

Vaccination of nulliparous gilts against porcine epidemic diarrhoea can result in low neutralising antibody titres and high litter mortality

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ABSTRACT. Porcine epidemic diarrhoea (PED) is a disease caused by an alphacoronavirus and the symptoms include watery diarrhoea and vomiting, with more than 80% mortality amongst newborn piglets. The placentation in sows hinders the transference of antibodies to the foetus, therefore, the vaccination of pregnant females and transference of antibodies to piglets through colostrum are essential to protect them against virus particles. The aim of the study was to determine whether vaccination of nulliparous gilts could induce a high colostrum antibody titre and lower litter mortality, in comparison with vaccinated multiparous sows previously exposed to the virus. Samples of colostrum were obtained from 11 nulliparous gilts with two previous vaccinations (inactivated vaccine) and from 9 multiparous sows with three or more vaccinations (inactivated vaccine) that had been exposed to the virus. The IgG antibody titre was determined through anti-PED enzyme-linked immunosorbent assays (ELISA) and the neutralisation of antibodies was evaluated through plaque reduction neutralisation tests (PRNT). The colostrum of nulliparous gilts, when compared to the multiparous sows, presented a lower anti-PED IgG antibody titre as well as fewer neutralising antibodies. Furthermore, the piglets of multiparous sows experienced higher survival in comparison with those of nulliparous gilts ($P<0.01$), and mortality was dependent on the 'farrowing' variable ($P<0.01$). In conclusion, these results show that vaccinating nulliparous gilts does not increase the survival of their piglets in comparison with multiparous sows and that the IgG titres and neutralising antibodies are significantly lower in the former. These results suggest that a modified vaccine strategy is needed for nulliparous gilts to increase piglet protection.

Key words: vaccine, PED, nulliparous, colostrum.

INTRODUCTION

Porcine epidemic diarrhoea (PED) is a disease caused by a +ssRNA virus from the *Coronaviridae* family, genus *Alphacoronavirus* (Jung *et al* 2020). It causes watery diarrhoea, vomit, anorexia, and depression in pigs of all age groups. In production units with naïve individuals, which have not been infected nor vaccinated, this disease causes morbidity of up to 100% and 80-100% mortality in newborn piglets, the youngest being the most vulnerable due to their slow enterocyte replacement (Shibata *et al* 2000, Song and Park 2012, Park and Shin 2014, Jung and Saif 2015).

This disease was first identified in May 2013 in the USA (Stevenson *et al* 2013). That same year it spread to Canada and the first outbreak in Mexico was described

in 2014, in the State of Mexico. This disease has not been controlled or eradicated in many states of Mexico (Trujillo-Ortega *et al* 2016).

Amongst adult pigs, the most effective adaptative immune response is given by secretory IgA antibodies in the intestinal mucosa and serum IgG antibodies that can cross the epithelial mucosa (Horton and Vidarsson 2013). The type of placentation in sows hinders the transference of antibodies to the foetus, therefore, the transference of antibodies through colostrum and milk is essential for the survival of lactating piglets (Borghesi *et al* 2014). The sow secretes colostrum which contains IgG as the most abundant immunoglobulin during the first 48 hours after farrow, then colostrum is gradually replaced by milk in which secretory IgA is predominant. It is known that maternal antibodies can protect piglets up to 14 days against PED through the neutralisation of virus particles, which stops them from entering their target cell (enterocytes), however, this can vary depending on the sow's level of immunity (Salmon *et al* 2009, Chattha *et al* 2014, Langel *et al* 2016, 2020). To increase this protection, vaccines are designed to promote the transference of immunity to piglets through colostrum and milk. Many studies have reported the effectiveness of using inactivated vaccines in increasing antibodies and diminishing piglet mortality. Even though vaccines have been administered for a few months after the emergence of the disease, studies indicate only a decrease of prevalence from 4.4 to 1.4% in two years at herd-level (Ajayi *et al* 2018) and in some cases the prevalence remain steady between 50 and 60% (Zhang *et al* 2019). In addition, most of these vaccines are inactivated and administered parenterally and

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significant field efficacy has not been observed (Gerdtz and Zakhartchouk 2017, Rapp-Gabrielson *et al* 2014).

Therefore, it is important to establish whether there are differences in antibody protection against PED according to the number of vaccinations received or previous exposure to the disease. In this study, we evaluated the response between vaccinated nulliparous gilts and vaccinated and previously exposed multiparous sows, and the association with the titre of neutralising antibodies that are produced after vaccination. It is also important to determine whether the titre of neutralising antibodies is correlated with piglet mortality.

MATERIAL AND METHODS

PIG PRODUCTION UNIT

Colostrum samples were obtained at a pig production unit with a farrow-to-finish system in Puebla, Mexico from a total of 20 females: 11 nulliparous gilts and 9 multiparous sows. According to the sample size calculated in the program “OpenEpi: Open Source Epidemiologic Statistics for Public health” for comparing two means with the results reported by Poonsuk *et al* (2016), we needed at least 4 sows per group for an 80% of power (Poonsuk *et al* 2016). Nulliparous gilts were vaccinated twice before farrowing; the first dose was administered 4 weeks before farrowing and the second dose 2 weeks after. Multiparous sows had been vaccinated at least three times, firstly during their first pregnancy following the same protocol as nulliparous and a booster vaccination was applied after the second pregnancy 2 weeks before each farrowing. An inactivated vaccine was delivered intramuscularly in all cases. The production unit was being affected by a yearly PED outbreak. This protocol was approved by the Institutional Subcommittee for the Care and Use of Experimental Animals (DC2018/2-4).

EVALUATION OF PIGLET MORTALITY UNTIL WEANING DURING A PED OUTBREAK

To calculate the pre-weaning mortality, the reports of deaths amongst piglets that were born alive and had died during lactation were evaluated after weaning.

ANTIBODY DETERMINATION

Colostrum. At least 8 mL of colostrum was obtained for each sow. Colostrum was treated following the Gomes protocol with the following modifications: centrifuged at 6000g for 60 minutes and inactivated at 56 °C for 30 minutes (Gomes *et al* 2011).

Anti-PED IgG antibodies determination through ELISA (anti-PED immunoenzymatic assay). An immunoenzymatic assay was standardised in which a 96 well Maxisorp plate

was coated with PED from the isolate described below. Each well was coated with 50 µL of a 1 µg/mL viral protein in a carbonate buffer solution (pH 9.6) and incubated for 18 hours at 4 °C. Fifty µL of a sample (50 µL of a 1:300 colostrum serum/phosphate buffer solution) were added to each well, followed by incubation for 2 hours at 30 °C. HRP (horseradish peroxidase) protein A was added as a secondary conjugate at a 1:8000 dilution in 50 µL of phosphate buffer solution, and the samples were incubated for one hour at 30 °C. The plates were read 20 minutes after TMB application through a 650 nm filter.

As a quantifiable positive control, 4 mL of serum from two sows that had been immunised against PED on multiple occasions and previously exposed to the virus circulating in the state of Puebla, were precipitated with ammonium sulphate and later dialysed. This precipitate was used to construct a standard curve covering 120 µg/mL to 0.23 µg/mL total protein content. The optical densities of the standard curve and sample readings were analysed with the AssayZap software. It was modelled on a positive sigmoid curve and it was accepted based on the value of $b > 0.80$ on three repeated occasions.

DETERMINATION OF NEUTRALISING ANTIBODIES

Cell line. For this assay, VERO cells, passage number 25, were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco 12800-017) with 10% foetal bovine serum at 37 °C with 5% CO₂.

Virus isolation. A section of the small intestine was obtained from a piglet, also from the state of Puebla, that tested positive for PEDv through RT-PCR. It was macerated in 500 µL of DMEM and later filtered and used to inoculate VERO cells with 5 mL of infection medium, composed of DMEM with 10 µg/mL trypsin (TBO626) and 1% penicillin-streptomycin-neomycin (100x 15640-055). This mixture was incubated for 72 hours until the characteristic cytopathic effect (syncytia) was observed on more than 50% of the cell monolayer. After incubation, 500 µL of the supernatant were inoculated into another VERO cell culture with infection medium. This procedure was repeated four times. In the end, the presence of the virus was confirmed through end-point RT-PCR with the Verso 1-step RT-PCR ReddyMix kit following the instructions of the manufacturer using the primers 5’-GAATGCAAAACCCCAGAGAA-3’/5’-GTGTCAACCACCATCAACAGC-3’, which amplify a 156 bp fragment of the viral nucleocapsid, with an annealing temperature of 55 °C.

Virus titration. After the fifth pass, the virus was titrated through a plaque assay. In each well of a 12-well plate, 100,000 VERO cells were plated, and 24 hours later with 80% confluency the virus was inoculated at 1:2, 1:10, 1:100, 1:200, and 1:1000 dilutions with infection medium onto them. The plate was incubated for 2 hours

EVALUATION OF PIGLET MORTALITY FROM BIRTH TO WEANING DURING A PED OUTBREAK

The percentage of piglet mortality during lactation was evaluated for each female and the cause of death was determined by clinical signs and a positive RT-PCR from a pool of intestinal samples. The litters of five nulliparous gilts had 100% mortality and the litter of another had 50% mortality. Meanwhile, the litter of one multiparous sow had 100% mortality and another had 43% mortality (figure 3A). Overall, the litters of nulliparous gilts had 41.8% mortality while mortality in the litters of multiparous sows was 15.63% (figures 3B and 3C).

The litters of nulliparous gilts tend to experience higher mortality (figure 3), therefore, we decided to evaluate the dependence of piglet mortality to the farrowing variable and its risk, as well as the number of dead piglets in each group, with a piglet survival curve. A χ^2 test for independence concluded that there was no independence between mortality and farrowing ($P < 0.01$). The relative risk between mortality and nulliparity was 2.675 (95% CI=1.700-4.212).

A significant difference was found in piglet survival between nulliparous gilts and multiparous sows ($P < 0.01$). The average survival time of the piglets of nulliparous gilts was 16.479 (15.170-17.789) days, compared to 18.627 (17.679-19.575) days among piglets from multiparous sows (figure 4).

Since multiparous sows present a higher antibody titre, a correlation between that variable and piglet survival is to be expected. A correlation analysis between the number of

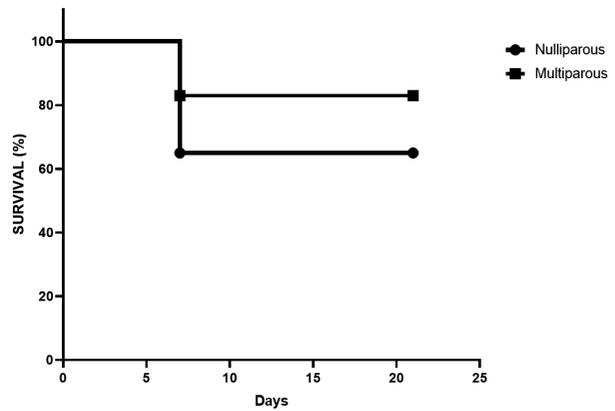


Figure 4. Kaplan-Meier survival curve of piglets born from nulliparous gilts and multiparous sows until weaning (at 21 days old) in a pig production unit undergoing an active PED outbreak $P < 0.01$.

weaned piglets and the amount of anti-PED IgG antibodies as determined through ELISA was carried out, finding near-significant results ($P = 0.055$).

DISCUSSION

In this study, the level of antibodies in the colostrum of nulliparous gilts was lower than that of multiparous sows, despite two immunisation treatments and having been exposed to the virus before farrowing. Furthermore, a low titre of antibodies was correlated with the neutralisation index, suggesting that females which have been immunised following this vaccination schedule may not generate an appropriate response. These results disagree with those reported by Paudel *et al* (2014) who evaluated the vaccination and revaccination of nulliparous gilts with three different protocols: two immunisations using inactivated virus, two immunisations using attenuated virus, or the first immunisation using active virus and the second immunisation using inactivated virus; the best response was obtained with two immunisations using inactivated virus (Paudel *et al* 2014). It is worth noting that in that study, the neutralising capacity of the antibodies generated was not measured and neither was the protection they provided to litters during outbreaks.

In this study, multiparous sows which had been previously exposed to the virus and vaccinated showed a higher antibody titre when compared to nulliparous gilts that had been vaccinated but not previously exposed to the virus. These results are consistent with those obtained by Shwartz (2016), who evaluated the efficacy of an inactivated virus vaccine in two different groups, females previously exposed to the virus (vaccinated and unvaccinated) and females that had not been exposed to the virus (vaccinated and unvaccinated). In that study, a difference in serum antibodies was found across all groups, with females that had been both exposed to the virus and vaccinated showing

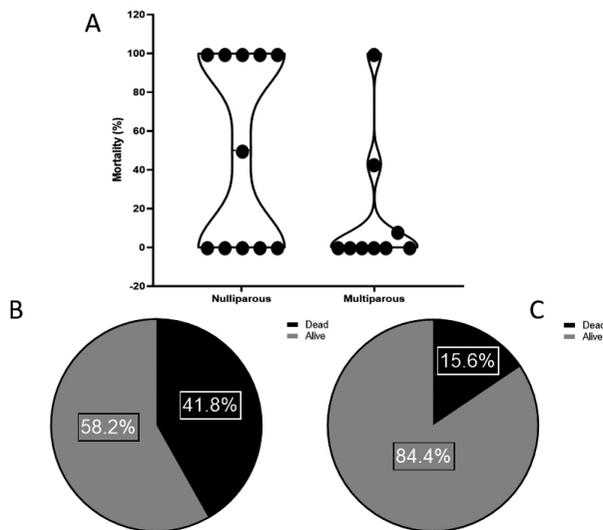


Figure 3. a) Violin plot showing piglet mortality percentage due to PED in nulliparous gilts and multiparous sows. Each dot represents one female's litter. b) Pie chart showing the percentage of piglets from nulliparous gilt litters that died due to PED. c) Pie chart showing the percentage of piglets from multiparous sow litters that died due to PED.

the highest average IgG antibody titre. As for IgA, only previously exposed groups had levels above the threshold for positivity. However, for an adequate antibody (IgG and IgA) titre in colostrum to be attained, a field exposure to the virus was necessary regardless of vaccination. This was reflected in piglet mortality, as the litters of females that had not been exposed to the virus had 100% mortality regardless of vaccination, while those of previously exposed females who had not been vaccinated had less than 10% mortality¹. In our study, piglet mortality among nulliparous gilt litters was not 100% in all litters but was nonetheless significantly higher than that of multiparous sow litters. This could be the result of gilts not being exposed to the field virus before receiving the vaccination.

Around 20% of females are replaced annually in pig production systems. These females undergo quarantine before being immunised against various diseases, including PED. If immunisation is not effective, replacement females and their litters remain susceptible to the disease and newborn piglets may suffer high mortality and morbidity (Kweon *et al* 2000). Colostrum and milk are the most important sources of protection for piglets, particularly of neutralising antibodies (Langel *et al* 2016). This study compared the neutralising capacity of the colostrum of vaccinated nulliparous gilts and multiparous sows, finding that the former had a lower neutralisation index (IN = 10.00) in comparison with the latter (IN = 26.16). Furthermore, a correlation with the results of the ELISA assay of 0.86 was found, similar to that reported by Oh *et al* (2005) (84.2%) across 1,024 serum samples. Song *et al* (2007) immunised sows with an attenuated virus vaccine orally and intramuscularly and found that colostrum antibody titres and their neutralising capacity were correlated in both groups, however, upon challenging their piglets, mortality amongst those born from females that had been vaccinated orally was 13%, while the litters of intramuscularly vaccinated females experienced 60% mortality. This could be due to the production of IgA in animals immunised through mucosae and could also explain why the females in our study, which had been exposed to the virus (multiparous sows), were able to better protect their litter, similar to the results by Song *et al*. To demonstrate this, IgA levels in females should be measured.

In our study, the litters of nulliparous gilts experienced on average 50% mortality, while those of multiparous sows averaged 17.66% mortality. However, Rapp-Gabrielson *et al* (2014) administered an inactivated virus vaccine and a placebo in a farm undergoing an active PED outbreak and saw a 90% decrease in mortality among the vaccinated

group when compared to the placebo. Nonetheless, piglet mortality in the placebo group was only 6.3%, which does not reflect the conditions of an outbreak such as the one in our study. Studies by Mogler (2014) and Crawford (2015) reported that subunit vaccines reduce the clinical signs and viral excretion post-challenge amongst weaned piglets. They observed that mortality in litters of nulliparous females was reduced to 69% after three pre-delivery immunisations, compared to 91% in the control group. Previously exposed females showed similar results, with piglet mortality going down from 59% to 45% with vaccination. These mortality rates are similar to those reported in our study, however, the infection in that study was controlled and not due to a natural outbreak (Crawford *et al* 2015, Mogler *et al* 2014, Rapp-Gabrielson *et al* 2014).

The results obtained in this study show that mortality is dependent on farrow number, with nulliparous gilt litters being at the highest risk. Furthermore, a statistical difference in survival length was observed, even when some nulliparous gilt litters showed no mortality. It is known that the main source of infection amongst newborn piglets is the mother's faeces due to the excretion of virus particles via this route. This factor, along with the number of antibodies transferred to piglets by females, will determine the development of this disease in piglets. Brown *et al* (2019) measured the excretion of PED viral particles amongst four nulliparous gilt groups with four different treatments: not previously exposed to the virus (Control), previously exposed to the virus (Nv), previously vaccinated twice with an inactivated virus vaccine (Pre), and vaccinated twice after being challenged with the virus (Pos). They found that only the group that had been previously exposed to the virus showed a shortened period of virus excretion, with only 10% of individuals still being positive after three weeks and a relative risk of 4.022 compared to the control. In contrast, more than 40% of females in vaccinated groups was still excreting the virus on the fourth week. Variations in mortality seen in our study could be the result of the different period of time in which each female was infected as the production unit was undergoing an uncontrolled active outbreak of the disease (Brown *et al* 2019). However, as the viral load of each female upon delivery was not determined, this variable cannot be correlated to antibody titres and piglet mortality and also the amount of antibodies that the new-borns adsorb was not determined.

In this study, we used only one isolated virus to determine the quantity of antibodies. Nonetheless, Lara-Romero *et al* (2018) characterised the spike gene from several isolates collected in México, showing a 99% homology between them (Lara-Romero *et al* 2018). Moreover, Wang *et al* (2016) found 55% of cross neutralisation between antibodies generated against the G1 and the G2 strain (Wang *et al* 2016).

In conclusion, this study shows that the colostrum of nulliparous gilts, despite being vaccinated twice, has

¹ Schwartz TJ, Rademacher CJ, Gimenez-Lirola LG, Sun Y, Zimmerman JJ. 2016. Evaluation of the effects of PEDV vaccine on PEDV naïve and previously PEDV exposed sows in a challenge model comparing immune response and preweaning mortality. *American Association of Swine Veterinarians*, 363-366. Available at https://www.aasv.org/foundation/research/Schwartz_QReport_2015_12.pdf

lower antibody counts and neutralising activity than the colostrum of multiparous sows. This suggests a poor response to vaccination in nulliparous sows, associated with a lesser passive immunity amongst piglets and lower survival in the litters. Multiparous sows showed a good antibody response to PED that is most likely associated with the multiple vaccinations or the previous exposure to the disease. Therefore, to improve immune response in sows the vaccines should be applied along with immune modulators that protect since the first delivery, in addition to the regular biosafety measures employed in pig commercial farms to reduce viral dissemination in the farrowing area.

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