Non-specific effects of maternal and offspring rabies vaccination on mortality and antibiotic use in a Danish pig herd: A randomized trial

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Highlights

- Rabies vaccine have been suggested to have beneficial non-specific effects in humans and dogs.
- In rabies virus-free pigs, early life rabies vaccine was not associated with overall reduced mortality.
- There was indication of a sex-differential rabies vaccine effect in litters of non-vaccinated sows.
- Prior sow vaccination significantly modified the rabies vaccine effect in females.

Abstract

***Introduction*:** Human non-live vaccines have been associated with detrimental non-specific effects (NSE), particularly in females. A large trial found 2-fold increased overall mortality in girls receiving a new malaria vaccine compared to the rabies vaccine used as a coontrol; a beneficial NSE of the rabies vaccine was proposed. Conversely, in dogs increased mortality was seen in females but not males following rabies vaccination of puppies born to immunized

mothers. We investigated NSE of non-live rabies vaccine in piglets and the potential modifying effect of maternal priming with rabies vaccine.

Methods: In a Danish herd of commercial rabies virus-free pigs, 575 pregnant sows (2–3 weeks before scheduled farrowing) and 5747 of their offspring (median 6-day-old) were allocated (1:1) to non-live rabies vaccine (Versiguard rabies vet) or no rabies vaccine. Outcomes were overall mortality and antibiotic treatment until departure from the nursery (approximately age 12 weeks/30 kgs).

Results: Until weaning, overall offspring mortality was 2.2% (127 piglets died, rabies vaccine: n = 69; control: n = 58), the proportion ratio (PR) being 1.19 (95% confidence interval: 0.84–1.68). Until end of follow-up, mortality was 4.1% (233, rabies vaccine: n = 115; control = 118, PR: 0.97 (0.76–1.25)). Prior sow rabies vaccination did not affect piglet mortality. For mortality as well as risk of antibiotic treatment before weaning, there was indication of a beneficial effect of rabies vaccine in female piglets, but a negative effect in (castrated) male piglets from rabies-naïve sows. Prior sow vaccination significantly modified the vaccine effect estimate in female piglets toward a detrimental effect of rabies vaccine on treatment risk. These effects had waned by 12 weeks of age.

Conclusion: The study did not support the hypothesized beneficial NSE of rabies vaccine. Although under-powered for subgroup analyses, the study indicated effect modification by sex and maternal vaccination. Results could be different in a herd with higher mortality and infectious burden.

Keywords: Non-specific effects; Heterologous immunity; Vaccine; Pigs; Rabies vaccine

1 Introduction

A large number of studies in humans have found that vaccines may affect resistance to other infections than the targeted disease; a phenomenon called *non-specific effects of vaccines* or heterologous immunity[1]. The non-specific effects may be beneficial, thereby decreasing susceptibility to other infections, or they may be detrimental, i.e. increasing susceptibility to other diseases. Common human vaccines with beneficial non-specific effects include the live vaccines bacillus Calmette-Guérin (BCG) against tuberculosis[2] , measles vaccine[3] and oral polio vaccine (OPV)[4] . In contrast, non-live vaccines like the inactivated diphtheria-pertussis-tetanus (DTP) vaccine may have detrimental non-specific effects, particularly in females [5,6].

Recently it was proposed that rabies vaccine has beneficial non-specific effects, protecting against non-rabies infections. The hypothesis was presented in the wake of a phase III trial of a malaria vaccine, the RTS,S/AS01 vaccine, in which rabies vaccine was used as a comparator vaccine in the control arm of the children enrolled at age 5–17 months [7]. The trial found that girls in the control arm had a remarkable lower overall mortality compared with the malaria vaccine arm, which may indicate a detrimental effect of the malaria vaccine in girls, as has previously been observed for other non-live vaccines[8]. Alternatively, it was proposed by Gessner *et al.* that the results indicated a beneficial non-specific effect of the rabies vaccine[9]. To corroborate their hypothesis, the paper referred to two previous murine studies reporting a protective effect of a rabies vaccine against death from a subsequent inoculation with *Klebsiella*

pneumoniae[10] or herpes virus infection[11], respectively, and an observational study in South African dogs[12], in which rabies vaccination was associated with reduced overall mortality[9].

Revisiting the latter observational data, a strong reduction in overall non-rabies related mortality of 56% (95% confidence interval = 16–77%) was observed in 0–3 months old dogs[13]. The analyses, however, may have suffered from misclassification bias due to differential retrospective owner recall of vaccination status. Live dogs may have been more likely to be reported as having been vaccinated due to legal requirements for rabies vaccination, whereas dead dogs were less likely to be reported as rabies vaccinated due to poor owner recall in these circumstances. The implication would be an exaggerated benefit of the rabies vaccine. Interestingly, in the same setting in a recent randomized placebo-controlled trial of rabies vaccination of 6 weeks old puppies all born to vaccinated dams, rabies vaccination was not associated with a reduced overall mortality (HR = 1.35 (0.83–2.18), but a three-fold higher mortality risk in female puppies (HR = 3.09 (1.24–7.69))[14].

Pigs are increasingly used as a model organism in experimental medicine due to the physiological similarity to humans[15]. For the study of non-intensive interventions, pigs are readily available in large numbers due to the large agricultural industry in Denmark and elsewhere. In addition, whereas a wide array of other pathogens is endemic to the herds, Danish pigs are rabies-free, thus facilitating the investigation of *non-specific* effects of rabies vaccine. As commercial pigs routinely receive different vaccines it is relevant to investigate NSE of vaccines in pigs for improved porcine health and to serve as a model organism for human medicine. In the present study we aimed to investigate if early age rabies vaccination reduced the overall mortality and risk of antibiotic treatment in a commercial Danish pig herd. Specifically, we hypothesized that rabies vaccine shortly after delivery compared to no rabies vaccine reduces all-cause mortality in piglets by 28% from vaccination to the piglets exit from the nursery, typically at 12 weeks of age or when reaching a weight of 30 kg.

2 Materials and methods

2.1 Population and setting

The study was conducted in a Danish commercial certified "specific pathogen free" (SPF)-herd, but with positive SPF status for *Mycobacterium hyopneumoniae* and *Actinobacillus pleuropneumoniae* serotype 12, comprising a farrow-to-finish herd of Danish Landrace/Danish Yorkshire crossbreds and paternal line Duroc. Enrolments started in September 2018 and the last observation was registered in April 2019. All enrolment procedures and rabies vaccinations were performed by two technicians of the study team, whereas daily observations of death and antibiotic treatment were recorded by the local staff on the herd.

2.2 Enrolment procedure sows

Pregnant sows were allocated 1:1 to receive Versiguard Rabies Vet vaccine or no rabies vaccine 2–4 weeks before expected day of delivery, ensuring a balanced distribution in respect to parity. On the same day, in accordance with the local vaccination schedule, sows received inactivated vaccines Entericolix Vet, Boehringer Ingelheim Animal Health, against *Escherichia coli* and *Clostridium perfringens* ; Porcilis Glässer Vet, MSD Animal Health, against *Haemophilus parasuis* ; and Porcilis Ery Parvo + Lepto, MSD Animal Health, against *Erysipelothrix rhusiopathiae,* Porcine parvovirus, *Leptospira interrogans* subsp.

2.3 Enrolment procedure piglets

Assessment for eligibility and enrolment of newborn piglets born to enrolled sows took place on a weekly basis on a fixed weekday. Piglets were assessed from 2 days of age; piglets with congenital disorders or piglets that were not viable were excluded from enrolment. Ten piglets per sow were included; the first 5 handled piglets were allocated to the same treatment, either rabies vaccination or no rabies vaccination; the next 5 eligible piglets were allocated to receiving the alternative treatment. The order of treatment designation (rabies vaccine vs no rabies vaccine) alternated for every new litter, hence rabies vaccine was allocated to the first 5 piglets in every second litter. Sex of the piglet was recorded and piglets were ear tagged with a unique identifier number. Piglets in the litter not enrolled remained in the pen together with their enrolled peers (of which half were rabies vaccinated).

2.4 Vaccinations

The vaccine under study was Versiguard Rabies Vet, Zoetis Finland Oy (batch no. 105824 A03), administered as 1 ml standard dose intramuscularly behind the ear base. The active ingredient is inactivated rabies virus, strain SAD Vnukovo-32 (minimum 2.0 International Units), with adjuvants aluminium hydroxide (2.0 mg) and thiomersal (0.1 mg). No placebo or comparator product was used in the control group. No other vaccines were administered to the piglets during the study.

2.5 Treatment and observations

Except for the enrolment procedures, all piglets were treated according to local practice, including tail docking of all piglets and castration of male piglets, usually 2–5 days after delivery. In the farrowing unit, the local staff routinely assessed piglets for sign of weakness (including indicators of low palpable temperature, fuzzy hair, decreased activity or inappetence). If deemed necessary, weak piglets were relocated to foster sows in order to optimize welfare, health, growth and survival of the piglets. Relocation to sows of the same sow treatment group were given priority; and if not possible piglets were relocated to the alternative treatment group. In rare cases, if the relocated piglets could not be accommodated within the enrolled pens, the piglets were placed with sows outside of the study pens.

For piglets placed outside the study pens, death was recorded by ear tag (without assignment of date or cause of death); antibiotic treatments were not recorded for these piglets.

Piglets were weaned and transferred to the nursery around 3–4 weeks of age according to routine in the herd (Fig. 1), where they remained until achieving a body weight of approximately 30 kg, usually around 12 weeks of age, whence they left the nursery and the study. All piglets, including those moved out of the study pens before weaning in the farrowing unit, were transferred upon weaning to the same nursery section.

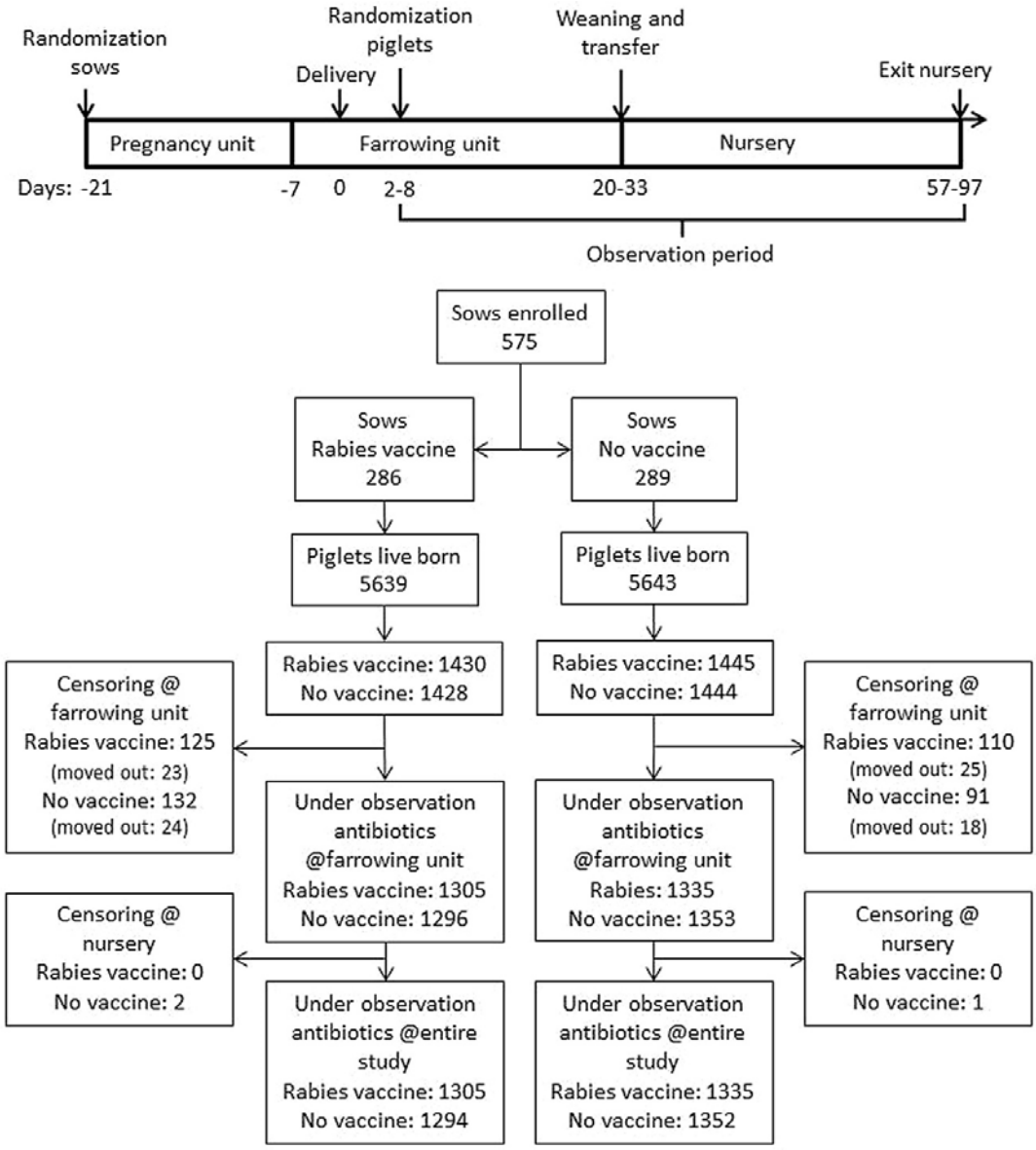

Fig. 1. Flow chart of the study. In the main analysis, for death as the outcome, all piglets were included; for the risk of antibiotics treatment, piglets were censored if the observations were conflicting, if essential data was missing or if the piglets were relocated to foster sows outside the study pens (moved), as indicated on the chart. Depending on where the irregular observations were placed, censoring was applied for the analysis of events in the farrowing unit alone or in the farrowing unit and nursery combined, respectively.

Piglets were followed daily by the local staff, who were blinded to the treatment. Health-related observations were noted in a designated form. In case of deceased piglets, suspected cause of death was assessed by the farm staff and categorized as one of following: Crushing, Starvation, Diarrhoea, Arthritis, Septicaemia, Omphalitis/Hernia, Respiratory infection, Other.

Treatment upon indication was categorized as the following: Respiratory ailment, Diarrhoea, Arthritis, Omphalitis/Hernia, Other.

2.6. Safety study

As the rabies vaccine is licensed in Denmark for use in animals from 12 weeks of age, a safety study was conducted before initiating the main trial (Supplementary Material). The safety study did not identify vaccine-related adverse reactions.

2.7 Sample size assumptions

We aimed to enroll 6000 piglets in total. Data on the mortality specifically from 1 week of age to 12 weeks is not available for the present study herd nor generally for commercial farms. Moreover, the mortality rate and causes of death may be subject to large variation by the specific herd, season and other factors. Two recent reports systematically conducted autopsies in suckling piglets and weaned piglets from a larger number of farms[16,17]. Although the analysis was not broken down in the specific period of interest for the present investigation, combining the two reporting estimates suggests that the mortality from 5 days of age to exit of the nursery with infection related primary or secondary diagnoses was 6%. Similar estimates were obtained from a small study on pre-weaning mortality counting daily mortality by major cause from birth to 21 days of life[18]. The observational study of rabies vaccine in dogs found a reduction in overall non-rabies related mortality of 56% (95% confidence interval = 16–77%) from 0 to 3 months of age[13] There is no data on rabies vaccine in pigs. With a power of 0.8 and alpha of 0.05, to be able to show a 28% reduction in our primary outcome overall mortality from 6% in non-vaccinated piglets to 4.3% in vaccinated piglets, we would need 2839 pigs per arm, in total 5678 pigs. This would allow a loss to follow up or incomplete data of 5% of the enrolled piglets.

2.8 Data validation and analysis

Data was double entered by technicians from the study team. Logic inconsistencies and extreme observations were identified, and if not resolvable, the piglets were flagged and censored in the analysis, where the uncertainty may affect the estimate. In sensitivity analyses, flagged piglets were included (Supplementary Table S1).

For the data validation, we employed a hierarchy of data validity, where observations of death overruled other logic inconsistencies, reasoning that the incorrect record of ear tags was less likely for dead (still) piglets compared to live (moving) piglets. Relocation was noted for the piglets individually in respect to date, origin and destination of litter (sow number). If a piglet was observed in a litter different from the biologic mother, but data on relocation to the foster litter was missing, the piglets were excluded from the main analysis of antibiotic treatment, but included in sensitivity analyses (Supplementary Table S1).

Primary analysis as stipulated in the protocol was effect of vaccination of piglets on mortality until 12 weeks of age or departure from the nursery. In secondary analyses, the effects of vaccination on number of animals receiving antibiotics treatment until 12 weeks of age or departure from the nursery as well as the effect on mortality and treatment rates until weaning were investigated. As per protocol, outcomes were stratified by sex and sow vaccination including test of interaction with piglet vaccination. Due to unforeseen inconsistent recording of exit from the nursery time-based survival analyses could not be carried out; the present analysis therefore puts emphasis on the observations until weaning.

Data was analysed in STATA 12 (StataCorp LP, College Station, Texas) using Poisson regression with robust errors providing proportion ratios (PR) with 95% confidence intervals. *Multiple testing was not corrected for.*

2.9 Ethics

The study was approved by the Danish Medicine Agency, protocol no. 2017103225.

3 Results

3.1 Population

In total, 575 sows and 5747 piglets were included in the trial (Fig. 1); sows and piglets (Table 1) were comparable on the available background parameters in respect to sow and piglet randomization. Overall, 51.6% of piglets were male, median age at randomization was 6 days (10–90%-tile: 2–8) and 83.3% were tail-docked (and castrated for males) before randomization.

3.2 Death overall

Total number of deaths during the entire study was 233 (4.1%); with no difference between rabies vaccinated and control piglets, PR = 0.97 (0.76–1.25) (Table 2). Of these, 127 piglets died in the farrowing unit, the PR being 1.19 (0.84–1.68) comparing rabies vaccinated with controls (Table 3). Of the total number of deaths, 210 (n = 104 rabies vaccinated and n = 106 controls) were observed in the study stables and were assigned a presumptive cause of death; the majority was crushed (29%) or assigned the category 'other' (31%) (Supplementary Table S2).

3.3 Antibiotic treatment overall

In total, 2564 (48.5%) of the piglets received individual antibiotic treatment during the entire follow-up, with no difference between the rabies vaccinated and control piglets, PR = 1.00 (0.95–1.06) (Table 4); 363 piglets received antibiotic treatment in the farrowing unit, PR = 1.08 (0.88–1.31) comparing rabies vaccinated with controls (Table 4).

3.4 Effect modification by sex

Mortality ratios for rabies vaccine vs no rabies vaccine in the farrowing unit did not differ between males and females (p = 0.38 for interaction between rabies vaccination and sex) (Table 3). For the antibiotic treatment, rabies vaccine was associated with increased risk in males (PR = 1.31 (1.00–1.72)); this trend was not seen in females, resulting in an interaction between rabies vaccination and sex (p = 0.04 for interaction) (Table 4). Combining death and antibiotic treatment, vaccinated males had a significantly larger risk of having the composite outcome (PR = 1.39 (1.10–1.75)); for females the PR was 0.89 (0.68–1.15), p = 0.01 for interaction between rabies vaccination and sex) (Table 4).

Table 1
Sow and piglet background factors.

	Sow control		Sow vaccine	
Sows, n	289		286	
No. of previous litters, median	3 (1–6)		3 (1–6)	
Days from randomisation to farrowing, median (p10-p90)	23 (18–28)		23 (18–28)	
Total litter size (live + dead), median (p10-p90)	22 (18–26)		22 (17–26)	
Live born, median (p10-p90)	20 (16–24)		20 (16–24)	
Stillborn, median (p10-p90)	2 (0–5)		2 (0–4)	
	Sow control		**Sow vaccine**	
Piglet	**Control**	**Vaccine**	**Control**	**Vaccine**
Piglets, n	1444	1445	1428	1430
Male piglets, % (n)	52.3 (755)	50.0 (722)	51.8 (740)	52.2 (747)
Age at randomisation, days median (p10-p90)	7 (2–8)	7 (2–8)	6 (2–8)	6 (2–8)
Tail docking before randomisation, % (n)	84.1 (1214)	84.1 (1215)	82.5 (1178)	82.5 (1180)
Tail docking days before randomisation, median (p10-p90)	2 (2–4)	2 (2–4)	2 (2–4)	2 (2–4)

Table 2
Mortality during the entire follow-up, by randomization group.

	Control	**Vaccine**	**Vaccine vs Control**	
	deaths/n	deaths/n	PR (CI 95%)	p
All	118/2872	115/2875	0.97 (0.76–1.25)	
Female	52/1377	44/1406	0.83 (0.56–1.23)	
Male	66/1495	71/1469	1.09 (0.79–1.52)	p = 0.29
Sow control	57/1444	58/1445	1.02 (0.71–1.45)	
Sow vaccinated	61/1428	57/1430	0.93 (0.66–1.33)	p = 0.74
Sow vaccination[1]	115/2889	118/2858	1.04 (0.81–1.33)	
Sow control:female	30/689	21/723	0.67 (0.39–1.15)	
Sow control:male	27/755	37/722	1.43 (0.88–2.33)	p = 0.04
Sow vaccinated:female	22/688	23/683	1.05 (0.59–1.87)	
Sow vaccinated:male	39/740	34/747	0.86 (0.55–1.35)	p = 0.59
interaction piglet#sow#sex[1]				p = 0.07
	Male	**Female**	**Male vs Female**	
Sex (male/female)[3]	137/2964	96/2783	1.34 (1.04–1.73)	

The relative risk of mortality in the entire follow-up (farrowing unit and nursery combined), comparing rabies vaccinated with controls, was analysed with Poisson regression using robust errors giving proportion ratios (PR) presented with 95% confidence intervals.
1) Comparing piglets from rabies vaccinated sows vs rabies non-vaccinated sows irrespective of piglet vaccination.
2) Test for three-way interaction between piglet vaccination, sow vaccination and sex of piglet.
3) Comparing males with females irrespective of piglet vaccination.

Table 3
Mortality in the farrowing unit, by randomization group.

	Control	Vaccine	Vaccine vs control	
	deaths/n	deaths/n	PR (CI 95%)	p
All	58/2872	69/2875	1.19 (0.84–1.68)	
Female	24/1377	24/1406	0.98 (0.56–1.72)	
Male	34/1495	45/1469	1.35 (0.87–2.09)	0.38
Sow control	26/1444	31/1445	1.19 (0.71–2.00)	
Sow vaccinated	32/1428	38/1430	1.19 (0.75–1.89)	0.99
Sow vaccination[1]	57/2889	70/2858	1.24 (0.88–1.75)	
Sow control:female	13/689	9/723	0.66 (0.28–1.53)	
Sow control:male	13/755	22/722	1.77 (0.90–3.49)	0.07
Sow vaccinated:female	11/688	15/683	1.37 (0.64–2.97)	
Sow vaccinated:male	21/740	23/747	1.08 (0.61–1.94)	0.63
interaction piglet#sow#sex[2]				0.10
	Female	**Male**	**Male vs Female**	
Sex (male/female)[3]	48/2783	79/2964	1.55 (1.08–2.20)	

The relative risk of mortality in the farrowing unit only, comparing rabies vaccinated with controls, was analysed with Poisson regression using robust errors giving proportion ratios (PR) presented with 95% confidence intervals. In total, n = 127 events and 5747 piglets included.

[1] Comparing piglets from rabies vaccinated sows vs rabies non-vaccinated sows irrespective of piglet vaccination.

[2] Test for three-way interaction between piglet vaccination, sow vaccination and sex of piglet.

[3] Comparing males with females irrespective of piglet vaccination.

Table 4
Mortality and antibiotic treatment in the farrowing unit and nursery during the entire follow-up, by randomisation group.

	Mortality + treatment in farrowing unit[4]		**Ever treated in farrowing unit**[5]		**Ever treated all time**[6]	
Events	475		363		2564	
Individuals	5323		5289		5286	
Censoring	424		458		461	
	Vaccine vs Control		**Vaccine vs Control**		**Vaccine vs Control**	
	PR (CI 95%)	p	PR (CI 95%)	p	PR (CI 95%)	p
All	1.13 (0.96–1.35)		1.08 (0.88–1.31)		1.00 (0.95–1.06)	
Female	0.89 (0.68–1.15)		0.86 (0.64–1.16)		1.00 (0.92–1.08)	
Male	1.39 (1.10–1.75)	0.01	1.31 (1.00–1.72)	0.04	1.01 (0.93–1.09)	0.85
Sow control	1.00 (0.79–1.27)		0.92 (0.70–1.21)		0.98 (0.91–1.06)	
Sow vaccinated	1.30 (1.01–1.66)	0.14	1.28 (0.96–1.72)	0.10	1.02 (0.94–1.11)	0.50
Sow vaccination[1]	0.99 (0.84–1.18)		0.93 (0.76–1.14)		0.98 (0.93–1.04)	
Sow control:female	0.55 (0.37–0.81)		0.51 (0.33–0.80)		0.96 (0.86–1.07)	
Sow control:male	1.56 (1.13–2.15)	<0.001	1.44 (1.00–2.08)	<0.001	1.01 (0.91–1.13)	0.46
Sow vaccinated:female	1.38 (0.96–1.99)		1.41 (0.93–2.14)		1.04 (0.93–1.17)	
Sow vaccinated:male	1.23 (0.88–1.72)	0.64	1.17 (0.78–1.76)	0.54	1.00 (0.90–1.12)	0.64
interaction piglet#sow#sex[2]		0.001		0.04		0.40
	Male vs Female		**Male vs Female**		**Male vs Female**	
Sex (male/female)[3]	1.20 (1.01–1.42)		1.10 (0.90–1.34)		0.99 (0.94–1.05)	

The risk of mortality or antibiotic treatment comparing rabies vaccinated with controls was analysed with Poisson regression using robust errors giving proportion ratios (PR) presented with 95% confidence intervals.

[1] Comparing piglets from rabies vaccinated sows vs rabies non-vaccinated sows irrespective of piglet vaccination.
[2] Test for three-way interaction between piglet vaccination, sow vaccination and sex of piglet.
[3] Comparing males with females irrespective of piglet vaccination.
[4] Composite outcome of deaths and treatments (single occurrence), excluding n = 424 piglets with uncertain observations for antibiotic treatment observations.
[5] Excluding n = 458 piglets with uncertain observations for antibiotic treatment observations.
[6] Excluding n = 461 piglets with uncertain observations for antibiotic treatment observations.

3.5 Effect modification by sex and sow vaccination

Sow vaccination did not substantially modify the effect of rabies vaccination on the risk of death of the piglets in the farrowing unit (Table 3). However, the trend for a sex-differential effect of piglet vaccination as presented above for antibiotic treatment was enhanced in non-vaccinated litters, in which rabies vaccination was associated with a lower risk of death and antibiotic treatment in the farrowing unit (composite outcome) in females (PR = 0.55 (0.37–0.81)), and an opposite association in males (PR = 1.56 (1.13–2.15), $p < 0.001$ for interaction between rabies vaccination of piglets and sex) (Table 4). The associations were comparable, although weaker for the outcome of death in the farrowing unit (Table 3). In contrast, among piglets of vaccinated sows, there was no sex-differential effect of rabies vaccination, although in females, rabies vaccination tended to be associated with increased mortality and risk of antibiotics use in the farrowing unit (for the composite outcome, PR = 1.38 (0.96–1.99)). This resulted in a significant 3-way interaction between rabies vaccination of piglets, sow vaccination and sex of piglet for the composite outcome, $p = 0.001$ (Table 4). The sex- and sow vaccination-dependent pattern on mortality persisted until end of follow-up, albeit the effect estimates were decreased (Table 2).

3.6 Sensitivity analyses

A subset of the piglets was moved out of the study stables before weaning or had missing information in respect to movements. Excluding these piglets increased the effect estimate of rabies vaccine on mortality in the farrowing unit in males from naïve sows (PR = 2.41 (1.06–5.51)) (Supplementary Table S3).

Including piglets with conflicting observations in the analysis of antibiotics treatment in the farrowing unit did not substantially affect the estimates from the censored analysis (Supplementary Table S3).

4 Discussion

In a Danish herd of production pigs, following piglets from randomization at around one week of age until departure of the nursery around 12 weeks of age or 30 kg, rabies vaccine was overall not associated with differential mortality or use of antibiotics.

Although the overall mortality was lower than expected, several important observations were made in subgroup analyses: First, before weaning, rabies vaccination was associated with a significant 31% increased risk of antibiotic treatment in males; for mortality in males, the effect estimate was in the same order of magnitude (35%) although with low precision. Second, the detrimental effect of rabies vaccination in male piglets was particularly seen for litters of rabies-naïve sows, whereas maternal vaccination abrogated the detrimental effect of piglet rabies vaccination. Third, this effect modification by maternal vaccination was opposite for female piglets, for which the protective effect of rabies vaccination against risk of death and antibiotic treatment in litters of non-vaccinated sows was reversed by maternal vaccination.

Importantly, the effect estimates for antibiotic treatment and mortality pointed in the same direction in the subgroup analyses, although the magnitude of the effects and particularly the statistical power were of variable strength.

4.1 Strengths and weaknesses

The randomized design achieved a balanced distribution of background parameters between the treatment groups. Danish pig farms, including the present, are rabies virus-free, hence any vaccine-specific effects on mortality or morbidity can be excluded. The local staff who recorded antibiotic treatments and deaths was blinded to the intervention throughout the study. No comparator vaccine was used; a comparator vaccine may have NSE itself, thereby complicating the interpretation of any finding.

Overall, the mortality rate in the herd was significantly lower than stipulated in our a priori sample size calculation. We therefore have limited power to draw any conclusions as also clearly reflected in the broad confidence intervals of the estimates. To that end, it should also be noted that p-values were not adjusted for multiple testing. Antibiotic treatment and (infectious) death clearly have overlapping causes. In turn, antibiotic treatments also reduce the mortality risk. We would expect that some piglets who would otherwise have suffered the mortality outcome now instead experience the alternative antibiotics outcome. We have combined these in a composite outcome, while acknowledging that the mortality outcome comprised both infectious and non-infectious related death.

The underlying causes of death were assessed by use of non-invasive inspection by the local staff, which did not receive focused training prior to the trial. The validity of this assessment could not be verified. By experience, the credibility of such assessment is low. In addition, the true cause of death may indeed be an interplay involving multiple infectious and non-infectious, and mutually exacerbating, conditions. E.g. piglets weakened by infectious or non-infectious conditions may be at larger risk of crushing. The suspected cause of death should therefore be interpreted with caution.

It is customary to relocate excess piglets to foster sows if the litter size exceeds the nurturing capacity of the biological mother, or specifically upon indication of lack of thriving of the piglet. Hence, relocated piglets are often relatively weak. The study staff was instructed to prioritize relocation of piglets to litters within the same sow randomization group, secondarily to the alternative sow randomization group. On some occasions, however, the relocated piglets could not be accommodated in foster litters located in the study farrowing unit. In total, 90 piglets (rabies vaccine, n = 48; control, n = 42) were recorded as removed from the study farrowing unit and transferred to foster sows in pens outside the study. Death was still observed and noted for these piglets as they maintained their study-identifiable ear tag, whereas antibiotic treatment was not recorded. Not surprisingly, there was an excess mortality among piglets moved out of the study; however, this should not have affected the mortality analyses as all ear tags were read. While unrecorded, the piglets moved out of the study may also have received excess antibiotics treatment. For a subset of piglets, the relocation was uncertain due to missing record of sow destination or origin. These piglets were therefore excluded from the analysis of antibiotic treatments, but remained in the mortality analyses. In sensitivity analyses these exclusions were nullified. Noteworthy, the directions of the effect estimates remained the same with and without the exclusions.

Unfortunately, an exit date from the farrowing unit and the nursery was not consistently registered, preventing us from conducting time-based survival analysis. The duration of observation time was therefore not accounted for in the analysis of risk. As weight is a determining factor for weaning and transfer from the nursery, piglets with reduced growth usually remain longer in the farrowing and nursery, and therefore contribute more risk

compared to thriving piglets. Hence, although this reflects natural conditions in a pig herd, an effect of vaccination may therefore be accentuated.

4.2 Comparisons with previous studies

Although the Versiguard Rabies Vet vaccine is licensed for use in pigs, to the best of our knowledge, this is the first report addressing non-specific effects of rabies vaccination in this species. Rabies vaccine has previously been associated with reduced all-cause mortality in an observational South African study in dogs[13]. Following up on this, a subsequent randomized placebo-controlled trial in the same setting in 6-week-old dogs followed up until 12 weeks of age, however, found no overall survival benefit of rabies vaccination (HR = 1.35 (0.83–2.18), with a significant effect modification by sex (p = 0.02), as female puppies observed a 3-fold increased mortality following rabies vaccination (HR = 3.09 (1.24–7.69)); the HR in males was 0.79 (0.41–1.53)[14]. Notably, the trial enrolled puppies only born to rabies vaccinated dams. The findings of a detrimental effect of rabies vaccine in female puppies from vaccinated mothers are in alignment with the present observations in pigs, although the effect estimates in pigs were more modest and did not reach statistical significance in any of the outcome measures.

Recently, rabies vaccine has received increased attention for its proposed NSE. Rabies vaccine was used as a compactor vaccine in a large multicenter phase III trial in African infants and young children of a new candidate vaccine against malaria, the RTS,S vaccine from GSK[7]. The trial included two age groups, either randomized to receiving RTS,S or a comparator vaccine. The comparator vaccine in children enrolled at 5–17 months was rabies vaccine (Verorab, Sanofi-Pastur) and meningococcal serogroup C conjugate vaccine (Menjugate, Novartis) in infants enrolled at age 6–12 weeks. Intriguingly, the trial found higher meningitis incidence in malaria vaccinated children aged 5–17 months compared to the control group. Gessner *et al.* proposed that the differential risk was caused by a beneficial non-specific effect of the rabies vaccine protecting infants against meningitis[9]. Worryingly, receipt of the malaria vaccine was also associated with a 2-fold increased all-cause mortality and an increased risk of fatal malaria in girls, but not in boys[19]; the association was seen in both age groups, also when another vaccine was used as a control vaccine, leading to the alternative hypothesis that malaria vaccine had a detrimental non-specific effect in girls[8]. It may be relevant to note that the present Versiguard Rabies Vet vaccine and the Defensor 3 vaccine used in the trial in dogs[14] were alum-adjuvanted, whereas the Verorab vaccine used in the control arm of RTS,S trial was not adjuvanted[7]. The implication of alum adjuvant on NSE is unclear.

Early animal experimental studies have suggested beneficial non-specific effects of rabies vaccine. A study found that inoculation of mice with attenuated live rabies virus enhanced resistance to subsequent experimental infection with *Klebsiella pneumoniae*[10]. An experiment from the 1960′s in mice found that multiple inoculations of live attenuated rabies virus protected against herpes virus infection up to 90 days after immunization[11]. As can be seen, however, these early murine studies used live virus in contrast to the more recent studies of rabies immunization. The available evidence from the majority of human studies of NSE of vaccines strongly indicate that the live/non-live status of the vaccines is a crucial determinant of whether the NSE is detrimental or beneficial[20]. The live vaccines BCG, oral polio, measles and smallpox vaccines are associated with decreased all-cause morbidity or mortality[21], whereas the non-live DTP-containing vaccines[5,22] are associated with increased morbidity or mortality. Hence, inferences from the investigations of live vaccines may not be applicable for non-live rabies vaccines.

The modulating effect of maternal immunity on the NSE of vaccines has been described for the live vaccines MV and BCG. BCG vaccination of children from BCG vaccinated mothers had a significantly beneficial NSE on infectious hospitalization in Denmark[23] whereas there was no significant effect of BCG on children from non-vaccinated mothers; similarly, in Guinea-Bissau the effect of having a BCG scar on mortality risk was significantly stronger in children born to mothers with a BCG scar compared to mothers without a BCG scar[24], whereas MV immunization reduced all-cause mortality more in infants who were immunized in the presence of maternally-derived anti-measles antibodies compared to vaccination in the absence of maternally-derived anti-measles antibodies[25,26]. Similarly, boosting the child with a live vaccine enhances the beneficial NSE[27]. The non-live vaccines influenza vaccine and pertussis-containing vaccines are given in pregnancy as well as to the offspring. Maternal pertussis vaccine has been associated with an increased risk of chorioamnionitis[28]. To our knowledge there is no investigation of the modulating effect of maternal immunity on the NSE of pertussis-containing vaccines or other non-live vaccines in infants.

For the specific effects, however, it is known that maternal immunity generally dampens the humoral response to immunization of both live and non-live vaccines. For live measles vaccine, vaccination in the context of passive immunity is known to dampen the specific humoral response to vaccination[29]. Such attenuating effect of maternally-derived antibodies on human infant humoral vaccine responses has also been observed for the non-live viral vaccines inactivated polio vaccine[30] and hepatitis B vaccine[31], and this is corroborated by a series of animal experimental studies[29], including studies in pigs of non-live *Haemophilus parasuis* immunization[32]. In humans and pigs alike, the development of active cell-mediated immunity may not be compromised by the presence of maternal immunity despite blunted humoral responses, as observed for *Mycobacterium hyopneumoniae* vaccination of piglets from immunized dams[33] or live measles vaccine in humans[29]. For the rabies vaccine, there is no data in humans or pigs, but interestingly in hamsters, maternal vaccination attenuated the protective specific effect of inactivated rabies vaccine of pups against live rabies infection[34]. In contrast, an experiment in puppies (dogs) born to rabies vaccinated dams found that single immunization at two weeks of age with either inactivated adjuvanted rabies vaccine or recombinant replication-incompetent viral vector vaccine expressing rabies antigens was fully protective against live rabies virus challenge, despite low anti-rabies titers at time of challenge[35].

One mechanism common to both live and non-live vaccines may be the interaction of specific antibodies with the vaccine, which may form immunological complexes and promote the opsonization of the vaccine antigen by phagocytic cells, thereby enhancing antigen intracellular processing and cross-presentation, driving the T cell immunological response.

In contrast to humans, IgG is not transferred transplacentally in pigs[36]; instead, the gut is permeable for uptake of colostral antibodies within 24 h of birth[37]. Maternally derived lymphocytes have also been identified in suckling piglets[38]. Hence, both maternally-derived humoral and cellular passive immunity may play a role in pigs in the effect modification by maternal vaccination. It is noteworthy that the present study was able to detect an effect of maternal immunization with non-live rabies vaccine, since all sows in both treatment arms also received three additional non-live vaccines simultaneously with the randomized allocation to rabies vaccine or no rabies vaccine. The implication of these non-live vaccines could not be investigated in the present study design. However, although the evidence is very limited, it has been indicated from previous studies in humans that the transgenerational priming for NSE in the offspring is vaccine-specific[23,26]. More studies are needed on the transgenerational NSE of

vaccines, including whether heterologous vaccine may impact on NSE of vaccines in the offspring.

The nucleocapsid protein of the rabies virus carries superantigen properties, being a strong driver of T cell expansion without prior anti-rabies memory[39,40]. Preceding immunological studies of the rabies vaccine have shown that rabies specific antibodies may enhance the presumed superantigen effect[41]. This is in keeping with the suggested effect modification by sow vaccination in the present study, although it remains unexplained why the indicated effect modification by maternal priming was much stronger in female than male piglets, and in addition pointed in opposite directions. The potential interaction between maternal and offspring immunity on NSE of vaccines has hitherto not been investigated systematically in an epidemiological study including randomization of the mother.

In the above mentioned RTS,S malaria vaccine trial, participating children were most likely rabies-naïve. Translating the findings from the present study in pigs to humans, girls in the older age group who were allocated to the control arm of the RTS,S trial may have benefitted from receiving the rabies vaccine in contrast to the RTS,S intervention arm. The 2-fold excess female mortality among RTS,S recipients also seen for the youngest participants[8] cannot be explained, however, by potential beneficial NSE of rabies vaccine, as the meningococcal vaccine was used as the comparator for this age group[7]. A very recent randomized controlled trial of non-live Rabivax-S vaccine in human adult volunteers[42]did not find an overall effect on self-reported infectious disease symptoms, although rabies vaccine was associated with a lower incidence of self-reported diarrhea episodes in males but not females. The study was underpowered, however, to support an interaction analysis with sex (Knobel D, manuscript in revision).

5 Conclusion

The present randomized controlled trial in a rabies-free pig herd found no overall beneficial effect of non-live rabies vaccine on risk of all-cause mortality or antibiotic treatment. The study indicated effect modification by maternal immunity and sex of the piglet. Vaccination tended to be detrimental in males, but beneficial in females in litters from non-vaccinated sows, whereas maternal vaccination reversed the beneficial effect in female piglets to a detrimental one. The results support that rabies vaccine has non-specific effects on health, although the limited power in subgroup analysis prompts a cautious interpretation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data statement

Data is available upon request to the corresponding author.

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