# INFORMATION FOR THE PUBLIC

## -ENGLISH-

# LABORATORIOS HIPRA, S.A.

## Information for the public

Evaluation of the safety and the efficacy of a live vaccine in the control of porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae*

European Notification number

B/BE/11/V3

The release of genetically modified organisms (GMOs) in the environment is strictly regulated at European level by Directive 2001/18/EC and at Belgian level by the Royal Decree of 21 February 2005 "regulating the deliberate release and/or marketing of GMOs or products that contain GMOs into the environment".

To ensure the safe use of GMOs, the provisions of the Royal Decree above stipulate that the release of GMOs for experimental aims is prohibited without prior consent from the competent Minister. The decision is based on a thorough evaluation of the biosafety of the planned release, which is conducted by the Biosafety Advisory Council, composed of different Scientific Committees grouping independent experts from Belgian Universities and governmental institutes.

To acquire the necessary authorization from the competent minister, the Company LABORATORIOS HIPRA, S.A. submitted an application dossier to the competent authority. On the basis of the advice of the Biosafety council, the competent minister could grant a permission to the company LABORATORIOS HIPRA, S.A. to conduct experiments with *Actinobacillus pleuropneumoniae* genetically modified live vaccine as stipulated in the application PENDING.

The release will take place at different locations in Flanders.

It is expected to start within 3 months from the authorization for the release.

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# GENERAL INFORMATION

***Description of the genetically modified micro-organism (GMM)***

The strain HP-3276 included in the vaccine PB-116 is a modified live *Actinobacillus pleuropneumoniae* strain. *Actinobacillus pleuropneumoniae* (thereafter “App”) is a Gram-negative bacterium which causes porcine pleuropneumonia, a worldwide distributed infectious disease responsible of important economic losses in the swine industry.
The strain HP-3276 is characterized by modification of specific genomic sequences corresponding to virulence factors of App. These result in a App strain with reduced toxicity while its immunoprotective capacity remains unchanged. The strain HP-3276 lacks pathogenic activity and constitutes an attenuated strain when compared to the pathogenic properties of the parent strain. It is therefore suitable for developing a live attenuated vaccine against swine pleuropneumonia.

The vaccine PB-116 is intended for use in pigs at eight weeks of age and the vaccination program includes a second dose to be administered 3 weeks later.

***Type and purpose of the envisaged trial***

Once confirmed the safety and the efficacy of the vaccine in pigs under laboratory conditions, the aim of the field study is to assess the safety and the efficacy of the vaccine in the field, as it is required by the EU legislation.
This is will be multicentric study in which pigs from several farms in Flanders will be vaccinated by intramuscular route with the test vaccine according to the vaccination program that is to be proposed for the product. In addition to pigs vaccinated with PB-116, each farm will contained a control non-vaccinated group of pigs.
Farms where pneumonic outbreaks caused by *Actinobacillus pleuropneumoniae* occurred prior to the study are selected.
Safety parameters as general reactions or adverse events will be monitored following each vaccination and during all the study. Local reactions will be also evaluated at different time points throughout the study.
The efficacy will be mainly focused on the clinical signs and the mortality.
The clinical trial will be monitored by veterinarians.

# RESEARCH/DEVELOPMENT ACTIVITIES

***Previous Development Activities:***

***Target Animal Safety***
The safety of the vaccine was confirmed by means of several laboratory studies, in which an overdose, the administration of repeated doses, the spread of the vaccine strain from vaccinated to non-vaccinated pigs and reversion to virulence were assessed.

a) Administration of an overdose, a single dose, a repeated dose, and spread of *Actinobacillus pleuropneumoniae* live vaccine PB-116 strain.

The objectives of this study were to evaluate the safety of the administration of an overdose, a single dose and a repeated dose of the PB-116 vaccine and also to evaluate its transmission from vaccinated to non-vaccinated animals.

A group of pigs was firstly vaccinated with an overdose of the vaccine followed by another 2 single dose administrations. Another group was vaccinated with the basic vaccination program (2 doses separated by three weeks).

The results obtained showed a transient increase in rectal temperature may occur after vaccination. In most cases, this increase in temperature is below 1.5ºC and it is always spontaneously resolved within 24 hours. Furthermore, there were no clinical signs related to the vaccination of the pigs used. These results confirm the safety of PB-116 even when an overdose and a repeated dose are administered.
The results also confirmed that the vaccine strain do not spread from vaccinated to non-vaccinated animals.

b) Dissemination of the *Actinobacillus pleuropneumoniae* HP-3276 strain in vaccinated animals after intramuscular administration.

In this study, the distribution of the *Actinobacillus pleuropneumoniae* HP-3276 vaccine strain in animals vaccinated by intramuscular route was assessed.

For the study, twenty 8-weekd old pigs of the minimum age recommended in the Summary of Product Characteristics (8 weeks old) were used. All the animals received one dose of vaccine by intramuscular route.
After vaccination, dissemination of the App HP-3276 vaccine strain was analysed in the animals' bodies at predetermined intervals. Different tissue samples were tested in order to identify if the vaccine strain was present. Samples from the body secretions were also analysed with the same aim.

From the results presented in this study, it can be concluded that HP-3276 vaccine strain dissemination is very poor and just located in tonsil and inoculation point nodes during the fourth week after vaccination. Therefore, the vaccine strain has been only detected in a few samples, with a so low incidence, that dissemination of the *Actinobacillus pleuropneumoniae* vaccine strain HP-3276, after its administration by intramuscular route, is considered almost negligible.

c) Dissemination of the *Actinobacillus pleuropneumoniae* HP-3276 strain in vaccinated animals after intranasal administration.

This study was performed to evaluate the distribution of the *Actinobacillus pleuropneumoniae* HP-3276 vaccine strain in vaccinated pigs after intranasal administration, and to obtain from this vaccinated animals tissue samples as the first passage samples to be further used in the reversion to virulence assay.

Intranasal route was assessed since this is the natural way by which this microorganism can enter the body and so it would be the most likely route to lead to reversion to virulence.

For the study, 5 groups of two 8-weeks old pigs each were vaccinated with master seed of the App vaccine strain HP-3276. All the animals received a 10-fold overdose. The objective of the first part of this study was to assess the most likely period and organs from which the vaccine strain could be recovered after intranasal administration. With this aim, necropsy of the animals and sample collection was performed at predetermined intervals.
In addition, general clinical signs and body temperature were recorded before vaccination, 4 hours later and daily during the period of time studied in order to assess the safety of the vaccine strain.

No relevant clinical signs were observed during the entire study period., although one animal of group 1 showed fever and slight dyspnoea 4 hours post-vaccination. Although symptomatology was no longer observed in this animal 1 day after vaccination it was euthanized, and it was confirmed that no App lesions were observed at necropsy.

Concerning the dissemination of the vaccine strain, the results obtained in this study are in agreement with those obtained in the previous one since, in both cases, either by intranasal or intramuscular route, dissemination of the vaccine strain was very poor.

With regard to the other objective of this study, three samples positive to HP-3276 strain were obtained, and a pool of these samples was further used to continue with the reversion to virulence study.

Moreover, the results obtained enable us to conclude that the vaccine strain, when administered intranasally, can be recovered from tonsils, nasal and salivary glands for a period no longer than 3 days from vaccination. This information was taken into account when designing the study on reversion to virulence.

d) Study of the reversion to virulence of the HP-3276 *Actinobacillus pleuropneumoniae* vaccinal strain.

The objective of this study was to examine the potential for reversion to or increase in virulence of the App vaccinal strain HP-3276 after several passages in target animals.
Three animals per group were used in this study. Only four groups were required since the vaccine strain was not further recovered. The animals used were of the minimum age recommended in the Summary of Product Characteristics (8 weeks).
The vaccine strain recovered from the previous study was used. Notwithstanding, the first passage had been obtained from samples of animals vaccinated with a 10-fold overdose of the master seed by intranasal route.
In the following passages vaccinations should be performed by intranasal route and swab samples of tonsils, nasal and salivary glands should be taken from these organs 1 to 3 days after vaccination. Necropsy should be performed 3 days post-vaccination and tissue samples should be also taken.
The presence of the vaccine strain in nasal, salivary glands (spittle) and tonsil samples was analysed by seeding swab samples in different medium plates, followed by PCR design to identify the vaccine strain.
In case of recovery, the recovered material after each passage was to be used as the inoculum for the next passage and was also administered by intranasal route.

Passages should be undertaken as long as vaccine strain was isolated. When there was no recovery, a reconfirmation should be performed by administering the last recovered passage to 11 animals.
In addition, general clinical signs and body temperature should be recorded before each vaccination, 4 hours later and daily during the period of time studied in order to assess the safety of the vaccine strain. Lung lesions were assessed in all the animals at necropsy.

The vaccine strain could not be recovered after the inoculation of the first passage to the animals of group B3, so the test was repeated with 11 animals (group B11). The experiment was considered to be completed at this point since the same result was obtained after inoculating the first passage in group B11.
The results obtained show that in none of the animals of either group (B3 and B11) was fever or clinical symptomatology observed after the administration of the recovered passage 1. Moreover, no typical App lesions were present in lungs of any of the animals of any group.

In addition, the impossibility of recovering the vaccine strain from any of the tested organs or body fluids of the animals inoculated with the first passage indicates that the transmission of the vaccine strain from vaccinated to unvaccinated animals is limited and the likelihood of reversion to virulence is considered null. The overall results concerning rectal temperatures, clinical signs, lung examinations and vaccine strain recovery obtained in the animals inoculated with the first passage enable us to confirm that the vaccine strain HP-3276 does not show an increase in or reversion to virulence.

***Efficacy assessment under laboratory conditions***

To date, the following laboratory test has been performed on the efficacy of PB-116.

Study on the efficacy of the PB-116 bacterial live vaccine against infection with *Actinobacillus pleuropneumoniae* serotype 2.

The objective of this study was to demonstrate the efficacy of the vaccination scheme of the PB-116 vaccine against pleuropneumonia caused by *Actinobacillus pleuropneumoniae* serotype 2. A total of 30 8-week-old pigs were used.

Half of the animals were vaccinated by intramuscular route according to vaccination schedule. The other group of 15 animals was administered sterile PBS, and kept as controls. After each vaccination, pigs were observed for 21 days.
Three weeks after the last vaccination, all pigs were challenged intranasally with a suitable quantity of an *Actinobacillus pleuropneumoniae* serotype 2 strain, virulent enough to produce typical signs of the disease.

After challenge, animals were observed for 7 days and general clinical signs were recorded daily. Severely ill control animals were sacrificed to avoid unnecessary suffering. At the end of the observation period, all surviving animals were euthanized and a post-mortem examination of the lungs was performed.
The parameters to assess the efficacy of the vaccine were the reduction in lung lesions, clinical signs and mortality in the vaccinated group when compared to the unvaccinated group.

Regarding safety data obtained from this study, as previously observed in the safety trials, a slight increase in rectal temperature may be observed after vaccination but these transient rises are always spontaneously resolved within 24 hours without treatment. Furthermore, the administration of two doses of PB-116 vaccine only caused a slight inflammation at the inoculation site in a very few animals, which disappeared within 24 hours. Concerning systemic reactions, some animals showed slight prostration between 4 and 6 hours post-vaccination, which was no longer observed 24 hours after the administration of PB-116.

Clinical signs after challenge were clearly milder in the vaccinated group, in comparison to control group, in which all animals were clearly affected. The same pattern was observed in regard with lung lesions at post-mortem examination. Moreover, mortality only occurred in the non-vaccinated group.

Therefore, the vaccinated group was significantly different from the unvaccinated group in terms of mortality, lung lesion incidence and score. These results, demonstrate the protective effect of the PB-116 vaccine against porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* serotype 2.

***Knowledge and experience obtained in previous development activities***

The *Actinobacillus pleuropneumoniae* HP-3276 strain has been used in confined conditions for studies to evaluate its effectiveness and safety as a vaccine for pigs. The results of these experiments are summarized in the previous sections. In short, it was found that the modified strain is able to protect against virulent *Actinobacillus pleuropneumoniae* infections, while it was confirmed the safety of an overdose and of the repeated administration of a single dose of the vaccine, a very limited capacity for transmission of the live vaccine strain from vaccinated to non-vaccinated animals, a very low distribution of the strain in the vaccinated animals and that the vaccine strain does not revert to virulence.

***Future activities***

The planned trial implies the deliberate release of the vaccine strain by intramuscular inoculation on fattening pigs from several farms, more specifically in buildings with appropriate means of isolation. Intramuscular administration is the usual form of vaccination of pigs. It is also the method used in controlled laboratory trials (safety and efficacy trials described above.)

The purpose of the deliberate release is to confirm the safety (lack of adverse side effects) and efficacy (protection of animals vaccinated against swine pleuropneumonia) of *Actinobacillus pleuropneumoniae* strain HP-3276 (see detailed protocol at the Clinical Trial Protocol , reference CD-2008-CB-003).

This study is one more step in the development of this vaccine. Finally, when all data are compiled, the marketing authorisation of the vaccine will be applied to the European authorities.

## BENEFITS

Different approaches were discarded during the development of the product PB-116 up to obtain a live vaccine that successfully confers the required immunoprotective response against infection, with minimum of adverse reactions. As demonstrated by efficacy data available, the administration of the vaccine according to the recommended instructions of use allows a significant reduction of the clinical signs, the pulmonary lesions and the mortality in vaccinated pigs.

## RISKS

Three aspects should be analysed when assessing the risk of the use of a vaccine: environmental safety, animal safety and public safety.

Impact of the use of PB-116 in the environment.

In the event of erratic use of the PB-116 vaccine, the only ingredient that could pose environmental problems would be the active component of the vaccine, the HP-3276 strain of *Actinobacillus pleuropneumoniae*. However, the possibility of environmental exposure of the active ingredient is negligible taking into account the following factors:

A - The recommended intramuscular route of administration prevents spread of the vaccine immediately after administration, which does not occur with other routes of administration such as intranasal or spray route, which may pose a risk to other animal species due to its inevitable spread.

B – Due to the low persistence of strain HP-3276 in vaccinated animals, it can be isolated only rarely from vaccinated animals. Thus, spread from intramuscular route can be considered virtually nil.

C - Although the vaccine strain could persist for a few days in the vaccinated pigs, there is no possibility of transmission to other pigs in contact with the former, since it did not spread to unvaccinated pigs (sentinels) in contact with vaccinated pigs. This further indicates that, if the inoculation is done correctly, there can be no dissemination of the antigen to the environment. Finally, the risk of transmission to other animal species is also very low as long as the vaccine is handled according to the recommendations contained in the Summary of Product Characteristics.

Possibility that the HP-3276 vaccine strain intervenes in the pathogenesis of other diseases in animals and man.

The PB-116 vaccine should not pose any problem in species other than the target species as long as the instructions in the leaflet accompanying the containers are followed and, as noted above, the only possibility of transmission is through accidental inoculation.

In case of accidental release of the vaccine strain HP-3276 into the environment, it would be necessary to discuss the possibility of whether this strain causes disease to other animal species and humans.

The chances of the strain being involved in the pathogenesis of human disease are non-existent. It is considered that the man is not susceptible to infection with *Actinobacillus pleuropneumoniae*. Infection has not been demonstrated either by virus isolation or serology in suspected cases. In short, to date, it has not been demonstrated that human beings be a host for *Actinobacillus pleuropneumoniae*, and thus, the HP-3276 strain used in the preparation of PB-116 would not be pathogenic for humans.

As for the possibility that the vaccine virus is involved in the pathogenesis of animal diseases, although the organism is distributed on five continents, the number of hosts is limited: no seroconversion has been demonstrated in any wild species and clinical signs of disease have been observed only in pigs; therefore, it is not expected that the HP-3276 strain would cause any clinical symptoms in animals other than pigs.

On the other hand, exposure of other species by means other than the use of contaminated needles is highly unlikely, since the vaccine strain is not spread.

## CONTAINMENT, CONTROL AND MONITORING MEASURES

***Control of GMM and Gene spreading***

From data of the studies performed, it can be stated that the possibility of dissemination into the environment of the vaccine strain HP-3276 is negligible, provided the person administering the vaccine follows the instructions and precautions specified in the leaflet accompanying the containers, and even in the event of accidental exposure, it would be very unlikely to spread to environment since the strain does not spread.

***Genetic stability of the GMM***

The genetic stability of the HP-3276 strain has been tested by performing 5 serial subcultures in a specific medium and genomic extraction in each pass. The PCR differential technique was subsequently used to detect whether there was a reversion towards the original genotype in any of the passes.

Identification of the recombinant genome is made by a PCR reaction that amplifies a fragment from the mutant version of one of the genes present in the modified strain. All passes show the amplification of a fragment that corresponds to the fragment from the mutant version of the gene present in the modified strain, thus demonstrating the genetic stability of the same.

Furthermore, the identification of the vaccine strain after the first passage performed in the study of reversion to virulence, also confirmed the genetic stability of the vaccine strain.

Genomic recombination is a phenomenon that can occur spontaneously in nature and can occur both between virulent field strains, and between field strains and vaccine strains. While it has been reported that, under natural conditions on pig farms, recombination between viral strains can occur, in no case has spontaneous genetic recombination between bacteria or between bacteria and viruses been demonstrated.

With regard to the strain that concerns us, recombination with other strains is not expected due to the short persistence of the strain in the vaccinated pigs and the impossibility of being isolated from the first passage performed.

However, in the event spontaneous genetic recombination between two bacterial strains were to occur, it would be essential that the same target tissue was infected simultaneously by two different strains, regardless of whether these strains are attenuated or virulent.

When vaccinating with any vaccine strain, it is impossible to tell if an animal was previously latently infected with a virulent strain. To do so, it would be necessary for all the pigs from one farm to undergo immunosuppressive treatments for some time, and even so, in most cases, it would not be possible to detect them. Hence, there is always the risk of vaccinating a pig that is latently infected, regardless of the vaccine used.

Nevertheless, the possibility that *in vivo* recombination were to create more virulent strains is virtually non-existent. Some authors, as Henderson *et al.*, 1991, have noted that, "The recombinants obtained by recombination of vaccine strains with virulent strains do not appear to be more virulent than field strains or more virulent than the parental strain that gave rise to the recombinant, because they maintain the working copies of all known virulence genes". "Therefore", conclude the authors, "Recombination does not create supervirulent strains".
Regarding the possibility of genomic recombination between vaccine strains and virulent field strains in immunosuppressed animals, there is currently no data in the world literature. However, we understand that genomic recombination can occur in any case, both in healthy animals and in immunocompromised animals and that, although immunosuppression of animals may act as a factor that facilitates the replication of bacteria in the target tissues of the animal, it does not - in any case - benefit or reduce the possibility of recombination, since the two phenomena are interdependent.

Therefore the possibility of genomic recombination of the vaccine strain HP-3276 with other strains can be considered negligible.

Finally, in spite of all the factors a spontaneous recombination occurred, the probability that the strain HP-3276 manifests the expression of unexpected features is unlikely, given that it does not incorporate genomic sequences donated by another micro-organism that could give rise to phenomena of genetic recombination and expression of unexpected features.

***Destruction of GMM containment material***
Vials, syringes, needles, tubes and other materials in contact with the GMO will be sterilized by incineration in the same farm.

***Training requirements***
The Veterinarian that will administer the vaccine has a wide experience in vaccinating pigs intramuscularly. This person will be trained hoe to handle the recombinant vaccine and to decontaminate the environment of vaccination during any spill of the vaccine.

***Emergency situations***

1. Methods and procedures for controlling the GMOs in case of unexpected spread.

In the unlikely event of an uncontrolled spread of GMOs, all animals on the farm would be slaughtered and all of the premises would be fumigated with a 10% solution of formaldehyde. There will be no entry of new animals until at least 15 days following spraying.

2. Methods for decontamination of affected areas, for example, eradication of the GMOs.

The slaughtered animals would be destroyed by incineration. A 10% solution of formaldehyde (a chemical agent suitable for inactivation of the GMO) would be applied topically on the farm. Ultraviolet light from sunlight would contribute to inactivation of the vaccine strain, due to its bactericidal properties on this organism.

3. Methods for disposal or sanitation of plants, animals, soil, etc., exposed during or after the spread.

The procedures to be carried out on animals, on the farm and the effects of ultraviolet light (sunlight), would guarantee the suitable elimination of the GMO if this was necessary.

4. Methods of isolation of the area affected by the spread.

The characteristics offered by the farms, and the poor dissemination properties of this bacteria administered intramuscularly, the isolation of the area is guaranteed from the beginning of the trial. For these reasons, the isolation method is considered appropriate in the event of an unexpected spread.

5. Plans for protecting human health and the environment in the event of an undesirable effect.

For all the reasons explained, mainly the absence of infection by *Actinobacillus pleuropneumoniae* in human species or other species apart from pigs, and the limited persistence time of the GMO, no potential danger for humans or for the environment in general is expected. The only undesirable effects which, despite being extremely unlike, could occur theoretically, would be in the target animals. In the event of an emergency, them would be eliminated.

***Other containment, control and monitoring measures***

Not applicable.

***Responsibilities of the notifier***

The consent that could be given to the notifier by the competent Minister stipulates that the notifier takes complete civilian liability regarding the damage that could be caused by the deliberate release to the health of human animals and environment.

***Inspection by the public authorities***
Inspectors are in charge of inspecting the trials for compliance with the conditions specified in the consent and to investigate potential breaches of the consent. In case where mismanagement or fraud is identified specific sanctions will be imposed.

***Activity report***
At the end of the trial an activity report prepared by the notifier needs to be delivered to the competent authority. This activity report includes at least the following data:

- The site and period of release.
- The precise nature of the actually released GMMs.
- The aims of the trial.
- The measures that were taken to prevent unwanted release of transgenic material.
- If applicable the measures that were taken to protect the subject (patient/animal) during administration of the GMM-containing study drug.
- If applicable the measures that were taken to protect the relatives of the treated patients.
- The measures that were taken to protect the workers who had to manipulate the GMM containing material.
- The method used for the destruction of the unused or contaminated material.
- The results obtained during the trial.
- An overview of the monitoring of patient/animal for GMM shedding.
- An overview of the monitoring of GMM or recombinant DNA in the environment.

## REFERENCE

Henderson, L. M., Levings, R. L., Davis, A. J., Sturtz, D. R. 1991. "Recombination of Pseudorabies virus vaccine strains is swine". *Am. J. Vet. Res., vol 52, no. 6, pp. 820-825*.

# Recombination of pseudorabies virus vaccine strains in swine

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## SUMMARY

We report here genetic recombination between 2 USDA-licensed vaccine strains of pseudorabies virus co-inoculated into swine. The vaccine strains, one of which was a conventionally attenuated strain and the other, a genetically engineered deleted strain containing a negative immunologic marker, had complementary genomes. Co-inoculation resulted in the creation of novel strains of pseudorabies virus containing negative immunologic markers with restored virulence genes. Plaque-purified recombinant progeny viruses were found in 2 litters of pigs in which both strains were co-inoculated IM, a litter in which both strains were co-inoculated oronasally, and a litter in which the conventionally attenuated strain was inoculated oronasally and the genetically engineered strain was inoculated IM. Recombinant phenotypes and recombinant restriction fragment patterns were observed. The creation, spread, and potential misdiagnosis of these types of recombinant strains could disrupt control and eradication programs that are based on the serologic identification of swine infected with potentially virulent strains of pseudorabies virus.

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Pseudorabies virus (PRV, Aujeszky disease virus, suid herpesvirus-1) causes disease in a number of mammalian species and is responsible for considerable economic loss in the swine industry. Infection may result in signs ranging from clinically inapparent latent carrier states to fatal respiratory tract or neurologic disease syndromes. Infection of pregnant sows may lead to abortion or neonatal death. Recovery from PRV infection often results in the establishment of a latent carrier state in the trigeminal ganglia, as well as the development of humoral and cell-mediated immunity. Viral transmission is possible from clinically normal carrier animals following reactivation throughout the lifetime of the host animal. Vaccination with killed or modified-live vaccines may lessen economic losses, but may not prevent subsequent infection, viral replication, or the establishment of a latent infective state.[1-3]

Currently, there are a number of modified-live PRV vaccines licensed by the USDA. These include the conventionally attenuated Bartha and Bucharest vaccine strains and the newer genetically engineered vaccine strains.[4] The genetically engineered vaccine strains, as well as some of the conventionally attenuated strains have mutations or deletions in nonessential glycoprotein genes that are useful as negative immunologic markers, including gX, gI, gIII, and gp63.[5] Many of these vaccines have companion diagnostic kits also licensed by the USDA, that allow serologic differentiation between animals exposed to the matching vaccine strains with negative immunologic markers and animals exposed to virulent field strains of PRV.[6,7] The companion vaccine-diagnostic kit pairs provide a useful tool for eradication or control programs.[8]

The PRV genome encodes a number of virulence factors, including gI and gIII, which act synergistically with gp63, and thymidine kinase (Tk).[9-11] Other virulence factors may also be encoded. Strain virulence also is influenced by host species.[12] Substantial genomic variation of virulence-associated factors, as well as antigenic factors, exists between vaccine strains of PRV, all of which have reduced virulence for swine.

The Tk gene encodes an enzyme that is active in the pyrimidine synthesis salvage pathway and is essential for DNA replication in cells in which the primary pyrimidine synthesis pathway is inactive or nonfunctional. The natural endogenous activity of Tk in differentiated neural tissue is low. Viral-encoded Tk facilitates acute infections of neurons, permitting viral replication and contributing to the virulence of Tk-positive PRV strains.[13] The modified-live genetically engineered vaccine strains licensed prior to 1990 have deletions in the Tk gene. This results in reduced virulence, as well as reduced frequency of reactivation of virus from latent infections although latency may still be established.[3,14] All conventionally attenuated modified-live vaccine strains have intact functional Tk genes and contain deletions or mutations in genes encoding other virulence-associated factors. The restoration of an intact Tk gene to a strain containing a negative immunologic marker may, therefore, result in creation of a strain with restored virulence and unexplored antigenic characteristics. The possibility of such an event exists as a result of recombination between genetically engineered vaccine strains and either conventionally attenuated vaccine strains or field strains of PRV.[15]

Recombination is an early event in PRV replication, unlike recombination in herpes simplex viruses, which occurs throughout the infective process. Homologous recombination is known to occur between multiple molecules of PRV DNA within a host cell before DNA replication.[16] Little intermolecular or intramolecular recombination can be detected between parental DNA and

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progeny DNA accumulating in the cells.[16] The inverted repeats promote recombinational events that lead to the isomerization of the unique sequences that they bracket.[17] However, intermolecular recombination is not an essential step in the process of viral DNA synthesis or the maturation of concatameric DNA. Co-infection of host cells with 2 strains of PRV during the early phases of DNA replication may lead to creation of recombinant genomes. Restriction fragment pattern (RFP) analyses are useful for differentiation of PRV strains[18] and may provide evidence of homologous recombination.

The focus of the study reported here is the potential recombination between strains of PRV resulting in the restoration of virulence genes to vaccine strains containing negative immunologic markers in swine. We have recently reported recombination between vaccine strains of PRV resulting in the restoration of virulence to strains containing the same negative immunologic markers as the vaccine strains in the in vitro studies and in sheep, a sensitive host animal.[15] Exposure of sheep to PRV leads to fulminating infections and high viral titers, a condition that would increase the occurrence of the co-infective events required for homologous recombination. It was unknown whether swine, a more restrictive host and the target of eradication and control programs, would restrict replication sufficiently to reduce recombinant events detected, using serologic differentiation of animals exposed to avirulent strains from animals exposed to virulent strains of PRV. The purpose of the study reported here was to determine whether recombination of PRV in swine could occur and disrupt the control and eradication programs.

## Materials and Methods

*Cell culture source*—Four cell lines were obtained: Madin-Darby bovine kidney (MDBK) cells (ATCC CCL 22), embryonic primary swine kidney (SKp) cells, porcine kidney (PK-15) cells (ATCC CCL 33), and L-mouse cells lacking a functional Tk (LMTK−) gene (ATCC CCL 1.3).

*Media*—Maintenance media for MDBK, SKp, LMTK−, and PK-15 cells was Eagle minimal essential media (MEM). One percent sodium pyruvate was added for PK-15 cells, and 5% fetal bovine serum was added for growth media.

*Characterization procedures*—The Tk assay, β-galactosidase assay, restriction endonuclease digestion, and agarose gel electrophoresis were performed as described previously.[15]

*Virus titration and plaque purification*—Virus was titered on confluent MDBK cells, using a standard plaque assay in 6-well tissue culture dishes. Reference virus (NVSL 87-2 Aujeszky strain) of known titer was assayed with each titration. Plaque purifications were performed by selection of individual plaques from the β-galactosidase assay followed by expansion on confluent MDBK cells in 25-cm$^2$ flasks.

*Virus isolation*—Suspensions of the tissues were inoculated onto SKp cell cultures. Two serial passages on SKp cells were made, with regular observation for evidence of viral-induced cytopathic effects. At the end of the second passage, the cultures were stained with a polyvalent antiserum and anti-porcine immunoglobulin fluorescent antibody conjugate.

*Virus strains*—Vaccines were rehydrated following manufacturer's instructions, using half the suggested diluent. Inocula were half the suggested volume, resulting in 1 normal vaccine dose/1 ml of rehydrated vaccine. For proprietary reasons, identification of the vaccine strains cannot be included. Additionally, the 2 strains chosen for this study were useful because of the differences in selectable phenotypic characteristics. We know of no reason not to believe that all strains will recombine. Virus strain A is a USDA-licensed Bartha-derived conventionally attenuated modified-live vaccine strain. It has an intact functional Tk gene and known deletions or mutations in the gI and gp63 genes. Strain A has a Tk-positive, gX-positive, β-galactosidase-negative phenotype. Virus strain E is a USDA-licensed genetically engineered vaccine strain with deletions in the Tk and gX genes and an *Escherichia coli* lacZ marker insertion, resulting in a Tk-negative, gX-negative, β-galactosidase-positive phenotype. The gX deletion serves as a negative immunologic marker (Fig 1 and 2).

*Virus inoculation*—Three-day-old pigs from PRV-seronegative sows were used to assess the ability to detect specific recombinant virus phenotypes following co-inoculation of 2 vaccine strains of PRV. Three days after farrowing, pigs were inoculated with 2 standard vaccine doses of PRV/pig. Exposure was either oronasal (ON) or IM in the large muscle of the rear limb, on the basis of random assignment. All principals in a litter received the same viruses. Each litter included a contact control that was not inoculated. Beginning on postinoculation day (PID) three, 2 pigs were euthanatized daily, using IV injection of T61 and postmortem examinations were conducted. Tissues were collected and virus isolation was attempted. Following isolation of PRV, Tk-positive recombinants were selected by 4 passages through LMTK− cells in HAT media. Each isolate was serially plaque-purified 3 times, using the β-galactosidase assay. Phenotypes of pure cultures were confirmed, and DNA was purified, digested with restriction endonuclease, and subjected to electrophoresis

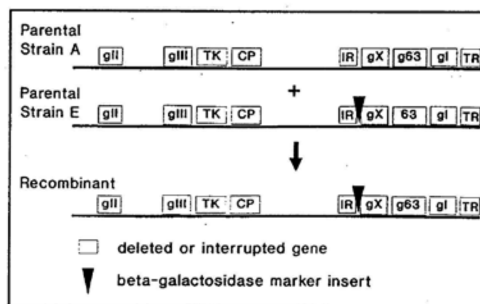


Figure 1—Diagrammatic map of parental pseudorabies virus (PRV) vaccines strains and potential recombinant with restored virulence and the same negative immunologic markers as vaccine strains.

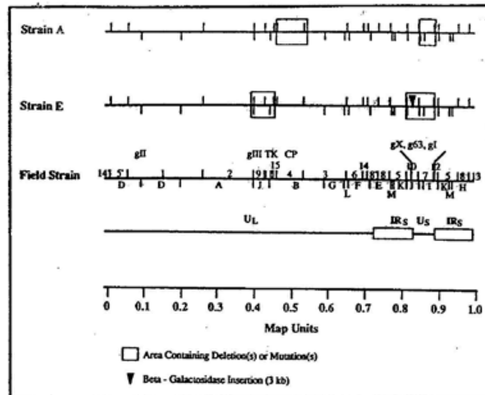


Figure 2—Restriction maps of parental vaccine strains A and E and a field strain of PRV. Areas between upward marks represent *Bam*HI fragments and areas between downward marks represent *Kpn*I fragments. Distances are given in map units.

in 1% agarose gel. The RFP from isolates were compared with RFP of both parental strains to confirm that recombinant phenotypes were attributable to homologous recombination.

Control litter AA pigs each received 2 standard vaccine doses of vaccine strain A. Four pigs were inoculated ON, 4 were inoculated IM, and 1 was an uninoculated contact control. Control litter EE pigs each received 2 standard vaccine doses of vaccine strain E. Five pigs were inoculated ON, 5 were inoculated IM, and 1 pig was an uninoculated contact control. Litter NN (n = 8) was co-inoculated ON with a standard vaccine dose of each strain A and strain E mixed in 1 vial. Litter NM (n = 8) was co-inoculated ON with a standard vaccine dose of strain A and IM with a standard vaccine dose of strain E. Litter MM (n = 9) was co-inoculated IM with a standard vaccine dose of each strain A and strain E, with both vaccines being mixed in a single vial. Litter MM′ principals (n = 6) were 12-day-old pigs co-inoculated IM with 1 standard vaccine dose each of strain A and strain E, similar to litter MM. Two pigs were uninoculated contact controls. Although this litter was 12 days old, all other conditions were similar to the 3-day-old litter. Litter MM′ was inoculated to better assess the clinical signs observed in litter MM. New vials of vaccine were rehydrated. Two of the recombinant isolates were inoculated into 3-day-old pigs to assess virulence restoration of a single isolated recombinant. Two pigs of 1 litter were inoculated ON with $10^6$ plaque-forming units of a recombinant isolated from the MM litter; 2 of the litter were uninoculated contact controls.

## Results

Clinical signs were not observed in any of the pigs in litter AA and no lesions were observed. Virus was isolated from the brain tissue of 1 of the ON inoculants, and from other tissues (lung, liver, spleen, or tonsil) of all of the ON inoculants, as well as 1 of the IM inoculants. Virus was not isolated from the tissues of the other 3 IM inoculants. The isolated viruses were identical to the parental vaccine strain in both Tk and β-galactosidase phenotype, as well as RFP. Notable lesions were not observed in any of the pigs in litter AA.

One IM inoculant from litter EE displayed slight limping, which was attributed to an injury. Clinical signs were not observed in any of the pigs in this litter. Notable lesions were not observed in 3 of the ON inoculants. One ON inoculant had moderate diffuse splenic lymphoid hyperplasia and 1 ON inoculant had mild splenic lymphoid hyperplasia. Notable lesions were not observed in the IM inoculants. Virus was isolated from the tonsil, spleen, or brain tissue of 3 of the ON inoculants and from lung tissue of 1 of the IM inoculants. In all cases, viruses isolated from this litter were identical to the parental vaccine strain in Tk and β-galactosidase phenotype, as well as in RFP.

Clinical signs were not observed in litter NN, except mildly high temperatures (Fig 3). Lesions compatible with PRV infection were observed in all of the pigs. Virus was isolated from lung, liver, spleen, or tonsil, as well as from brain tissue of all 7 inoculated pigs (Table 1). In all cases, Tk-positive, β-galactosidase-positive recombinants were observed. Virus was also isolated from the lung tissue of the contact control. This virus had a Tk-positive, β-galactosidase-negative phenotype, similar to parental strain A, with similar *Bam*HI RFP (Fig 4).

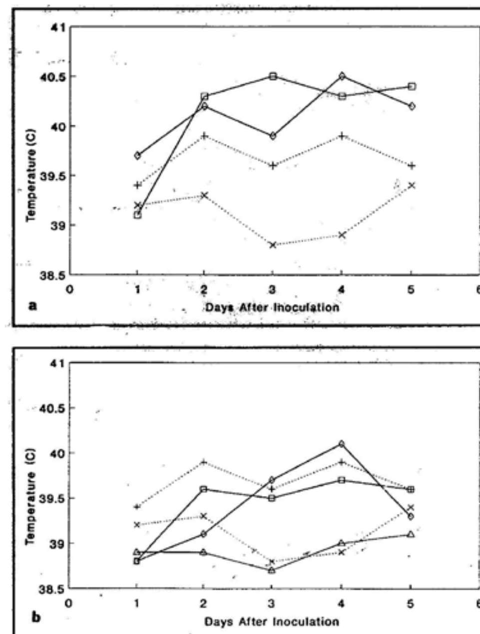


Figure 3—Mean daily temperatures of pigs following IM co-inoculation (a) and IM-ON and ON-ON co-inoculation (b). Temperatures are means of 3 or more pigs. Temperatures of control pigs are means of all clinically normal contact controls.

Table 1—Virus isolation from pigs inoculated with strains of pseudorabies virus vaccines or uninoculated controls

| Litter identification | No. of pigs in group | Inoculation Dose/route/strain | Tissue type from which virus was isolated | | Tk | β |
|---|---|---|---|---|---|---|
| | | | Neural | Other | | |
| AA | 4 | 2X/ON/A | 1 | 4 | + | − |
| | 4 | 2X/IM/A | 0 | 1 | + | − |
| | 1 | Uninoculated | 0 | 1 | + | − |
| EE | 5 | 2X/ON/E | 2 | 3 | − | + |
| | 5 | 2X/IM/E | 0 | 1 | − | + |
| | 1 | Uninoculated | 0 | 0 | ... | ... |
| NN | 7 | 1X/ON/A; 1X/ON/E | 7 | 7 | + | + |
| | 1 | Uninoculated | 0 | 1 | + | − |
| NM | 7 | 1X/ON/A; 1X/IM/E | 2 | 7 | + | + |
| | 1 | Uninoculated | 0 | 0 | ... | ... |
| MM | 8 | 1X/IM/A; 1X/IM/E | 5 | 8 | + | + |
| | 1 | Uninoculated | 0 | 1 | + | −* |
| MM′ | 4 | 1X/IM/A; 1X/IM/E | 1 | 2 | + | + |
| | 2 | Uninoculated | 0 | 0 | ... | ... |

* Restriction fragment pattern of recombinant virus strain differs from that of parental virus vaccine strain.
Tk = thymidine kinase phenotype; β = β-galactosidase phenotype; ON = oronasal.

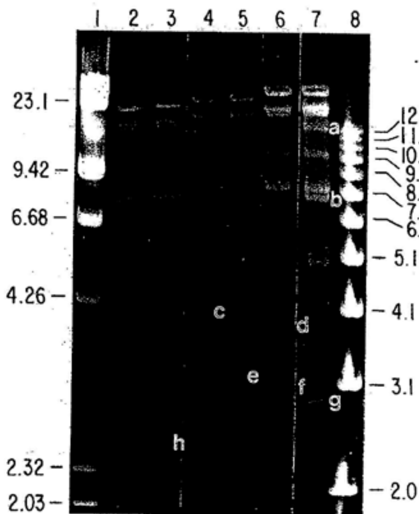


Figure 4—*Bam*HI restriction endonuclease patterns of parental and recombinant viruses. Lane 1: lambda *Hin*dIII standards. Lane 2: parental strain A. Lane 3: brain isolate from litter AA ON inoculant. Lane 4: brain isolate from litter MM inoculant. Isolate differs from parental strain A at bands c and d, and differs from parental strain E at bands a, b, c, e, g, and h. Lane 5: brain isolate from litter MM inoculant. Isolate differs from parental strain A at bands b and c, and differs from parental strain E at bands a, c, d, e, and g. Lane 6: brain isolate from litter NN. Isolate differs from parental strain A at bands b, f, and h, and differs from parental strain E at bands a, d, e, f, and g. Lane 7: parental strain E. Lane 8: 1 kb DNA ladder standard.

Clinical signs were not observed in litter NM. Notable lesions were not observed, but virus was isolated from the lung, spleen, liver, or tonsil of each of the inoculants, as well as from brain tissues of 2 of the 7 inoculants. Four of the non-neural tissue isolates and 1 of the brain isolates were Tk-positive, β-galactosidase-positive recombinant strains. Isolates from the remaining 2 pigs were phenotypically similar to the Tk-positive. β-galactosidase-negative parental strain A; RFP similar to parental strain A were observed for these 2 isolates (Fig 3).

Clinical signs in litter MM included limping, depression, and markedly high temperatures observed on PID 2 in all 8 inoculated pigs. Acute clinical signs were observed in all inoculated pigs on PID 3, including markedly high temperatures, dyskenesia, depression, shivering, and caudal paralysis. Mild clinical signs were observed in the contact control pig on PID 3, and virus was isolated from a pool of spleen and lung tissue of this isolate. Perineural edema of the cauda equina was observed in all inoculants. Notable lesions were not observed in the contact control. Virus was isolated from the spleen, lung, liver, or tonsil of all inoculants and the contact control, as well as from neural tissue of 5 of the inoculants. Neural tissue virus isolates from all inoculants included Tk-positive, β-galactosidase-positive recombinants (Fig 3). The RFP analyses of some of the recombinants isolated from these pigs confirmed that selected strains RFP differed from parental strains RFP (Fig 4). On the basis of seroconversion of the sow in litter MM, it was determined that shedding from the pigs to the sow occurred, although it is not known which strain (vaccine or recombinant) was shed.

Mild clinical signs (marked temperature increase, depression, and respiratory tract signs) were observed in all 4 inoculants of litter MM′. Clinical signs were not observed in the 2 contact controls. Gross lesions suggestive of PRV infection were not observed. Tk-positive, β-galactosidase-positive recombinant viruses were isolated from a tonsil, spleen, and lung pool of 1 of the inoculants, as well as from a lung and liver pool and neural tissue of another of the inoculants (Fig 3).

A marked temperature increase was observed in all pigs inoculated with recombinants isolated from the MM litter. Other clinical signs were not observed. There were no signs of spread to the contact controls. Virus isolation was attempted from 3 of the inoculants and 1 contact control, and PRV was isolated from neural tissue, as well as from spleen, liver, and lung of these 3 inoculants. Five of another litter were inoculated IM with $10^6$ plaque-forming units of a brain isolate from litter MN; 2 uninoculated contact controls remained. A marked increase in temperature, as well as a slight cough and pruritus were observed in these pigs. Postmortem examinations were conducted on 2 of the inoculants, as well as on 1 contact control. Notable lesions were not observed in the contact control and 1 inoculant. Slight perineural edema of the cauda equina of 1 of the inoculants was observed. All other pigs were observed for 2 weeks. Both litters recovered from the mild clinical signs.

## Discussion

On the basis of our findings, we conclude that genetic recombination between strains of PRV occurs in swine. The isolation of plaque-purified phenotypic recombinants from the neural tissue of swine co-inoculated with 2 vaccine strains of PRV is important. Recombinant RFP provide further evidence in those cases in which phenotypic characteristics are similar to a parental strain. Previous studies[15] have demonstrated that multiple recombinant events are likely. We have not attempted to map the site(s)

of recombination because the number of isolates studied is insufficient to establish recombination "hot spots." The procedure used in this study was used to purify only a single isolate of 1 specific phenotypic recombinant. Also, all RFP that differed from both parental strain RFP were considered to be recombinant RFP with no attempt to determine crossover sites or number of apparent crossovers. We have not attempted to establish the relative virulence of recombinant strains. The restoration of a functional intact Tk gene to a vaccine strain of PRV may restore full virulence, but in many recombinants there will be diversity in a number of virulence factors. We establish the formation of recombinants here; the importance of any single recombinant is not the focus of this study.

Swine are a highly resistant host species. Host defense systems are expected to provide a selective advantage to recombinant strains with restored virulence. Immunologically immature host animals provide increased opportunities for recombinant events to occur because viral replication is less restricted than that in immunologically competent hosts. Regardless of immunologic maturity, we find recombinants are easily detected in the neural tissue of swine simultaneously exposed to standard vaccine titers of more than 1 strain of PRV under these experimental conditions. Previous studies have demonstrated that recombination between 2 strains co-infecting a host cell is a frequent event early in the infective process prior to DNA replication.[16] Although it is possible that acute clinical signs were in fact attributable to complementation, rather than to recombination with restoration of virulence factors, it is unlikely that 2 complementary strains, replicating in tandem, would reach the brain tissue in swine after IM inoculation in the rear limbs, with no recombinant event occurring between the 2 strains prior to tissue culture inoculation. Because isolation techniques begin with tissue culture amplification of PRV present, we cannot be positive that the recombinant events did not occur in tissue culture, rather than in vivo; again, clinical signs and the severity of lesions observed suggests that recombinant events occurred prior to viral replication in neural tissue. Although immunologic maturity may affect the number of recombinants detected, it appears that recombinant events occurred in 3- and 12-day-old pigs. Some vaccine strains are licensed for use in 3-day-old pigs; if virulent recombinants are formed and shed from immunologically immature pigs, mature swine may be susceptible to the newly created strain.

These results are not unexpected. Recombinant strains of other viral agents are known to naturally form in vivo, including the generation of intertypic recombinants.[19,20] Avirulent herpes simplex viruses have been shown to generate lethal recombinants in vivo.[21] Also, evidence of possible recombinant events has been reported between PRV strains in swine.[22]

Of particular importance is the finding of recombinant virions in swine exposed to conditions that might be found in field situations (litters NN and NM), particularly in those situations in which swine are concurrently exposed to 2 strains. These conditions may occur if more than 1 strain of virus is shed at a time. Vaccination during a herd epizootic of PRV is likely to create conditions in which individual animals may be exposed to high titers of both the vaccine strain and the virulent field strain. Although conditions found in litter MM are not likely to be found in a field situation, the use of an oronasal vaccine during an epizootic could result in the creation of conditions similar to those in litter NN, whereas the use of an IM vaccine would mimic the conditions in litter NM. There is evidence of recombinant events occurring under both conditions. Animals simultaneously exposed to 2 strains of PRV should be monitored carefully. During acute infection and following recovery from clinical signs, it is possible that clinically normal carrier animals may shed virulent recombinant PRV containing negative immunologic markers. These recombinants could have an impact in field situations in which serologic responses are the determining factor in allowing movement of animals between herds or in lifting of quarantine restrictions. Those responsible for the interpretation of test results must consider the possibility of recombination if the clinical findings do not match the serologic findings.

A recent report on the ability of 2 different strains of an α-herpesvirus (bovine herpesvirus-1) to establish latency in the same tissue[23] suggests that it may be possible for a recombination event to occur in a host animal exposed to more than 1 strain, even when a substantial length of time separates the exposures. Although this has not yet been tested with PRV in swine, it may be prudent to restrict the exposure of herds to only 1 vaccine strain. The establishment of a latent infective state and the ability of the virus to replicate and shed throughout the lifetime of the host animal may facilitate recombination between herpesvirus strains that have replicated in a host animal at different times.

The ability to efficiently reconstitute PRV from subgenomic parts within a host cell through overlap recombination[24] suggests that defective interfering particles, which contain genomes of less than unit length, may be capable of contributing to the variation in genomic structure of the progeny viruses. Modified-live vaccines containing pieces of PRV genome inserted into foreign vectors also may contribute to the genetic material available for recombination within a host. Additionally, before the use of herpesviruses as vectors for multivalent vaccines, the consequences with respect to recombinant events must be assessed. Recent reports[25] of increased virulence of vaccine vectors attributable to the insertion of DNA encoding foreign proteins suggest that foreign inserts must be evaluated within the genetic background in which they might be found. The impact with respect to virulence of a different genetic background on the foreign insert, as well as that of a foreign gene on a different genetic background, must be considered before the release of new genetically engineered microorganisms. Failure to carefully evaluate the potential interactions of new strains of viruses before environmental release could lead to undesirable consequences.

Many genotypic combinations may result from recombinant events. Diagnostic tests that are based on serologic responses to glycoprotein gene-deleted vaccine strains may not be able to differentiate vaccinates from animals infected with recombinants of this kind.[26] Recombinants detected did not appear to be more virulent than field strains of PRV, nor are they expected to be more virulent, because field strains retain functional copies of all known virulence genes. Therefore, recombination is not expected to create a supervirulent strain. However, the ability of vaccine strains to recombine in vivo with either different

vaccine strains or field strains to produce virulence-restored strains containing the same negative immunologic markers as vaccine strains is of concern for control programs that are based on serologic differentiation of swine exposed to vaccine strains from swine exposed to virulent strains of PRV. The ability to detect all animals that could be carrying virulent strains is crucial if eradication and control programs are to function efficiently. In addition, the restoration of an intact Tk gene to a vaccine strain containing a negative immunologic marker would be expected to increase the likelihood of reactivation from latent infections and may also have an impact on eradication programs.

Considerable improvement in herd health, as well as economic savings have resulted from the use of vaccines to control PRV. Although the use of subunit vaccines in which antigenic proteins elicit a protective serologic response without the introduction of intact genetic material and concomitant possibility of establishment of a latent infective state may alleviate concerns about the consequences of recombination, there are no such vaccines currently licensed. The use of inactivated virus vaccines containing negative immunologic markers with compatible companion diagnostic kits presents an option in which recombination should not present a problem. Modified-live virus vaccines that are based on the use of novel strains of PRV have made possible the differentiation of animals exposed to an assortment of strains of PRV, a condition that greatly decreases the costs of eradication programs. Without the use of these tools, eradication efforts may not be practical. If the usefulness of these unique strains is to be retained, care must be taken to reduce the likelihood of creation and spread of virulent recombinant strains containing the same negative immunologic markers as vaccine strains of PRV.

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