

## Vaccine Development Using Recombinant DNA Technology

### *Animal Agriculture's Future through Biotechnology, Part 7*

#### ABSTRACT

Development of vaccination as a tool in fighting disease has resulted in the potential to combat almost all infectious agents affecting people and animals. The ultimate objective of vaccination is to induce an immune response that subsequently recognizes the infectious agent and fights off the disease. Current public health threats posed by the potential spread of highly infectious disease agents between animals and humans, as well as the emergence of new diseases, impact animal agriculture significantly. Animal vaccinations are among the most effective, successful tools for dealing with these concerns.

This Issue Paper provides a brief historical overview of vaccine development and describes three basic categories of newer, recombinant vaccines: live genetically modified organisms, recombinant inactivated ("killed") vaccines, and genetic vaccines. Separate sections evaluate the development of vaccines for cattle, sheep, and goats; swine; poultry; fish; and companion animals. Within each category, the authors describe the vaccines that are commercially available, outline the recent advances in recombinant vaccines for the control of specific infectious diseases, and discuss the future of vaccines for animal diseases.

Future research needs to focus on vaccine delivery methodology. In addition, new efforts need to target the development of vaccines for economically important diseases for which no currently available vaccines exist, and diseases for which poorly effective vaccines are currently in use. Advances in recombinant DNA technology, in knowledge of the host immune response, and in the genetic makeup of disease agents will lead to new vac-



**In laboratory tests, a vaccine injection with recombinant DNA is used to immunize chicks against coccidia. (Photo courtesy of the USDA Agricultural Research Service.)**

cines against diseases that currently have few or no control measures.

#### INTRODUCTION

Infectious animal diseases continue to rank foremost among the significant factors limiting efficient production in animal agriculture. In addition, infectious agents that are transmitted from animals to humans by way of food and water present an increasing threat to the safety and security of the world food supply and continue to affect human health significantly. Awareness is increasing that animal agriculture could

lose the use of several important antimicrobial agents and drug classes for two reasons: (1) increased resistance among pathogens and (2) public health threats posed by the potential spread of antimicrobial-resistant *zoonotic*<sup>1</sup> microbes. Consequently, new approaches are needed to develop improved tools and strategies for prevention and control of infectious diseases in animal agriculture. Among the most effective and successful of these tools are animal vaccines.

<sup>1</sup> Italicized terms (except genus and species names) are defined in the Glossary.

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Using *recombinant deoxyribonucleic acid (rDNA) technologies*, scientists have been able to develop three types of recombinant vaccines: (1) live genetically modified organisms, (2) recombinant inactivated (“killed”) vaccines, and (3) genetic vaccines. These vaccines no longer cause disease, but still induce a strong immune response. Paralleling the development of new, more efficacious, stable, and safe recombinant vaccines has been the study of vaccine delivery methods and *immunostimulating adjuvant* compounds that enhance the immune response.

Advances in gene discovery of animal pathogens can be expected to identify new proteins and metabolic pathways, thereby providing a foundation for improved understanding of pathogen biology and, ultimately, aiding in the design of new and effective therapies. New treatments, whether vaccines or new drugs, must rely on more than empirical methods of discovery and must be based on a fundamental knowledge of pathogen biology and genetics.

This Issue Paper first provides a brief historical overview of vaccine development and describes the three basic categories of recombinant vaccines. The following sections evaluate the development of vaccines for cattle, sheep, and goats; swine; poultry; fish; and companion animals. Within each category, the authors describe the vaccines that are commercially available, outline the recent advances in recombinant vaccines for the control of infectious diseases, and discuss the future of vaccines for animal diseases.

## HISTORY AND OVERVIEW OF VACCINE DEVELOPMENT

The history of vaccination dates back to the 1798 studies by Edward Jenner, an English physician who used cowpox virus to immunize people against smallpox (Jenner 1798). Almost 200 years later, the comprehensive smallpox vaccination program established by the World Health Organization eventually led to the worldwide eradication of that disease. That success story is proof of the tremendous potential of vaccination and has led to the development of vaccines against almost all infectious agents affecting people and animals. The ultimate objective of vaccination is to induce an immune response that subsequently recognizes the infectious agent and fights off the disease.

Vaccination usually is accomplished with either weakened or attenuated live agents; with inactivated agents that no longer can cause disease; or with selected, *immunogenic* parts of the disease agent called subunit vaccines. Traditional methods of creating vaccines include using a similar agent that does not cause disease, such as Jenner’s cowpox virus, or passing a pathogenic disease agent through a laboratory host system to weaken or attenuate the agent. Inactivating the disease agent with one or more chemicals also can be used to create vaccines. In addition, extracting, purifying, and using one or more parts of the disease agent can be used to induce a protective immune response.

An immune response is stimulated when a foreign substance called an *antigen* is encountered by the immune

system. The animal’s immune system has the ability to distinguish between a foreign substance, such as the proteins in a virus or bacterium, and its own proteins. It does not matter whether the foreign proteins are from a disease agent or a vaccine against the disease agent, the immune response is similar: when the animal encounters the virus or bacteria again, the immune system recognizes it and, ideally, responds to protect the animal from the disease.

Although vaccination has saved countless lives, it can have both favorable and unfavorable consequences. Certain vaccines—specifically, live vaccines—can revert back to pathogenic organisms and produce disease or, in some instances, even death. The development of rDNA technologies has provided new ways of attenuating disease agents by modifying their genetic makeup, or genomes, to create safer, more efficacious vaccines.

The genome of all living beings is made up of many genes that define the characteristics of the organism. The genetic material consists of nucleic acids (DNA and ribonucleic acid [RNA]) that carry and convey genetic information through their bases (adenine, cytosine, guanine, and thymine); in RNA, thymine is replaced by uracil). The bases are uniquely ordered to make up the sequence of the particular gene. Modifying or deleting the genes responsible for causing disease in an organism can be accomplished in the laboratory using rDNA technologies.

Typically, rDNA technology refers to laboratory methods used to break and recombine DNA molecules from differ-

ent organisms. The terminology also has grown to include laboratory methods used for gene isolation, sequence modification, nucleic acid synthesis, and gene cloning.

Using rDNA technologies, scientists can isolate a disease agent, reduce it to its basic components, examine its genetic makeup, and modify it so that it no longer causes disease but still induces a strong immune response. Methods of extracting and purifying genes from extremely small amounts of even the tiniest organisms have become routine for many laboratories. Once extracted, the nucleic acids can be modified and the genes reinserted into the organism to produce a vaccine that is attenuated and/or capable of inducing better immunological protection.

Vaccine development using rDNA technologies requires a thorough understanding of the disease agent, particularly the antigens critical for inducing protection. In addition, it is important to understand the *pathogenicity* of the disease agent and the immune response of the host, to ensure that the vaccine induces the appropriate immunological reaction. Increasingly, genetic information from both microbial genomics studies and *proteomic studies* is being used to gain a better understanding of the interactions between the disease agent and the host. Nonetheless, vaccines developed through recombinant technologies are designed to be safer, more efficacious, and/or less expensive than traditional vaccines.

## Categories of Vaccines

Recombinant vaccines fall into three basic categories: live genetically modified organisms, recombinant inactivated vaccines, and genetic vaccines (Ellis 1999).

### Live Genetically Modified Vaccines

The first category—live genetically modified vaccines—can be viruses or bacteria with one or more genes deleted or inactivated, or they can be vaccines carrying a foreign gene from another disease agent, which are referred to as vaccine *vectors*. Deletion or gene-inactivated vaccines are developed to attenuate the disease agent. Generally two (double-knockout) or more genes are deleted or inactivated so the vaccine

remains stable and cannot revert to a pathogenic agent (Uzzau et al. 2005).

Developing a vaccine of this type requires knowledge of the gene(s) responsible for pathogenicity and assumes that those genes are not the same genes governing viability and the ability of the modified organism to induce an immune response. Examples of gene-deleted vaccines include a *Salmonella* vaccine for sheep and poultry and a pseudorabies virus vaccine for pigs.

Another relatively recent method of creating a live genetically modified vaccine is to use an infectious clone of the disease agent. An infectious clone is created by isolating the entire genome of the disease agent (usually viruses) in the laboratory. This isolated or cloned genome can be specifically and purposefully modified in the laboratory and then used to re-create the live genetically modified organism.

Vector-based vaccines are bacteria, viruses, or plants carrying a gene from another disease agent that is expressed and then induces an immune response when the host is vaccinated. For viral and bacterial vectors, the vaccine induces a protective response against itself (the vector) as well as the other disease agent. Foreign genes must be inserted into the genome of the vaccine vector in such a way that the vaccine remains viable.

The first commercial vaccine vector was VectorVax FP-N (Zeon Corporation, Japan), a vaccine primarily used in turkeys; it consists of a fowl pox vaccine virus that carries genes from Newcastle disease virus. Other agents used as vectors of foreign genes are *Salmonella*, herpesviruses, adenoviruses, and adeno-associated viruses. Edible plant-derived vaccines take advantage of the ability of some antigens to induce an immune response when delivered orally. Foreign genes from disease agents have been inserted into potatoes, soybeans, and corn plants and fed to animals; the expressed proteins from those foreign genes immunized the animals against the disease agent (Streatfield 2005).

### Recombinant Inactivated Vaccines

The second category—recombinant inactivated vaccines—are subunit vaccines containing only part of the whole organism. Subunit vaccines can be *synthetic peptides* that are synthesized in

the laboratory and represent the most basic portion of a protein that induces an immune response. Subunit vaccines also can consist of whole proteins extracted from the disease agent or expressed from cloned genes in the laboratory. Several systems can be used to express recombinant proteins, including expression systems that are cell free or that use whole cells. Whole-cell expression systems include prokaryotic (bacteria-based) systems such as *Escherichia coli*, and eukaryotic (mammalian, avian, insect, or yeast-based) systems.

The baculovirus expression system is a widely used eukaryotic system because it is applicable to many different proteins and because relatively large amounts of protein can be produced. Baculovirus expression systems are engineered specifically for expression of proteins in insect cells. It is important to express proteins of disease agents with the greatest possible similarity to the authentic molecule so that the proper immune response is induced when the proteins are used as a vaccine. Baculovirus expression systems are effective for properly modifying recombinant proteins so they are antigenically, immunogenically, and functionally similar to the native protein.

Another type of recombinant subunit vaccines, called virus-like particles (VLPs), can be created when one or more cloned genes that represent the structural proteins of a virus are expressed simultaneously and self-assemble into VLPs. These VLPs are immunogenic (i.e., they look like the virus on the outside, but cannot replicate because they do not contain any genetic material on the inside).

Because subunit vaccines do not replicate in the host, they usually are administered (injected) with an adjuvant, a substance that stimulates the immune system of the animal to respond to the vaccine. The mechanisms of action of many adjuvants are poorly understood; consequently, they usually are selected on a trial-and-error basis. Adjuvants can enhance the response to a vaccine by protecting the vaccine from rapid degradation in the animal; these are usually oil-based adjuvants. Or, they can attract so-called antigen-presenting cells, which process and deliver antigens to the immune system. Adjuvants also can be molecules that enhance the immune



response by stimulating immune cells directly, or by stimulating immune-modifying and immune-strengthening or *immunopotentiating* substances called *cytokines*. In addition, adjuvants can be a combination of these types.

### Genetic Vaccines

The third category of recombinant vaccines is referred to as genetic vaccines because they are actually DNA alone. Genetic or DNA vaccines usually are circular pieces of DNA, called plasmids, which contain a foreign gene from a disease agent and a promoter that is used to initiate the expression of the protein from that gene in the target animal (Rodriguez and Whitton 2000). Plasmids can be maintained in bacteria (usually *E. coli*) and have been designed to accept foreign genes for expression in animals. The recombinant plasmids containing a foreign gene are purified from the bacteria, and the “naked” DNA is injected directly into the animal, usually intramuscularly or intradermally (into the skin). The animal’s cells take up the DNA, and an immune response is induced to the protein expressed from the foreign gene.

In addition to genes coding for immunogenic proteins, genetic vaccines also have been designed to include different immune-stimulatory genes that trigger different compartments of the immune system, depending on the type of immunity desired. Unique features of DNA vaccines are intrinsic sequences embedded in the DNA, so-called *CpG motifs*. These unmethylated motifs were shown to act as an adjuvant, stimulating the innate immune responses and enhancing the effectiveness of the vaccine.

The following sections describe commercially available recombinant vaccines for ruminants, swine, poultry, fish, and companion animals. In addition, each section addresses recent advances in recombinant vaccines for control of infectious agents in those animals, as well as future vaccine technologies being explored for animal health and protection.

## RECOMBINANT VACCINES FOR CATTLE, SHEEP, AND GOATS

Clinically and economically significant diseases of cattle include the bovine

respiratory disease complex (shipping fever) in feedlot cattle, calf-scours and respiratory diseases in cow-calf operations, and abortions. Pathogens commonly vaccinated against in cattle are *Leptospira* sp., *E. coli*, *Clostridia* sp., *Mannheimia haemolytica* (also sheep and goats), *Haemophilus somnus*, infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD), bovine respiratory syncytial virus (BRSV), parainfluenza-3 (PI-3), rotavirus, and bovine coronavirus (BCoV). Two weeks before weaning/arrival at the feedlot, cattle typically are vaccinated against respiratory disease and clostridial infections, as part of prearrival conditioning. Vaccines used in sheep and goats include products for “overeating disease” caused by a bacterium, *Clostridium perfringens* types C and D, tetanus toxoid, sore mouth (contagious ecthyma) caused by poxvirus, diarrhea caused by *E. coli*, infectious causes of abortion in sheep (*Campylobacter fetus* and *Chlamydia psittaci*), footrot in sheep caused by two bacteria (*Bacteroides nodosus* and *Fusobacterium necrophorum*), and PI-3 respiratory disease in lambs. Nearly all vaccines for cattle, sheep, and goats currently licensed by the U.S. Department of Agriculture (USDA) were developed using conventional technologies.

### Commercially Available Recombinant Vaccines

Recombinant bovine vaccines have not been licensed for use in the United States. The European Union, however, has approved a naturally occurring glycoprotein E (gE)-deleted IBR vaccine for its IBR eradication program. Infectious bovine rhinotracheitis is a herpesvirus responsible for respiratory disease in feedlot cattle as well as for reproductive diseases, conjunctivitis, and nervous disorders. Deletion of the gE gene attenuates the IBR virus and prevents it from being shed in bovine secretions, limiting its transmission. This modified-live, naturally occurring, gene-deleted vaccine is immunogenic and safe for cattle of all ages.

An important advantage of this vaccine is that the gE-deleted vaccine virus and wild-type IBR viruses can be differentiated by the polymerase chain reaction, a method of amplifying very small amounts of nucleic acid to detectable levels (Schynts et al. 1999). Thus, the

vaccinated cattle can be distinguished from naturally infected animals, which is critical for eradication of this disease.

There are no commercially available recombinant vaccines used for sheep and goats in the United States.

### Recent Advances

Bovine viral diarrhea virus (BVDV) causes respiratory, enteric, and eye infections, as well as abortions, in cattle. The virus is thought to play a role in bovine respiratory disease complex because it causes an immunosuppression that leads to bacterial pneumonia (shipping fever). The E2 surface *glycoprotein* is a major immunogen on the virus, and DNA vaccination using the E2 protein gene of BVDV provides protection against the disease (Nobiron et al. 2003).

A more recent study found that DNA vaccination with the E2 protein gene followed by a boost vaccination with recombinant E2 protein provided good protection against the pathogenic NY-strain of BVDV (Liang et al. 2006). Another genetic vaccine tested in cattle used RNA from a vaccine strain of BVDV in a microparticle bombardment method (Vassilev, Gil, and Donis 2001). The vaccinated cattle developed protective neutralizing antibodies. In addition, a genetically engineered E2-deleted BVDV isolate, which can be propagated efficiently in an E2-expressing cell line in the laboratory, will infect and induce an immune response in cattle. But because it is lacking E2, it cannot produce infectious virus particles, making it extremely safe (Reimann, Meyers, and Beer 2003).

Likewise, a *gene-deleted* vaccine for BRSV does not produce infectious virus particles after vaccination. Bovine respiratory syncytial virus, a cause of pneumonia, plays an important role in shipping fever of cattle. The G spike protein located on the surface of the virus is one of the major immunogenic viral proteins. Genetically engineered BRSV lacking the G spike protein gene still can be propagated in cell culture, and vaccinated calves produce neutralizing antibodies, but no infectious virus particles are formed (Schmidt et al. 2002).

Capripox causes diseases such as sheep pox and goat pox in domestic small ruminants. It also can cause lumpy skin disease in cattle and possibly in buffalo. Capripox vaccine is a poxvirus

being used as a vaccine vector for two important sheep and goat diseases—rin-derpest and peste-des-petits-ruminants. Those diseases are caused by a paramyxovirus that is not endemic in the United States.

## Future Developments

The process of distinguishing infected from vaccinated animals, known as the DIVA approach, is essential for disease eradication programs. The use of gene-deleted or *marker vaccines* provides a method to distinguish vaccinates from naturally exposed animals by testing for antibodies directed against the marker in the vaccine or proteins unique to the wild-type virus. One example is a BVDV gene-deleted vaccine that was modified to inactivate the RNase gene within the viral glycoprotein E gene. That deletion resulted in an attenuated virus, which could be an effective vaccine against BVD (Meyer et al. 2002). Control programs targeting the removal of persistently infected animals use vaccination as part of an overall strategy to achieve a status of BVDV-free. Those programs would benefit greatly from such a marker vaccine.

Vaccines for *Mannheimia haemolytica*, the major pathogen involved in shipping fever in cattle and pneumonia in sheep and goats, have focused on its *leukotoxin*, which is the primary *virulence* factor. The leukotoxin gene has been cloned and expressed in cell lines as well as in plants, and the recombinant protein induces leukotoxin-neutralizing antibodies (Lee et al. 2001). *Immunodominant epitopes* on a major *M. haemolytica* surface lipoprotein, designated PlpE, also have been identified as potential vaccine candidates, and antibodies against them are effective in complement-mediated *M. haemolytica* killing. A recombinant PlpE protein was expressed in the laboratory, added to a commercial *M. haemolytica* vaccine, and shown to enhance protection in calves (Confer et al. 2006).

Bovine coronavirus causes calf diarrhea, or scours, infecting the entire length of the intestinal tract. Recombinant vaccines against BCoV are difficult to develop because it is hard to reproduce the viral proteins accurately in the laboratory. Viral *hemagglutinin-esterase* (HE) and *spike surface proteins* have been used as subunit vaccines

against BCoV, but only partial protection has been induced. A recombinant human adenovirus vector expressing the BCoV HE immunogen was developed, and in a mouse model, antibodies induced against HE were detected in serum and lung washes. It is not clear if this vaccine vector will be effective against BCoV in cattle (Yoo et al. 1992).

Bovine rotavirus (BRV) is responsible for scours in approximately 30% of calf viral enteritis cases. The BRV spike surface protein is cleaved by trypsin into the viral proteins VP5 and VP8. The immunogenic VP8 subunit induces antibodies that can neutralize the virus in the laboratory, making it a good candidate for a subunit vaccine.

A unique protein expression system has been developed using a tobacco mosaic virus vector in tobacco plants. The tobacco mosaic virus vector containing the VP8 BRV gene is used to infect the tobacco plant, and the expressed recombinant VP8 protein is extracted from the plant leaves. After intraperitoneal (IP) inoculation of the material containing the VP8 recombinant protein, antibody responses were detected against BRV in mice and protected the mice against infection with BRV (Perez Filgueira et al. 2004). Moreover, triple-layer VLPs (complete BRV particles have three layers) have been produced using viral proteins expressed using the baculovirus system. Those VLPs were found to induce *lactogenic antibodies*. But like all VLPs, they are safe in that they do not replicate in the host because they do not contain any genetic material.

*E. coli* with the K99 *fimbrial antigen* commonly causes diarrhea in young cattle, sheep, and goats. FanC is the major subunit of this immunogenic protein. Agrobacterium-mediated transformation, a method of delivering foreign genes to plants, has been used successfully to express the FanC subunit of K99 in soybeans as a first step to developing an edible vaccine (Piller et al. 2005). When mice were injected with protein extracted from the soybeans, they developed FanC-specific antibodies and cytotoxic T-cell responses, which demonstrates that the protein expressed in plants can function as an immunogen. It is not clear, however, whether oral vaccination that works in animals with a monogastric digestive tract, such as mice, will work in cattle or sheep, which have a ruminant digestive tract.

A raccoon pox virus expressing a rabies virus glycoprotein has been tested in sheep. In addition, subunit vaccines against parasites such as the cestode parasite *Taenia ovis* in sheep and ectoparasites like ticks have been developed for sheep and goats.

## RECOMBINANT VACCINES FOR SWINE

In the United States, approximately 75% of swine producers vaccinate against common swine pathogens including *Mycoplasma* sp.; porcine reproductive and respiratory syndrome virus; *Erysipelas*; *E. coli* scours; parvovirus; *Leptospira* sp.; H3N2 and H1N1 swine influenza; and bacterial rhinitis caused by *Pasteurella*, *Bordetella*, or *Mycoplasma*. Primarily, conventional vaccines are the ones licensed and used in commercial swine. But rDNA vaccines are being developed to further decrease or eliminate economically important diseases in domestic swine and to prevent reintroduction of these pathogens from feral swine populations.

## Commercially Available Recombinant Vaccines

The DIVA approach, as discussed for cattle, also has been used for the eradication of pseudorabies virus (PRV) in commercially grown pigs in the United States. Pseudorabies is an acute, highly fatal disease of pigs. Although it is caused by a herpesvirus (not the virus that causes rabies), the clinical signs are similar to rabies. Pseudorabies virus glycoprotein I (gI) and glycoprotein X (gX) gene-deleted vaccines were used for PRV eradication in the United States. Piglets intranasally immunized with the gene-deleted vaccine can be distinguished from infected animals by a differential enzyme-linked immunosorbent assay (ELISA) that detects the presence of antibodies against gI and/or gX, indicating that the animals were exposed to pathogenic PRV (Swenson, McMillen, and Hill 1993). The USDA-licensed gene-deleted vaccine pairs well with the PRV *differential ELISA test* (IDEXX Laboratories, Westbrook, Maine). As of May 2005, the United States achieved pseudorabies-free status (Stage V), and vaccination no longer is practiced in commercial swine.



## Recent Advances

Progressive atrophic rhinitis in swine is caused by a *Pasteurella multocida* toxin and is characterized by nasal turbinate bone degeneration, distortion of the nose and face, and nasal hemorrhage. Inactivated toxin, or toxoid, which is used as a vaccine, can lose some of its immunogenic properties when it is inactivated. To circumvent that problem but still maintain a safe vaccine, scientists tested fragments of recombinant toxin expressed in *E. coli* for the ability to protect against progressive atrophic rhinitis. Vaccination of piglets resulted in toxin-neutralizing antibodies. Sows immunized with recombinant subunit toxin had high serum antibodies, and their progeny had improved survival after exposure to a lethal dose of authentic toxin compared with animals immunized with conventional toxoid vaccine (Liao et al. 2006).

## Future Developments

The coronavirus transmissible gastroenteritis virus (TGEV) causes severe diarrhea in piglets. The major antigenic protein of TGEV is the spike glycoprotein. Attempts to faithfully re-create these antigenic regions in recombinant vaccines have proved difficult, but several vaccines hold promise. Recombinant pseudorabies virus carrying the TGEV spike gene was constructed and may have potential as a recombinant vaccine against both pseudorabies and TGEV.

In addition, *Lactobacillus casei* and *Salmonella enterica* (serovar typhimurium) expressing the TGEV spike, or portions of the spike protein, have been shown to induce neutralizing antibodies in serum and intestinal secretions of pigs and mice, respectively. A DNA vaccine, an adenovirus serotype 5 vector, and plant-derived vaccines containing the spike or nucleoprotein genes of TGEV also have been constructed and shown to elicit humoral and cell-mediated immune responses in mice.

One of the big problems with inducing immunity in young animals is the presence of maternally derived antibodies against the antigen. To overcome antibody interference of vaccination in swine, a human adenovirus 5 vector, delivering the hemagglutinin and nucleoprotein of swine influenza virus, is

being developed. Because piglets do not have maternal antibodies against human adenovirus 5, the interference observed with conventional swine influenza vaccines does not occur (Wesley and Lager 2006). But if sows are vaccinated with the human adenovirus 5 vector, the maternal antibodies passed to their progeny will interfere with vaccination because human adenovirus 5 is susceptible to neutralization by those antibodies.

Swine mycoplasmal pneumonia is caused by the bacterium *Mycoplasma hyopneumoniae*, which is worldwide in distribution. The disease is a significant burden on the swine breeding industry because it causes losses associated with severe respiratory disease and predisposes pigs to secondary invaders that can cause fatal infections. Although inactivated whole-cell vaccines (bacterins) are safe and currently the most effective means of controlling the disease, they are not always efficacious against *M. hyopneumoniae* because it is difficult to induce an immune response that protects against the clinical signs and lesions associated with this disease.

Thus, rDNA technology is being used to produce subunit vaccines containing the P97 adhesin protein responsible for adherence of the bacterium to swine ciliated epithelial cells. Adhesion is important for virulence, and both local and systemic antibodies have been produced in mice to a recombinant subunit vaccine containing a portion of P97 (the R1 repeat region) coupled to highly immunogenic enterotoxin subunit B from *E. coli* (Conceicao, Moreira, and Dellagostin 2006).

## RECOMBINANT VACCINES FOR POULTRY

The U.S. consumption of poultry meat exceeds consumption of either beef or pork. Currently, the U.S. poultry industry is the world's largest producer and exporter of poultry meat. The tremendous numbers of animals raised in relatively confined environments promotes the spread of infectious disease agents. The fact that individual animals are of so little value makes it economically unfeasible to attempt to treat sick birds. Consequently, the poultry industry—more than any other industry—relies on vaccines to prevent outbreaks

of infectious disease. Nearly all these poultry vaccines are conventional vaccines that have worked well in general. But avian pathogens continue to change and develop ways to evade the immunity induced by the current vaccines. Thus, there is an ongoing race to find or develop new vaccines.

## Commercially Available Recombinant Vaccines

Currently, there are 11 U.S. licensed rDNA poultry vaccines (Table 1). Ten of these vaccines are live recombinant viral vectors designed to deliver specific genes to stimulate the host's immune system. Seven of these use an attenuated fowl pox virus (FPV) as a vector to deliver selected pathogen genes. Three use an attenuated vaccine, Marek's disease virus (MDV), or a closely related non-pathogenic turkey herpesvirus as a vector. Surprisingly, only one license is for a bacterial pathogen, *Salmonella*, which is a double-knockout mutant resulting in a stable attenuated bacterium. Another interesting observation is that the list of pathogens being targeted by rDNA vaccines is small compared with the list of actual poultry pathogens.

Not all poultry pathogens pose the same degree of risk to the industry. One excellent source of information on current poultry pathogens that pose the greatest risk for the poultry industry is the research priorities list established by the Cooperative State Research, Education, and Extension Service, a USDA granting agency. Their 2006 high-funding priority list includes avian *Clostridium perfringens*, Marek's disease, poult enteritis complex (a multifactorial disease affecting young turkeys), avian influenza, and exotic Newcastle disease. The list indicates that current conventional and recombinant vaccines designed to protect against these diseases are not very efficacious. It seems that the industry needs better vaccines than those listed in Table 1 for MDV disease, avian influenza, and exotic Newcastle disease, and that vaccine companies and researchers should consider developing recombinant vaccines for *C. perfringens* and poult enteritis complex.

## Recent Advances

Earlier reviews listed numerous reports of recombinant vaccines under

**Table 1. Currently licensed rDNA poultry vaccines<sup>1</sup> (Source: Data provided by Michel Carr, USDA-Animal and Plant Health Inspection Service.)**

Company	Pathogen	Vector
Lohmann Animal Health	<i>Salmonella typhimurium</i>	Double-deletion mutant
Merial Select	AIV, FPV	FPV
Merial Select	NDV, FPV	FPV
Merial Select	IBDV, MDV	HVT
Intervet	MDV	HVT
Intervet	MDV, NDV	MDV
Biomune Co.	AEV, FPV, LTV	FPV
Biomune Co.	NDV, FPV	FPV
Biomune Co.	FPV, MG	FPV
Biomune Co.	AEV, FPV, MG	FPV
Biomune Co.	FPV, LTV	FPV

<sup>1</sup>AIV, avian influenza virus; NDV, Newcastle disease virus; IBDV, infectious bursal disease virus; MDV, Marek's disease virus; AEV, avian encephalomyelitis virus; FPV, fowl pox virus; LTV, laryngotracheitis virus; HVT, turkey herpesvirus; MG, *Mycoplasma gallisepticum*.

development (Jackwood 1999). One of the simplest recombinant vaccines to construct is a subunit vaccine containing only the immunogenic part of the infectious agent. As an example, researchers have identified *gametocyte* antigens from coccidia that can be administered as a subunit vaccine (Belli et al. 2004). Other researchers have shown that adding immune system modifiers/enhancers, such as *interleukins* or *gamma interferon*, to subunit vaccines can decrease *oocyst* shedding further and induce protective intestinal immunity against coccidiosis (Ding et al. 2004).

In general, subunit vaccines are not as efficacious as whole organism vaccines. One exception seems to be the subunit vaccine developed against the adenovirus that causes egg-drop syndrome. A subunit vaccine using just a portion of the adenovirus fiber protein was shown to be significantly more efficient than the full-length fiber protein (Fingerut et al. 2003).

Despite some success with subunit vaccines, more effort is being applied to making knockout mutants by deleting virulence genes and using the resulting attenuated pathogens as a vector to deliver immunogenic gene products. In one example, the UL0 gene of infectious laryngotracheitis virus (ILTV) was shown to be nonessential for growth in cell culture. Deletion of UL0 resulted in an attenuated virus that could be used as a vaccine to protect birds against challenge with pathogenic ILTV. When UL0 was replaced by the hemagglutinin gene from a pathogenic avian influenza virus, the resulting recombinant virus vector

protected against both ILTV and influenza virus (Veits et al. 2003).

The large genome of the FPV virus, and its ability to incorporate large fragments of foreign DNA, makes FPV-based vaccine vectors a popular choice. Recently, FPV vaccine vectors were made to express antigens from avian influenza (Qiao et al. 2003), infectious bursal disease virus (Butter et al. 2003), and MDV disease (Lee, Hilt, and Jackwood 2003). Future recombinant vaccine efforts will continue the process of refining vectors and inserting evermore immunogenic gene products. But newer, more imaginative approaches to vaccine development ultimately will be needed.

## Future Developments

Advancements in technology and a growing knowledge of avian pathogens are allowing researchers to explore novel approaches to vaccine development. One approach that certainly will increase with time is the use of *immunomodulators*. In mammalian systems, unmethylated CpG motifs were shown to function as vaccine adjuvants, which stimulate innate immune responses. In poultry, CpG *oligonucleotides* were shown to enhance the resistance of chickens to coccidiosis (Dalloul et al. 2004). Other adjuvants, such as starch microparticles, also may prove to be useful (Rydell, Stertman, and Sjöholm 2005).

The DNA vaccines based on bacterial plasmids have been available for many years, are produced easily, and are safe. But DNA-based vaccines often have not been very effective or practical

in poultry. The primary problems have been to deliver sufficient quantities of DNA to elicit an effective immune response, and to mass-vaccinate thousands of birds in a flock. One promising approach currently being studied is the use of lipids or other micro-DNA-encapsulating compounds to protect and deliver the DNA directly to the host cell (Oshop et al. 2003).

Virus-like particles are safe, immunogenic vaccines, but few VLP poultry vaccines have been tested. A VLP vaccine for infectious bursal disease has been described by Rogel and colleagues (2003). That vaccine was produced by expressing viral proteins (VP2, VP4, and VP3) in *E. coli*, which assembled into VLP and protected chickens against infectious bursal disease. Although the vaccine was injected intramuscularly, which is not a practical delivery mechanism for large flocks of commercial poultry, the vaccine does show promise for the future.

One of the most recent technologies adapted to inhibit pathogen growth has been the use of small interfering RNAs (siRNA), which can be synthesized easily and designed to target specific genes, resulting in the inhibition of gene expression. The technology is still new, however, and several problems remain to be addressed adequately. One problem is to protect the siRNA molecule from nuclease degradation; a second is to deliver the siRNA molecules to the appropriate tissues where the pathogen is replicating. A third problem is the inability to predict which of several possible siRNA molecules will function to inhibit gene expression. Often, several different siRNAs must be evaluated experimentally before an effective construct can be identified. Nevertheless, RNA interference has been used successfully to inhibit influenza virus replication in cell lines and embryonated chicken eggs, and it serves as a proof of concept (Ge et al. 2003). Undoubtedly, the use of siRNA technology in future recombinant poultry vaccines will increase.

More important than addressing new technologies, future recombinant vaccines must address the needs of the poultry industry. Injection of individual birds is too time-consuming and not economically feasible. Vaccines should be developed that can be administered to

multiple birds at one time.

Even better would be vaccines that can be administered to the embryo, currently the industry standard for vaccine delivery. Researchers should evaluate their new vaccines in maternal antibody-positive birds. In the field, nearly every day-old chick has maternal antibodies, and testing vaccines in maternal antibody-negative birds does not represent a true picture of the efficacious nature of the vaccine.

In many instances, current vaccines can prevent disease but do not prevent replication of the pathogen and the subsequent transmission of the disease agent from one animal to another, known as horizontal spread. Future recombinant vaccines also must prevent shedding.

Finally, new vaccines should target economically important diseases for which there are no available vaccines, or for which the current vaccines are not very effective.

## RECOMBINANT VACCINES FOR FISH

Vaccines have been used for almost 20 years in commercial aquaculture. The economic finfish vaccine targets include bacterial and viral pathogens affecting marine and freshwater fish. The major marine species of farmed fish include salmon, trout, cod, halibut, cobia, turbot, seabass/seabream, yellowtail, groupers, and snappers. Freshwater species include catfish, tilapia, and carp. Fish generally are vaccinated as fingerlings through immersion, oral administration, or IP.

Salmonids represent the most important segment of the fish vaccine market. Farmed salmon smolts are hand-vaccinated by IP injection during the period when they are transferred from freshwater hatcheries to marine sea cages. The major bacterial diseases include *Yersinia ruckeri* (ERM), *Aeromonas salmonicida* (*Furunculosis*), *Vibrio* species, *Renibacterium salmonicida* (BKD), *Flavobacterium columnare*, *Nocardia* species, *Streptococcus iniae*, *Lactococcus garvieae*, *Psirickettsia salmonis* (SKS), and *Edwardsiella ictaluri* (ESC). Virus pathogens commonly vaccinated for are *infectious haemopoietic necrosis virus* (IHN), *infectious salmon anemia virus* (ISAV), *infectious pancreatic necrosis virus* (IPNV), and

*viral haemorrhagic septicaemia virus* (VHSV).

## Commercially Available Recombinant Vaccines

There currently are no recombinant vaccines registered for fish in the United States. Norway, however, has approved a recombinant IPNV vaccine (Midtlyng et al. 2003); Chile has approved subunit recombinant vaccines for salmon rickettsial septicaemia (SRS) and IPNV; and Canada has approved a DNA vaccine for IHN in farm-raised Atlantic salmon.

The IPNV vaccines are based on the VP2 protein, which is the major component of virus particles and an important antigen. The SRS vaccine contains a major antigenic surface protein, OspA, fused to a measles T-cell stimulating peptide and must be injected. *Psirickettsia salmonis* is refractive to antibiotics and historically has responded poorly to live/live attenuated or single protein vaccination.

Canada's IHN vaccine was the first DNA vaccine registered for use in any animal. A viral coat protein carried on a DNA plasmid and injected behind the salmon's dorsal fin protects northern-farmed salmon against this potentially devastating disease. The plasmids enter muscle cells where the antigenic viral protein is produced, which eventually triggers antibody production and white blood cell (T-cell) stimulation, providing protection against future exposure to the virus. Plasmid DNA is not integrated into the chromosome, and after a few months the cells containing the plasmids die, leaving virtually no traces other than the immunological barriers. This vaccine was a significant step in controlling this disease because attenuated modified live IHN vaccines have been difficult to produce.

## Recent Advances

Viral hemorrhagic septicemia is caused by a rhabdovirus that affects fish worldwide and causes significant mortality in trout. The recombinant, injectable subunit vaccine produced in insect cells infected with a modified baculovirus and containing the major antigenic glycoprotein of the virus was efficacious, but it was never registered with the USDA for commercial use because of high costs (de Kinkelin et al. 1995).

Subsequent experimentation with a DNA vaccine carrying the glycoprotein gene was shown to induce a protective antibody response (Boudinot et al. 1998) and has been remarkably effective. This vaccine may find a niche in protecting trout from this virus.

Infectious salmon anemia virus is a highly infectious enveloped *orthomyxo-like* virus causing acute mortality, primarily in Atlantic salmon. Studies on 160 isolates show that there exist up to eight different combinations of hemagglutinin A and neuraminidase subtypes. As in the closely related influenza A virus, antigenic drift occurs frequently with these highly variable, constantly mutating surface glycoproteins, which are the most important antigens. Conventional vaccines do not provide full protection from the disease, but research on both DNA-based vaccines as well as VLPs shows some promise (Miller and Cipriano 2002).

Infectious pancreatic necrosis virus is a *birnavirus* affecting salmonids worldwide. Live attenuated and killed vaccines are not 100% effective and are expensive. The bi-segmented, double-stranded viral RNA genome includes two structural *capsid proteins*, VP3 and VP2. The VP2 induces neutralizing antibodies, but there is wide antigenic diversity between field strains and serotypes. All isolates, however, show some cross-reaction. Recombinant protein subunit vaccines have been produced, but evidence is controversial as to their overall protective efficacy. Current research is focusing on DNA-based vaccines containing the entire viral polyprotein and the addition of CpG motifs to achieve immunostimulatory activity (Mikalsen et al. 2004).

## Future Developments

Antiviral DNA vaccines continue to receive considerable attention as a new approach and as a solution to fish diseases that are refractive to traditional vaccines. The main advantage of the vaccines is that they mimic a natural viral infection whereby the host reliably reproduces a single viral protein that induces a protective immune response. Production of viral proteins in the natural host elicits both cellular (T-cells) and humoral (antibodies) immune responses, indicating that these vaccines have a high degree of efficacy (Kim et al. 2000;



Leong et al. 1995, 1997).

A major obstacle in the progress of DNA vaccines has been delivery of sufficient quantities to elicit protective immune responses. Subunit vaccines also will continue to be developed with multiple antigens (proteins) expressed from a single genetic construct. Progress in adjuvants, manufacturing, and vaccine delivery technologies will be critical for both DNA and subunit vaccines. Oral vaccination delivered in feed would be the preferred method of vaccination for fish. Different approaches to protect the antigen (liposomes, biofilms, alginate beads) from gastric neutralization have yielded promising results.

## RECOMBINANT VACCINES FOR COMPANION ANIMALS (DOGS, CATS, AND HORSES)

The vaccine market for companion animals is growing more rapidly than any other sector of animal health. This growth is caused partly by demographic changes in the human population and partly by the high value associated with companion animals. Currently, companion animals are vaccinated routinely for a variety of diseases. In the future, vaccinations also will address nonpathogenic conditions such as reproduction, as well as cancer and periodontal health.

Canine vaccinations include those for *Leptospirosis*, kennel cough (*Bordetella bronchiseptica*), Lyme disease (*Borrelia burgdorferi*), rabies, distemper, adenovirus, parainfluenza virus, and parvovirus. Feline vaccinations target feline herpesvirus, feline calcivirus, feline panleukopenia, rabies, feline leukemia (FeLV), and feline immunodeficiency virus. Horses commonly are vaccinated for tetanus, rabies, equine influenza, and herpesvirus infections. Depending on the geographic region, however, they also may be vaccinated for West Nile virus (WNV), equine encephalitis viruses (eastern equine encephalitis virus, Venezuelan equine encephalitis virus, western equine encephalitis virus), rotavirus, and equine viral arteritis.

### Commercially Available Recombinant Vaccines

A poxvirus from a canary was developed into a virus vector for use in

nonavian species. Canarypox does not replicate in nonavian species, making it absolutely safe, although it is an excellent vaccine in mammals because it completes one round of replication in nonpermissive cells and induces a robust immune response in the vaccinee. The development of both humoral (antibodies) and cell-mediated (T-cells) immunity and the duration of protection induced by canarypox vectors in mammals are well known (Paoletti 1996). In addition, prior exposure to canarypox in nonavian species does not seem to interfere with subsequent vaccinations.

A number of commercially available canarypox-vectored vaccines have been developed by Merial (Duluth, Georgia). Canine vaccines include RECOMBITEK Lyme for vaccination against Lyme disease, RECOMBITEK Canine Distemper, RECOMBITEK Ferret Distemper (approved for use in ferrets), and PURVAX Canine Rabies.

Commercially available canarypox-vector-based feline vaccines include PUREVAX Feline Rabies, EURIFEL Feline Leukemia, and PUREVAX Feline Leukemia NF. Feline vaccinations are influenced strongly by the appearance of vaccine-associated injection site muscle cell tumors called sarcomas, which are associated epidemiologically with traditional FeLV vaccines and killed rabies vaccines that contain strong adjuvants. The canarypox-vectored vaccine is administered subcutaneously and does not contain an adjuvant, markedly decreasing the inflammation at the injection site, which is believed to be the cause of the injection site sarcomas. The PUREVAX NF canarypox virus carrying the FeLV subgroup A env and gag genes can be given transdermally in the skin without adjuvant to decrease the inflammation possibly associated with the development of injection site sarcomas.

Several canarypox vector recombinant vaccines are commercially available for horses. West Nile virus is a mosquito-borne virus that emerged in the United States in 2002 and caused disease in nearly one-third of all horses. RECOMBITEK WNV (Merial) is a WNV vaccine for horses that induces protective immunity but does not replicate in a horse, making it completely safe. A canarypox-based recombinant vaccine for equine influenza (ProteqFlu, Merial) contains two different canarypox

vectors—one carrying the immunogenic hemagglutinin gene from a U.S. strain of equine influenza and the other carrying the hemagglutinin gene from a European strain of equine influenza virus. The ProteqFlu-Te (Merial) vaccine contains the equine influenza canarypox vectors in a tetanus toxoid diluting agent.

## Recent Advances

A second-generation Lyme disease vaccine based on the C-terminal fragment of the OspA protein of *B. burgdorferi* has been developed to avoid the side effects associated with the whole protein, and as a result, is a safer vaccine. A unique structure-based approach was used to determine the three-dimensional makeup of the protective epitopes and generate a stable antigen for use in the vaccine (Koide et al. 2005). Structure-based methods of vaccine development are a promising new approach that will allow scientists to target critical immunogenic antigens specifically for incorporation into safer, more efficacious vaccines.

Long-term survival after vaccination against malignant melanoma—a skin cancer—in dogs with advanced disease has shown some promise. The so-called *xenogeneic* DNA vaccine carries a human tyrosinase-encoding gene that, when expressed in the vaccinated dogs, results in cytotoxic T-cell responses that function to reject the tumor (Bergman et al. 2006).

Zona pellucida glycoproteins can be used as immunogens for contraception. Contraceptive vaccines based on zona pellucida glycoproteins have been studied for more than 10 years, but recent advances in recombinant vaccine technology—specifically recombinant proteins, synthetic peptides, and DNA vaccines—have led to longer-lasting immunocontraceptive effects and less ovarian pathology (Gupta, Chakravarty, and Kadunganattil 2005). In the future, contraceptive vaccines likely will substitute for the castration of domestic pets, farm livestock, and wild animals.

## Future Developments

Recent research in companion animal vaccines has focused on recombinant vaccine development for parasitic diseases. Resistance to drugs used to control nematodes has led to the study of various secreted larval antigens for elic-

iting protective immunity. Preliminary data in mice for protection against hookworms, which affect dogs and cats as well as people, used DNA vaccines and recombinant hookworm proteins expressed in the laboratory—as well as unique route, delivery, and adjuvant formulations—to induce protection.

Other approaches are being used to identify immunogenic molecules that also are protective against extremely complex protozoan parasites. These approaches include identification of genes in parasites linked to escape from the immune response and the use of microarrays and immune selection to map immunoprotective antigens in parasites. These techniques are being used to identify potential vaccine candidates for the coccidian parasites that cause enteric disease in almost all animals.

## CONCLUSIONS

Vaccines induce an immune response in the animal host that subsequently recognizes infectious agents and helps fight off the disease; vaccines must do this without causing the disease. Using recombinant DNA technologies, scientists have been able to develop live genetically modified organisms, recombinant killed vaccines, and genetic vaccines that no longer cause disease yet induce a strong immune response. Developing vaccines using rDNA technologies requires a thorough understanding of the disease agent, particularly the antigens critical for inducing protection and the factors involved in causing disease. In addition, it is important to understand the immune response of the host to ensure that the vaccine induces the appropriate immunological reaction.

Paralleling the development of new, more efficacious, stable, and safe recombinant vaccines is the study of vaccine delivery methods. In addition to using conventional delivery routes such as oral, intranasal, intradermal, transcutaneous, intramuscular, and IP, scientists are experimenting with needle-free systems and vaccine strategies that allow mass vaccination of many individuals simultaneously.

Another active area of research is the study of compounds with the potential to enhance the immune response to vaccines. These approaches include incorporating immunomodulating com-

pounds into vaccines that can affect the type of immune response directly and immunopotentiating compounds that strengthen the immune response. The antigenic pathway can thus be modulated to stimulate the appropriate arm of the immune response to provide solid protection. Also, new compounds that indirectly stimulate the immune response (such as microparticles and adjuvants) are being studied. These compounds are designed to present antigens to the immune system in such a way that optimal stimulation is achieved.

The promise of better vaccines to benefit animal agriculture and companion animals through rDNA technology is becoming a reality. A number of recombinant vaccines are available commercially, and many more are projected to be available soon, so the future of recombinant vaccines is bright. New efforts need to focus on delivery methodology as well as development of vaccines for economically important diseases for which no currently available vaccines exist or for diseases where poorly effective vaccines are currently in use. Advances in rDNA technology, in knowledge of the host immune response, and in the genetic makeup of disease agents will lead to new vaccines against diseases for which no control measures currently exist.

## GLOSSARY

**Adjuvant.** A compound mixed with a vaccine to enhance the immune response.

**Antigen.** A substance (usually a protein) that is foreign to the animal and stimulates a specific immune response.

**Birnavirus.** A bi-segmented RNA virus (e.g., infectious bursal disease virus).

**Capsid proteins.** Proteins that make up the shell of a virus particle that contains the genetic material.

**Challenge.** The process of infecting an animal with a disease agent to test for protective immunity.

**CpG motif.** A genetic element contained in DNA vaccines that stimulates the immune system.

**Cytokine.** Substances produced by cells of the immune system of the animal that function to stimulate or modify the immune response (e.g., interferon

and interleukins).

**Differential ELISA test.** Detection of antibodies specific to either the vaccine or the disease agent by the enzyme-linked immunosorbent assay.

**Epitope.** A molecular region on the surface of an antigen capable of eliciting an immune response and of combining with the specific antibody produced by such a response.

**Fimbral antigen.** Proteins that make up the hairlike organelles on the surface of bacteria responsible for motility.

**Gametocyte.** A cell that makes up one of the developmental stages in the reproduction of coccidiosis.

**Gamma interferon.** A cytokine that enhances the antimicrobial properties of the immune response.

**Gene-deleted.** An organism with one or more genes removed from the genome to render it nonpathogenic so it can be used as a vaccine.

**Glycoprotein.** A biomolecule composed of a protein and a carbohydrate.

**Hemagglutinin-esterase.** A cell attachment protein on the surface of some viruses that recognizes sialic acid residues on the cell surface and has both the ability to agglutinate red blood cells (hemagglutinin) and receptor-destroying activity (esterase) thought to release the virus from the cell surface.

**Immunodominant epitopes.** Regions of a protein that stimulate the production of antibodies.

**Immunogenic.** Having the ability to stimulate the immune response.

**Immunomodulators.** Compounds capable of stimulating or down-regulating the immune response.

**Immunopotentiating.** Strengthening of the immune system.

**Immunostimulating.** Capable of stimulating an immune response.

**Interleukins.** Cytokines produced by cells of the immune system that function to enhance inflammation and activate the immune response.

**Lactogenic antibodies.** Antibodies secreted in milk that are important for providing protection to progeny.

**Leukotoxin.** A virulence factor secreted from some bacteria that kills host phagocytic cells in a species-specific manner.



**Marker vaccine.** A recombinant organism containing a foreign gene in which, when used as a vaccine, the foreign gene or antibodies to the expressed protein from the foreign gene can be detected. Marker vaccines or animals receiving marker vaccines can be detected with specific diagnostic tests.

**Oligonucleotides.** A linear nucleic acid fragment (not necessarily DNA) consisting of 2–10 nucleotides joined by phosphodiester bonds.

**Oocyst.** Egg stage of the coccidian parasite.

**Orthomyxo-like virus.** A virus similar to viruses in the family *orthomyxoviridae* (e.g., influenza).

**Pathogenicity.** The ability to cause disease.

**Proteomic studies.** Studies on the entire protein makeup of a cell, including protein–protein interactions, protein modifications, protein functions, and the genes involved in expression of those proteins.

**Recombinant deoxyribonucleic acid (rDNA) technologies.** Denotes any manipulation of DNA in the laboratory including, but not limited to, cloning, polymerase chain reaction, restriction enzyme digestion, ligation, nucleic acid replication, reverse-transcription, and ribonucleic acid synthesis.

**Refractive.** Not affected by. (In this context, antibiotics do not have an inhibitory effect on the organism.)

**Spike surface proteins.** Petal-like projections on the surface of a coronavirus particle that are involved in attachment of the virus to the host cell, among other things.

**Synthetic peptides.** Short 2–30 amino acid molecules synthesized through chemical reactions in the laboratory.

**Vectors.** In recombinant DNA technology, it can be (1) a self-replicating molecule of DNA that serves to transfer a gene or foreign DNA fragment from one organism to another (usually bacteria) or (2) a virus or bacteria containing a foreign gene that is used to vaccinate an animal.

**Virulence.** The severity of the disease caused by an infectious agent.

**Xenogenic.** Derived or obtained from an organism of a different species.

**Zoonotic.** Relating to a disease that is communicable from animals to humans under natural conditions.

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