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Assessment of Replication and Virulence of Attenuated Pseudorabies Virus in Swine

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ABSTRACT

A nonclinical study was conducted to characterize the replication behavior of a modified live gE-deleted pseudorabies virus (PRV MS+1) in swine and potential for reversion to virulence after animal passages. Two to 3 week-old weaned pigs, negative for PRV, were maintained in isolation and challenged by intranasal instillation. For the first passage, 6 pigs were given 1 mL of PRV MS+1 ($10^{7.3}$ $TCID_{50}$/mL) and 2 were necropsied at 3, 4 and 5 days post-inoculation (PI). Brain and secondary lymphoid tissues were collected, homogenized and the supernatants individually pooled for virus isolation, and PRV was recovered from each sample. No clinical signs of PRV infection were observed, but each pig had a nasal swab suspect or positive for PRV. For the second passage, 5 pigs were given 1 mL of the homogenate of mixed tissues from 1 animal in the previous passage (PRV at $10^{1.9}$ $TCID_{50}$/mL). At 5 days PI, all pigs were necropsied, and PRV was not recovered from their tissue homogenates or nasal swabs, and no clinical signs were observed. During a second attempt at a second passage, tissue homogenates from all pigs in the first passage (PRV at approximately $10^{1.7}$ $TCID_{50}$/mL) were pooled and used to inoculate 15 pigs with 2 mL for 3 consecutive days. Ten pigs were monitored for clinical signs and seroconversion through 21 days PI, and 5 pigs were necropsied at 5 days PI. No clinical signs or PRV antibodies were detected in the 10 monitored pigs, and no PRV was recovered from the homogenates or nasal swabs of the 5 necropsied pigs. Thus, no evidence of reversion to virulence was demonstrated in pigs given the attenuated PRV.

Keywords : Pseudorabies, Virulence, Reversion, pigs

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Introduction

Pseudorabies virus (PRV), porcine herpesvirus 1, is an important pathogen that causes Aujeszky's Disease in swine [1,11,13]. The virus is an enveloped DNA virus, a member of the *Alphaherpesvirus* subfamily, and is immunologically related to bovine herpesvirus 1 and herpes simplex virus 1 [10]. Like other alpha-herpesviruses, PRV can establish latent infections in ganglionic neurons, and can be reactivated due to stress and infect commingled animals [2,7]. The infection in pigs is detectable by demonstrating the presence of virus or virus-specific antibody using enzyme-linked immunosorbent assay, serum virus neutralization test, immunofluorescence microscopy of tissues, or via nucleic acid amplification using the polymerase chain reaction [9,19,21].

Swine serves as the principal reservoir for PRV, and the virus is an ubiquitous organism that adversely impacts swine production worldwide [1,11,13]. The resulting disease in PRV-naive piglets is generally acute and clinical signs include lethargy, pyrexia, incoordination, muscle spasms, excessive salivation, convulsions and death. Infected mature animals demonstrate poor growth associated with respiratory symptoms, and pregnant swine may reabsorb or abort their litters, or deliver mummified, stillborn or feeble piglets. Infection spreads principally among commingled animals by direct contact with acutely or latently infected animals, by airborne transmission of virus in nasal secretions, or by contact with environmental contamination. Clinical disease can be experimentally induced in piglets by intranasal inoculation of virulent PRV.

Endemic disease is difficult to control and no effective treatment is available for swine displaying clinical signs of infection with PRV. Currently, healthy animals are routinely immunized with inactivated (killed) or modified live virus (including those that are gene-deleted) vaccines to minimize clinical disease and death loss. Modified live vaccines incorporate attenuated bacteria or virus as immunogens and there is concern that, after vaccination, such organisms may revert back towards virulence during replication in the host [3,4,6,12,14]. As a result, "back-passage" studies are recommended to evaluate the genetic stability of live

bacterial or viral seeds to assure that such organisms, albeit attenuated, will not regress to virulent forms after being administered to the target species or when spread by contact to commingled animals [15]. The following investigation was conducted to determine the potential of a modified live gE-deleted PRV to revert to virulence after multiple passages in PRV-naive pigs.

Materials and Methods

Animals

Crossbred, weaned, approximately 2- to 3-week-old pigs were purchased from a commercial farm free of PRV as needed. All pigs were determined to be serologically negative for PRV and porcine reproductive and respiratory syndrome virus (PRRSV) upon arrival to animal facility, and were maintained in strict isolation throughout the investigation.

Pseudorabies virus

A modified live, gE (g1)-deleted PRV was used to inoculate the initial group of pigs. The virus (PRV MS+1) represented a first passage in cell culture from a vaccine master seed (PRVac, PRVac Plus, Pfizer, Inc., USA).

Experimental design

A multiple-passage study in animals was conducted in central Iowa USA and in accordance with Good Laboratory Practices for nonclinical studies [18]. For each passage, pigs were screened approximately 1 week prior to virus challenge for PRV and porcine reproductive and respiratory syndrome virus (PRRSV) based on serology. Additionally, the day before challenge, a blood sample and nasal swab were collected from each animal and tested for PRV by serology and qualitative virus isolation, respectively. For the first animal passage of the virus, 6 pigs (Animal Nos. 1 and 3-7) were given a 1 mL intranasal inoculation (0.5 mL / naris) of the PRV MS+1 ($10^{7.3}$ TCID50/mL). Subsequently, the pigs were observed twice daily for clinical signs of PRV infection (or Aujeszky's disease) and body temperatures were recorded daily. At 3, 4 and 5 days post-inoculation (PI), nasal swabs were collected from available animals and 2 pigs were randomly selected for necropsy on each day. At necropsy, the entire brain and stem, spleen, pharyngeal tonsils, and retropharyngeal and bronchial lymph nodes were collected, immediately placed on ice, and homogenized separately. Thereafter, the resulting homogenates were pooled for each pig and stored frozen ($\leq$-70℃) until assayed for PRV titers. For the second passage, 5 pigs were monitored as previously described and then given a 1 mL intranasal inoculation (0.5 mL / naris) of pooled filter-sterilized tissue homogenate obtained from 1 pig (Animal No. 7) in the first passage. That animal, necropsied 5 days PI, had demonstrated PRV in the pooled tissues at a rate of $10^{1.9}$ TCID50/mL and had nasal swabs positive for PRV on 3 consecutive days (i.e., 3-5 days PI). At 5 days PI of the second passage, nasal swabs were collected from all 5 pigs, and the animals were necropsied. At necropsy, the same tissues were harvested, processed and assayed as described above for the first passage.

A second attempt at a second animal passage was made using pooled tissue homogenates obtained from all 6 pigs during the first viral passage which was determined to contain PRV at approximately $10^{1.7}$ TCID50/ml. Fifteen pigs were monitored as previously described and challenged intranasally with 2 mL (1 mL / naris) for 3 consecutive days. At 5 days PI, nasal swabs were collected from 5 randomly selected pigs that were then necropsied. Once again, the same tissues were harvested, processed and assayed as described above for the previous passages. The 10 remaining pigs were observed twice daily for clinical signs of PRV infection and body temperatures were recorded daily through 21 days PI. At the end of that interval, blood samples were collected and the sera were assayed for circulating antibodies specific for PRV.

To ensure that no significant genetic changes occurred in PRV MS+1 during animal passage, a genetic comparison of the modified live challenge virus and the viruses recovered from pigs in the first passage was performed by restriction fragment length polymorphism (RFLP) analysis.

Serology

Blood samples collected during the study were processed to serum and stored frozen ($\leq$-20℃) until tested. Sera were assayed for antibodies to PRV and PRRSV using a virus neutralizing (VN) test and/or commercially available enzyme-linked immunosorbent assay (ELISA) kits (IDEXX Laboratories, Inc., Maine, USA). The VN test was performed in 96-well microtitration plates using PK-15 cells as the indicator. Serum samples were heat-inactivated at 56℃ for 30 minutes prior to performing the test and serially diluted 2-fold using minimum essential medium, Eagles salt (MEM, Sigma Chemical Co., St. Louis, USA) in 96-well plates. One hundred microliters of PRV (Shope strain) at a rate of 100 TCID50/0.1 mL were added to each well containing an equal volume of each sample dilution. Plates containing virus-serum mixtures were incubated at 37℃ for 60 minutes. One hundred microliters of the cell suspension prepared in MEM supplemented with 2% fetal calf serum (FCS) and 2 mM glutamine (GIBCO/BRL, Grand Island, NY, USA) at a concentration of 4×10^5 cells/mL was then added to each well containing the virus-serum mixture. After a 72-hour incubation, the cells were monitored for cytopathic effect (CPE) typical of PRV. Virus neutralizing antibody titers were expressed as the highest dilution in which no visible CPE was detected.

Enzyme-linked immunosorbent assays were performed using procedures recommended by the manufacturer (IDEXX Laboratories, Westbrook, ME, USA). Samples with S/P (sample/positive control) ratio of > 0.4 were considered positive for PRV and PRRSV, respectively.

Virus isolation and quantitation

The presence and level of PRV in swabs and mixed tissue homogenates were determined by a microtitration infectivity assay using PK-15 cells as the indicator. Swabs were collected from the nares, placed on ice for transport, and stored frozen (≤-70℃) within approximately 1 hour post-collection in 3 mL of MEM supplemented with 2% FCS, 2 mM glutamine, 10 g/mL amphotericin B (Fungizone®), 50 g/mL gentamicin, 100 IU/mL penicillin, and 100 g/mL streptomycin. Prior to assay, each swab sample was quickly thawed at 37℃, vigorously vortexed, and centrifuged at approximately 1,500 × *g* for 10 minutes.

All tissue samples were homogenized (20% w/v) with Earles balanced salt solution (Sigma Chemical Co.) immediately after collection. All homogenates were centrifuged at approximately 1,500 × *g* for 10 minutes. Tonsil homogenates were filtered through 0.22 m membrane filters to eliminate bacterial contamination. The resulting supernatants were pooled for each pig and frozen (≤-70℃). The individual pooled tissue supernatants were assayed for PRV.

For the assay, all samples were 10-fold serially diluted in MEM. One hundred microliters of each undiluted and diluted sample were inoculated onto confluent monolayers of PK-15 prepared in 96-well plates and incubated for 24 to 36 hours. Each dilution was run in 8 wells of a 96-well plate. Inoculated cells were further incubated for up to 7 days, monitoring characteristic CPE. At the end of 7 days, all cells were fixed with 80% acetone solution and the presence of PRV was confirmed by immunofluorescence microscopy. Virus titer in each sample was calculated using the Kärber [17, 18] or Reed-Muench method [17, 19], and expressed as 50% tissue culture infective dose per mL (TCID50/mL). Samples (undiluted) were considered to be negative for PRV after 2 blind passages.

Restriction fragment length polymorphism (RFLP)

A genetic comparison of the PRV MS+1 and the viruses recovered from porcine tissues during the first passage was made by RFLP analysis. The PRV samples were passaged once or twice in Madin-Darby Bovine Kidney (MDBK) cells to obtain sufficient viral particles, and the PRV MS+1 and 5 of the 6 tissue-reisolated viruses (i.e., back-passaged in Animal Nos., 1, 3-5 and 7) were propagated sufficiently for RFLP testing. Virus recovered from 1 pig (Animal No. 6) failed to adequately grow in culture for the analysis. Subsequently, DNA from each available virus sample was extracted, purified, and quantitated following the procedures of Whetstone [20] with the following modifications. Samples were incubated in sodium dodecylsulfate and proteinase K overnight instead of for 1 hour, and the DNA was extracted twice with TE-saturated phenol instead of once. Approximately 1 g of DNA was precipitated in 10% 3M sodium acetate and 2.5 volumes of 100% ethanol at -20℃ [17]. The DNA was pelleted by centrifugation in a microcentrifuge for 30 minutes, dried and resuspended in 16 L of sterile distilled deionized water. The DNA was digested using the following 6 restriction enzymes: *Bam* HI, *Eco* RI, *Hind* III, *Kpn* I, *Pst* I, and *Xba* I (New England Biolabs, Beverly, Massachusetts, USA) according to the manufacturer's recommendations. The digested DNA was extracted with TE-saturated phenol and chloroform (1:1) and the aqueous layer was electrophoresed on a 0.8% agarose gel in TBE buffer (0.045M Tris-acetate, 0.001 M EDTA and 0.445 M boric acid, pH8), at 35 V (constant voltage) for 15 hours. The RFLPs were visualized with ethidium bromide on a UV transilluminator. Additionally, for each comparison with a restriction enzyme, molecular weight standards, uninfected MDBK cells, and undigested viral DNA were prepared and included in the analysis. The resulting band patterns were photographed and compared among viruses for genetic differences.

Results

First Viral Passage in Pigs

For the initial virus challenge, 6 pigs were given 1 mL of the PRV MS+1 at approximately 107.3 TCID50/mL, which was approximately 15,000 greater than the established minimum immunizing dose (i.e., approximately 103.1 TCID50/mL). Subsequently, virus was reisolated from the tissue homogenates of 6 pigs necropsied at 3, 4 or 5 days PI (N=2 pigs/day), and the PRV titers from those resulting supernatants ranged between 101.7 to 102.2 TCID50/mL. Further, each animal had a nasal swab sample that was either suspect or positive for PRV on at least 1 sampling day PI. We were able to demonstrated the presence of PRV in the sample but unable to quantitate probably due to very low amount of virus. However, no clinical signs of PRV infection, including pyrexia, were observed in that group.

Second Viral Passages in Pigs

Subsequently, the tissue homogenate obtained for 1 animal necropsied at 5 days PI was used to challenge pigs during the second animal passage. That inoculum was selected because the resulting PRV titer was 101.9 TCID50/mL and because the nasal swabs collected from that animal at 3, 4 and 5 days PI were each positive for PRV. That inoculation quantity was approximately 16 fold less than the established minimum immunizing dose.

Pseudorabies virus was not recovered from the tissue homogenates nor from the nasal swabs collected from any of the 5 pigs necropsied at 5 days PI during the second passage. Furthermore, no clinical signs of PRV infection, including pyrexia, were observed. Since no virus was reisolated, pooled tissue homogenates obtained from all 6 pigs during the first animal passage were used to inoculate 15 pigs during a second attempt at a second in-vivo passage. The inoculum was determined to contain PRV at approximately 101.7 TCID50/ml. Those animals were challenged with 2 mL, as opposed to 1 mL in the previous

passages, and for 3 consecutive days, as opposed to once. As a result, the total PRV challenge was approximately $10^{2.2}$ $TCID_{50}$/mL, a quantity that was approximately 4 fold less than the established minimum immunizing dose.

No PRV was recovered from tissue homogenate pools nor from the nasal swab samples obtained from any of the 5 pigs necropsied at 5 days PI during, what proved to be, the ultimate animal passage. Further, there was no seroconversion to PRV among the remaining 10 pigs monitored through 21 days PI. Finally, no clinical signs of PRV infection were observed in any of the 15 pigs observed (i.e., 5 pigs monitored for 5 days PI prior to necropsy and 10 pigs monitored for 21 days PI) during the observation period.

RFLP analysis

RFLP analysis using *Bam* HI (Fig. 1), *Eco* RI (Fig. 2A), *Hind* III (Fig. 2B), *Kpn* I (Fig. 2C), *Xba* I (Fig. 2D) and *Pst* I (Fig. 3) to contrast the PRV MS+1 and the 5 viruses reisolated after back-passage, did not revealed any changes in the number and pattern of DNA fragments of viruses reisolated from tissues in comparison to PRV in the inoculum (i.e., PRV MS+1), strongly indicating that all the viral genomes were retained same during the animal passage.

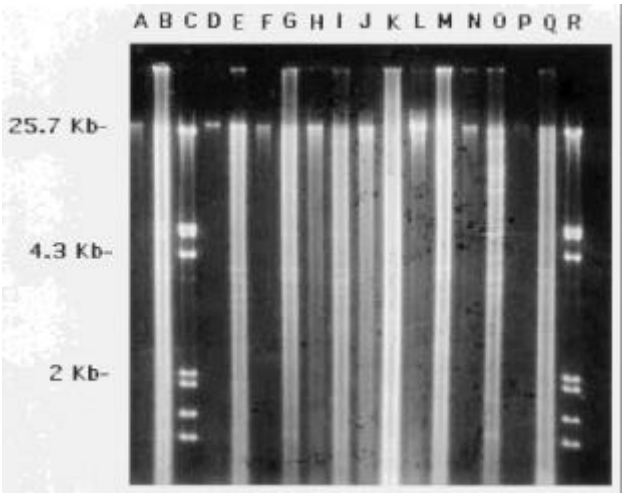

Figure 1. Restriction fragment length polymorphism analysis of attenuated PRV inoculum (MS+1) and back- passaged virus using *Bam* HI. No differences were observed in the RFLP among the MS+1 and the other 5 back- passaged viruses. Lane A = undigested back-passaged virus from Animal No. 7; Lane B = digested back-passaged virus from Animal No. 7; Lane C = molecular weight standards; Lane D = undigested MDBK cells; Lane E = digested MDBK cells; Lane F = undigested MS+1 virus; Lane G = digested MS+1 virus; Lane H = undigested back-passaged virus from Animal No. 1; Lane I = digested back-passaged virus from Animal No. 1; Lane J = undigested back-passaged virus from Animal No. 3; Lane K= digested back-passaged virus from Animal No. 3; Lane L = undigested back-passaged virus from Animal No. 4; Lane M = digested back-passaged virus from Animal No. 4; Lane N = undigested back-passaged virus from Animal No. 5; Lane O = digested back-passaged virus from Animal No. 5; Lane P = undigested back-passaged virus from Animal No. 6; Lane Q = digested back-passaged virus from Animal No. 6; Lane R = molecular weight standards.

Discussion

The objective of this study was to characterize the replication of attenuated PRV in pigs and determine the susceptibility of an attenuated PRV to revert to virulence after multiple passages in PRV-naive pigs under experimental conditions. The PRV evaluated was a modified live, gE-deleted virus obtained after 1 passage in-vitro from a master seed virus. After intranasal instillation of pigs with that virus, a minimal level of PRV was recovered from brain and secondary lymphoid tissues, as well as from nasal secretions collected post-inoculation, demonstrating that the PRV MS+1 was able to replicate, but to a limited degree, in pigs as expected for a modified virus. However, no clinical signs of PRV infection were observed in any of those pigs, indicating the attenuation of its pathogenicity.

Furthermore, no PRV was recovered from the tissues or nasal swabs collected from pigs in a second passage which were challenged with the supernatant containing PRV from 1 animal inoculated in the previous passage. Again, no clinical signs of PRV infection were observed. Those observations demonstrated that the back-passaged virus was not able to establish infection and replicate beyond 1 animal passage, when low levels of the reisolated virus were administered.

To ensure that the pigs were adequately challenged with PRV beyond the first passage, tissue supernatants obtained from PRV-positive pigs in the first passage were combined and used to inoculate pigs for 3 consecutive days. This approach was deemed appropriate, as opposed to culturing the re-isolated virus in-vitro to obtain a higher titer, to preclude artificially altering the attenuation, or lack thereof, of the challenge virus. Further, the USDA reversion-to-virulence study guidelines used provided that virus reisolated between animal passages could be concentrated, but in-vitro propagation between passages was prohibited [15]. At 5 days PI, 1 group of pigs was necropsied and PRV was not recovered from their tissues, confirming the failure of the virus to replicate during the second passage. Further, a separate group of pigs monitored for 21 days PI failed to present with clinical signs of PRV infection and failed to seroconvert. Thus, those pigs also confirmed the failure of the virus to replicate in the host beyond a single passage.

Finally, a genetic comparison of the modified live PRV and virus reisolated from the tissues of pigs challenged in the first passage was made by RFLP. No changes in the pattern of DNA fragments (number and size) were observed

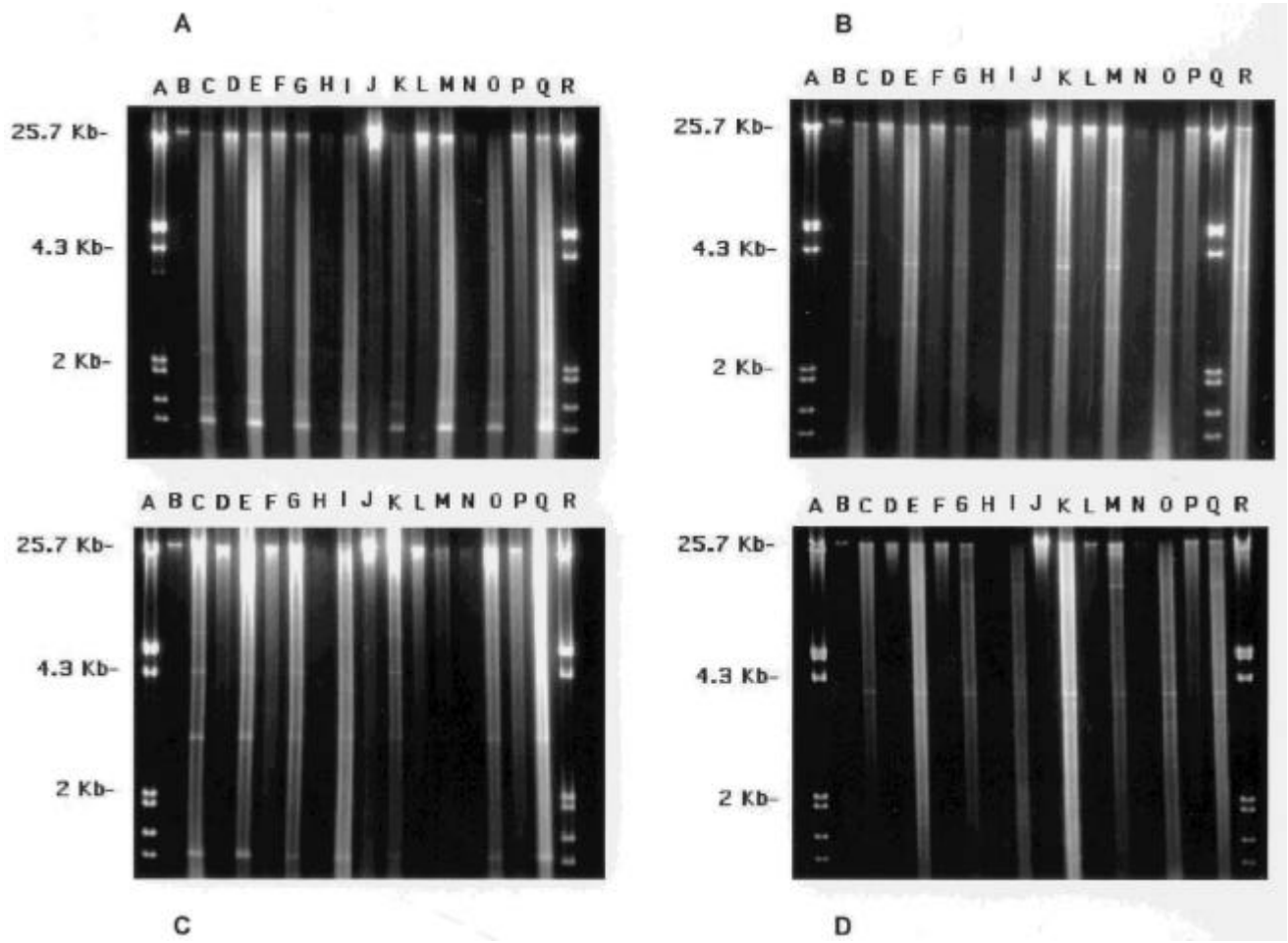

Figure 2. Restriction fragment length polymorphism analysis of attenuated PRV inoculum (MS+1) and back-passaged virus using *Eco* RI (A), *Hind* III (B), *Kpn* I (C), and *Xba* I (D), respectively. No differences were observed in the RFLP among the MS+1 and the other 5 back-passaged viruses. Lane A = molecular weight standards; Lane B = undigested MDBK cells; Lane C = digested MDBK cells; Lane D = undigested MS+1 virus; Lane E = digested MS+1 virus; Lane F = undigested back-passaged virus from Animal No. 1; Lane G = digested back-passaged virus from Animal No. 1; Lane H = undigested back-passaged virus from Animal No. 3; Lane I = digested back-passaged virus from Animal No. 3; Lane J = undigested back-passaged virus from Animal No. 4; Lane K= digested back-passaged virus from Animal No. 4; Lane L = undigested back-passaged virus from Animal No. 5; Lane M = digested back-passaged virus from Animal No. 5; Lane N = undigested back-passaged virus from Animal No. 6; Lane O = digested back-passaged virus from Animal No. 6; Lane P = undigested back-passaged virus from Animal No. 7; Lane Q = digested back-passaged virus from Animal No. 7; Lane R = molecular weight standards.

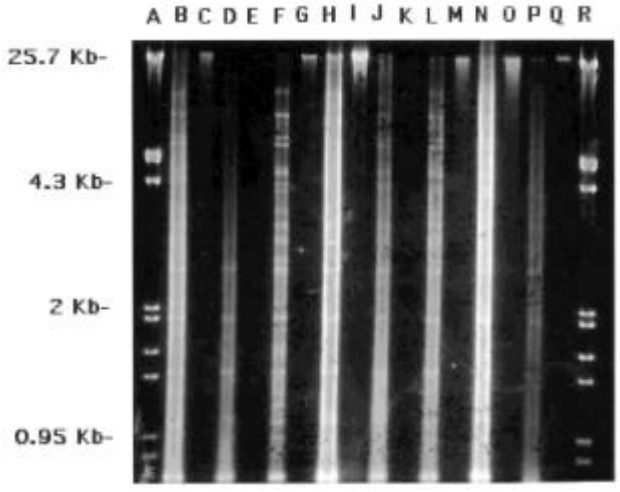

Figure 3. Restriction fragment length polymorphism analysis of attenuated PRV inoculum (MS+1) and back-passaged virus using *Pst* I. Lane A = molecular weight standards; Lane B = digested back-passaged virus from Animal No. 7; Lane C = undigested back-passaged virus from Animal No. 7; Lane D = digested back-passaged virus from Animal No. 6; Lane E = undigested back-passaged virus from Animal No. 6; Lane F = digested back-passaged virus from Animal No. 5; Lane G = undigested back-passaged virus from Animal No. 5; Lane H = digested back-passaged virus from Animal No. 4; Lane I= undigested back-passaged virus from Animal No. 4; Lane J = digested back-passaged virus from Animal No. 3; Lane K = undigested back-passaged virus from Animal No. 3; Lane L = digested back-passaged virus from Animal No. 1; Lane M = undigested back-passaged virus from Animal No. 1; Lane N = digested MS+1 virus; Lane O = undigested MS+1 virus; Lane P = digested MDBK cells; Lane Q = undigested MDBK cells; Lane R = molecular weight standards.

among those viruses when 6 different enzymes were used to digest the samples. Thus, the viral genomes tested were similar or the same.

The study demonstrated that the modified live virus did not replicate beyond 1 passage in susceptible pigs, as evidenced by no positive virus isolation or seroconversion. It was also demonstrated that there were no subsequent DNA changes in the virus or reversion to virulence after that passage.

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