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Review article

Mucosal delivery of vaccines in domestic animals

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Abstract – Mucosal vaccination is proving to be one of the greatest challenges in modern vaccine development. Although highly beneficial for achieving protective immunity, the induction of mucosal immunity, especially in the gastro-intestinal tract, still remains a difficult task. As a result, only very few mucosal vaccines are commercially available for domestic animals. Here, we critically review various strategies for mucosal delivery of vaccines in domestic animals. This includes live bacterial and viral vectors, particulate delivery-systems such as polymers, alginate, polyphosphazenes, immune stimulating complex and liposomes, and receptor mediated-targeting strategies to the mucosal tissues. The most commonly used routes of immunization, strategies for delivering the antigen to the mucosal surfaces, and future prospects in the development of mucosal vaccines are discussed.

mucosal / vaccine / livestock / animals / review

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1. INTRODUCTION

Infectious diseases remain the major cause of death and economic losses in animals. One way to reduce this is by vaccination. Although immunization has had a great impact on the economics of livestock production and on animal suffering, today's vaccines produced by conventional means are less than optimal in many respects including virulence, safety and efficacy. Moreover, there is a need to develop new vaccines for new emerging diseases. Thus, novel vaccines and alternative routes of immunization are needed to meet future challenges in the control of human and livestock diseases. Here we will review current strategies for mucosal vaccination and recent progress in this field.

2. RATIONALE FOR MUCOSAL VACCINATION

The primary reason for using a mucosal route of immunization is that most infections affect or initiate the infectious process at the mucosal surfaces, and that in these infections, mucosal application of a vaccine is often required to induce a protective immune response [69]. Prime examples of such infections include gastrointestinal infections with enterotoxigenic *E. coli* (ETEC), rotavirus or calicivirus, and respiratory infections with *Mycoplasma*, influenza virus or respiratory syncytial virus. For most of these infections the induction of local immunity at the site of infection is crucial for optimal protection. The induction of secretory IgA (S-IgA) represents the main effector mechanism of the local adaptive immune response and thus, represents the primary goal for most mucosal vaccines. In addition to S-IgA other immunoglobulins, such as IgG are transudated across the mucosal surface. This is especially important for respiratory and genital infections since the transudation of antibodies is more easily facilitated at these mucosal surfaces than for example in the intestinal tract [69]. In addition to humoral immunity, the induction of cytotoxic T cells (CTL) represents another important goal of mucosal vaccines. However, to date only very few experimental vaccines have been demonstrated to induce CTL so far, most of these vaccines being live attenuated vaccines. Interestingly, it was recently demonstrated that transcutaneous immunization resulted in the induction of mucosal CTL in mice [14]. More research will be necessary to confirm this observation in domestic animals. Other reasons for using mucosal routes of immunization include the practicability of administering the vaccine without injections, and therefore the reduction of injection site reactions, and the possibility of vaccination at a very early age of life in the presence of passively acquired maternal antibodies (Tab. I) [15].

3. GENERAL CONSIDERATIONS FOR MUCOSAL VACCINES

Immunity at the mucosal surfaces is mediated by the mucosa-associated lymphoid

Table I. Advantages of mucosal routes of immunization.

Induces protective immunity at the site of infection
Induces both systemic and mucosal immunity
Effective in the presence of maternal antibodies
No injection site reaction, no needles required
Readily administered (i.e. oral vaccines combined with feed)

tissues (MALT), which represent the largest immune compartment within the body. The main functions of the MALT are to (i) protect the mucosal membranes against infection and colonization with pathogenic microorganisms, (ii) to tolerate antigens derived from ingested food, airborne matter and commensal microorganisms, and (iii) to prevent the development of any potentially harmful immune responses against these antigens in case they breach the mucosal lining [69]. To achieve these functions, the MALT are equipped with highly specific mechanisms, which are discussed in greater detail throughout this special issue of Veterinary Research. However, certain mechanisms are of relevance for the development of mucosal vaccines. For example, it is clear that an early interplay between innate and adaptive immunity is essential for effective immunity against invading pathogens [68]. In fact, it is the first contact between pathogen and innate immune system, for example through recognition via *Toll like receptors* (TLR), complement receptors (CR), C-type lectins, or nucleotide-binding oligomerization domain (NOD) receptors NOD 1 or NOD 2, that determines the outcome of the immune response [54, 98, 127]. Pattern recognition molecules (PRM) for these highly conserved pathogen-associated molecular patterns (PAMP) are found on various types of immune cells including antigen-presenting cells, lymphocytes, and epithelial cells within the mucosal tissues. Of special importance, however, are mucosal dendritic cells (DC), which express a wide variety of PRM [30, 92, 99]. Signaling through these receptors leads to recruitment and activation of DC, which then can either sample antigen directly from the external surfaces or receive it from highly specialized M cells [78, 96, 115, 131, 132]. Within the lymphoid follicle the matured DC then present the antigen to lymphocytes to induce a mucosal immune response. Depending on the initial innate stimulus, the DC can "imprint" the effector cells to selectively home back to certain mucosal sites and shift the type of the immune response to a either Th1 or Th2 type of immune response [30, 99]. Interestingly, the ability of intestinal DC to polarize the immune response is conditioned by IL-10 and IL-6 secreting epithelial cells. In the intestine, this typically results in promotion of a Th2-type of an immune response. Thus, mucosal DC are essential in the early decision making process at the mucosa and in controlling homeostasis versus inflammation [132]. Although not demonstrated in domestic animals yet, it is clear that the stimulation of DC by certain components of the vaccine (adjuvant) represents an important issue to consider for future vaccine formulations (Tab. II).

Table II. Characteristics of an ideal mucosal vaccine.

Safe, no side effects in adult and newborn animals
Stable in gastrointestinal microenvironments
Readily dissolved and administered (spray, injector, oral), small volume
Cross mucosal barrier
Retain antigen at the mucosal surface by targeting to mucosal epithelial cells
Stimulate both innate and adaptive immunity at the mucosa
Contain delivery-systems that greatly enhance vaccine uptake
Ensure homing of effector cells to the site of infection
Induce long term immunity

Following their priming in lymphoid tissues, effector cells leave the site of induction and migrate to the effector site, which is generally the lamina propria at the mucosal surfaces. Effector cell trafficking is crucial for the communication among the various compartments of the mucosal immune system leading to the concept of the "common" mucosal immune system. However, it is clear that beyond the "common" level indeed the mucosal immune system is highly compartimentalized. In fact, some of the mucosal compartments favor the trafficking of effector cells from some inductive sites to certain

Table III. Compartmentalization of the mucosal immune system in mice and humans.

Route	Effective response in	Non-effective response in
Oral	Small intestine (proximal), ascending colon, mammary and salivary glands	Distal large intestine, genital mucosa, tonsils
Rectal	Rectum	Small intestine, proximal colon
Nasal or tonsilar	Upper airway, regional secretions, genital mucosa	Gut
Vaginal	Genital mucosa	
Skin	Gut (?)	

effector sites. Cell trafficking between compartments is facilitated by a complex network of interactions mediated by mucosal addressins, integrins, and chemokines (CC) allowing the tissue-specific migration of immune cells from the inductive to the effector site [85]. Chemokine receptors (CCR) and mucosal homing molecules, such as the $\alpha_4\beta_7$-integrin, are found on effector lymphocytes, and in fact the expression of these receptors is controlled by the imprinting by mucosal DC. For example, in mice only DC derived from Peyer's patches and mesenteric lymph nodes were able to induce expression of $\alpha_4\beta_7^+$ and CCR9$^+$ on CD8$^+$ T cells leading to site specific homing of these effector cells to the small intestinal lamina propria [74, 109]. CCR9, the receptor for the thymus-expressed chemokine (TECK, CCL 25), is expressed on effector B and T cells and mediates the selective migration of these effector cells to the small intestine in humans and mice [19, 87, 120]. Interestingly, CCR9$^{-/-}$ knockout mice display reduced numbers of plasma cells in the small intestinal lamina propria and impaired ability to respond to oral vaccination [119]. Thus, chemokines and their receptors are important molecules in linking inductive sites with particular effector sites ("compartmentalization" of the mucosal immune system). As a result, certain routes are more favorable for inducing immunity at the desired effector site (Tab. III). This knowledge should be taken into consideration for the design of every novel type of vaccine that is to be used for mucosal immunization.

4. TYPES OF VACCINES FOR MUCOSAL DELIVERY

Almost all types of current vaccines including live attenuated, inactivated, subunit and DNA vaccines have been tested for their effectiveness as a mucosal vaccine in a wide variety of species. However, only about 20 vaccines are currently licensed for mucosal delivery. Examples are listed in Table IV. Numerous vaccines are currently in an experimental stage of development, and it is expected that many of them will hit the market in the near future. In general, vaccines containing live microorganisms (live attenuated or vector vaccines) are more effective for mucosal delivery since they rely on the viral or bacterial mechanisms of invasion. However, because of the perceived lower safety profile of these live vaccines and the chance of reversion to virulence via recombination between vaccine and wild type strains [103, 157], novel types of vaccines such as DNA vaccines or recombinant proteins are currently being tested in animal models. It should be emphasized that genetic deletions in virulence genes provides a safer vaccine than conventional attenuation. However, since the effectiveness of these types of vaccines is expected to be much lower than live vaccines, potent adjuvants and delivery systems are needed to enhance the uptake of the vaccine antigen and to increase the induction of immunity. Promising candidates for mucosal adjuvants include bacterial toxins and their non-toxic mutants, bacterial cell wall components, CpG oligonucleotides

Table IV. Currently licensed mucosal vaccines in domestic animals (in North America).

Species	Diseases/pathogens
Bovine	**BHV-1** (i.n.); **rotavirus** (oral); **coronavirus** (oral)
Ovine	–
Porcine	**Transmissible gastroenteritis virus** (i.n.); **rotavirus** (oral); ***Bordetella bronchiseptica*** (i.n.)
Equine	**Equine influenza virus** (i.n.); ***Streptocoocus equi*** (i.n.)
Canine	**Canine adenovirus** 2 (i.n.); **parainfluenza virus** (i.n.); ***Bordetella bronchiseptica*** (i.n.)
Feline	**Feline calicivirus** (i.o.); **feline rhinotracheitis** (i.o.)
Poultry	**Turkey adenovirus** (oral); **infectious bronchitis virus** (oral, i.n., i.o., spray); **Newcastle virus** (oral, i.n., i.o., spray); **infectious bursitis virus** (oral, i.n., i.o., spray); **chicken herpesvirus** (oral, i.n., i.o., spray); **turkey herpesvirus** (oral, i.n., i.o., spray); **reovirus** (oral, i.n., i.o., spray); ***Bordetella avium*** (oral, spray); ***Pasteurella multocida*** (oral)

(CpG ODN), and antimicrobial peptides, also called cationic host defense peptides. However, the broad variety of mucosal adjuvants and their mechanisms of action are discussed in another paper of the special issue of Veterinary Research [32].

5. ROUTES OF IMMUNIZATION

The observation that the various compartments of the mucosal immune system are in permanent contact with each other resulted in a wide variety of possible routes of immunization including oral, intranasal, rectal, vaginal, and intraocular (Tabs. III and IV). Nevertheless, immunization of livestock species and poultry is often limited to oral and intranasal immunization for reasons of practicability. Companion animals, however, and even dairy cattle could be theoretically immunized via all of these routes. As outlined above, immunization via some of the routes favors the accumulation of effector cells in certain compartments. For example, oral vaccination results in strongest immunity in the small intestinal tract, whereas intranasal immunization results in optimal immunity in the respiratory tract (Tab. III). Although some of these observations have not been confirmed yet in domestic animals, one can assume that the compartments of the mucosal immune systems are connected to each other in a similar fashion in domestic species. Thus, depending on the disease and the species to be vaccinated, future mucosal vaccines should be administered according to the site where maximal immunity is needed, and the practicability of vaccination via that specific route.

6. STRATEGIES FOR MUCOSAL DELIVERY

One of the greatest challenges in vaccinology today is the development of novel mucosal vaccines and vaccine formulations that are safe, effective, and yet cost effective (Tab. III). A variety of delivery systems including live vectors, microparticles, and liposomes have been developed for this purpose. Combined with novel strategies to target vaccines to the mucosal surfaces these provide tremendous opportunities to address this challenge. Here we will summarize recent progress on live vectors, microparticulate delivery systems, and novel strategies to target vaccines to the intestinal mucosa.

6.1. Live vectors

One of the most effective ways to deliver vaccines to mucosal surfaces is with live vectors that actually infect the mucosal surfaces. Both viral and bacterial vectors have

Table V. Licensed vector vaccines.

Product	Backbone	Technology	Indication	Company
Raboral	Vaccinia	Vector	Rabies	MERIAL
TROVAC/NDV	Fowlpox	Vector	NDV	MERIAL
TROVAC/AIV H5	Fowlpox	Vector	AIV H5	MERIAL
Recombitek	Canarypox	Vector	Distemper	MERIAL
Purevax	Canarypox	Vector	Rabies	MERIAL
Eurifel FeLV	Canarypox	Vector	FeLV	MERIAL
Eurifel RCCP FeLV	Canarypox	Vector	Feline Combo	MERIAL
ProteqFlu	Canarypox	Vector	Equine Flu	MERIAL
ProteqFlu-Te	Canarypox	Vector	Equine Flu/tetanus	MERIAL
Recombitek WNV	Canarypox	Vector	WNV	MERIAL
Purevax FeLV NF	Canarypox	Vector	FeLV (transdermal)	MERIAL
Gallivac IBDV	Canarypox	Vector	IBDV	MERIAL
HVT-MDV	HVT	Vector	MDV	Intervet
HVT-MDV-NDV	HVT	Vector	MDV/NDV	Intervet
HVT-IBDV	HVT	Vector	IBDV	Biomune
MeganVac-1	Salmonella	Deletion mutant	Salmonella	Avant

NDV (Newcastle disease virus); AIV (avian influenza virus); FeLV (feline leukemia virus); WNV (West Nile virus), MDV (Marek's disease virus); IBDV (infectious bursal disease virus), HVT (turkey herpesvirus). This table was presented by MERIAL at the Marker Vaccine Meeting, Sheman Conference Centre, Ames, IA, April 4–6, 2005. We are thankful to Drs R. Nordgren, J.-C. Audonnet, and H. Hughes, MERIAL.

been developed for vaccine delivery in humans and animals. In general, live vectors allow the delivery of recombinant proteins expressed within the vector itself, or genetic information either integrated into the genome or as plasmid DNA. Genomic and proteomic approaches have helped in understanding the structure and function of a wide variety of infectious agents and through the use of genetic engineering, we are now able to generate a select number of live vaccines that are safer and possibly more effective than conventional vaccines (Tab. IV). By introducing multiple gene deletion mutations in a directed way in the genome of an infectious agent, one can virtually eliminate the agent's ability to cause disease and reversion to virulence, as well as make room for the insertion of genes encoding multiple vaccine antigens. Once an agent is attenuated it can be used to carry genes or plasmid DNA for other pathogens and immunomodulators (e.g. cytokines) thus making it possible to immunize animals to produce protective immunity to various disease organisms at one time.

An ideal live vector would have the following features: (i) non-pathogenic to animals and humans, (ii) easy to manipulate, (iii) relatively easy and cost effective to produce, (iv) contain stable genome, (v) contain well defined sites for insertion of foreign genes, (vi) easy to deliver, (vii) no integration into the host genome, (viii) induce both mucosal as well as systemic immune responses when delivered orally or intranasally. While these are very stringent criteria one can hope that because of the rapid progress in this field these will be achievable in the near future. Indeed many live vectored vaccines are already licensed (Tab. V).

6.1.1. Bacterial vectors

Live bacterial carrier vaccines also have great potential as novel mucosal vaccines,

Table VI. Examples of live bacterial vectors.

Vector	Pathogen / Antigen	Tested in	Reference
Salmonella spp.	*E. tenella* / SO7 :TA4	Chicken	[125]
	E. coli / HL Toxin B	Mice	[39]
	L. monocytogenes	Mice	[33]
	T. parva	Calves	[56]
	Shigella spp.	Mice	[152, 164]
	Corynebacterium diphteriae	Mice	[118]
	PRV	Mice	[142]
Corynebacterium pseudotuberculosis	*Anaplasma marginale* / ApH	Sheep	[107, 108]
	Dichelobacter nodosus / protease		
	Babesia bovis / 11C5		
	Tania ovis / 45W		
Shigella flexneri			
Listeria monocytogenes			
Bacillus anthracis	*Clostridium perfringens* / Iota toxin	Mice	[146]
Lactococcus lactis	IBDV / VP2	Chicken	[38]
	BRV / NSP4		[44]
Erysipelothrix rhusiopathiae YS-1	*Mycoplasma hyponeumoniae* / P97	Pigs	[144]
Bacillus Calmette-Guerin	PRRSV / GP5; M	Mice/pigs	[10, 11] [153]
	Toxoplasma gondii / GRA1	Sheep	
E. coli	PRV	Mice	[143]

IBDV (infectious bursal disease virus), BRV (bovine rotavirus), PRRS (porcine respiratory and reproductive syndrome virus).

with attractive advantages over present-day injectable vaccines [53, 158]. For example, live attenuated bacteria such as *Salmonella* or *Shigella* can target mucosal tissues and deliver the antigen specifically to antigen-presenting cells via bacterial secretion systems [4, 55, 57]. Other bacterial vectors have been demonstrated to carry plasmid DNA across the mucosal surfaces [34, 37, 94, 147] and more recently, bacterial ghosts were demonstrated to represent effective means to deliver plasmid DNA [40]. The first live attenuated bacterial vectors such as *Salmonella* spp. and *Shigella* spp. were created by inserting transposon mutations into the bacterial genomes [151]. These mutations resulted in safer bacteria for delivering a variety of foreign antigens to animals [53, 151, 158]. However, since these initial "proof of concept"-studies various bacterial delivery systems have been optimized for the delivery of recombinant antigens as well as plasmid DNA (Tab. VI) [53].

In general, live recombinant bacteria appear to be attractive vehicles of vaccine delivery as they (i) are inexpensive and can be easily produced economically, (ii) can be delivered orally or intranasally, (iii) can accommodate many different foreign genes, thus protecting animals against several pathogens simultaneously, (iv) can induce both humoral and cellular immune responses and (v) adverse effects can be controlled with antibiotics if necessary. However, one of the major concerns regarding the use of bacterial vectors particularly those derived from attenuated strains is the stability of the recombinant phenotype and the potential reversion to full virulence [101]. In addition, although a number of bacterial vectors

Table VII. Examples of herpesvirus vectors.

Vector	Pathogen / Antigen	Tested in	Reference
BHV-1	PRV / gC	Swine, Cattle	[84]
	FMDV / VP1, Cp-epitope	Cattle	[82, 83]
	BVDV / E2	Cattle	[86]
	BRSV / G	Cattle	[138, 156]
HVT	NDV / HN;F	Chicken	[130]
	MDV /	Chicken	[130]
	IBDV / VP2	Chicken	[35, 162]
PRV	HCV / E1	Swine	[167]
FHV	FCV / prCapsid	Cats	[176]
	T. gondii / ROP2	Cats	[106]

BHV (bovine herpesvirus)-1, HVT (herpesvirus of turkey), PRV (pseudorabies virus), FHV (feline herpesvirus of turkey), FMDV (foot and mouth disease virus), BVDV (bovine viral diarrhea virus), BRSV (bovine respiratory syncytial virus), NDV (New castle disease virus), MDV (Marek's disease virus), IBDV (infectious bursal disease virus), HCV (hog cholera virus), FCV (feline calcivirus), *T. gondii* (*Toxoplasma gondii*).

are capable of inducing humoral and cellular immune responses to passenger antigens, it still remains to be seen if preexisting immunity to bacterial vector will affect booster immunization [101].

Currently, a number of bacterial vectors are being evaluated for delivering vaccine antigens and DNA plasmids to mucosal surfaces for inducing potent immune responses (Tab. VI) [33, 34, 37, 158]. These include commensal microorganisms such as *Lactobacillus, Lactococcus, Staphylococcus, Streptococcus* species, and attenuated pathogenic microorganisms such as *Salmonella, Shigella, BCG, Corynebacterium, Bacillus, Yersinia, Vibrio, Erysipelothrix* and *Bordetella* species [34, 37, 93, 133, 147, 148, 151]. Although significant progress has been made in developing and evaluating bacterial vectors for inducing protective immune responses, there are not many reports of their application to vaccinate veterinary species (Tab. VI).

6.1.2. Viral vectors

Viral vectors represent another potential strategy for efficient delivery of vaccine antigens across the mucosal surfaces [140, 175]. Today, a number of viruses including poxviruses, herpesviruses and adenoviruses have been used as viral vectors for veterinary vaccines (Tabs. V and VII–X) [175]. Efficacy of a viral vector is mainly determined by its host range and tropism, the ability to replicate in the target animal, and the expression of the foreign antigen. Both, the requirement of post translational modifications including proper folding and processing of the antigen and the detailed characterization of viral genomes have led to the development of genetically modified viruses as vaccine delivery vehicles for use in the veterinary field [140, 175]. However, in contrast to the human field, viral vectors for veterinary application are currently designed only for the delivery of vaccines, but not for targeting tumors or delivering genes for gene-therapy. A number of viral vectors are currently being evaluated for mucosal delivery including DNA viruses such as adenoviruses, pox viruses and herpesviruses, and RNA viruses such as Newcastle disease virus (NDV), Venezuelan equine encephalitis (VEE), Semliki forest virus (SFV) and a few retroviruses (Tabs. V and VII–X). For example, a recombinant alphaherpesvirus pseudorabies (PRV) carrying the E1 glycoprotein of the classical swine fever virus

Table VIII. Examples of poxvirus vectors.

Vector	Pathogen / Antigen	Tested in	Reference
Canary pox virus	CDV / HA; F	Dogs	[121]
		Siberian polecat	[172]
		Ferrets	[171]
	WNV / PrM; E	Horses	[105]
Modified vaccinia	FCV / M	Cats	[65]
Virus Ankara	EIV / HA; NP	Horses	[21]
	T. gondii / ROP2	Cats	[134]
Capri pox	Pdp ruminants / HA	Goats	[36]
Fowl pox	IBDV / VP2	Chicken	[22, 25, 66]
	HEVT / hexon	Turkeys	[41]
	BVDV / E2	Mice	[20]
	AIV / H7,H1	Chicken	
Swinepox virus	PRV	Pigs	[166]

CDV (canine distemper virus), WNV (West Nile virus), FCV (feline coronavirus), EIA (equine influenza virus), *T. gondii* (*Toxoplasma gondii*), Pdp ruminants (peste des petits ruminants), IBDV (infectious bursal disease virus), BVDV (bovine viral diarrhea virus), HEVT (hemorrhagic enteritis virus of turkey).

Table IX. Examples of adenovirus vectors.

Vector	Pathogen / Antigen	Tested in	Reference
BAdV-3	BHV-1 / gD	Cattle, Cotton rats	[129, 178]
	BVDV / E2	Cotton rats	[12, 177]
OAV	*Tinea ovis* / 45W	Sheep	[135]
PAdV-3	CSFV / E2	Swine	[62]
	PRV / gD	Swine	[63]
PAdV-5	TGEV / S	Swine	[163]
CAdV-2	CDV / HA; F	Dogs	[47]
CELO	IBDV / VP2	Chicken	[49]
FAdV-8 FAdV-10	IBV / S1	Chicken	[75]
	IBDV / VP2	Chicken	[141]
HAdV-5	PRCV / S	Swine	[23]
	TGE / S	Swine	[161]
	Rabies / G	Skunk	[174]
	Rabies / G	Dogs	[126]
	BCV / HE	Cotton rats	[5]
	FIV / env	Cats	[60]
	PRV / gD	Swine	[2]
	PRV / gD	Rabbit / Mouse	[43]
	BVDV / E2	Cattle	[42]
	BHV-1 / gD	Pigs	[59]
	PRRSV / GP5	Pigs	[52]

BAdV (bovine adenovirus)-3, PAdV (porcine adenovirus)-3;-5, CAdV (canine adenovirus) -2, CELO (cell associated lethal orphan), FAdV (fowl adenovirus)-8;-10, HAdV (human adenovirus)-5, BHV (bovine herpesvirus)-1, BVDV (bovine viral diarrhea virus), BRSV (bovine respiratory syncytial virus), CSFV (classical swine fever virus), PRV (pseudo- rabies virus), TGEV (transmissible gastroenteritis virus), CDV (canine distempervirus), IBDV (infectious bursal disease virus), IBV (infectious bronchitis virus), PRCV (porcine respiratory coronavirus), BCV (bovine coronavirus), FIV (feline immuno deficiency virus), PRV (pseudorabies virus), BVDV (bovine viral diarrhea virus), BHV (bovine herpesvirus)-1, PRRSV (porcine respiratory and reproductive syndrome virus).

Table X. Examples of other viral vectors.

Vector	Pathogen / Antigen	Tested in	Reference
VEE	EAV / G(L); M	Horses	[8]
	Anthrax / PA	Mice	[88]
SFV	LIV / ME; NS	Sheep	[112]
	IBDV / VP2; VP2,4,3	Chickens	[124]
NDV	IBDV / VP2	Chicken	[71]
FFV	FCV / capsid Pr	Cats	[139]

VEE (Venezuelan equine encephalitis), SFV (Semliki forest virus), NDV (Newcastle disease virus), FFV (feline foamy virus), EAV (equine arteritis virus), LIV (Looping ill virus), IBDV (infectious bursal disease virus), FCV (feline calcivirus).

(CSFV) was used by van Zijl et al. [167] to protect pigs against challenge infection with CSFV. Kit et al. [82] used the bovine herpesvirus type 1 (BHV-1) to deliver the viral protein (VP) 1 of foot-and-mouth disease virus (FMDV) to young calves, and Kweon et al. [86] used BHV-1 as a vector for delivering the glycoprotein E2 of the bovine virus diarrhea virus (BVDV) to calves. A recombinant BHV-1 vector was also used by Schrijver et al., who delivered the bovine respiratory virus glycoprotein G to calves [138]. Other examples for herpesviral vectors include the herpesvirus of turkeys (HVT), which has been used to deliver glycoproteins from Newcastle disease virus (NDV) and Marek disease virus (MDV) [130] and the infectious bursal virus (IBDV) VP2 [35] [162]. The feline herpesvirus 1 (FHV) was used for delivering antigens from the feline calicivirus (FCV; [176]) or *Toxoplasma gondii* [106]. Poxviruses represent another type of promising viral vectors. Indeed, canary pox based vaccines have been licensed already or are in the process of getting licensed (Tab. V). Thus, live viral vectors represent powerful tools for delivering vaccine antigens across mucosal surfaces. However, concerns regarding their use are related to safety and stability of the vaccines, the applicability, as well as their effectiveness in the presence of pre-existing immunity. Thus, other strategies for inducing protective immunity at the mucosal surfaces such as particulate delivery systems may prove to be very complementary for vaccinating livestock.

6.2. Particulate delivery systems

In general, non-replicating antigens such as proteins and killed vaccines are poorly immunogenic when given mucosally. This has been attributed to degradation of antigen or inefficient uptake by APC in the mucosae. Studies in laboratory animals have clearly demonstrated that particulate delivery systems can significantly improve the immunogenicity of mucosally administered antigens. However, there are only limited studies in domestic animals and the potential of mucosal immunization with killed antigen remains largely unexploited in veterinary medicine. We will briefly review the delivery of antigens to mucosae using particulate delivery systems (microparticles, immune stimulating complex (ISCOM) and liposomes), and where possible highlight investigations in domestic animals or where antigens from pathogens of veterinary importance have been used.

6.2.1. Microparticles

The enhanced immune responses observed with the use of microparticles for mucosal delivery is related to protecting the antigen from degradation and increasing antigen uptake into specialized mucosa-associated lymphoid tissues [116]. Also, microparticles facilitate presentation by APC via both

MHC class I and MHC class II restricted processing and presentation pathways [102]. Uptake of microparticles occurs predominantly by M cells in the follicle-associated epithelium overlying the Peyer's patches in mice and domestic animals [13, 81]. Interestingly, DC extend their dendrites between epithelial cells to sample antigen in the lumen [131], but the significance of this pathway in particle uptake is unclear. However, the poor uptake of microparticles is a major limitation of all microparticle delivery systems which results in suboptimal responses. Approaches such as targeting microparticles to M cells using lectins [48] as well as incorporating adjuvants such as CpG ODN can improve the potency of microparticles as delivery vehicles.

6.2.1.1. Polymers

A variety of polymers can be made into microparticles including Poly-lactide-co-glycolide (PLG), alginate, polyphosphazenes and starch. PLG is a biodegradable aliphatic polyester used in humans as suture materials and has been extensively investigated for the delivery of micro-encapsulated vaccines. A variety of antigens have been encapsulated in PLG microparticles with successful induction of protective immune responses [117, 165]. Interestingly, oral immunization of mice with ovalbumin (OVA) encapsulated in PLG microparticles induced serum IgG and intestinal IgA responses to a level similar to that induced with cholera toxin (CT) adjuvants [117]. This is significant given that CT is the most potent and widely investigated mucosal adjuvant. Mucosal immunization with antigens encapsulated in PLG microparticles protected animals against challenge with mucosal pathogens. Mice orally immunized with antigen in PLG microparticles were better protected against oral challenge with *S. typhimurium*, than those immunized intraperitoneally (IP) with antigen in Freund's adjuvant [3, 45]. Interestingly, a single oral immunization with fimbriae from *Bordetella pertussis* encapsulated in PLG microparticles protected mice from intranasal challenge with the bacteria [76], indicating that the microparticle delivery system effectively stimulated the common mucosal immune system to protect against infection at a distant mucosal site. PLG microparticles have also been evaluated with DNA vaccines. Mice orally immunized with rotavirus VP4, VP6 and VP7 DNA vaccines in PLG microparticles elicited both rotavirus-specific antibodies and protection against challenge with rotavirus [28, 67]. Since encapsulation of plasmid DNA causes significant damage to the DNA as a result of shear, adsorption of DNA on the surface of PLG microparticles can circumvent this problem. Intranasal (IN) immunization of mice with DNA encoding HIV-1 gag adsorbed on the surface of cationic PLG microparticles induced antibody and cell-mediated immune responses locally and systemically, but no responses were seen with naked DNA [145]. A single IN immunization of mice with synthetic malaria SPf66 vaccine in PLG microparticles greatly improved and sustained systemic Th1 and Th2 responses over conventional alum adjuvant [24]. This suggests that mucosal immunization with microparticles has the potential to deliver the next generation of vaccines against many different diseases. Besides mice, PLG microparticles have also been evaluated in other species. Three of ten adult humans immunized via an intestinal tube with enterotoxigenic *E. coli* (ETEC) antigen (CFA/II) in PLG microparticles were protected, but all ten unimmunized controls developed clinical disease [154]. In contrast, oral immunization of pigs with ETEC antigens in PLG microparticles did not induce any significant antibody response or reduction in *E. coli* shedding, perhaps reflecting differences in immunization procedures [46]. Torche et al. [159, 160] demonstrated phagocytosis of PLG-particles by porcine macrophages in vitro, and assessed their uptake in the intestine via M cells. A single oral immunization in 2-week-old chickens with formalin-inactivated *S. enteritidis* in PLG microparticles elicited a significant

intestinal S-IgA antibody response and protected birds against oral and intramuscular challenge [91]. The use of PLG microparticles may be limited by the fact that PLG is soluble only in organic solvents, which may denature critical epitopes required for inducing protection. Poly-ε-caprolactone (PEC) microparticles, like PLG, are biodegradable polyesters. Oral immunization of mice with an antigen from *Brucella ovis* in PEC microparticles protected mice against IP challenge while antigens in PLG did not [114]. However, a study by [9] using *Schistosoma mansonii* antigens did not support this conclusion.

6.2.1.2. Alginate

Alginate is a naturally occurring carbohydrate (kelp) which polymerizes into particles upon contact with divalent cations. Oral immunization of cattle with the model antigen OVA in alginate microparticles enhanced IgA- and IgG-immune responses in the respiratory tract, providing evidence that oral immunization with microparticles can stimulate the common mucosal immune system in a large animal [17]. Calves immunized IN with porcine serum albumin (PSA) in alginate microparticles developed high level of anti-PSA IgG1 antibodies in serum, nasal secretions and saliva [128]. In contrast, oral immunization did not induce any significant responses, suggesting that IN was more efficient for induction of nasal and serum responses. Rabbits orally immunized with *P. multocida* antigens encapsulated in alginate microparticles developed significantly higher nasal IgA responses [18]. Increased protection against *P. haemolytica* and *Streptococcus pneumoniae* challenge in mice was demonstrated by encapsulating antigens in alginate microparticles following oral immunization [80]. These studies clearly show that alginate microparticles are effective for mucosal delivery of vaccines in small and large animals.

6.2.1.3. Polyphosphazenes

Polyphosphazenes are synthetic biodegradable and water-soluble polymers (no need for organic solvents) with potential as vaccine delivery systems. Procedures for the preparation of poly[di(carboxylatophenoxy)phosphazene] (PCPP) microparticles appear to be relatively simple [122]. IN immunization of mice with tetanus toxoid or influenza antigens in PCPP microparticles induced significant increases in serum IgG in mice [122, 123]. Considering that PCPP microparticles are relatively easy to produce in an aqueous environment, and the polymer has adjuvant activity as well, investigations in large animals are warranted.

6.2.1.4. Other biodegradable polymers

Antigens from *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* coated with acrylic resins (cellulose acetate phthalate and methacrylic acid) to protect them from the low pH of the stomach and released in intestines have been tested and shown efficacy when given orally to swine [89, 90]. Fimbriae of ETEC in a cellulose-based delivery system reduced disease in experimentally infected pigs [150].

6.2.2. ISCOM

ISCOM is a small 40 nm nanoparticle composed of saponin (adjuvant), lipids and antigen, and has been described as an antigen delivery system because it not only has adjuvant activity and but also the ability to target APC [111]. ISCOM is a versatile delivery system that allows incorporation of additional adjuvants and targetting molecules. The mechanisms whereby ISCOM are so effective in inducing immunity is related to the ability to stimulate innate immunity by producing cytokines such as IL-12 and modulation of antigen uptake via recruitment of APC [51, 149]. Furthermore, ISCOM target antigens to both the endosomal and cytosolic pathways resulting in both MHC class I and MHC class II restricted immune responses [110]. Numerous studies indicate that ISCOM enhance immune responses to a variety of vaccine antigens in laboratory and domestic animals, and ISCOM are now included in

injectable commercial veterinary vaccines [18, 111]. However, there is also evidence that ISCOM have great potential in the delivery of antigens to the mucosae. Mowat et al. [97, 113] showed OVA incorporated into ISCOM given orally induced serum antibodies, mucosal IgA, Th1 and Th2 CD4 T cell responses and MHC I restricted CTL activity. Mice fed OVA in ISCOM prior to feeding with tolerogenic doses of OVA had elevated OVA-specific antibody responses, indicating that ISCOM may deviate immune response in favor of immunity rather than tolerance [97, 113]. Additionally, OVA in ISCOM induced the recruitment of DC and macrophages into the mesenteric lymph nodes and recruitment of macrophages and B cells in Peyer's patch tissues [51]. Lovgren et al. [95] showed that mice immunized IN with influenza ISCOM were protected against subsequent challenge with influenza virus.

Intranasal immunization with respiratory syncytial virus (RSV) antigens in ISCOM induced high levels of IgA in the upper respiratory tract and in the lungs of mice [70]. Antigens from *Mycoplasma mycoides* subspecies *mycoides*, a cause of severe lung disease in cattle, were incorporated in ISCOM and induced mucosal and systemic IgG1, IgG2a and IgG2b antibody responses in mice [1]. Dogs immunized IN with *Echinococcus granulosus* antigens in ISCOM induced high mucosal IgA responses but no systemic antibody responses [26]. Based on these reports it appears that ISCOM fulfill some important attributes of an effective mucosal delivery system; it contains an adjuvant (saponin) and allows incorporation of additional adjuvants, stimulates the innate immune system and recruits and targets antigen to DC. By incorporating targeting molecules into the ISCOM it should be possible to further enhance their utility as mucosal delivery systems [111]. Further investigations in domestic animals using relevant antigens are warranted to fully exploit the potential of this technology in veterinary medicine.

6.2.3. Liposomes

Liposomes are phospholipid vesicles which enhance immune responses primarily by increasing antigen uptake and presentation [61]. Liposomes have been used extensively in oral and IN delivery of antigens in mice [104]. Since uptake of liposomes by the GALT following oral delivery is a major concern [179], some recent investigations have focused on improving uptake of liposomes and the co-delivery of adjuvants as a way of improving mucosal responses. In this regard, conjugation of liposomes with recombinant B subunit of cholera toxin (rCTB) significantly enhanced mucosal IgA and serum IgG compared to responses in mice immunized orally with unconjugated liposomes [64]. Similarly, the presence of IgA on liposomal surfaces increased uptake of liposomes into the PP mucosa, and the local rectal and colonic IgA responses to ferritin following rectal immunization of mice was about 5-fold higher than those induced by uncoated liposomes [180]. The addition of CT to a liposome-antigen formulation also enhanced responses to ferritin [180]. Addition of the adjuvant monophosphoryl lipid A to VTEC antigen formulated in liposomes induced significant systemic and mucosal antibody responses in mice immunized orally [155]. IN immunization of mice with a commercial inactivated *Yersinia pestis* vaccine in liposomes resulted in significant enhancement of IgG and IgA titers in lung and nasal washes of immunized mice [7]. Interestingly, IN immunization of mice with liposome-encapsulated plasmid DNA encoding influenza hemagglutinin conferred complete protection while all mice immunized with naked plasmid died [169]. Chickens immunized by intraoccular, oral and IN delivery with liposome-associated whole cell extract of *Salmonella enterica* serovar enteritidis developed significant serum and intestinal IgG and IgA antibody responses whereas those immunized with antigen alone had no detectable antibody responses. Interestingly, intraoccular immunization of

chickens elicited better responses than both IN and oral immunization [50]. Apparently intraocular immunization circumvents acids, enzymes and dilution effects encountered by antigen in the respiratory and gastrointestinal tracts.

Liposomes have been successfully used to co-deliver antigen and IL-12 by IN delivery [6]. An alternative to co-delivering cytokines is to use liposomes containing cytokine-inducing immune modulators such as CpG ODN. This would reduce the potential toxicity often associated with large doses of cytokines. Indeed, the adjuvant activity of CpG ODN was augmented by liposomal delivery in mice immunized IN against influenza and hepatitis B viruses [77].

6.3. Other strategies to enhance vaccine uptake

6.3.1. Lectin-mediated targeting

The efficacy of mucosal vaccines is currently limited by very inefficient delivery of the antigen to the mucosal surfaces. Thus, various strategies to selectively target antigens to M cells or enterocytes have been developed in small rodents [73]. One of the most promising current strategies is the use of lectins that specific recognize sugar-residues on the apical surfaces of M cells. For example, the *Ulex europeus* 1 (UEA1), a lectin specifically for α-L-fucose residues, binds almost exclusively to the apical surface of mouse Peyer's patch M cells. UEA1-coated antigens (OVA) were specifically targeted to the M cells in murine Peyer's patches after administration into ligated intestinal loops [48], and resulted in enhanced immunity against OVA. Similarily, incorporation of wheat germ agglutinin lectin (WGA) also enhanced uptake of polymerised liposomes by Peyer's patch, albeit at lower levels than UEA1 [27]. Giannasca et al. reported that an M cell selective lectin administered via the intranasal route targeted to and was endocytosed by respiratory hamster M cells in vivo [58] and Roth-Walter et al. showed that lectins from *Aleuria aurantia* can be used to target M cell for oral allergen immunotherapy [136, 137]. Novel strategies for targeting DNA vaccines to intestinal and respiratory M cells are based on using the M cell specific reovirus protein σ1 as a carrier [170, 173]. *Yersinia pseudotuberculosis* invades via M cells in vivo and this is mediated by interaction between bacterial invasin with cell surface β_1-integrins on M cells [29]. Hussain and Florence mimicked this microbial strategy and showed that *Yersinia* adhesion protein invasion could be used to improve uptake of nanoparticles [72]. However, it remains to be formerly proven whether this strategy can result in significant enhancement of immune responses.

6.3.2. Antibody-mediated targeting

Other strategies for targeting the mucosal surfaces include antibody-mediated targeting to mucosal homing molecules such as the mucosal-addressin cellular adhesion molecule (MAdCAM-1). Mc Kenzie et al. vaccinated mice with OVA and demonstrated that even after parenteral immunization the targeting to MadCAM enhanced OVA-specific antibody responses in gut and serum [100]. Bonifaz et al. [16] recently reported that antibody-mediated targeting of antigens to maturing dendritic cells via the DEC-205 receptor increased the efficiency of mucosal and systemic immunity in mice.

6.3.3. Receptor-mediated targeting

Another strategy of targeting intestinal surfaces is to use antigen-specific receptors. For example, the *E. coli* fimbriae protein (F4) binds to the F4 receptor (F4R) on the surface of porcine intestinal cells [31]. Interaction between ligand and receptor is required for pathogenesis, and only F4R+ pigs develop disease, and interestingly also immunity against *E. coli*. Thus, a possible strategy for mucosal vaccines would be to fuse a vaccine antigen to the F4 in order to retain the antigen within the intestinal tract

and facilitate antigen uptake via M cells and dendritic cells. In fact, Verdonck et al. recently demonstrated that chemically conjugated F4 to human serum albumin (HAS) resulted in enhanced HSA-specific IgA- and IgG responses [168]. Finally, it has also been possible to express a fragment of the non-glycosylated E2 antigen of classical swine fever in *E. coli* and administer the inclusion bodies orally to mice. All vaccinated mice developed IgG and IgA antibodies against the E2 [79]. This is an interesting observation since systemic immunization with inclusion bodies generally is ineffective in inducing immune responses. It is possible that the proteolytic cleavage in the intestine combined with the particulate nature of the inclusion bodies allows uptake and processing of the antigen suitable for induction of immunity.

7. CONCLUSION

The advent of molecular biology and our understanding of the critical antigens involved in inducing protection from infectious diseases has allowed researchers and companies to identify and produce large quantities of putative protective antigens from almost any pathogen. However, the major challenge to effective vaccination now lies in formulation and delivery of these antigens. Our thesis is that even with the best antigen identification production systems, the vaccines will not reach their full potential unless they are formulated and delivered properly. Thus, economic losses and animal suffering will continue.

Since over 90% of all pathogens, regardless of which species, enter and initiate infection at mucosal surfaces, the best target for effective vaccines is the mucosal surface to reduce the ability of the pathogen to get established. Thus, the mucosal surface should be our site for immunization since systemic immunization rarely induces mucosal responses. As a result, systemic immunity, if effective, can only prevent disease but have little impact on the initial infection process. Even if mucosal immunization does not totally eliminate infection, mucosal antibody limits the degree of replication and shedding of the pathogen, thereby, reducing the pathogen load in the environment and consequently dramatically reducing the rate of herd infection and transmission of disease through the herd.

The recent advances in mucosal immunization, especially in rodents are very encouraging since they demonstrate that mucosal immunization is feasible. Whether all vaccines will be given mucosally is debateable since some management systems will make intranasal immunization very difficult. However, one could envisage initial systemic immunization followed by intranasal boosting, thereby, achieving the full benefit of mucosal immunization with only half the challenge of intranasal immunization. A second challenge is the duration of immunity at mucosal surfaces. Since mucosal immunization induces both mucosal and systemic immunity even if mucosal immunity wanes upon exposure to a mucosal pathogen, there will be a rapid response both systemically and mucosally.

Possibly the most exciting development in the past few years is the knowledge that the most effective antigen-presenting cells have specific cell markers. This is allowing us to directly link antigens to antibodies or lectins that specifically bind to the dendritic cell, thereby ensuring that the quantity of antigen needed to induce immunity is extremely small. This makes the production of expensive antigens feasible for livestock vaccine due to the extremely low quantities of antigen acquired (antigen sparing). We envisage that such approaches will become common place in livestock vaccines being developed over the next decade. Thus, we will not only be able to target the vaccines to mucosal surfaces, but ensure that these vaccines are further targeted to the antigen-presenting cells that are monitoring the events occurring at these mucosal surfaces. It is interesting that such targeting can occur with live vectored vaccines, as well as with

subunit and even DNA-based vaccines. Furthermore, by designing formulation delivery systems which focus the immune response to either give a balanced immune response or one skewed to either Th1 or Th2 depending on the pathogen of interest, we can target the response as needed for maximum protection and reduce the consequences of infection from most pathogens. As a result of the recent advances, we are on the brink of unprecedented opportunities to reduce animal suffering, improve the economics of livestock production, and reduce the spread of many infectious diseases from animals to humans. This latter factor is becoming extremely important as the livestock industry finds itself at the crossroads with our continued urbanization. Historically, over 50% of all human diseases were considered zoonotic, however, currently, a full 80% of the new emerging diseases are zoonotic. As our global population increases, there will be more pressure on the agricultural industry to reduce all types of contaminants arising from livestock including infectious diseases.

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