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Abstracts

Recent outbreaks of PED and its pathogenicity in China

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Starting in the October 2010, a remarkable increase in PED emerged in China in an outbreak characterized by high mortality rates among suckling piglets, resulting in substantial economic losses. This etiologic agent was new variant PEDV strains. In this paper, the new variant strains were introduced in three parts: origin, pathogenicity and genetic features. Hoping this introduction could provide new information for understanding the pathogenicity of new variant PEDV strains and the control measures of PED.

Porcine epidemic diarrhea virus (PEDV) infection in the United States: Etiology, epidemiology, pathogenesis and immunoprophylaxis

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PEDV (family *Coronaviridae*, genus *Alphacoronavirus*) causes acute diarrhea/vomiting, dehydration and high mortality in seronegative neonatal piglets. The fecal-oral route is the main means of PEDV transmission, although aerosolized PEDV remains infectious. For the last three decades, PEDV infection has resulted in significant economic losses in the European and Asian pig industries, but in 2013–2015 the disease has also emerged or re-emerged in the US and worldwide. The PED epidemic in the US, from April 2013 to 2014, led to the loss of >10% of the pig population. Since 2013–2014, the porcine deltacoronavirus, genetically distinct but clinically and pathologically similar to PEDV, has also emerged and influenced PEDV-infected pigs nationwide.

The original PEDV strains identified during the initial outbreak were genetically closed to the Chinese strains (China/2012/AH2012). Since 2013–2014, novel PEDV strains (OH/OH851) have been also found in the US. The novel strains with multiple deletions and insertions in their S gene (so called S INDEL strains), which clustered closely with Chinese strain HBQX-2010, rather than AH2012, were found to possess low nucleotide identity in their 5'-end S1 region and high nucleotide identity in the remaining S gene, compared to the original US PEDV strains. Possible recombination events involving strain(s) from China may have contributed to a rapid evolution of US PEDV. The S INDEL strains have been found globally. In German farms with PED caused by S INDEL strains, severe clinical disease and frequent deaths of neonatal piglets were observed. A comprehensive understanding of the pathogenic characteristics of the strain is further needed.

The original US PEDV strains acutely infect villous epithelial cells of the entire small and large intestines although the jejunum and ileum are the primary sites of infection. Initial infection and replication of PEDV in the villous epithelial cells, frequently affecting the entire villous epithelium, and subsequent vacuolar degeneration and necrosis of infected cells acutely occur for 12–24 hours after exposure, accompanied by occurrence of proliferating crypt cells to replace the necrotic cells. PEDV infections cause acute, severe atrophic enteritis accompanied by viremia that leads to severe diarrhea and/or vomiting, followed by extensive dehydration and metabolic acidosis as the major cause of death in nursing piglets. PEDV antigens were evident in the villous or crypt epithelial cells of the small and large intestines and antigen-presenting cells in Peyer's patch, spleen, and mesenteric lymph node, whereas they were not detected in other organs.

PED is the most devastating in nursing piglets causing 100% morbidity and 50–100% mortality. The several mechanisms by which PEDV infection induces greater disease severity and deaths in nursing versus weaned pigs, have been defined. Infected nursing pigs showed early severe clinical

disease and villous atrophy and fecal virus shedding, whereas a longer incubation period of PEDV was required for weaned pigs to show fecal virus shedding or lesions and clinical disease. There was an innate immune deficiency in functional natural killer cells in the ileum and blood of nursing piglets that may have contributed to the greater susceptibility of nursing pigs to PEDV infection. Several anatomical factors that may influence a longer recovery from disease in nursing pigs include: 1) the slower turnover of enterocytes in neonatal piglets compared to weaned pigs; and 2) the anatomically less developed large intestine that may increase the vulnerability to dehydration; and 3) lack of crypt stem cells and lower numbers of proliferating crypt cells in the small intestine, compared to weaned pigs, leading to the slow turnover of enterocytes and contributing to the slow recovery and greater susceptibility of nursing pigs to PED (diarrhea and dehydration).

In the US, currently, a killed virus, adjuvanted vaccine from Zoetis, Inc. and a RNA particle-based vaccine from Harrisvaccines, Inc. were conditionally licensed to immunize pregnant swine (IM, 2 doses at 2 or 4–5 weeks before farrowing) to protect seronegative pregnant swine; however, no detailed efficacy of these vaccines are reported. Globally, pregnant swine have been vaccinated using live attenuated PEDV strains via an intramuscular or oral route, but induction of complete protection has never been observed in the nursing piglets. Moreover, whether the live vaccine strain is genetically stable and remains non-infectious in the fields should be studied. To select optimal US vaccine strain(s), studies are needed to define whether there is antigenic cross-reactivity between the original US and S INDEL strains, and other variants concurrently circulating in US swine. The time taken until dehydration of PEDV-infected nursing piglets is too short to enable the animals to recover from the disease through naturally occurring epithelial cell renewal by crypt stem cells. Pharmacological or biological mediators that promote stem cell regeneration or maturation would be also promising targets to shorten the time for epithelial cell renewal. Combined use of preventive (vaccination) and therapeutic interventions would synergistically reduce PEDV death losses.

Recent Outbreaks and emergence of mutants of Porcine Epidemic Diarrhea Viruses (PEDV) in Korea

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Porcine epidemic diarrhea (PED) virus was first reported in South Korea in 1992 and has since been circulating and exhibiting considerable genetic diversity. During 2013, PED outbreaks reoccurred in South Korea; however, the emerging PEDVs in these outbreaks were not variants of previous Korean isolates or attenuated vaccine strains. During October 2013–June 2014, 30 intestinal samples of dead piglets and 16 fecal swabs were found to be PEDV positive. One field strain of PEDV (named BM1) was successfully adapted for growth on Vero cells. This virus was isolated from BM farm of 60 sows, which had not vaccinated against PEDV. Pigs of all ages from the farm showed clinical symptoms of diarrhea with 100% deaths among suckling piglets and 10% among sows. Examination at necropsy revealed that the dead piglets of BM farm were covered with brown blotches of dried scour material and their stomachs were filled with undigested milk. Thin, translucent small intestines containing yellow fluid were also observed. The BM1 PEDV field isolate induced cytopathic effects of rounded shape within 48 hours at passage 10. The presence of PEDV in the cell culture was confirmed by immunofluorescence assay, which showed the specific fluorescence signal. Beside of microscopic observation, the increment in the quantity of viral RNA was observed to increase as the number of passages increased, from 30,325 copies/μl ($C_T = 16.11$) at passage 2 to 418,000 copies/μl ($C_T = 13.77$) at passage 10. The infective titers of the BM1 isolate increased from $10^{4.7}$ TCID₅₀/ml (at passage 2) to $10^{7.9}$ TCID₅₀/ml (at passage 10). The complete S gene of BM1 (KP861982) was sequenced for genetic characterization. The S gene has 4,161 nt in length and encodes 1,386 aa. The spike protein of the BM1 isolate showed substitutions at neutralizing SS6 epitope from LQDGQVKI to SQSGQVKI but identity at the SS2 and 2C10 neutralizing epitopes. The genetic relationship of the BM1 isolate with other PEDVs in the world was inferred from a codon-based alignment of 409 sequences of the complete S gene. The maximum likelihood phylogenetic tree was constructed by the FastTree program with the general time reversible nucleotide substitution model. The phylogeny constructed on the basis of the complete S gene showed that the BM1 isolate belongs to subgroup 2a, genogroup 2 of PEDV. This isolate clustered closely with emergent PEDV strains in the United States, showing 99.2%–99.7% identity with PEDVs of North American strains. This observation was repeated by the phylogenetic inference of the complete N gene. The branching pattern clearly showed that BM1 is genetically less related (92.9–93.4% identity) to the live vaccine strains derived from genogroup 1 and used currently to prevent PEDV infections in South Korea. And as an additional study, since 1992, porcine epidemic diarrhea virus (PEDV) has become one of the most important porcine diarrhea associated viruses in South Korea. We performed large-scale study of the incidence of PEDV in pigs with diarrhea in South Korea, and consequently identified and characterized a novel PEDV variant with large genomic deletion.

Outbreaks and control measures of PED in Japan

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On October 1, 2013, outbreak of PED was confirmed in Okinawa prefecture for the first time in seven years in Japan, and then the outbreaks expanded to the 37 prefectures including Ibaraki prefecture, Kagoshima prefecture and Miyazaki prefecture. Number of cases had increased rapidly since March, 2014. Eventually, over 100 new cases per week were reported in the second week of April, 2014. Although the strengthening of control measures and getting warmer weather led to decrease the number of cases temporarily, the outbreaks occurred in 38 prefectures and 817 cases in total, and caused death of 420 thousand weaning pigs from November 2013 to August 2014 (“2013 epidemic season”). After 2013 epidemic season, upsurge of PED cases has not been reported as in 2013 epidemic season, 233 and 30 cases are reported in 2014 epidemic season and 2015 (from September to the first week of December) respectively. The control measures for PED in Japan such as strengthening of hygiene management on farm, promotion of vaccination, sharing of related information and prevention of cross-contamination at livestock relating facilities have been implemented steadily through mutual cooperation among concerned parties. These control measures are working well, and consequently massive outbreaks have not occurred as in 2013 epidemic season. However, control measures for PED should keep to be taken as the PED cases are still reported sporadically.

Strategies to control against the outbreaks of PED in Europe

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Swine producing areas are increasingly affected by a range of emerging infectious diseases. The emergence of PEDV USA in 2013 has raised many questions on introduction and rapid transmission of this pathogen across North America but has also raised concerns from other swine production areas at that time not affected by PEDV. Initially PEDV has been recognized more than three decades ago in the UK and was characterized by outbreaks of diarrhea in feeder and fattening pigs. Subsequently, the disease spread to other European countries. Around 1976, the age tropism of the disease appeared to change as acute diarrhea was also appearing in suckling pigs in addition to feeders and fattening pigs. During this time, the clinical disease was first named “epidemic viral diarrhea” or PED. Widespread PED epidemics were prevalent throughout Europe including swine producing countries such as Belgium, England, Germany, France, the Netherlands, and Switzerland during the 1970s and 1980. During subsequent years, PED epidemics became rare and PEDV was more often associated with single outbreaks. In general it was assumed that the immunity in the sow population was high enough to protect pigs from clinical signs associated with PEDV infection. However, a PEDV epidemic was observed in Italy in 2005–2006 emphasizing that PEDV was still present and epidemics may occur at any time. In January of 2014, PEDV associated with high mortality was first recognized in the Ukraine. Subsequently, PEDV outbreaks associated with watery diarrhea in piglets were also seen in Germany, Portugal, France, the Netherlands and Belgium. In essentially all cases, a PEDV genogroup 2 S-INDEL strain was identified similar to strains circulating in the US but different from the PEDV prototype genogroup 2 strains. When characterized in pigs in the US, genogroup 2 S-INDEL strains have been found to be less pathogenic compared to prototype strains and this somewhat milder clinical disease course has also been noted in the European field. During the initial European outbreak of PEDV in the 1970ies, pregnant sows were deliberately exposed to the intestinal contents of dead infected pigs. However this practice commonly known as “feedback” or “controlled exposure” is currently not recommended in Europe. Therefore, control of PEDV in Europe is largely limited to improving biosecurity. There are efforts to implement a commercial inactivated sow vaccine in several European countries but there is only limited information available to date. In summary, PEDV has been re-emerging in Europe but appears to have an altered virulence different from what has been observed in North America and Asia in recent years. Control measures such as improving biosecurity appear in many cases sufficient to control the disease.

Development and application of the effective vaccine and other strategies against recent porcine epidemic diarrhea outbreaks

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In April 2013 porcine epidemic diarrhea virus (PEDV) was detected for the first time in the United States and within months the virus was detected throughout all major swine producing regions of the US. The virus caused severe diarrhea in young piglets leading to high mortality. Within the first year of the epidemic about 50% of the sows in the US became infected resulting in a 7-8% loss in total pig production. Interestingly, the US PEDV isolates were almost identical to contemporary Chinese PEDV isolates. The initial control measures for PEDV in the US included a controlled oral exposure of the sow herd to infectious virus, and an increased cleaning and sanitation of transport vehicles and facilities. Controlled exposure or “feedback” involved inoculating the entire sow herd with infectious material (feces collected from sick and infected piglets) in an attempt to stimulate active immunity against PEDV in the sow herd. The goal was to develop immunity in the sow and transfer that immunity to pigs at birth through colostrum. In most acutely affected herds this practice would take several weeks to induce a protective immunity in sows that would provide clinical protection to newborn piglets. The duration of this sow immunity is not known. In 2014, two PEDV vaccines became commercially available. One was an inactivated whole virus vaccine; the second vaccine was a non-replicating viral vector that expresses a portion of the PEDV spike gene. To date there is limited experimental data available on the efficacy of either vaccine. However, both have been used extensively in the field to vaccinate sows and there are anecdotal reports supporting the use of the vaccines to reduce PEDV losses. During the second year of the epidemic the incidence of new cases declined dramatically although there was a small, but steady reporting of new cases indicating horizontal spread of the virus among swine farms continued, but at a much reduced rate. It is not clear why this occurred, but the reduced spread of the virus to naïve herds may be related to intense sanitation efforts of transport vehicles and restrictions on movement of people and fomites among farms. During the second, and now third year of the emergence of PEDV in the US, there are reports of re-breaks in previously infected herds, but these events are less severe than the original disease break. It is not clear if the re-breaks represent a pocket of naïve animals (replacement sows), or waning of immunity in the sows. In most swine herds there is a high replacement rate for sows where frequently 50% of the sow herd is replaced every year. It would be expected that these herds would have an unstable herd immunity to many endemic swine diseases including PEDV. Current strategies to control PEDV still involve “feedback” to inoculate replacement gilts, increased biosecurity practices that include extensive cleaning of barns, transport vehicles, restrictions on movement of people, and frequent testing of feed additives in an effort to reduce the threat of introducing new PEDV isolates to a farm through the purchase of feed. Commercial vaccines are still being used although their use may be reduced in an effort to save money in herds that do not currently have clinical disease. At the time of this writing, pork producers in the US are hoping PEDV will continue to fade away, however, winter is considered to be the best season for the virus and there is great concern that with the high replacement rate for sows, PEDV may become a significant problem again during the winter of 2015/2016.

Efficacy of the PED vaccine evaluation in Korea

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Porcine epidemic diarrhea virus (PEDV) is an infectious, highly contagious virus of swine, which belongs to the Coronaviridae family (Pensaert *et al.* 1978). PEDV was first reported in Belgium and the United Kingdom in 1978 and is an etiological agent of acute entero-pathogenic diarrhea in swine (Debouck & Pensaert 1980). Several PEDV vaccines, which differ in their genomic sequence, mode of delivery, and efficacy, have been developed (Bernasconi *et al.* 1995). In Europe, the disease caused by PEDV has not been of sufficient economic importance to focus on vaccine development. Therefore, trials for vaccine development have primarily been carried out in Asian countries where PEDV outbreaks have been so severe that the mortality of the newborn piglets was significantly increased. In Japan, a commercial vaccine consisting of attenuated virus of cell culture-adapted PEDV (P-5V) has been administered to sows since 1997. Although these vaccines are considered efficacious, not all sows develop solid lactogenic immunity (Usami *et al.* 1998). Prophylactic vaccines capable of preventing the initial stages of viral infection usually induce neutralizing antibodies against the viral pathogen (Noda *et al.* 1987). Other candidate vaccines have focused on the CO-26K fragment equivalent (COE) domain located on the spike protein that is reported to contain neutralizing epitopes against PEDV (Chang *et al.* 2002). Information on PEDV mucosal immunity has typically been limited. De Arriba *et al.* used the enzyme-linked immunospot (ELISPOT) technique to characterize the isotype-specific antibody-secreting cells in mucosal and systemic-associated lymphoid tissues in pigs inoculated with PEDV (De Arriba *et al.* 2002a). There have also been reports of immune response of the pigs exposed to PEDV antigens expressed by plants (Bae *et al.* 2003; Kang *et al.* 2005; Hou *et al.* 2007). The efficient vaccine and vaccine program for PEDV should focus on several criteria; including factors related the reduction of virus shedding in piglets and the details of the mucosal immunity to PEDV. In this study, we evaluated and compared antibody response in sera from different vaccination program using killed and live vaccines.

In this study, the antibody level of killed and live vaccines against porcine epidemic diarrhea virus (PEDV) were evaluated and compared. The highest levels of virus-specific IgG and IgA in serum and colostrum from sows, as well as serum from piglets, was found in the group that was vaccinated with killed virus (K/K), while the lowest levels were found in the group vaccinated with live vaccine (L/L). Protein enzyme-linked immunosorbent assay (pELISA) carried out on serum samples further illustrated the neutralizing activity of the S3 region, the carboxy-terminal of spike protein. The K/K group presented the highest neutralizing activity and colostrum showed the highest protection level with the pronounced neutralizing activity in piglets. In addition, the K/K group produced the highest level of antibodies against the structural proteins, spike protein and nucleocapsid protein. These findings clearly suggest that the K/K vaccination program for PEDV.

Characterization of porcine epidemic diarrhea (PED) virus field strains

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Porcine epidemic diarrhea (PED), which is characterized by lethal diarrhea in newborn piglets and caused by intestinal infection by PED virus, a member of the family *Coronaviridae*, the genus *Alphacoronavirus*, has rapidly spread throughout pig farms in Japan with serious economic losses since the emergence at the end of 2013. In the 2014–2015 season, we were involved in a governmental project, Strategic Improvement project of the national Surveillance and Diagnosis system for Animal, supported by the Ministry of Agriculture, Forestry and Fisheries of Japan: the object of the project was to collect PED virus field isolates circulating in Japan and to virologically characterize the isolates. During the course of the project, we received intestinal tissue specimens of PED-affected pigs from several livestock hygiene service centers in Japan and isolated seven PED viruses from the specimens collected in four prefectures. To genetically characterize PED viruses circulating in Japan, we determined the nucleotide sequences of the full-length spike (S) genes, which encode a critical antigenicity determinant S protein, from our seven isolates, as well as six contemporary PED viruses isolated by livestock hygiene service centers in two prefectures. We then phylogenetically analyzed the determined 13 sequences with counterparts from the representative PED virus isolates all over the world. To gain further insight into the antigenicity of these Japanese isolates, we constructed 3D structures of the PED virus S protein based on the deduced amino acid sequence by means of a homology modeling approach and identified the spatial position of the amino acids that show variations between the isolates tested. In addition, the antigenic differences between the recent isolates and a vaccine strain were investigated by means of serological assays. In this presentation, our results of these studies will be described.

Discovery of Inhibitory Materials against PEDV Corona Virus from Medicinal Plants

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Coronaviruses (CoVs) are one type of enveloped viruses with single-stranded RNA genome. They contain the largest genome to date, ranging from approximately 26 to 32 kilobases. During CoVs replication, genes encoding the viral structural proteins, including nucleocapsid (N), membrane (M), spike (S), and envelope (E), are key elements for viral integrity. CoVs infections are a major cause of enteric, respiratory, and central nervous system diseases at both animals and humans. An outbreak of SARS-CoV infections in 2002–2003 caused severe acute respiratory syndrome (SARS) of more than 8,000 individuals and 774 fatalities worldwide. Middle East Respiratory Syndrome coronavirus (MERS-CoV), which were occurred Saudi Arabia in 2012, shows a recent outbreak of a completely novel respiratory virus. MERS on May in 2015 abruptly broke out in Korea and 36 patients of infected 186 persons were died. These experiences in Korea suggest that MERS outbreak or other CoVs-infectious diseases should be prepared to control for the protection of human health.

There are four common CoVs infecting animals, including porcine epidemic diarrhea virus (PEDV), canine coronavirus (CCV), avian infectious bronchitis virus (IBV), and transmissible gastroenteritis coronavirus (TGEV). PEDV can spread via vertical transmission through lactation or the fecal-oral route. By targeting the epithelial cells of the small intestine PEDV can cause severe mucosal atrophy and malabsorption, and result in acute and lethal diarrhea in piglets. PEDV infection can cause a high mortality in piglets and fattening swine with almost 100% for one-week old piglets. Recently, new PEDV was noticed in USA for the first time in May 2013, and its outbreak affects 23 US states by the end of January of 2014. PEDV infection in US swine industry caused severe watery diarrhea, variable vomiting, dehydration, and high mortality in affected swine, especially neonatal piglets resulting severe economic loss. In concerning about the control and eradication of PEDV infection, generous efforts have been made with major focus on preventive therapy by vaccination. However, as an oral vaccine for preventing PEDV infections is still not able to significantly alter the duration of virus shedding, an effective control strategy may be the benefit to prevent such infections.

Natural products have been regarded as a rich source of novel chemical entities with unique pharmacological activities. During the course of our antiviral screening programs from natural products for PEDV, we screened strong inhibitory materials by using of virus-induced cell death method by PEDV. A CPE assay, RT-PCR analysis, western blot analysis, and immunofluorescence assay supported that some candidates from natural products showed potent and promising inhibitory effects on PEDV replication by targeting key structural protein synthesis and relevant gene expression. These studies provided a new class of natural scaffolds that may be able to be developed as potential anti-PEDV agents via inhibiting viral replication. The clear SARs outlined will facilitate the further structure optimization of this compound class for developing novel antiviral agents. Furthermore, human corona viruses, like SARS or MERS, from the same Coronaviridae family share a similar replication mechanism with PEDV. Therefore, the anti-PEDV molecules obtained in this study will also be used for further investigation against the fatal human coronaviruses, including SARS or MERS.

Efficacy of the Porcine Epidemic Diarrhea Live Vaccine

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Porcine epidemic diarrhea (PED), which is caused by oral infection with the PED virus (PEDV), is mainly characterized by watery diarrhea and has an extremely high fatality rate in suckling piglets less than 1 week of age. The outbreak of PED in Japan has been reported since the early 1980's. The live vaccine was developed using Japanese isolates and obtained marketing approval in November 1996. Thus, outbreaks of nationwide PED that began in late 2013 were the first time to face the case for this vaccine.

The target cells of PEDV *in vivo* are epithelial cells of the gastrointestinal tract, especially those of the small intestine. The virus is thought to partially enter the bloodstream via the lymphoid tissue under the mucous membrane, and the viral genome may be transiently detected in multiple organs. However, no secondary replication of the virus is seen in those organs; following replication in the gastrointestinal epithelial cells, the virus is excreted mainly through feces, which then becomes a new source of infection. Post-weaning pigs may often allow them to be asymptomatic even after forced oral inoculation of the virus. However, clinical symptoms, such as anorexia or diarrhea, may transiently occur if pigs under stress conditions (e.g., pigs infected with other pathogens, farrowing, or in overcrowded housing environments) are exposed to PEDV.

The PED vaccine was designed based on the following concept: an immunized sows with a live vaccine in order to stimulate active production of PEDV-specific neutralizing antibodies for passive transfer via colostrum and milk to suckling piglets. As long as suckling piglets receive sufficient neutralizing antibodies in milk, they are protected from disease. Thus, the prevention of PEDV infection is not because of the maternal antibody in the colostrum but because of the continuous intake of milk containing a high level of neutralizing antibodies during the suckling period. This intake prevents the onset of PED and alleviates the symptoms, which are indications of the vaccine, and in the event that this continuous intake is not possible, the piglet is likely to be infected by the virus and is consequently susceptible to severe infection. A sow with PEDV antibody-negative or with inadequate immunity exhibits clinical symptoms with decreased lactation upon exposure to the large volume of virus, and the vaccine may not always be fully effective in such cases.

Our vaccine strain was established as an attenuated virus using serial passage cell cultures system. This serial passaging attenuates the pathogenesis of the strain. Therefore, no symptoms are seen in piglets even if the virus is orally inoculated, and also no virus is excreted in the feces of pigs intramuscularly injected with the vaccine.

A challenge experiment using a wild-type strain with genotype that newly emerged in Japan in 2013 was performed in piglets born from sows immunized (im, twice) with the vaccine during their pregnancy. Alleviated clinical symptoms and increased survival rate was more commonly observed in piglets taking milk from immunized sows than in piglets in the non-immunized control group. The amount of viral genome in the organs of immunized piglets decreased significantly after the challenge. The neutralizing antibody titer was higher in herds where the sows were inoculated with the PED vaccine than not inoculated, and there have been cases where the period of onset of PED has been short. In conclusion, this vaccine is thought to be an effective measure against the new strain.

In order to develop a more effective PED vaccine in the future, a thorough understanding of PED

pathology is required as a mucosal infectious disease. A new PED vaccine should be developed to induce advanced active immunity in sows without causing a serious condition in them. It also should concomitantly ensure the protection from disease in suckling piglets by a lactogenic immunity.