed to non-neutralizing antibodies, a process described in dengue virus infections [12]. Finally, another issue with inactivated vaccines is overcoming the differentiation of infected and vaccinated animals not to interfere with the surveillance diagnostics. While formaldehyde reacts primarily with proteins, β-propiolactone (BPL) and binary ethylenimine (BEI) modify mainly DNA or RNA so BPL is expected to maintain a high immunogenicity during the inactivation of viruses. However it has been reported that BPL may also and react to some amino acids including cysteine, methionine, and histidine so certain modification of proteins may also affect the immunogenicity of BPL vaccines. Similarly BEI has been also shown to react with proteins [13]. This compound is used widely for the inactivation of foot and mouth disease virus (FMDV) in the preparation of vaccines. Nonetheless, inactivated vaccines remain as a leading methodology for vaccine production (both for human and veterinary use) in part due to the effectiveness of adjuvants (mainly aluminum salts) in the vaccine formulations overcoming the main issue of limited immunity. In fact, this technology may benefit from other inactivation approaches such as the use of hydrogen peroxide or protonating compounds, such as diethylpyrocarbonate (DEPC). Hydrogen peroxide could inactivate both DNA and RNA viruses (vaccinia virus, LCMV, WNV and YFV) with little damage to the antigenic structure, thus minimizing the effect on immunogenicity. More interestingly, this inactivation approach rendered vaccines able to induce both humoral (neutralizing antibodies) and cellular immune responses including WNV and LCMV specific CD8+ cytotoxic T-cells [14, 15]. Using a histidine-protonating agent such as DEPC it was reported the abolishment of vesicular stomatitis virus (VSV) infectivity and pathogenicity in mice. These animals survived a further lethal challenge and this protection was associated to the induction of neutralizing antibodies [16] although no further reports have arose since the first description. In spite of the advances made in different technologies for stimulating the immune responses the classic inactivation methodology is still broadly used to manufacture many vaccines for veterinary use, in part since manufacturers need to balance carefully the investment needed to adapt their traditional manufacturing processes to the new technologies and the

expected profitability. Other classical inactivation techniques by physical methods have been exposure to several types of radiation: thermal, electromagnetic or ionizing. UV radiation has been one of the most used in human vaccine manufacturing.

5 Type II Vaccine Technologies

Live attenuated virus vaccines are among the most successful forms of vaccines particularly with regards to immunogenic character. The ability to replicate makes these vaccines stronger inducers of innate responses, a feature that critically may influence the outcome of the acquired immune responses as discussed above. Several veterinary and companion animal vaccines are based on attenuated viruses and these types of vaccines are used in the human side as well. The common feature shared by attenuated virus vaccines is the lost of virulence factors while the immunogenicity is maintained. Traditional methods for development of attenuated vaccines were the serial passage or propagation of the virus in heterologous cell cultures or in brain tissue from rodents, suckling mice, rabbits or goats, in particular for veterinary use. Propagation in different tissue usually ends up with a change of tropism. For example, hepatotropic viruses passaged in brain tissues were unable to replicate in liver though acquired neurovirulence. A different approach is to induce mutations with mutagenic compounds such us nucleoside analogous. Temperature sensitive mutants grown at lower temperatures were then unable to replicate at normal temperatures in the hosts. The main advantages of attenuated vaccines over inactivated or killed vaccines or subunit vaccines can be related with a wider presentation of epitopes since, obviously, more proteins will be expressed as a consequence of virus replication into the infected host cell (in the infected cell protein fragments will be presented through MHC-I), and also with the possibility of administration by similar or natural routes of infection (i.e., nasal/mucosal route for influenza vaccines). The immune responses elicited are also similar to that of infections, including triggering of innate immune responses, as well as humoral and/or cellular responses. Importantly, the costs of generation and manufacturing these types of vaccines are usually affordable for the veterinary vaccine markets. On the other side, possible disadvantages of attenuated virus vaccines are the genetic instability, allowing reversion to virulence or lost of replicating phenotype, problems related with immunosuppressed individuals, or deleterious effects of some attenuated vaccines when used in gestating animals. This usually accounts for those vaccines obtained by methods in which the inactivation process is not fully controllable or understood (i.e., serial propagation in tissue culture). Table 4 summarizes advantages and disadvantages between killed and attenuated vaccines. For diseases affecting

several species a vaccine that is safe for a specific ruminant host might not be safe for swine. In general terms it is generally accepted that inactivated vaccines offer less safety problems than attenuated vaccines. Advances in the knowledge of pathogen biology, immunology and molecular biology allowed to carry out more rational vaccine designs so novel alternatives to the attenuated type of vaccines have been developed. Particularly for RNA viruses, the generation of reverse genetic systems (i.e., the ability to rescue fully infectious virus from cloned viral genomes and transcripts) [17] has allowed to develop novel attenuated vaccines with enhanced safety features. For DNA viruses defining virulence and/or immunomodulatory genes allowed its deletion by homologous recombination techniques [18]

In most of the cases the modification of these genomes allowed the introduction to these vaccines of an important characteristic for veterinary vaccines: the possibility of differentiate infected from

Table 4
Most recognizable pros and cons of inactivated and attenuated vaccines

Inactivated vaccines		Attenuated vaccines	
PROS	**CONS**	**PROS**	**CONS**
No risk of infection	May potentiate disease (paramyxovirus, lentivirus, coronavirus vaccines)	Systemic and local immune activation. Humoral and cellular immune responses	Presence of adventitious agents
No residual adventitious agents	Parenteral administration (No mucosal immunity)	Durable immunity	May cause illness
	Low rate of CTL responses	Effective immunity	May loose attenuation
	Low immunity	Low cost of production	Spread to contacts
	Need boosting doses	Easy administration	May loose infectivity
	Expensive manufacturing	Herd immunity (most if vaccine spreads)	Storage limited Risk for pregnancy
		Single dose administration	Interference with live virus (preexisting immunity). Presence of defective interfering particles
			Discrimination of vaccinates and infected animals more difficult
			Immunosuppression

vaccinated animals [19]. This is particularly important when surveillance diagnostic is implemented for example to maintain the condition of a disease-free country.

6 Type III Vaccine Technologies

Once identified, protective antigen fractions or components from whole pathogens can be isolated and/or produced by cloning and expression in heterologous systems (bacterial, yeast, plant, eukaryotic cell). We include in this category both subunit particulate and nucleic acid vaccines. With this approach the specificity of the immune response generated is maximized but the magnitude of the immune response tends to be lower than that of attenuated vaccines. Thus, immune adjuvants, targeting strategies or prime boost regimens might be considered to enhance the immune responses.

Subunit vaccines have several advantages over conventional attenuated vaccines in particular regarding safety and production. Most used systems to produces subunit vaccines are based on bacteria, yeast, insect or mammalian cells. More recently other systems based on non-fermentative approaches such as live organisms have been developed, particularly plants or insects. In plants two main alternatives have been developed, either genetically modified or expressing transiently antigens encoded by plant virus or bacterial vectors. In live insects (Lepidoptera) recombinant baculoviruses can be used to infect insect larvae and transgenic silkworms can be also generated (reviewed in [20]). A particular feature of subunit vaccines is the possibility of generation of virus like particles (VLPs) by co-expression of capsid proteins constituent of virions, but devoid of ribonucleoproteins. Like the viral capsids, the VLPs are composed of a geometrically arranged array of proteins, forming repetitive structures against which soluble antibodies and/or B-cell receptors can interact with high avidity. These structures are thus good inductors of T-cell independent responses. In addition the VLPs can be also internalized and processed by APCs to induce both Th and CTL responses, therefore having the potential to stimulating broader immune responses than monomeric forms of protein subunits. Another advantage of VLPs is that they can be produced in a variety of expression systems (baculovirus, poxvirus, alphavirus replicons, plants, Salmonella, E. coli, yeasts, and so on) and can be engineered in order to even express foreign epitopes or immune-stimulatory molecules in the form of chimeric-VLPs, or by covalent linking of immunomodulators (either linear or cyclic peptides, haptens, glycans). VLPs can be obtained from enveloped viruses by budding from cells expressing the VLP components (such in the case of influenza virus). A more specialized technique

is the reconstitution of viral envelopes in unilamelar liposomes, termed virosomes. These synthetic structures can be also complemented with immune-stimulatory conjugates or even heterologous molecules such as DNA, siRNA, antibody fragments (reviewed in [20]). Perhaps from the veterinary vaccine perspective, the generation of VLP subunit vaccines and derivatives is being hampered by the higher costs for production precluding a more generalized use as a vaccine production technology.

Instead of using whole proteins as antigens, immunogenic epitopes previously identified allows to design synthetic peptides to direct more specifically the immune response. Known B and T-cell peptides and combinations can be included in a peptide vaccine design [21]. One of the advantages of peptides over subunit protein vaccines is the simple production, storage and distribution, as well as the flexibility to introduce modifications or mutations (for those highly changing viruses). In spite of these advantages, peptide vaccination is not yet a generalized approach since it needs a deeper knowledge of the protective immune responses in the host species and the intrinsic lower immunogenicity of peptides over whole proteins. However immunogenicity can be enhanced by multimerization strategies [22] or by the use of micro/nano particulate delivery of covalently attached peptides including or not targeting signals to facilitate interaction with immune cell receptors.

Genetic vaccines were discovered upon gene therapy experiments by Wolff and Felgner when intended to deliver DNA into muscle cells by using cationic lipids containing DNA [23]. In fact DNA uptake was produced even in the absence of lipids and expressed the encoded protein. Thus transcriptional units encoding HA antigens were placed under control of a viral promoter (CMV) so a DNA vaccine against influenza was first described in 1993 [24]. Usually DNA vaccines are delivered by intramuscular or intradermal injections. In the first case muscle cells can be directly transfected and express the protein. Dendritic cells present in the interstitial spaces could uptake the soluble antigen, or take up cells killed by the vaccine, or even being transfected directly. On the other hand, the cytosolic expression of the protein enables its MHC-I processing in either muscle or dendritic cells. MHC upregulation is one of the consequences of innate immunity stimulation by unmethylated CpG motifs upon TLR-9 receptor engagement. The main advantages of DNA vaccines are the ease to design and produce, allows differentiation of vaccinated and infected animals (DIVA), antigen is processed naturally, mimicking the immune response induced by virus replication thus stimulating the development of both cellular and humoral immune response. Finally, as with other vaccine strategies, DNA allows combining several antigens, targeting signals, or immunostimulatory molecules (cytokines and

chemokines) to improve the immune response elicited. DNA vaccination has been so far successful in mice models of disease. The only DNA vaccines licensed to date have been against WNV in horses and VHS in salmonids [20]. However, experimental DNA vaccination in large animals against livestock viral diseases still needs further optimization in order to achieve stronger immune responses (the amount of plasmid needed for immunization may represent a serious disadvantage). This handicap could be addressed by the use of stronger promoters, replicon based plasmids (Alphavirus), increasing plasmid uptake efficiency or by co-delivery of immune-stimulatory molecules. Nonetheless, it remains a very attractive way for a rationale design of vaccines, combining the simplicity of production and the potential use in combined vaccine approaches such as prime boost.

7 Type IV Vaccine Technologies

Recombinant viral vectors constitute a very important platform for vaccine design and experimental vaccination approaches. Virtually any infectious, non-pathogenic, virus can be used to express foreign genes, provided a system for recombinant incorporation and expression has been developed. This has been achieved for different RNA viruses that were previously attenuated by using reverse genetics systems or in DNA virus by means of homologous recombination techniques. Among the DNA viruses used to deliver vaccine antigens Poxvirus (from both orthopoxvirus and parapoxvirus genus), Herpesvirus, Adenovirus and Baculovirus have been the most widely used in experimental vaccine trials. The main advantage of DNA viruses over RNA viruses is related with the higher stability of DNA genomes, greater insertional sites and availability of BAC-DNA clones available making engineering and rescuing of recombinant virus a conventional laboratory task. Additional features include the cytoplasmic replication (with the exception of herpesviruses) and the induction of long lived humoral and cellular immune responses, with emphasis on the strong CD8-T-cell activation that is mediated by attenuated poxvirus and adenovirus infections. On the RNA virus side, several viruses from different families have been used as foreign gene carriers: Alphavirus, Bunyavirus, Coronavirus, Flavivirus, Paramyxovirus, Retroviruses, Rhabdovirus [25]. This has been possible by the establishment of reverse genetics technologies allowing the rescue of infectious virus from a copy of its genome. Paramyxoviruses are very potent inducers of humoral and cellular immune responses conferring complete long-life protection when used as attenuated vaccines. They allow interchange of nucleoproteins or envelope glycoproteins between related family

members giving rise to chimeric viruses for use as bivalent marker attenuated vaccines. In addition they can accommodate additional genetic information for expression of foreign antigens maintaining stability during propagation in cell culture, therefore they can be used also to immunize against pathogenic paramyxovirus and other infectious agents [25]. Attenuated rhabdoviruses (generated by manipulation of the viral glycoprotein and phosphoprotein and/or genome order rearrangement) offer similar characteristics for use as a vector for delivery of foreign genes, including the induction of innate and adaptive immune responses. One additional advantage of this type of vectors is the absence of seropositivity in both human and animal populations [25]. Replication deficient alphavirus have been also modified to express foreign antigens for use as vaccines, and in cancer and gene therapy studies. An interesting characteristic of alphaviruses is the induction of mucosal protective immune responses [26, 27]. For some bunyavirus the identification of virulence genes nonessential for growth in vitro allowed replacement for reporter genes or other viral antigens [28, 29]. As attenuated viruses they are capable of sustain limited replication in the host's enabling the initiation of innate immune responses against the transgene. All this examples outline the number of strategies than can be selected when designing attenuated vector vaccines as well as the possibility to design marker vaccines to elicit protection against several virus pathogens simultaneously (multivalent vaccines).

8 New Approaches for Vaccine Design

The conventional approaches for vaccine design are often not sufficient to provide immunity against highly variable pathogens or when T-cell immunity is crucial for protection. Tools from molecular biology integrating systems biology (genomics, proteomics, structural biology) approaches allow researchers to identify ways to improve the quality of vaccines or identify repertories of potentially protective antigens. For example, high throughput sequencing can identify the presence of adventitious viral pathogens in commercial vaccines, or defective genomes in cell culture lines used for vaccine production. Structural modeling of the interaction of neutralizing antibodies and/or antibody fragments with antigen can uncover the molecular signatures defining protective epitopes (cryptic (hidden) epitopes or involving quaternary structures) being another approach for vaccine antigen (or antiviral compounds) design. Additionally, novel flow and mass cytometry technologies [30] may help to gain deeper knowledge of specific cell types involved in protective immune responses for each viral disease. Finally,

integrating data of vaccine trials, including vaccine antigens, adjuvant usage or in silico epitope prediction algorithms, may allow development of platforms for experimental vaccine antigen candidates [31]. Though these approaches are far from being generalized they hold promise on the future of rationale vaccine design for some relevant viral diseases [32].

9 Concluding Remarks

Transition of successful experimental vaccines to industrial production and manufacturing may become a bottle neck in vaccinology since veterinary vaccines need to fulfill several important requisites, among them environmental and safety issues, manufacturing costs and marketability prospects. Considering that most novel vaccine technologies (other than killed or attenuated vaccines) need to adapt the current production processes, many vaccines will never develop further enough to reach market. Nonetheless, animal vaccine research is a very attractive research field with many advantages and complexity over human vaccine field. Firstly, due the larger number of target animal species or segments (ruminant livestock, poultry, porcine, equine, companion animals, aquaculture, and other animal vaccines), secondly, the lack of deep knowledge in the immune mechanisms and lack of reagents adds more difficulties if immune response characterization is needed. The possibility to test the efficacy of the vaccine prototypes in the target species and study the immune response evoked is one important difference that can speed the process of vaccine development over that of human vaccines. Another important advantage is the possibility of testing more innovative approaches that can be further tested for human vaccine development.

The following chapters illustrate a number of different techniques to provide antigen delivery in order to develop vaccines against viral diseases. Though the number of techniques is not exhaustive, the ones showed can be considered most currently used by laboratory researchers in the field of animal health. The reader will find useful examples for application to a particular viral disease since most of the techniques can be virtually applied to any virus pathogen. Among them, representative protocols for each of the broad categories for vaccine technologies discussed above. More discursive chapters are also included related to different techniques and protocols for analyses of the immune responses, the use of adjuvants as an essential part of vaccines based on non-live organisms, and experiences on the use of DNA vaccination in large animals.

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