Le terrain comme support de la modélisation : d'une étude observationnelle en élevage aux essais expérimentaux

I. Etude de la dynamique de l'infection par le virus de l'hépatite E chez le porc et de ses facteurs de variation en conditions naturelles

La dynamique de l'infection par le HEV chez le porc conditionne directement la probabilité que le foie contienne des particules virales à l'abattage, c'est-à-dire qu'il présente un risque pour la santé publique. Une **grande variabilité de la dynamique infectieuse** est décrite dans la littérature (Salines *et al.*, 2017a) et n'est que **partiellement expliquée** à ce jour. En effet, si des facteurs de risque ont été identifiés à l'échelle de l'élevage, notamment en ce qui concerne la structure de l'élevage et les pratiques d'élevage, d'hygiène et de biosécurité (Di Bartolo *et al.*, 2008; Li *et al.*, 2009a; Jinshan *et al.*, 2010; Hinjoy *et al.*, 2013; Rutjes *et al.*, 2014; Walachowski *et al.*, 2014; Lopez-Lopez *et al.*, 2018), peu d'études ont décrit les variations des profils individuels d'infection par le HEV (de Deus *et al.*, 2008; Casas *et al.*, 2011a; Feng *et al.*, 2011), et encore moins se sont intéressées aux facteurs pouvant expliquer ces variations (Andraud *et al.*, 2014). De plus, à l'instar des hépatites E chroniques décrites chez des patients humains immunodéprimés, il est possible que des **pathogènes immunomodulateurs porcins**, comme le virus du syndrome dysgénésique et respiratoire porcin (SDRP) ou le circovirus porcin de type 2 (PCV2) – qui sont fortement prévalents dans la filière de production porcine et affectent à la fois la réponse immunitaire innée et adaptative du porc – influencent la dynamique de l'infection par le HEV chez le porc.

C'est dans ce contexte qu'un **suivi longitudinal de trois élevages porcins naisseurs-engraisseurs** a été réalisé. Le premier objectif de cette étude observationnelle était de **décrire, à partir de données individuelles, les profils d'infection par le HEV**. En parallèle, l'étude a permis d'évaluer *(i)* l'influence de **caractéristiques individuelles des porcelets** ou de

spécificités liées aux portées, *(ii)* le rôle de l'**immunité anti-HEV**, *(iii)* ainsi que l'**impact de co-infections avec le virus du SDRP et/ou le PCV2** sur la dynamique de l'infection par le HEV chez le porc. Cette étude a été publiée dans le journal *Transboundary and Emerging Diseases* (Salines *et al.*, 2019c).

Publication 2

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ORIGINAL ARTICLE

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Natural viral co-infections in pig herds affect hepatitis E virus (HEV) infection dynamics and increase the risk of contaminated livers at slaughter

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Abstract

Hepatitis E virus (HEV) is a zoonotic pathogen, in particular genotype 3 HEV is mainly transmitted to humans through the consumption of contaminated pork products. This study aimed at describing HEV infection patterns in pig farms and at assessing the impact of immunomodulating co-infections namely Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Porcine Circovirus Type 2 (PCV2), as well as other individual factors such as piglets' immunity and litters' characteristics on HEV dynamics. A longitudinal follow-up was conducted in three farrow-to-finish farms known to be HEV infected. Overall, 360 piglets were individually monitored from birth to slaughter with regular blood and faecal sampling as well as blood and liver samples collected at slaughterhouse. Virological and serological analyses were performed to detect HEV, PCV2 and PRRSV genome and antibodies. The links between 12 explanatory variables and four outcomes describing HEV dynamics were assessed using cox-proportional hazard models and logistic regression. HEV infection dynamics was found highly variable between farms and in a lower magnitude between batches. HEV positive livers were more likely related to short time-intervals between HEV infection and slaughter time (<40 days, OR = 4.1 [3.7–4.5]). In addition to an influence of piglets' sex and sows' parity, the sequence of co-infections was strongly associated with different HEV dynamics: a PRRSV or PCV2/PRRSV pre- or co-infection was associated with a higher age at HEV shedding (Hazard Ratio = 0.3 [0.2–0.5]), as well as a higher age at HEV seroconversion (HR = 0.5 [0.3–0.9] and HR = 0.4 [0.2–0.7] respectively). A PCV2/PRRSV pre- or co-infection was associated with a longer duration of shedding (HR = 0.5 [0.3–0.8]). Consequently, a PRRSV or PCV2/PRRSV pre- or co-infection was strongly associated with a higher risk of having positive livers at slaughter (OR = 4.1 [1.9–8.9] and OR = 6.5 [3.2–13.2] respectively). In conclusion, co-infections with immunomodulating viruses were found to affect HEV dynamics in the farrow-to-finish pig farms that were followed in this study.

KEYWORDS
hepatitis E virus, infection dynamics, PCV2, PRRSV, public health, risk factors

1 | INTRODUCTION

Hepatitis E virus (HEV) is a non-enveloped single-stranded RNA virus causing acute and occasionally chronic hepatitis in humans (Emerson & Purcell, 2003; Kamar et al., 2011). In industrialized countries, hepatitis E cases are mainly related to genotype 3 and 4 (HEV-3 and HEV-4) which are shared between humans and other animal species (Dalton, Bendall, Ijaz, & Banks, 2008; Purcell & Emerson, 2008). In Europe, HEV-3 is particularly prevalent in the pig population (Rose et al., 2011), with swine and human HEV strains being genetically very close (Bouquet et al., 2011; Meng et al., 1998). Moreover, some autochthonous cases in industrialized countries have been related to the consumption of raw or undercooked pork products, especially those containing liver (Colson et al., 2010; Moal, Gerolami, & Colson, 2012; Motte et al., 2012). Thus, hepatitis E is now considered as a foodborne zoonosis with domestic pigs recognized as one of the main reservoirs in developed countries (Dalton et al., 2008; Pavio, Meng, & Renou, 2010). The epidemiology of HEV in the pig-farming sector is far from being fully elucidated (Salines, Andraud, & Rose, 2017). Comparing outcomes of prevalence and seroprevalence studies evidences great variability between countries (Salines et al., 2017). Within a same study in a given country, the individual and farm-scale prevalence figures may also vary greatly (Rose et al., 2011). Within-farm and between-farm variability has been explored in several studies. For instance, de Deus et al. (2008), Feng et al. (2011) and Casas et al. (2011) individually followed a 45, 32 and 120 piglet sample from one Spanish, one Chinese and six Spanish farrow-to-finish farms respectively. They highlighted a great individual variability in ages at HEV shedding and immunological profiles. This heterogeneity may reflect a wide range of infection dynamics related to farm- or individual-specific risk factors which have only been sporadically explored to date. Farm-level observational studies have highlighted husbandry practices in terms of hygiene, biosecurity and rearing conditions as pivotal factors favouring HEV spread on farms (e.g. farm size, mingling practices, origin of drinking water, presence of a hygiene lock) (Hinjoy et al., 2013; Jinshan, Manglai, Takahashi, Nagashima, & Okamoto, 2010; Li et al., 2009; Walachowski et al., 2014). Between-farm pig movements and the contact network topology have also been found to influence the epidemiological HEV situation of farms (Salines, Andraud, & Rose, 2018). However, individual risk factors related to piglets' specific characteristics (e.g. gender) or inherited from their dam (e.g. litter characteristics such as number of mummified, live-born or weaned piglets, parity rank of the dam, maternal immunity) have not been investigated to date. Using mathematical modelling based either on experimental trials or on field studies revealed new insights on HEV infection dynamics (Andraud, Casas, Pavio, & Rose, 2014; Andraud et al., 2013; Salines et al., 2015). As such, a partial protection conferred by maternally derived antibodies (MDAs) was shown to delay HEV infection in growing pigs (Andraud et al., 2014). Immunomodulating swine pathogens, that are widespread in the pig population, may also affect HEV infection dynamics. PRRSV (Porcine Reproductive and Respiratory Syndrome Virus) was previously demonstrated to have a suppressive effect on the antiviral innate immunity by inhibiting the IFN-α response (Albina, Carrat, & Charley, 1998; Van Reeth, Labarque, Nauwynck, & Pensaert, 1999). Besides, as suggested by Loving, Osorio, Murtaugh, and Zuckermann (2015), this decreased IFN-α response could be involved in the delayed and low specific immune response characterizing PRRSV infection. Nonetheless, the immunosuppressive potential of PRRSV and its facilitating role for other viral/bacterial co-infection is still debated (Rahe & Murtaugh, 2017). Some compelling studies have shown yet that PRRSV infection could alter the immune response to viral infection or vaccination (Van Reeth, Nauwynck, & Pensaert, 2001; Suradhat et al., 2006). More specifically, PRRSV co-infection is likely to lead to chronic HEV infection (Salines et al., 2015), with apparently extended latency and infectious period, increased HEV faecal shedding and impaired humoral immune response. Another swine virus, the porcine circovirus of type 2 (PCV2), is known to modulate the immune response as well. PCV2 can cause PCV2- systemic disease also named post-weaning multisystemic wasting syndrome, which leads to severe B and T lymphocyte depletion in blood and lymphoid tissues (Kekarainen & Segales, 2015). PCV2 DNA genome is able to inhibit the production of IFN-α by stimulated plasmacytoid dendritic cells (Vincent et al., 2007). Some CpG motifs in the PCV2 genome have been shown to also inhibit the production of IFN-α by porcine peripheral blood mononuclear cells in vitro (Wikstrom, Fossum, Fuxler, Kruse, & Lovgren, 2011; Wikstrom et al., 2007). PCV2 also modulates the expression of another cytokine, the immunosuppressive interleukine 10 (IL-10) by increasing its production in vitro and in vivo (Darwich et al., 2003, 2008; Fort et al., 2010; Kekarainen, Montoya, Mateu, & Segales, 2008). This IL-10 under-expression may be due to the interaction between the capsid protein of PCV2 and gC1qR protein (also named p32, HABP, C1qBP) that is a membrane receptor of the C1q component of the complement system. This has been demonstrated in lung alveolar macrophages after PCV2 infection (Du et al., 2016). Thus, as PCV2 induces the production of IL-10 that is a cytokine affecting innate and adaptive immune response, PCV2 infection in pigs may affect HEV infection. However, to date, only few data report on HEV/PCV2 co-infection (Jackel et al., 2018; Martin et al., 2007; Savic et al., 2010). In these studies, PCV2 and HEV were simultaneously detected in pigs but no direct correlation between the two infections could be evidenced.

Given the risk HEV represents to public health, it is necessary to fully understand the conditions related to HEV transmission dynamics within an infected pig farm in order to mitigate the risk of introducing contaminated products into the pork chain. A longitudinal follow-up was therefore conducted in three French pig farms known to be HEV infected so as to describe the within herd HEV infection patterns at the individual pig level and to assess the impact of co-infections with PRRSV and/or PCV2, anti-HEV immunity and litters' and individual piglets' characteristics on HEV infection dynamics.

2 | MATERIAL AND METHODS

2.1 | Study design

2.1.1 | Ethical statement

This study was carried out in strict accordance with the guidelines of the Good Experimental Practices (GEP) standard dictated by the European Union. The study was conducted in accordance with the recommended procedure of the Anses/ENVA/UPEC (French Agency for Food, Environmental and Occupational Health and Safety/Ecole Nationale Vétérinaire d'Alfort/Université Paris Est Créteil) ethical committee (agreement #16 to the National committee for ethics in animal experimentation). ANSES-Ploufragan is approved for animal experimentation and is registered under certification number C-22-745-1 delivered by the official French veterinary services.

2.1.2 | Cohort study

A longitudinal follow-up study was conducted in three farrow-to-finish pig farms located in Brittany region (North-Western part of France) and followed over the 2011–2012 period. These farms were selected to be farrow-to-finish pig farms with a majority of growing pigs reared on site, and were previously identified as HEV infected in a national prevalence and seroprevalence survey (Rose et al., 2011). Before starting the study and to ensure that HEV has been circulating since the prevalence study, the HEV status of three farms was checked at the slaughterhouse by randomly sampling 20 pigs per batch at the slaughter line (blood and liver) with three repetitions for each farm. The serological positive results confirmed the HEV positivity of the farms (File S1). The three farms were also known to be PRRSV and PCV2 positive, but a PCV2 vaccination programme was implemented in growing pigs from Farm 2 using an inactivated vaccine based on a PCV2 strain belonging to PCV2a genogroup. Sows were vaccinated against PRRSV in the three farms using a modified live vaccine, the vaccination schedule being the same in the three farms (booster vaccination 20 days post-farrowing on average). No PRRSV vaccination was implemented in growing pigs from any farm under study. In these three farms, three successive pig batches were followed, a batch being defined as a group of contemporary piglets in the same physiological stage. Farm 1 had 310 sows, conducted in a 5-week management system (leading to 900 piglet batches, approximately), farm 2 had 230 sows conducted in a 3-week management system (leading to 500 piglet batches) and farm 3 had 218 sows conducted in a 3-week management system (leading to 300 piglet batches). In each batch, a representative sample of 10 sows was randomly selected stratifying on parity (gilts, parities 1–2, 3–4 and 5 or more). At farrowing, all the piglets from the selected sows were identified and four piglets per litter were randomly selected to be ear-tagged and tattooed, leading to a cohort of 40 piglets per batch. This sample size per batch enabled the detection of the infection at each sampling time at a prevalence threshold of 7% with 95% confidence. This selection process resulted in a sample of 120 piglets monitored per farm and overall 360 pigs were individually followed from birth to slaughter. Selected piglets could not be cross-fostered and remained with their native dam until weaning; this ensured they received only colostrum form their dam. Cross-fostering was allowed for the other littermates. The monitored piglets were reared with other piglets in the batch and subjected to the same practices as other piglets in the farm after weaning. Individual blood and rectal faecal swab samples were taken at 1, 6, 10, 14, 18 and 22 weeks of age. Faecal swab samples were kept frozen (−80°C) until use. Blood samples were also taken from the related dams one week after farrowing to assess the transfer of maternal antibodies to the piglets through colostrum. Blood samples were collected by jugular vein puncture, using evacuated tubes (Vacuette, Dutscher SAS) without additive. Serum was obtained by centrifugation of blood samples for 10 min at 3,500 *g* and stored at −20°C until subsequent analysis. At slaughterhouse, blood and liver samples were collected on these same pigs.

2.1.3 | Virological and serological analyses

HEV RNA extraction and quantification were performed on faeces and liver using real-time quantitative RT-PCR as described in Barnaud, Rogée, Garry, Rose, and Pavio (2012). Results were expressed in terms of Cycle threshold (Ct). The detection of anti-HEV antibodies in serum was performed using the HEV ELISA 4.0v kit (MP Diagnostics) according to the manufacturer's instructions. This ELISA test detects all classes of anti-HEV antibodies including IgG and IgM with a specificity of 98.8% (Hu et al., 2008). Samples were positive when the optical density (OD) at 450 nm wavelength obtained for the sample was higher than the threshold defined as the mean for negative controls +0.3.

The detection of anti-N-PRRSV antibodies was performed using PRRS X3 Ab ELISA tests (IDEXX Laboratories) according to the manufacturer's instructions. Results were expressed as sample to positive control (S/P) optical density ratios. A sample was considered positive when the S/P ratio was equal or higher than 0.4.

PCV2 DNA extraction and quantification were performed on serum using real-time PCR based on TaqMan technology as described in (Grasland et al., 2005). Results were expressed in genomic copy number per millilitre of serum (ge/mL).

2.2 | Statistical analyses

2.2.1 | Outcome definitions

For each of the 360 followed pigs, four outcome variables were defined:

- The estimated age at HEV shedding, calculated as the age at first positive faecal sample minus 7 days, to take sampling intervals into account.

- The estimated duration of HEV shedding period, calculated as the interval between the first and the last positive faecal samples, plus 14 days to take sampling intervals into account.
- The estimated age at HEV seroconversion of pigs having shed HEV, derived from the individual antibodies' kinetics fitted with cubic splines (Green & Silverman, 1994). The age at seroconversion was calculated as the age when the cubic spline crossed the threshold on the upward slope. For the particular cases of animals being seropositive all over the study period, the age at seroconversion was defined as the age at lowest OD.
- The HEV status of the liver (positive or negative) at time of slaughter.

2.2.2 | Explanatory variables

Thirteen explanatory variables were considered to be compared with the outcomes:

Individual piglet's and litter's characteristics

(i) piglet's sex (one should note that pigs were not housed separately depending on their gender, and that male were castrated); (ii) sow's parity (note that a sow that had never delivered piglets at the time of inclusion in the study was attributed a parity of 0); (iii) number of stillborn piglets in the litter; (iv) number of mummified piglets in the litter; (v) number of live-born piglets in the litter; (vi) number of ingoing piglets into the litter; (vii) number of outgoing piglets from the litter; (viii) number of weaned piglets in the litter; (ix) age at slaughter and time interval between infection and slaughter. Continuous variables were categorized according to their distributions (mean, median or other quantiles depending on the shape of the distribution), making sure that categories contained at least 10% of the whole sample.

Anti-HEV serological status

(i) anti-HEV piglet's antibody status at first week of age, categorized as absent (OD < threshold) or present (OD > threshold); (ii) anti-HEV sow's immunity one week after farrowing, categorized as absent (OD < threshold) or present (OD > threshold).

Co-infections with viruses

- Exposure to co-infecting pathogens: pig's status regarding PCV2 and PRRSV was recorded and pigs were categorized as PCV2 infected, PRRSV infected, or PCV2 and PRRSV infected, whatever the order of the infections.
- Sequence of co-infections: First, ages at HEV/PCV2/PRRSV infection were calculated as followed: (1) pig's age at HEV infection was calculated as the age at HEV shedding minus 14 days,

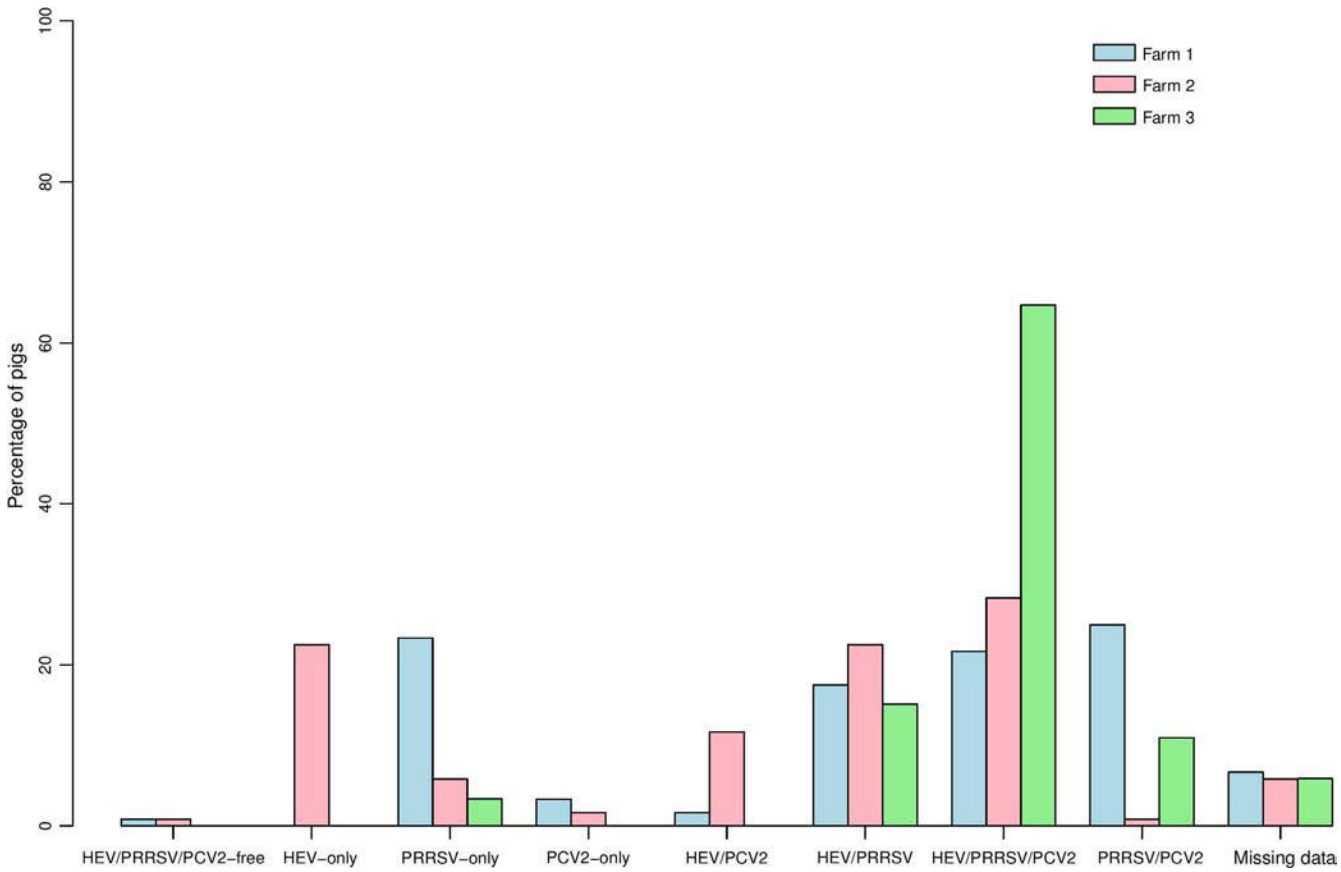

FIGURE 1 Cumulative incidence of HEV, PRRSV and/or PCV2 infections in the three French farrow-to-finish pig farms (3 batches per farm, *n* = 360 pigs)

corresponding to HEV latency period as described in Salines et al. (2015); (2) pig's age at PCV2 infection was calculated as the age at first PCV2 positive PCR minus 7 days, as described in literature (Andraud et al., 2008, 2009); (3) similarly to the calculation of the age at HEV seroconversion, the age at PRRSV seroconversion was derived from the individual antibodies' kinetics fitted with cubic splines; then, pig's age at PRRSV infection was calculated as the age at PRRSV seroconversion minus 7 days, as described in literature (Diaz, Darwich, Pappaterra, Pujols, & Mateu, 2005). Then, the sequence of infections was computed and four possible statuses were attributed to piglets: (i) infected by HEV first; (ii) infected by PCV2 first (i.e. PCV2 pre-infection) or during the HEV infection (considered as a PCV2 co-infection); (iii) infected by PRRSV first (i.e. PRRSV pre-infection) or during the HEV infection (considered as a PRRSV co-infection); (iv) pre- or co-infected both by PCV2 and PRRSV, whatever the order.

2.2.3 | Statistical models

Observed data of the age at HEV shedding, the shedding duration and the age at HEV seroconversion were fitted to different distributions (Weibull, lognormal and exponential) using the function 'fitdistcens' of the R package 'fitdistrplus'; the quality of fit was then evaluated using the Akaike Information Criterion (AIC) as described in Delignette-Muller and Dutang (2015). Finally, the average age at HEV shedding, shedding duration and age at HEV seroconversion were estimated using parametric survival regression with the previously selected distribution.

Cox-proportional hazard models were built to explore the link between (i) the explanatory variables and the age at HEV shedding; (ii) the explanatory variables and the age at HEV seroconversion; (iii) the explanatory variables and the duration of HEV shedding period. For this third model, the age at HEV shedding was included as a supplementary explanatory variable to account for the possible confounding effect of the age at HEV infection on the duration of HEV shedding. The influence of 'farm' and 'batch' variables on these three outcomes was also tested in cox-proportional hazard models and it appeared that 'farm' influenced the outcomes more than 'batch' (p-value < 0.0001 vs. >0.01 respectively). Thus, the 'farm' variable was included in the three models as a frailty effect (Proc PHREG in SAS (2014)) to account for non-independence of piglets within farms. The proportional hazard assumption of the Cox model were checked by (i) plotting the survival curves (Kaplan Meier estimate) and checking that they were not crossing; (ii) plotting the Log(-log SDF) versus time to check graphically and (iii) computing the Shoenfeld residuals to be plotted versus time.

A generalized estimating equation (GEE) logistic regression was performed to assess the link between the explanatory variables and the probability of livers being HEV positive at slaughterhouse. Again, the variability related to the 'farm' variable was greater than for the 'batch' variable. The 'farm' variable was therefore included

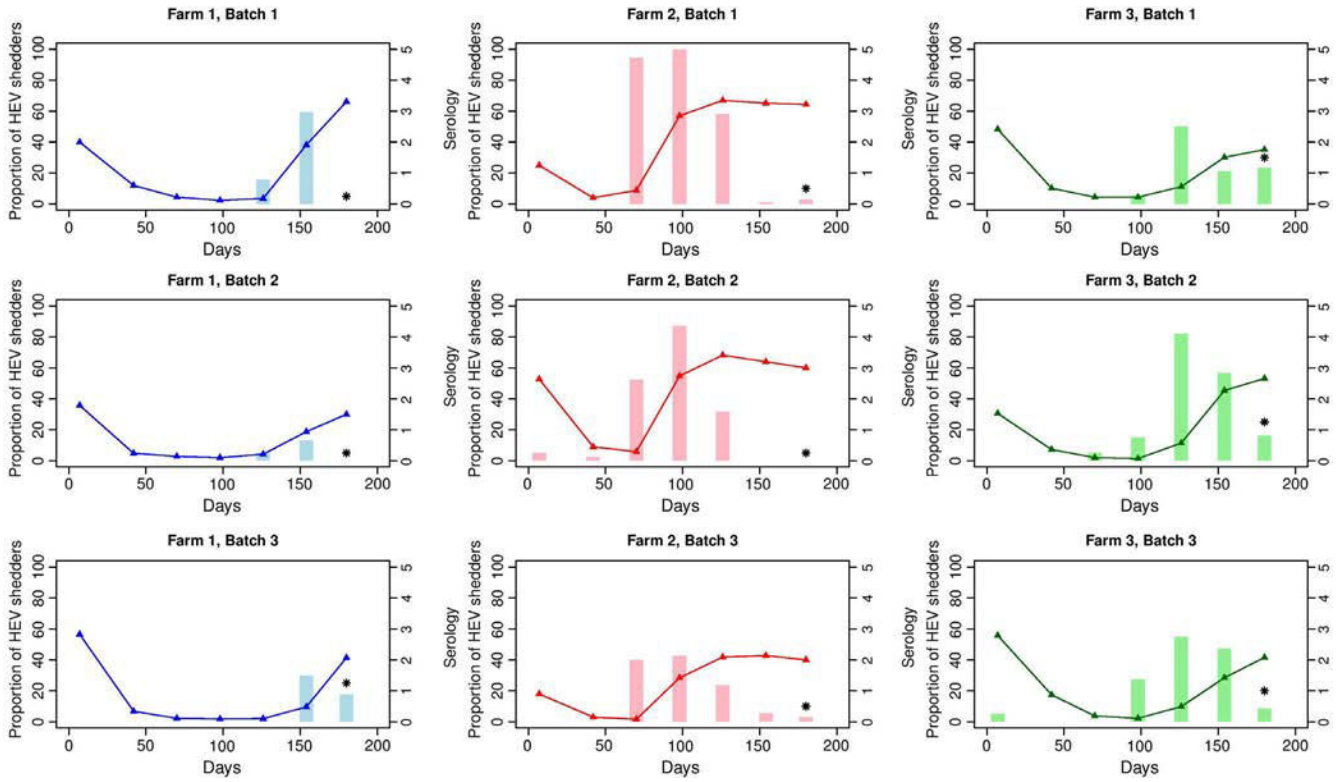

FIGURE 2 HEV course of infection in the three French farrow-to-finish pig farms (3 batches per farm, n = 360 pigs). Proportion of HEV shedders (bars) and HEV ELISA average optical density (lines) at 7, 42, 70, 98, 126, 154 and 180 days of age. The bar and star at 180 days stand for the proportion of HEV positive livers at slaughterhouse

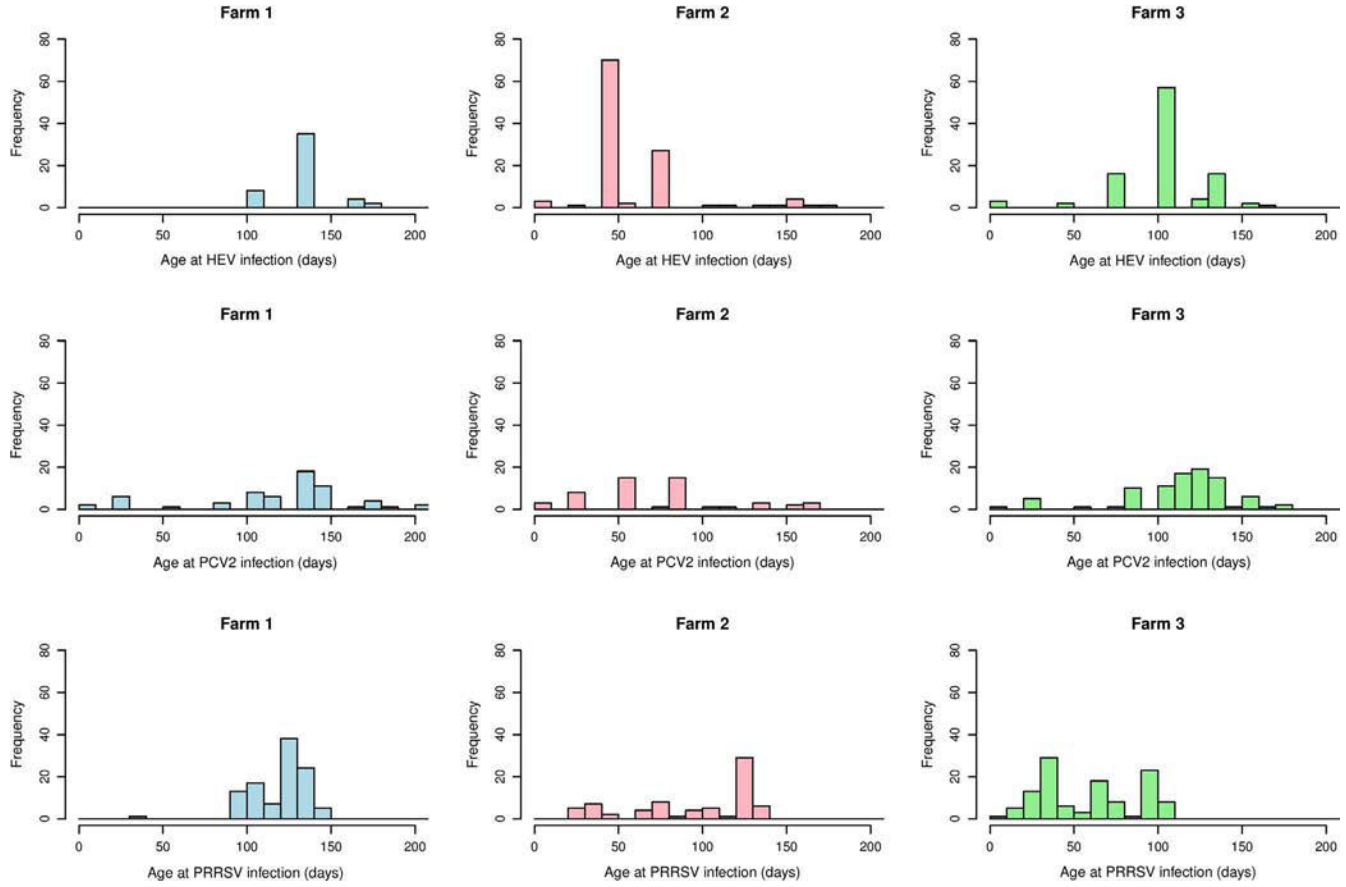

FIGURE 3 Distribution of the ages at HEV, PRRSV and PCV2 infection derived from a longitudinal follow-up in three French farrow-to-finish pig farms (3 batches per farm, n = 360 pigs)

as repeated statement (Proc GENMOD in SAS). These four models were all built as followed: first, a univariate analysis was conducted. All variables having a significant effect at univariate step ($p < 0.20$) were selected for a bivariate analysis aiming to remove too highly correlated variables. If variables did not show strong collinearity ($p > 0.05$), they were included in a multivariate model. A backward procedure was then applied until all remaining variables in the final model were significantly related to the outcome ($p < 0.05$).

3 | RESULTS

3.1 | HEV, PCV2 and PRRSV infection profile of the three farms

3.1.1 | Exposure to and sequence of infections

In the nine followed batches, 69.4% [95% Confidence Interval 64.4–74.2], 60.4% [55.0–65.4] and 84.1% [80.0–87.8] of pigs were found infected by HEV, PCV2 and PRRSV respectively (cumulative incidence of each virus), based on virological (HEV, PCV2) and serological (PRRSV) data (Figure 1). Co-infections were frequent, with variable proportions of co-infection cases depending on the farm. For instance HEV-only infection was only found in Farm 2 (representing 8.6% [5.9–12.0] of the 360 followed pigs and 22.5% [15.4–31.0] of the 120 piglets in Farm 2). Triple infections (i.e. detection of the three viruses or antibodies over a pig's life) were the most frequently encountered situation (53.7% [48.3–58.9] of the 360 pigs), especially in Farm 3 (64.7% [54.9–72.7] vs. 21.7% [14.7–30.1] and 28.3% [20.5–37.3] in Farms 1 and 2 respectively). Double infection cases were mainly PRRSV/HEV co-infections (e.g. 22.5% [15.4–31.0] of pigs in Farm 2). More precisely, 15.9% [12.2–20.0] of pigs were first infected by HEV, whereas 11.8% [8.5–15.4], 32.5% [27.7–37.6] and 39.8% [34.6–45.0] had pre- or co-infections with PCV2, PRRSV or both PCV2 and PRRSV respectively.

3.1.2 | HEV infection dynamics

The three studied farms exhibited variable HEV infection profiles (Figure 2). There were also differences between batches within a farm but to a less extent with more consistent patterns. HEV faecal shedding profiles differed greatly between farms i.e. late shedding in Farm 1 versus early shedding in Farm 2, with shedders as early as lactating period. Within-batch spread was also variable depending on farms, leading to heterogeneous prevalence figures: for instance up to 100% of pigs shed HEV in Farm 2, versus 60% in Farm 1 at most (Figure 2). The highest proportion of positive livers at slaughter was reached in Farm

TABLE 1 Effect of co-infections, immunity and litter characteristics on the age at HEV shedding (3 farrow-to-finish pigs farms, 3 batches per farm, *n* = 360 pigs)

			Univariate model		Multivariate model	
Variable	**Category**	***n***	**Hazard Ratio [95%CI]**	***p*-value**	**Hazard Ratio [95%CI]**	***p*-value**
Exposure to co-infecting pathogens			Likelihood-ratio $Chi^2 = 8.59$	0.035**		
	None	29	-	-		
	PCV2	22	0.63 [0.34–1.17]	0.14		
	PRRSV	105	0.68 [0.42–1.10]	0.11		
	PRRSV and PCV2	181	1.03 [0.64–1.68]	0.89		
Temporal order of co-infections			$Chi^2 = 43.44$	<0.01***	$Chi^2 = 25.52$	<0.01***
	HEV first	54	-	-	-	-
	PCV2 pre- or co-infection	40	0.56 [0.35–0.87]	0.011	0.64 [0.41–1.01]	0.056
	PRRSV pre- or co-infection	110	0.21 [0.13–0.34]	<0.01	0.28 [0.17–0.47]	<0.01
	PRRSV and PCV2 pre- or co-infection	135	0.20 [0.12–0.33]	<0.01	0.26 [0.15–0.46]	<0.01
Piglet's HEV serology (1 week of age)			$Chi^2 = 0.01$	0.92		
	Negative	114	-	-		
	Positive	243	0.99 [0.75–1.29]	0.92		
Sow's HEV serology one week after farrowing			$Chi^2 = 0.022$	0.88		
	Negative	108	-	-		
	Positive	251	1.02 [0.78–1.34]	0.88		
Sex			$Chi^2 = 1.93$	0.16*		
	Female	172	-	-		
	Male	187	1.20 [0.93–1.54]	0.16		
Sow's parity			$Chi^2 = 32.52$	<0.01***	$Chi^2 = 21.85$	<0.01***
	0–1	104	-	-	-	-
	2–3	88	1.22 [0.88–1.68]	0.23	1.36 [0.99–1.88]	0.059
	>4	167	0.51 [0.37–0.70]	<0.01	0.62 [0.44–0.85]	<0.01
Cross-fostering: number of ingoing piglets into the litter			$Chi^2 = 2.42$	0.30		
	0–1	207	-	-		
	2–5	84	1.32 [0.93–1.87]	0.12		
	6–12	68	1.22 [0.77–1.94]	0.39		
Cross-fostering: number of outgoing piglets from the litter			$Chi^2 = 3.22$	0.20*		
	0–4	260	-	-		
	5–7	67	0.71 [0.46–1.07]	0.10		
	8–12	32	0.66 [0.33–1.33]	0.25		
Number of weaned piglets in the litter			$Chi^2 = 4.34$	0.11*		
	7–10	83	-	-		
	11–12	200	1.06 [0.77–1.44]	0.73		
	13–14	76	1.49 [0.98–2.25]	0.06		
Number of liveborn piglets in the litter			$Chi^2 = 10.72$	<0.01***		
	0–11	68	-	-		
	12–14	112	0.67 [0.48–0.95]	0.03		
	15–18	179	0.58 [0.41–0.80]	0.001		

(Continues)

TABLE 1 (Continued)

Variable	Category	*n*	Univariate model Hazard Ratio [95%CI]	*p*-value	Multivariate model Hazard Ratio [95%CI]	*p*-value
Number of stillborn piglets in the litter			Chi^2 = 0.45	0.50		
	0–1	267	-	-		
	2–6	92	0.91 [0.68–1.21]	0.50		
Number of mummified piglets in the litter			Chi^2 = 3.31	0.069*		
	0	247	-	-		
	1–2	112	0.77 [0.59–1.02]	0.069		

Note: Summary statistics as obtained thanks to a cox-proportional hazard model with the 'farm' effect being included as a frailty effect.
Shaded areas represent variables that were not retained in the multivariate model.
***$p < 0.01$.
**$p < 0.05$.
*$p < 0.20$.

3 with more than 24% [0.11–0.42] of positive livers in the first batch (Figure 2), consistently with what was observed at the selection phase (File S1). This was associated with a late shedding peak, reached after 120 days of age, and a high proportion of HEV shedding pigs (Figure 2).

More precisely, the average age at HEV shedding was estimated to 124.5 days [95% CI 106.4–144.3], 92.9 days [84.4–102.3] and 137.0 days [130.3–146.1] in Farm 1, 2 and 3, respectively, with Weibull distributions (File S2). The average duration of HEV shedding at the pig level was estimated to 16.2 days [95% CI 14.6–17.9], 35.5 days [31.2–40.5] and 25.9 [22.4–30.1] in Farm 1, 2 and 3, respectively, with lognormal distributions (File S2).

3.1.3 | Ages at PCV2 and PRRSV infection and comparison with HEV dynamics

The infection profiles regarding the two co-infecting pathogens also differed greatly (Figure 3, Files S3 and S4). In addition to different ages at infection, the cumulated prevalence of PCV2 viraemic pigs was found higher in Farm 3 than in Farms 1 and 2 (up to 60% vs. 40% and 35% respectively), the lowest being observed in Farm 2. The distributions of the ages at PRRSV infection were more narrowly spread within farms but between-farm variability was also found, with PRRSV infections occurring much earlier in Farm 3 than in Farm 1. The comparison of the distributions of the ages at HEV, PRRSV and PCV2 infection also highlighted different profiles in the sequences of infections depending on farms, for example PRRSV infection occurred much earlier than HEV infection in Farm 3 for every batch.

3.2 | Factors affecting HEV infection features

3.2.1 | Age at HEV shedding

The univariate analysis showed that both exposure to and temporal order of co-infections were associated with the age at HEV shedding, as well as six out of the eight variables related to piglet's and litter's characteristics (Table 1). The multivariate model evidenced that a PRRSV or PCV2/PRRSV pre- or co-infection was associated with a higher age at HEV shedding similarly (Hazard Ratio = 0.28 [0.17–0.47] and 0.26 [0.15–0.46] respectively)). Sow's parity was also associated with the age at HEV shedding, with piglets from oldest sows (parity higher than 4) exhibiting later HEV shedding (HR = 0.62 [0.44–0.85]) (Table 1). Other individual or litter characteristics such as number of live-born and mummified piglets, cross-fostering or sex did not remain in the multivariate model.

3.2.2 | Age at HEV seroconversion

The univariate analysis showed an impact of the exposure to and the sequence of co-infections and of six variables reflecting piglet's and litter's characteristics (Table 2). According to the results of the multivariate model, males exhibited HEV seroconversion later than females (HR = 0.70 [0.53–0.91]) and HEV seroconversion was delayed in piglets from oldest sows (HR = 0.39 [0.27–0.55] for sows of parity higher than 4 vs. parity less than 1). A PRRSV or PCV2/PRRSV pre- or co-infection was also associated with a higher age at HEV seroconversion (HR = 0.53 [0.30–0.91] and HR = 0.41 [0.24–0.69] respectively) (Table 2).

3.2.3 | Duration of the HEV shedding period

The model evidenced a strong impact of the sequence of co-infections on the duration of the HEV shedding period, with a PCV2/PRRSV pre- or co-infection lengthening the shedding period (HR = 0.50 [0.32–0.79]). This variable was the only one related to this outcome (Table 3).

3.2.4 | HEV status of livers

From the results of the univariate analysis, both exposure to and sequence of co-infections were found to affect the probability of

TABLE 2 Effect of co-infections, immunity and litter characteristics on the age at HEV seroconversion of HEV shedders (3 farrow-to-finish pigs farms, 3 batches per farm, *n* = 249 pigs)

			Univariate model		Multivariate model	
Variable	**Category**	***n***	**Hazard Ratio [95%CI]**	***p*-value**	**Hazard Ratio [95%CI]**	***p*-value**
Exposure to co-infecting pathogens			Likelihood-ratio Chi^2 = 5.96	<0.01***		
	None	27	-	-		
	PCV2	16	0.26 [0.13–0.51]	<0.01		
	PRRSV	66	0.72 [0.43–1.20]	0.21		
	PRRSV and PCV2	136	0.59 [0.36–0.97]	0.04		
Temporal order of co-infections			Chi^2 = 20.21	<0.01***	Chi^2 = 12.69	<0.01***
	HEV first	54	-	-	-	-
	PCV2 pre- or co-infection	34	1.03 [0.66–1.60]	0.90	0.98 [0.62–1.53]	0.91
	PRRSV pre- or co-infection	71	0.45 [0.26–0.76]	<0.01	0.53 [0.30–0.91]	0.02
	PRRSV and PCV2 pre- or co-infection	90	0.35 [0.21–0.58]	<0.01	0.41 [0.24–0.69]	<0.01
Piglet's HEV serology (1 week of age)			Chi^2 = 1.52	0.22		
	Negative	79	-	-		
	Positive	169	0.84 [0.63–1.10]	0.22		
Sow's HEV serology one week after farrowing			Chi^2 = 1.24	0.27		
	Negative	74	-	-		
	Positive	175	0.85 [0.64–1.13]	0.27		
Sex			Chi^2 = 6.38	0.01**	Chi^2 = 7.03	<0.01***
	Female	119	-	-	-	-
	Male	130	0.71 [0.55–0.93]	0.01	0.70 [0.53–0.91]	<0.01
Sow's parity			Chi^2 = 34.37	<0.01***	Chi^2 = 32.99	<0.01***
	0–1	79	-	-	-	-
	2–3	76	1.01 [0.73–1.40]	0.95	0.93 [0.67–1.30]	0.67
	>4	94	0.40 [0.28–0.57]	<0.01	0.39 [0.27–0.55]	<0.01
Cross-fostering: number of ingoing piglets into the litter			Chi^2 = 5.36	0.07*		
	0–1	165	-	-		
	2–5	51	1.50 [1.06–2.13]	0.37		
	6–12	33	1.27 [0.80–2.01]	0.94		
Cross-fostering: number of outgoing piglets from the litter			Chi^2 = 4.14	0.13*		
	0–4	207	-	-		
	5–7	31	1.52 [0.99–2.33]	0.05		
	8–12	11	1.58 [0.73–3.42]	0.25		
Number of weaned piglets in the litter			Chi^2 = 0.08	0.96		
	7–10	67	-	-		
	11–12	136	1.00 [0.72–1.40]	0.98		
	13–14	46	1.05 [0.69–1.61]	0.81		
Number of liveborn piglets in the litter			Chi^2 = 14.79	<0.01***		
	0–11	55	-	-		
	12–14	80	0.93 [0.65–1.34]	0.71		
	15–18	114	0.55 [0.39–0.78]	<0.01		

(Continues)

TABLE 2 (Continued)

Variable	Category	*n*	Univariate model		Multivariate model	
			Hazard Ratio [95%CI]	*p*-value	Hazard Ratio [95%CI]	*p*-value
Number of stillborn piglets in the litter			Chi^2 = 0.18	0.67		
	0–1	183	-	-		
	2–6	66	1.07 [0.79–1.44]	0.67		
Number of mummified piglets in the litter			Chi^2 = 1.87	0.17*		
	0	171	-	-		
	1–2	78	1.21 [0.92–1.60]	0.17		

Note: Summary statistics as obtained thanks to a cox-proportional hazard model with the 'farm' effect being included as a frailty effect.
Shaded areas represent variables that were not retained in the multivariate model.
***$p < 0.01$.
**$p < 0.05$.
*$p < 0.20$.

liver being HEV-positive at slaughter, as well as six variables linked to piglet's and litter's characteristics. A short time-period between HEV infection and slaughter (<40 days) also increased the odds of a liver being HEV positive at slaughter (Odds Ratio = 4.07 [3.72–4.45]). The multivariate model evidenced that a PRRSV pre- or co-infection increased the risk of having positive livers at slaughter (OR = 4.10 [1.87–8.97]), particularly when combined with a PCV2 pre- or co-infection (OR = 6.49 [3.18–13.23]) (Table 4).

4 | DISCUSSION

Understanding the features and drivers of HEV infection dynamics on pig farms is crucial in order to implement HEV surveillance programmes and to assess and manage public health risks. Quite a few studies have investigated the dynamics of HEV infection at individual and collective levels in pig population in recent years (Berto, Mesquita, Hakze-van der Honing, Nascimento, & Poel, 2012; Casas et al., 2011; de Deus et al., 2008; Feng et al., 2011; Gardinali et al., 2012). The primary interest of our results lies in both describing and explaining within- and between-farm variability of HEV infection dynamics. To the best of our knowledge, this is the first individual follow-up conducted in several pig farms and monitoring HEV dynamics along with other co-infecting pathogens simultaneously. Though previous cohort studies exploring HEV infections have been already conducted in Spain and China, they only included a small number of animals, raised in a single batch from several farms (Casas et al., 2011) or in a single farm (de Deus et al., 2008; Feng et al., 2011); this limits the investigation of variation factors at farm, batch or individual levels. Other studies aiming to describe HEV course of infection consisted of biological samples taken at successive ages or production stages but from different pigs (Berto et al., 2012; Gardinali et al., 2012). In that sense, the monthly individual follow-up proposed here offered a unique opportunity to describe HEV course of infection while accounting for within and between-farm variability. A preliminary check of the status of the farms to be selected revealed that they were still contaminated by HEV 2 years after a large prevalence survey (Rose et al., 2011). Moreover, the infection profile in terms of prevalence of HEV-containing livers at slaughter time was consistent with the results obtained thereafter in the follow up study. It suggests a strong ability of HEV to maintain in farrow-to-finish farms and a good stability in terms of dynamics of infection. In addition to a descriptive analysis of HEV patterns, statistical models were built to explain features of HEV infection dynamics.

Several outcomes were considered to describe HEV dynamics: the age at HEV shedding, the age at HEV seroconversion, the duration of HEV shedding and the HEV virological status of the liver at slaughter time. These four parameters were chosen in order to accurately describe the infection pattern at an individual scale in terms of shedding and immune response; they were also relevant to inform on the related public health risk. In that sense, they offer a full view of HEV characteristics on pig farms. The degree of uncertainty due to the sampling design and to the calculation method of these parameters was taken into account. Indeed, samples were taken every month and the dates of events occurring in these time intervals were therefore uncertain. For instance if piglets were found shedder at one sample only, it was very unlikely that they shed the virus for only one day. To address this, it was considered that the age at HEV shedding was the age at first positive faecal sample minus 7 days and the duration of HEV shedding period was calculated as the interval between the first and the last positive faecal samples plus 14 days. By doing so, the individual shedding period was at least 14 days, which is consistent with literature data (Salines et al., 2015). The age at HEV infection was inferred using a fixed latency value, which may affect the results. However, choosing 14 days as latency duration is a careful choice: indeed, experimental trials have shown that latency may vary between 7 and 14 days (Andraud et al., 2013; Salines et al., 2015), choosing 14 days can thus lead to underestimating the number of co-infected pigs. It therefore confirms the above results regarding the effect of immunomodulating viruses on HEV infection dynamics. A

TABLE 3 Effect of co-infections, immunity and litter characteristics on the duration of HEV infectious period (3 farrow-to-finish pigs farms, 3 batches per farm, *n* = 249 pigs)

			Univariate model		Multivariate model	
Variable	**Category**	***n***	**Hazard ratio [95%CI]**	***p*-value**	**Hazard ratio [95%CI]**	***p*-value**
Exposure to co-infecting pathogens			Likelihood-ratio Chi^2 = 0.37	0.94		
	None	27	-	-		
	PCV2	16	1.08 [0.57–2.03]	0.82		
	PRRSV	66	1.02 [0.62–1.66]	0.95		
	PRRSV and PCV2	136	0.93 [0.58–1.51]	0.78		
Temporal order of co-infections			Chi^2 = 10.05	0.018*	Chi^2 = 10.05	0.018*
	HEV first	54	-	-	-	-
	PCV2 pre- or co-infection	34	0.92 [0.59–1.44]	0.72	0.92 [0.59–1.44]	0.72
	PRRSV pre- or co-infection	71	0.70 [0.44–1.11]	0.13	0.70 [0.44–1.11]	0.13
	PRRSV and PCV2 pre- or co-infection	90	0.50 [0.32–0.79]	<0.01	0.50 [0.32–0.79]	<0.01
Piglet's HEV serology (1 week of age)			Chi^2 = 0.014	0.90		
	Negative	79	-	-		
	Positive	169	1.02 [0.76–1.37]	0.90		
Sow's HEV serology one week after farrowing			Chi^2 = 0.37	0.54		
	Negative	74	-	-		
	Positive	175	0.91 [0.68–1.23]	0.54		
Sex			Chi^2 = 0.13	0.72		
	Female	119	-	-		
	Male	130	0.95 [0.72–1.26]	0.72		
Sow's parity			Chi^2 = 2.94	0.23		
	0–1	79	-	-		
	2–3	76	0.77 [0.54–1.10]	0.89		
	>4	94	1.03 [0.73–1.43]	0.12		
Cross-fostering: number of ingoing piglets into the litter			Chi^2 = 0.20	0.90		
	0–1	165	-	-		
	2–5	51	0.96 [0.67–1.37]	0.81		
	6–12	33	0.90 [0.57–1.43]	0.66		
Cross-fostering: number of outgoing piglets from the litter			Chi^2 = 2.83	0.23		
	0–4	207	-	-		
	5–7	31	0.70 [0.44–1.09]	0.12		
	8–12	11	1.11 [0.54–2.31]	0.77		
Number of weaned piglets in the litter			Chi^2 = 0.54	0.76		
	7–10	67	-	-		
	11–12	136	0.95 [0.68–1.33]	0.77		
	13–14	46	1.09 [0.71–1.67]	0.69		
Number of liveborn piglets in the litter			Chi^2 = 2.76	0.25		
	0–11	55	-	-		
	12–14	80	1.26 [0.86–1.85]	0.24		
	15–18	114	0.97 [0.67–1.40]	0.88		

(Continues)

TABLE 3 (Continued)

Variable	Category	*n*	Univariate model		Multivariate model	
			Hazard ratio [95%CI]	*p*-value	Hazard ratio [95%CI]	*p*-value
Number of stillborn piglets in the litter			Chi^2 = 0.01	0.92		
	0–1	183	-	-		
	2–6	66	0.98 [0.72–1.35]	0.92		
Number of mummified piglets in the litter			Chi^2 = 0.89	0.35		
	0	171	-	-		
	1–2	78	1.15 [0.86–1.53]	0.35		

Note: Summary statistics as obtained thanks to a cox-proportional hazard model with the 'farm' effect being included as a frailty effect and the age at HEV shedding being included as an explanatory variable. Shaded areas represent variables that were not retained in the multivariate model.
*$p < 0.05$.

sensitivity analysis would make it possible to consolidate the validity of our conservative method; it may show an even higher impact of PRRSV and/or PCV2 on HEV infection dynamics. The ages at seroconversion (HEV) or at infection (PRRSV) based on serological data were derived from modelling the antibodies kinetic curve by cubic splines to infer from the discrete sampling scheme the most likely seroconversion time. PRRSV serological data at day 180 were missing for Farms 2 and Farms 3. However, this did not affect the results: indeed, if pigs were PRRSV infected before HEV infection, they produced antibodies earlier than 180 days of age, therefore they have been detected at previous sampling points. Otherwise, they have been considered as HEV infected at first.

High between-farm variability of the HEV infection dynamics was evidenced, in contrast to more stable within-farm HEV pattern. Other studies also pointed a number of farm-specific factors that influence HEV infection features, for example farm size, genetic background, lack of hygiene measures, origin of drinking water, frequency of pig exchanges, etc. (Di Bartolo et al., 2008; Hinjoy et al., 2013; Jinshan et al., 2010; Li et al., 2009; Salines et al., 2018; Walachowski et al., 2014). This is the reason why the farm variable was included as a repeated and frailty effect in the GEE and survival models respectively. By doing so, it was possible to investigate the proper effect of other factors measured at the individual pig level and to extend our conclusions beyond the farm specificities. Several possible factors likely to explain features of HEV infection dynamics were considered: factors related to piglets' and litters' characteristics that had not been investigated to date; factors linked to anti-HEV immunity (in particular the effect of maternally derived antibodies) that had only been partially explored (Andraud et al., 2014); factors concerning immunomodulating pathogens that had only been studied in experimental conditions (Salines et al., 2015).

Pigs in Farm 1 got infected late and shed the virus for a short time period with a limited spread at the batch level, whereas pigs in Farm 2 got infected early and were shedders for a long time with a huge spread, and pigs in Farm 3 were infected late and shed the virus for a long time period with an important spread at the batch level as well. Some piglets in Farms 2 and 3 were found to shed HEV as early as lactating phase, suggesting the possible HEV transmission from sows to piglets at this stage. Interestingly, the proportion of shedding sows found at farrowing or one week later was 0, 4.8 and 4.8% in farms 1, 2 and 3 respectively (data not shown). In our study and on all three farms, 7.2% of pigs (26/360) had HEV positive liver at slaughterhouse versus 6.2% (6/96) in Casas et al. (2011). The prevalence of HEV positive livers varied between farms, with a high proportion of positive livers in Farm 3 (up to 24% in the first batch). Our model evidenced that the time period between HEV infection and slaughter affected the HEV liver status, with a time-interval lower than 40 days increasing the probability of livers being HEV-positive at slaughterhouse by a factor of 4 in the univariate model. The variability in the prevalence of positive livers is therefore consistent with the different HEV infection patterns depending on farms, with a high proportion of contaminated livers in Farm 3 where infection occurred late in association with long shedding and high spread among pigs.

Our study showed that males exhibited HEV seroconversion later than females and were also more likely having HEV-containing livers at slaughter. To the best of our knowledge, the association between sex and HEV infection characteristics has never been reported in pigs. However, this is consistent with epidemiological studies in human population showing a high male/female sex ratio, probably associated with host factors that are still unknown (Borgen et al., 2008; Ijaz et al., 2005; Lewis, Morgan, Ijaz, & Boxall, 2006; Mansuy et al., 2009; Said et al., 2009). Our models also evidenced that HEV shedding and seroconversion were delayed for piglets from a high parity sow. This may be related to the sow's immunological status as regard HEV, old sows having a better immunity than young ones (e.g. in terms of IgG quantity and/or affinity, or other non-specific antiviral factors), hence delivering a stronger maternal immunity to their piglets. In addition, among the 17 sows out of 90 found HEV positive in faeces at any sampling time, six were of parity higher than four (out of 38 sows of parity higher than four) four were of parity between one and four (out of 21 sows in this parity category) and seven were gilts (out of 21 gilts in total) (data not shown). There is a trend of more frequent HEV shedding in gilts compared to multiparous sows (OR = 2.9 [0.8–10.3], *p*-value = 0.06). It suggests a higher susceptibility of gilts to infection, favouring transmission to their

TABLE 4 Effect of co-infections, immunity and litter characteristics on the probability of liver being HEV-positive at slaughter (3 farrow-to-finish pigs farms, 3 batches per farm, *n* = 360 pigs)

			Univariate model		Multivariate model	
Variable	**Category**	***n***	**Odds ratio [95%CI]**	***p*-value**	**Odds ratio [95%CI]**	***p*-value**
Exposure to co-infecting pathogens			Likelihood-ratio Chi^2 = 5.49	<0.01***		
	None	29	-	-		
	PCV2	22	0.99 [0.67–1.44]	0.94		
	PRRSV	105	1.11 [0.24–5.30]	0.89		
	PRRSV and PCV2	181	0.98 [0.24–3.92]	0.97		
Temporal order of co-infections			Chi^2 = 48.63	<0.01***	Chi^2 = 34.09	<0.01***
	HEV first	54	-	-	-	-
	PCV2 pre- or co-infection	40	1.45 [0.19–11.10]	0.72	1.49 [0.22–10.35]	0.69
	PRRSV pre- or co-infection	110	4.06 [2.36–6.99]	<0.01	4.10 [1.87–8.97]	<0.01
	PRRSV and PCV2 pre- or co-infection	135	6.39 [3.70–11.03]	<0.01	6.49 [3.18–13.23]	<0.01
Piglet's HEV serology (1 week of age)			Chi^2 = 0.28	0.59		
	Negative	114	-	-		
	Positive	243	0.74 [0.25–2.23]	0.59		
Sow's HEV serology one week after farrowing			Chi^2 = 0.08	0.78		
	Negative	108	-	-		
	Positive	251	0.84 [0.24–2.93]	0.78		
Sex			Chi^2 = 12.99	<0.01***	Chi^2 = 136.91	<0.01***
	Female	172	-	-	-	-
	Male	187	1.29 [1.12–1.49]	<0.01	1.39 [1.32–1.47]	<0.01
Sow's parity			Chi^2 = 197.17	<0.01***	Chi^2 = 66.75	<0.01***
	0–1	104	-	-	-	-
	2–3	88	1.91 [1.38–2.65]	<0.01	2.49 [1.29–4.80]	<0.01
	>4	167	1.25 [0.65–2.39]	0.50	1.32 [0.49–3.57]	0.58
Cross-fostering: number of ingoing piglets into the litter			Chi^2 = 0.80	0.67		
	0–1	207	-	-		
	2–5	84	0.48 [0.054–4.19]	0.50		
	6–12	68	1.04 [0.32–3.41]	0.95		
Cross-fostering: number of outgoing piglets from the litter			Chi^2 = 22.50	<0.01***		
	0–4	260	-	-		
	5–7	67	1.29 [0.17–9.61]	0.80		
	8–12	32	0.82 [0.32–2.12]	0.69		
Number of weaned piglets in the litter			Chi^2 = 1.02	0.60		
	7–10	83	-	-		
	11–12	200	0.97 [0.63–1.49]	0.89		
	13–14	76	0.46 [0.08–2.63]	0.38		
Number of liveborn piglets in the litter			Chi^2 = 34.94	<0.01***		
	0–11	68	-	-		
	12–14	112	0.94 [0.21–4.12]	0.93		
	15–18	179	1.37 [0.23–8.01]	0.73		

(Continues)

TABLE 4 (Continued)

Variable	Category	*n*	Univariate model		Multivariate model	
			Odds ratio [95%CI]	*p*-value	Odds ratio [95%CI]	*p*-value
Number of stillborn piglets in the litter			Chi^2 = 6.04	0.014**		
	0–1	267	-	-		
	2–6	92	0.64 [0.45–0.91]	0.014		
Number of mummified piglets in the litter			Chi^2 = 23.45	<0.01***		
	0	247	-	-		
	1–2	112	0.49 [0.37–0.65]	<0.01		
Time period between HEV infection and slaughter			Chi^2 = 933.26	<0.01***		
	>40 days	187	-	-		
	≤40 days	59	4.07 [3.72–4.45]	<0.01		

Note: Summary statistics as obtained thanks to a generalized estimating equation (GEE) logistic regression model with the 'farm' effect being included as a repeated statement.
Shaded areas represent variables that were not retained in the multivariate model.ssss
***$p < 0.01$.
**$p < 0.05$.

piglets at an early stage. However, the two variables linked to anti-HEV immunity (serological status of the dam and piglets at one week post-farrowing) were not statistically associated with HEV infection features. Based on serological data from longitudinal studies in six pig herds, Andraud et al. (2014) showed that passive immunity delayed HEV infection of piglets by about six weeks in all but one farm on which the dynamics of infection were similar, whatever the animals' initial serological status. It suggests that beyond maternally derived passive immunity, other factors depending on host characteristics, farm-specific husbandry and hygiene practices have an impact on HEV transmission process.

Our models also showed a strong impact of a pre- or co-infection with PRRSV, alone or associated with PCV2 on all outcomes. This is in accordance with a previous experiment in which PRRSV was found to delay the age at HEV shedding with an increased latency period by a factor of 1.9, to delay the age at HEV seroconversion by a factor of 1.6, to lengthen HEV shedding period by a factor of 5 and to increase the probability of livers being HEV positive at 49 days post-infection (Andraud et al., 2013; Salines et al., 2015). The delayed age at HEV shedding evidenced in our study may be due either to a lengthened latency period or to a reduced sensitivity to infection. However, the extended latency period seems more likely, as the experimental trial conducted by Salines et al. (2015) showed that PRRSV co-infection extended the HEV latency period but also increased the susceptibility to HEV infection of pigs exposed to infectious particles in the environment and enhanced transmission of the virus between pigs. Our results suggest that PCV2 alone did not affect HEV infection dynamics as PRRSV. This may be related to specific characteristics of the infection dynamics observed in the farms under study. Hence, the average age at infection for PCV2 and HEV was very similar in almost the three farms under study whereas it was more different between PRRSV and HEV. This might be specific to these three farms and does not preclude a similar behaviour in all HEV/PCV2/PRRSV co-infected farms. Investigations in a larger sample of herds would be required to evaluate this assumption. As regards potential immune mechanisms specific to co-infecting viruses, further work would be needed as PCV2 and PRRSV both have a suppressive effect on the innate immunity but their specific impact on HEV infection is still unknown (Butler et al., 2014; Darwich & Mateu, 2012).

In conclusion, these results show that co-infections with viruses affecting pig immune response, mainly PRRSV – alone or associated with PCV2 – have a major impact on HEV dynamics. These intercurrent pathogens may lead to extended HEV shedding and chronic HEV infection, increasing the risk of having HEV contaminated livers at slaughter age. At the batch level, the sequence of infection both influence the extent of HEV spread between pigs and the average age at infection. Taken together all these individual- and population-based characteristics directly influence the prevalence of HEV-containing livers at slaughter time. Controlling these pig-specific pathogens may therefore be a major lever to mitigate public health risk related to HEV.

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CONFLICT OF INTEREST

The authors declare that no competing interests exist.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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II. Etude de la dynamique de l'infection par le virus de l'hépatite E chez des porcs co-infectés par un virus immunomodulateur en conditions expérimentales

Le suivi longitudinal de trois élevages de porcs naisseurs-engraisseurs a mis en évidence que **la co-infection naturelle de porcs par le virus du SDRP, seule ou couplée à une co-infection par le PCV2, est associée à une excrétion plus tardive du HEV dans les fèces, une durée d'excrétion fécale du HEV plus longue, une séroconversion vis-à-vis du HEV retardée, et une augmentation du risque de positivité du foie à l'abattoir**. Néanmoins, et malgré la prise en compte statistique de facteurs de confusion, cette étude en conditions naturelles ne suffit pas à conclure de manière certaine quant à la **relation de causalité** pouvant exister entre ces variables associées. C'est la raison pour laquelle des **essais expérimentaux** ont été réalisés chez des porcs EOPS (Exempts d'Organismes Pathogènes Spécifiques) afin d'**étudier de manière spécifique, en conditions contrôlées, l'effet de la co-infection par le virus du SDRP et par le PCV2 – séparément – sur la dynamique de l'infection par le HEV**.

Ces essais ont donné lieu à trois publications internationales : une avant la thèse dans *Veterinary Research* (Salines *et al.*, 2015b), et deux dans le cadre de la thèse dans *International Journal of Food Microbiology* (Salines *et al.*, 2018d) et *Veterinary Microbiology* (Salines *et al.*, 2019a). Si les articles dans *Veterinary Research* et *Veterinary Microbiology* s'intéressent à l'**influence des co-infections sur les paramètres généraux de la dynamique infectieuse** (**période de latence, période infectieuse, paramètres de transmission, statut du foie à l'abattage**), la publication dans *International Journal of Food Microbiology* traite plus spécifiquement de l'impact de la co-infection par le SDRP sur le **risque de présence du HEV dans le sang et les muscles des porcs co-infectés**. Deux articles ont aussi été publiés dans des revues nationales (Annexe 2 et Annexe 3) (Salines *et al.*, 2015a; Rose *et al.*, 2017).

Publication 3

Salines M., Barnaud E., Andraud M., Eono F., Renson P., Bourry O., Pavio N., Rose N., 2015. Hepatitis E virus chronic infection of pigs co-infected with Porcine Reproductive and Respiratory Syndrome Virus. *Veterinary Research*, 46:55.

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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Hepatitis E virus chronic infection of swine co-infected with Porcine Reproductive and Respiratory Syndrome Virus

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Abstract

In developed countries, most of hepatitis E human cases are of zoonotic origin. Swine is a major hepatitis E virus (HEV) reservoir and foodborne transmissions after pork product consumption have been described. The risk for HEV-containing pig livers at slaughter time is related to the age at infection and to the virus shedding duration. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is a virus that impairs the immune response; it is highly prevalent in pig production areas and suspected to influence HEV infection dynamics. The impact of PRRSV on the features of HEV infections was studied through an experimental HEV/PRRSV co-infection of specific-pathogen-free (SPF) pigs. The follow-up of the co-infected animals showed that HEV shedding was delayed by a factor of 1.9 in co-infected pigs compared to HEV-only infected pigs and specific immune response was delayed by a factor of 1.6. HEV shedding was significantly increased with co-infection and dramatically extended (48.6 versus 9.7 days for HEV only). The long-term HEV shedding was significantly correlated with the delayed humoral response in co-infected pigs. Direct transmission rate was estimated to be 4.7 times higher in case of co-infection than in HEV only infected pigs (0.70 and 0.15 per day respectively). HEV infection susceptibility was increased by a factor of 3.3, showing the major impact of PRRSV infection on HEV dynamics. Finally, HEV/PRRSV co-infection – frequently observed in pig herds – may lead to chronic HEV infection which may dramatically increase the risk of pig livers containing HEV at slaughter time.

Introduction

Hepatitis E virus is a non-enveloped single-stranded RNA virus causing an acute hepatitis in humans. It is mainly transmitted by the oro-fecal route and is responsible for clinical signs similar to hepatitis A virus infection [1]. Chronic cases have been described, mainly in immunocompromised patients [2,3]. Four HEV genotypes have been described. Genotypes 1 and 2 infect only humans and circulate in Asia, Africa and Central America in epidemic waves linked to the consumption of contaminated water [4–6]. Genotypes 3 and 4 are shared between humans and other animal species and are responsible for autochthonous sporadic cases in industrialized countries. In particular, the number of hepatitis E cases linked to genotype 3 has considerably increased in the last decade [6,7], in relation to better diagnosis. This genotype is highly prevalent in the swine population [8]. Some studies have shown that swine and human HEV strains are genetically very close [9] and HEV cross-species transmission has been proven [10,11]. Moreover, a number of autochthonous cases have been related to the consumption of undercooked pork meat, especially liver products [12–16]. Thus, hepatitis E is now recognized as a foodborne zoonosis for which domestic pigs are considered as the main reservoir in developed countries [7,17,18]. Understanding factors influencing the transmission dynamics of HEV in pig herds is crucial to limit the risk of an introduction of contaminated products in the food chain. Several studies have described experimental HEV infection trials via oral or intravenous route [19–24] but few studies were aimed at quantifying HEV transmission [20,25]. The results of these studies on HEV transmission were different than those observed in pig farms on the field, with the latent and infectious period estimates being generally longer

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than in experimental trials [26–28]. Moreover, a high variability of HEV infection dynamics is observed on pig farms and has not yet been fully explained [29]. Some factors affecting swine immune response may also influence the course of HEV infection. Porcine Respiratory and Reproductive Syndrome Virus (PRRSV) is a highly prevalent virus that impairs the immune response. It has been detected together with HEV in several studies but no evidence of a causal relationship has been shown to date [30–32]. Since chronic cases in humans are generally linked to immunosuppressive conditions [33–36], PRRSV might be suspected as a frequent co-factor affecting the features of HEV infection in pigs.

The impact of a PRRSV infection on HEV infection dynamics (in terms of viral shedding duration and quantity, transmission and humoral immune response) has therefore been studied through a transmission experiment involving HEV/PRRSV co-infection of specific-pathogen-free (SPF) pigs compared to an infection trial with HEV only that was previously led in our facilities, under the same conditions [25].

Materials and methods

HEV-only infection experiment

A transmission trial with HEV only has been carried out before the co-infection experiment [25]. The experiment was conducted in Anses air-filtered level-3 biosecurity facilities. Briefly, sixty-eight SPF Large-White piglets were used for the experiment. Eight pigs were kept as negative controls and the others were allocated to six rooms containing two pens per room. Rooms 1 to 3 were used to evaluate direct and environmental transmission, whereas Rooms 4 to 6 were used to examine between-pen transmission. The inoculated pigs received orally 10^8 ge (genome equivalent) under a volume of 10 mL of a genotype 3 HEV suspension (strain FR-SHEV3e, Genbank access number JQ953665). Individual fecal samples were collected four days before inoculation and three times per week from 0 to 39 days post-infection (dpi) when the pigs were killed for necropsy. Blood samples were collected twice a week during the same period and clinical signs and rectal temperature were monitored on a daily basis.

HEV/PPRSV co-infection experiment

Animal housing conditions and inoculation

The experiment was conducted in the same Anses air-filtered level-3 biosecurity facilities. Twenty five-week-old SPF Large-White piglets were included in the study; they were HEV and PRRSV free and they did not have any maternal antibodies against these two viruses. Pigs were housed in metallic flat decks with a punched floor for feces and urine evacuation. As in the field situation, fecal material could accumulate in the corners and was not removed during the trial. Three rooms were used: two negative control pigs were housed in Room 1 whereas the 18 remaining piglets were randomly allocated to 3 independent pens distributed in Room 2 and Room 3 (6 piglets per pen) stratifying on gender (3 males and 3 females per pen), weight and the litter they came from. Room 2 contained 2 pens separated by a solid partition to prevent contamination of a pen by the other one (Figure 1). The average weights at weaning (sd) were 9.5 kg (2.7), 9.3 kg (1.6), 9.3 kg (2.3) and 9.3 kg (1.4) for Controls and groups #1, #2 and #3 respectively. In each pen, 3 piglets were inoculated with both HEV and PRRSV at day 0. For inoculation, piglets to be inoculated were grouped in a pen and they were put in contact with their corresponding pen-mates at day 1. The 3 inoculated piglets received the following: (i) orally 10^8 ge under a volume of 10 mL of a genotype 3 HEV suspension (strain FR-SHEV3e, Genbank access number JQ953665) prepared according to the protocol previously described in Andraud et al. [25] (ii) and by nasal route 2.5 mL per nostril of a PRRSV suspension (strain PRRS-2005-29-24-1 "Finistere", genotype 1, subtype 1) titrating 10^5 $TCID_{50}$/mL. The experiment was performed in accordance with EU and French regulations on animal welfare in experiments. The protocol was approved by the Anses/ENVA/UPEC ethical committee (agreement #16 with the National committee for Ethics in animal experimentation).

Data collection

Individual fecal samples were collected three days before inoculation and three times a week until the end of the experiment (49 dpi). Blood samples were collected before inoculation and once a week until the end of the experiment. Clinical examination was also performed (clinical signs, rectal temperature, feces consistence, weight, food consumption and trough cleanliness were recorded daily). Euthanasia was carried out by intravenous injection of 1 g/50 kg live weight of Nesdonal® (thiopental-sodium, Merial, Lyon, France) followed by exsanguination. Necropsy was performed and liver samples were taken.

Because HEV is a zoonotic agent, strict biosecurity measures were applied to prevent any transmission from pigs to animal technicians.

Virology and serology analyses

HEV RNA quantification in fecal and liver samples was performed, after manual total RNA extraction, using real-time quantitative RT-PCR as described in Barnaud et al. [37] and Andraud et al. [25]. The results were expressed in terms of Cycle threshold (Ct). Standard quantification curves were produced by plotting the Ct values against the logarithm of the input copy numbers of standard RNA. Standard RNA was obtained after in vitro transcription of a plasmid pCDNA 3.1 ORF 2–3 HEV, as described in Barnaud et al. [37]. The results are expressed in genomic copy number per gram of feces (ge/g).

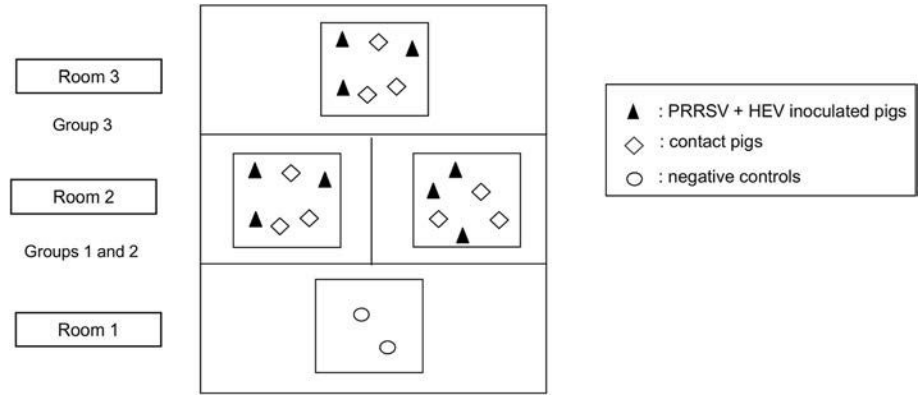

Figure 1 Experimental design of the co-infection experiment. Inoculated and susceptible contact animals are represented by black triangles and white diamonds, respectively. Rooms 2 and 3 contained three pens housing three HEV/PRRSV co-inoculated (black triangles) and three susceptible contact pigs (white diamonds). One negative control group was housed in Room 1.

The detection of anti-HEV antibodies was performed using the HEV ELISA 4.0v kit (MP Diagnostics, Illkirch, France) according to the manufacturer's instructions, except the serum quantity used (10 μL instead of 20 μL). This sandwich ELISA allows the detection of all antibody classes (IgG, IgM and IgA) and uses a recombinant antigen that is present in all HEV strains. Samples were positive when the optical density at 450 nm wavelength obtained for the sample was higher than the threshold defined as the mean for negative controls + 0.3.

PRRSV RNA detection in sera was performed using a real-time RT-PCR as described in Charpin et al. [38]. Briefly, RNA extraction was performed using the NucleoSpin® 8 virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA detection was then performed using the mix GoTaq 1-Step RT-qPCR System (Promega) supplemented with probes and specific primers of the target gene (ORF7 pan-PRRSV) and of the internal reference gene (swine Beta-Actin). The RT-PCR was performed on a Bio-Rad Chromo4 real-time PCR detection system (Bio-Rad) according to the following program: 50 °C for 30 min, 94 °C for 2 min followed by 45 cycles of 94 °C for 15 s and 60 °C for 30 s. The results are expressed in Ct.

Models

Estimation of durations related to HEV infection dynamics

The latent and infectious period durations and the duration of the period required to produce anti-HEV antibodies were estimated using survival data analyses. For each inoculated animal, the latent period was determined as the time elapsed between the inoculation day and the date of the first positive fecal sample for HEV RNA. The latent period after inoculation was fitted to a gamma distribution, from which the shape and scale parameters were estimated by the maximum log-likelihood method. A nonparametric bootstrap procedure was used to determine the 95% confidence interval of the parameter estimates.

A parametric model for survival data was built to estimate the duration of the infectious period, using the RT-PCR performed on livers after euthanasia as the last observation date. Two parametric models were tested (log-normal and Weibull distributions of survival times) and compared using the Akaike Information Criterion (AIC).

The impact of PRRSV co-infection on the time to HEV seroconversion was also studied with a parametric survival model applied to the data from the co-infection trial and the only HEV infection experiment [25]. The link between the earliness of the HEV antibody response and the duration of the infectious period was studied with a Cox model. The immune response was considered as absent or late if the delay before seroconversion was longer than 25 dpi, and as early if it was shorter than 25 dpi [39].

All analyses were performed using the R software (survreg and coxph functions) [40].

Quantification of HEV shedding, environmental accumulation and transmission

The distributions of HEV shed viral loads with time (with and without co-infection) are represented with box plot series. A linear mixed model (proc Mixed, SAS 9.3, [41]) which took into account repeated measurements with time was built to study the difference in the quantity of HEV shed particles between co-infected and non co-infected pigs.

The environmental load corresponds to the accumulation of viral particles in the environment through fecal shedding by infected animals, which is partially compensated by the clearance rate hereafter denoted δ. The clearance rate takes into account feces elimination

through the metallic flat deck and HEV intrinsic mortality in the environment. As described in Andraud et al. [25], for each pen (k) and every sampling time (t_i), the average quantity of genome equivalent shed in the environment per gram of feces was calculated with:

$$V_k(t_i) = \sum_j V_k^j(t_i)/N_k$$

where $V_k^j(t_i)$ represents the quantity of virus shed per gram of feces in pen k by pig j at time t_i and N_k the total number of animals in pen k. Thus the cumulated viral load in the environment of pen k between two sampling times t_i and t_{i+1} is given by the equation:

$$\begin{aligned} E_{ki} &= E_k(t_{i+1}) \\ &= \left(E_k(t_i) + \int_0^{\Delta t} V_k(t_i+u)e^{\delta u}du\right)e^{-\delta\Delta t}, \text{ with} \Delta t \\ &= t_{i+1} - t_i. \end{aligned}$$

Two HEV transmission routes were investigated in this study: (i) transmission due to direct contact between infected and naïve pigs; (ii) indirect transmission via an environmental reservoir of the virus in the pen. A Bayesian model similar to the one described in Andraud et al. [25] was used. Briefly, on each sampling interval $D_i = [t_i,\ t_{i+1}]$ of duration d_i, the probability for a susceptible pig j housed in pen k to escape infection is given by:

$$p_i^{(k)} = exp\left(-d_i\left(\beta_w \pi_i^{(k)} + \beta_E^{(w)}\frac{E_{ki}^{(w)}}{N}\right)\right),$$

where $\pi_i^{(k)}$ represents the proportion of shedding pigs in the time interval D_i located in pen k, $E_{ki}^{(w)}$ is the environmental pool of viral particles in time interval D_i in the pen, β_w is the within-pen transmission rate by direct contact and $\beta_E^{(w)}$ is the within-pen environmental transmission rate. For each pig j, the time interval in which the infection occurred was determined by estimating the latent period λ_j. Let $D_{I_j} = \left[t_{I_j},\ t_{I_j+1}\right]$ denote the time interval during which the first positive fecal sample was detected in pig j. The contribution of contact animal j in pen k to the likelihood model, i.e. the probability for its first positive fecal sample to stand in the interval $D_{I_j} = \left[t_{I_j},\ t_{I_j+1}\right]$ is:

$$L^{(j)}\left(D_I, \pi_w, E|\beta_w, \beta_E^{(w)}, \lambda, \delta\right) = \left\{\prod_{i=1}^{I_j} p_{i-1}^{(k)}\left(1 - p_{I_j}^k\right)\right\} \times f_{Lat}(\lambda_j,\ \alpha, s),$$

The probability of infection (given by the first term of the equation aforementioned) is weighted by the probability that the estimated latent period λ_j is consistent with the data observed in inoculated animals. f_{Lat} represents the prior distribution of the latent period based on the estimation of the latent period in inoculated animals. The global likelihood is given by:

$$L\left(D_I, \pi_w, E|\beta_w, \beta_E^{(w)}, \lambda, \delta\right) = \prod_{j=1}^{N_c} L^{(j)}\left(D_I, \pi_w, E|\beta_w, \beta_E^{(w)}, \lambda, \delta\right),$$

where N_c is the total number of contact pigs.

The direct and indirect transmission rates β_w and $\beta_E^{(w)}$ respectively, the latent period λ_j for each contact animal and the HEV clearance rate were estimated by Bayesian inference using Monte Carlov Markov Chain. An informative prior distribution based on Andraud et al. [25] was used for the environmental clearance rate δ, which was assumed to be normally distributed with mean 0.3 and standard deviation 0.075. The prior distributions of transmission parameters were based on the results obtained by Andraud et al. [25]; they were constructed such that the expected value is equal to the posterior mean and 33% of the prior mass covers the 95% confidence interval for parameters derived from data obtained by Andraud et al. [25,42] (normal distribution (−2,3) and (−13.5,5) for β_w and $\beta_E^{(w)}$ respectively). The prior distribution of the latent period in contact pigs was based on the distribution of the latent period in inoculated pigs (gamma distribution $\Gamma(26,2)$).

Parameter updating was performed sequentially by the Metropolis-Hastings algorithm. Three chains were run with random initial conditions, 110 000 steps per chain, a burnin of 10 000 steps and thinning parameter of 10. Convergence was assessed by visual inspection and diagnostic tests (autocorrelation, Heidelberger, Gelman-Rubin diagnostics).

The whole model was performed using the R software [40].

Results

HEV-only infection experiment

In this trial, the average HEV latent period in inoculated animals lasted 6.9 days (5.8; 7.9) and average infectious period lasted 9.7 days (8.2; 11.2) (Table 1) [25]. Direct transmission rate was estimated at 0.15 (0.03; 0.31) pigs per day and indirect transmission rate was estimated at $2{\cdot}10^{-6}$ g/ge/day ($1{\cdot}10^{-7}$; $7{\cdot}10^{-6}$) (Table 1) [25]. HEV serology results on individual blood samples for HEV-only infected pigs are presented in Additional file 1 [25].

HEV shedding and seroconversion in the context of HEV/PRRSV co-infection

HEV infection data are presented in Figures 2 and 3 for quantitative RT-PCR on fecal samples and serological results respectively. In our trial, all inoculated animals were infected by HEV. None of the 2 negative-control pigs excreted HEV from day 3 to day 49. Inoculated and contact animals started to shed HEV between 9 and 18 dpi and between 25 and 32 dpi respectively. All exposed individuals shed HEV until the end of the trial (49 dpi) (Figure 2). At

Table 1 Summary of the infectious dynamics parameters and comparison with data from the HEV-only infection experiment [25]

	HEV + PRRSV	HEV alone [25]
Latent period (days)	13.4	7.1
	(8.6; 17.1)	(3.2; 12.3)
Infectious period (days)	48.6	9.7
	(27.9; 84.6)	(8.2; 11.2)
Seroconversion period (days)	43.1	26.3
	(35.7; 52.2)	(23.5; 29.5)
Direct transmission ($days^{-1}$) β_w	0.70	0.15
	($1.2{\cdot}10^{-3}$; 3.67)	(0.03; 0.31)
Indirect transmission (g/ge/d) β_E^w	$6.6{\cdot}10^{-6}$	$2.0{\cdot}10^{-6}$
	($1.4{\cdot}10^{-10}$; $1.3{\cdot}10^{-4}$)	($1.1{\cdot}10^{-7}$; $7.0{\cdot}10^{-6}$)

β_w is the direct transmission rate, defined as the mean number of newly infected pigs generated by a single infectious individual in a fully susceptible population per day. β_E^w represents the within-pen transmission rates related to the environmental component, defined as the mean number of newly infected pigs per HEV genome equivalent per gram of feces in the environment (see text for more details). Numbers in brackets are the upper and lower limits of the 95% credibility interval.

the necropsy stage, 14 livers out of 18 were positive, the 4 negative livers being from contact pigs (Figure 2).

The detection of anti-HEV antibodies was performed on all groups of animals until 49 dpi (Figure 3). None of the negative controls showed anti-HEV antibody response. Only 4 inoculated animals out of 9 produced anti-HEV antibodies between 35 and 49 dpi, 3 in group 2 and one in group 3; none of the inoculated animals from group 1 seroconverted. Seven contact individuals out of 9 seroconverted between 42 and 49 dpi, two from groups 1 and 2 and all three contact animals from group 3 (Figure 3).

PRRSV infection and seroconversion in the context of HEV/PRRSV co-infection

All animals inoculated with PRRSV were viremic from the first sampling time (7 dpi). The viremia of contact animals started between 7 and 42 dpi. One contact individual did not show any detectable PRRSV viremia during the experiment (Figure 4). Finally, all animals except 2 contact individuals were viremic for PRRSV before HEV shedding was detected.

Regarding clinical data (data not shown), inoculated and contact animals showed hyperthermia (rectal temperature >40 °C) between 1 and 14 dpi and 14 and 28 dpi, respectively. Co-infected pigs necropsied at 49 dpi did not show any macroscopic lesion possibly linked to hepatitis.

Quantification of HEV infection dynamics parameters in the context of HEV/PRRSV co-infection

Convergence of MCMC was assessed through visual inspection and conventional diagnostic tests. Heidelberger and Geweke diagnostics failed to reject the convergence hypothesis, which was also supported by the Gelman-Rubin test based on three independent chains with a potential scale reduction factor (PSRF) close to 1.0 (≤1.02) (Additional file 2).

HEV latent and infectious periods

The duration of the latent period in pigs inoculated with HEV and PRRSV was fitted to a gamma distribution with shape parameter $\alpha = 25.7$ (11.6; 180.4) and scale parameter $s = 0.5$ (0.08; 1.1) leading to an estimated mean duration of the latent period of 12.9 days (12.8; 14.4). In contact animals, individual distributions of latent periods (Additional

	dpi	2	4	7	9	11	14	16	18	21	23	25	28	32	35	39	42	44	46	49	Necropsy (liver)
	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Group 1	Inoculated	0	0	0	$1.08.10^5$	0*	$2.23.10^4$	$2.23.10^5$	$1.24.10^6$	$2.62.10^6$	$1.38.10^7$	$1.35.10^7$	$5.96.10^6$	$8.27.10^6$	$7.51.10^6$	$2.95.10^7$	$4.43.10^7$	$8.02.10^6$	$8.76.10^6$	$1.49.10^7$	$1.46.10^6$
	Inoculated	0	0	0	0	0	$2.23.10^4$	$1.72.10^5$	$1.91.10^6$	$6.80.10^6$	$2.24.10^7$	$3.56.10^7$	$7.66.10^7$	$1.32.10^8$	$9.91.10^7$	$6.76.10^7$	$1.51.10^7$	$1.09.10^8$	$4.01.10^7$	$5.85.10^7$	$1.87.10^6$
	Inoculated	0	0	0	0	0	0	0	$1.50.10^5$	$1.53.10^6$	$5.85.10^6$	$1.14.10^7$	$1.30.10^7$	$4.06.10^7$	$3.99.10^7$	$9.27.10^7$	$4.28.10^7$	$5.02.10^6$	$7.20.10^7$	$6.40.10^7$	$1.02.10^6$
	Contact	0	0	0	0	0	0	0	0	0	0	$2.67.10^5$	$1.16.10^6$	$1.79.10^7$	$1.54.10^7$	$3.75.10^7$	$1.92.10^7$	$1.42.10^7$	$5.31.10^7$	$4.87.10^7$	$8.72.10^5$
	Contact	0	0	0	0	0	0	0	0	0	0	0	$2.91.10^5$	$3.68.10^6$	$3.51.10^6$	$6.24.10^5$	$1.53.10^5$	$4.91.10^5$	$1.17.10^6$	$3.49.10^5$	0
	Contact	0	0	0	0	0	0	0	0	0	0	$1.78.10^2$	$8.84.10^5$	$1.43.10^6$	$3.04.10^6$	$5.43.10^5$	$1.20.10^6$	$4.29.10^6$	$1.91.10^6$	$1.20.10^6$	0
Group 2	Inoculated	0	0	0	0	0	0	$1.56.10^5$	$1.09.10^6$	$1.57.10^6$	$2.81.10^6$	$1.67.10^7$	$2.69.10^7$	$8.71.10^7$	$6.33.10^7$	$4.44.10^6$	$3.92.10^6$	$1.64.10^7$	$9.92.10^5$	$1.45.10^5$	$1.56.10^3$
	Inoculated	0	0	0	0	0	0	$2.19.10^5$	$5.44.10^5$	$6.85.10^6$	$1.86.10^7$	$1.51.10^7$	$2.31.10^7$	$1.55.10^7$	$1.79.10^7$	$2.57.10^7$	$7.88.10^6$	$1.37.10^8$	$1.97.10^7$	$6.80.10^6$	$2.42.10^6$
	Inoculated	0	0	0	0	0	$1.03.10^5$	$6.97.10^5$	$5.05.10^8$	$3.85.10^7$	$4.20.10^7$	$3.96.10^7$	$6.72.10^7$	$1.25.10^8$	$1.39.10^8$	$5.93.10^7$	$8.10.10^7$	$1.32.10^8$	$1.13.10^7$	$4.86.10^6$	$3.59.10^5$
	Contact	0	0	0	0	0	0	0	0	0	0	$1.65.10^6$	0*	$1.45.10^6$	$4.12.10^6$	$4.94.10^6$	abs	$1.18.10^7$	$2.51.10^7$	$5.55.10^7$	$1.12.10^6$
	Contact	0	0	0	0	0	0	0	0	0	0	0	0	$1.82.10^6$	$5.38.10^6$	$8.40.10^6$	$4.43.10^6$	$8.37.10^7$	$1.43.10^7$	$5.24.10^6$	$1.69.10^6$
	Contact	0	0	0	0	0	0	0	0	0	0	0	0	$1.47.10^6$	$7.26.10^6$	$1.97.10^7$	$3.03.10^7$	$4.37.10^7$	$2.49.10^8$	$3.07.10^8$	$3.44.10^6$
Group 3	Inoculated	0	0	0	0	0	0	$2.37.10^5$	$3.12.10^5$	$3.88.10^6$	$6.75.10^6$	$7.98.10^6$	$1.05.10^7$	$4.21.10^7$	$2.64.10^7$	$4.26.10^7$	$1.51.10^7$	$2.90.10^7$	$1.60.10^7$	$2.08.10^8$	$3.24.10^8$
	Inoculated	0	0	0	0	0	0	0	$6.06.10^5$	$9.54.10^5$	$4.08.10^6$	$3.22.10^6$	$1.55.10^7$	$3.02.10^7$	$1.76.10^7$	$1.80.10^7$	$1.88.10^7$	$8.52.10^7$	$1.85.10^7$	$5.75.10^7$	$2.04.10^4$
	Inoculated	0	0	0	0	0	$8.04.10^4$	$4.35.10^5$	$2.26.10^6$	$1.14.10^7$	$2.25.10^7$	$1.73.10^7$	$2.37.10^7$	$3.66.10^7$	$3.17.10^7$	$1.04.10^7$	$4.39.10^6$	$4.09.10^7$	$1.43.10^7$	$5.61.10^7$	$9.63.10^5$
	Contact	0	0	0	0	0	0	0	0	0	0	0	0	$8.80.10^4$	$2.04.10^5$	$2.34.10^6$	$3.40.10^5$	$4.04.10^5$	$5.02.10^5$	$7.39.10^5$	0
	Contact	0	0	0	0	0	0	0	0	0	0	0	0	$8.26.10^6$	$1.50.10^7$	$8.47.10^7$	0*	$1.45.10^7$	$8.67.10^7$	$1.03.10^8$	$5.87.10^5$
	Contact	0	0	0	0	0	0	0	0	0	0	0	0	$1.31.10^6$	$8.42.10^5$	$1.12.10^6$	0*	$4.41.10^4$	$7.78.10^5$	$8.29.10^5$	0

Figure 2 HEV RNA quantification in fecal and liver samples from HEV/PRRSV co-infected animals and contact pigs. Quantitative HEV RT-PCR results on individual fecal samples (HEV copies/g of feces) at each sampling time and from liver samples at necropsy. Shaded zones correspond to periods in which infected individuals were considered infectious, corresponding to the time between the first and last HEV positive fecal samples for each animal. dpi: day post infection; *tested in duplicate; abs: missing.

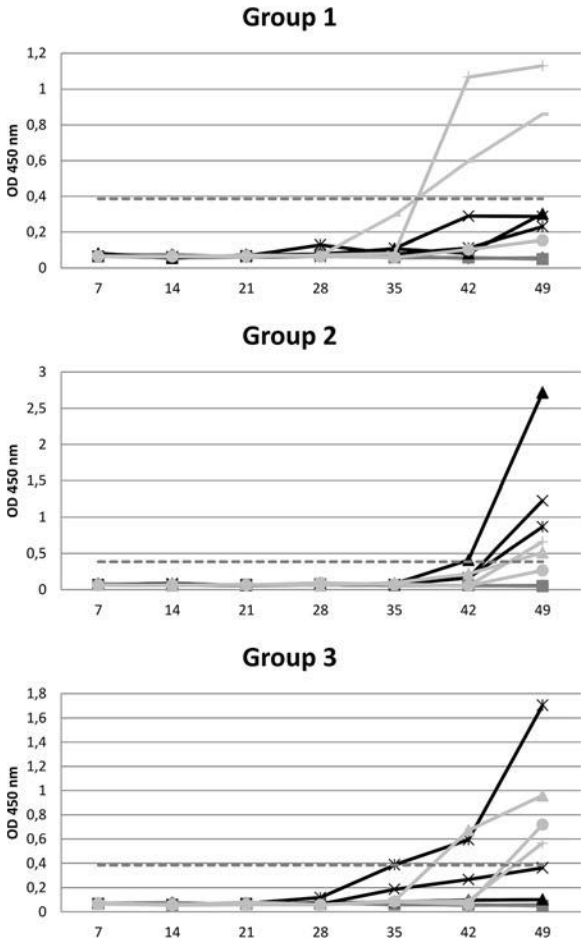

Figure 3 HEV serology results on individual sera samples from HEV/PRRSV co-infected animals and contact pigs. Optical density (450 nm) values of ELISA test HEV 0.4v per animal at different days post infection. For each group, inoculated animals are indicated in black ($n = 3$), contact pigs in light grey ($n = 3$) and negative control in dark grey ($n = 2$). The cut off value is indicated by a dashed grey line.

file 2) were merged to obtain a global distribution of the latent period, leading to a mean latent period duration of 13.4 days (8.6; 17.1) (Table 1).

The duration of the infectious period was fitted to a log-normal distribution, leading to an estimated mean duration of the infectious period of 48.6 days (27.9; 84.6) (Table 1).

Estimation of time to HEV seroconversion

Time-to HEV seroconversion was fitted to log-normal distribution, with means 43.1 days (35.7; 52.2) with PRRSV co-infection and 26.3 days (23.5; 29.5) with only HEV infection (Table 1). The duration of the infectious period was significantly associated with the earliness of the humoral immune response. An absent or late immune response was related to a lengthening of the infectious period duration showed by a delay in time-to end of shedding (Hazard Ratio HR = 0.35 (0.19; 0.64)) (Figure 5).

HEV shedding and accumulation in the environment

The distribution of the HEV shed viral load with time (with and without co-infection) is shown in Figure 6. PRRSV infection was found to be significantly associated with the increase of the quantity of HEV particles shed by inoculated animals ($P = 0.05$) from the linear mixed model accounting for repeated measurements. The interaction between time and PRRSV infection was also significant and positive, i.e. the impact of the PRRSV infection increased with time ($P = 0.04$). However, the effect of the PRRSV infection was not found to be statistically significant in contact animals ($P > 0.05$).

The viral load accumulated in the environment was modeled for each experimental pen (Figure 7). The environment was HEV-free until 15 to 20 dpi; then the environmental load increased and reached $1.0.10^8$ to $1.5{\cdot}10^8$ ge/g of feces until the end of the trial.

HEV transmission parameters

The results show that, in experimental conditions, one infectious pig was able to infect 0.70 pig per day by direct contact ($\beta_w = 0.70$ ($1.18{\cdot}10^{-3}$; 3.67)) (Table 1). The indirect transmission rate can be considered as the average number of animals that can be infected by a single genome equivalent present in the pen environment ($\beta_E^{(w)} = 6.59{\cdot}10^{-6}$ g/ge/day ($1.43{\cdot}10^{-10}$; $1.27{\cdot}10^{-4}$)). In other words, the inverse of $\beta_E^{(w)}$ corresponds to the average number of viral copy number of genome per gram of feces in the environmental pool required to infect one animal in one day, i.e. $1.51{\cdot}10^5$ ge/g/day ($7.86{\cdot}10^3$; $7.00{\cdot}10^9$) (Table 1).

Discussion

Several studies suggested a possible link between HEV and PRRSV infections [30–32]. Our study was aimed at evaluating the impact of PRRSV infection on hepatitis E dynamics of infection through an experimental HEV/PRRSV co-infection trial. As shown in Table 1, the comparison of the results with those derived from a previous infection trial with HEV alone [25] evidenced a modification of hepatitis E infection dynamics in the presence of PRRSV. Although the two trials were not carried out simultaneously, they were conducted under the same experimental conditions making the comparison of the results fully relevant (same experimental facilities, same handlers, pigs from the same SPF herd and genetically similar, same age of the animals, same sex ratio, same HEV strain, same dose, same inoculation protocol and same contact structure).

HEV shedding was delayed in case of PRRSV co-infection, with a latent period estimated to 13.4 days, against 7.1 days with HEV alone [25], i.e. an increase by a factor of 1.9. In the Bouwknegt et al. trial, the latent period was estimated at only 3 days in intravenously inoculated animals [20], confirming that the route of inoculation

	dpi	7	14	21	28	35	42	49
	Control	nt	nt	N/A	nt	N/A	N/A	nt
	Control	nt	nt	nt	N/A	N/A	N/A	N/A
Group 1	Inoculated	28.94	26.39	35.52	33.43	28.08	N/A	N/A
	Inoculated	30.74	29.27	33.43	39.25	N/A	N/A	N/A
	Inoculated	32.30	29.88	33.30	38.68	35.92	N/A	N/A
	Contact	31.76	29.16	29.64	28.96	37.73	N/A	N/A
	Contact	N/A	30.19	35.32	29.44	N/A	N/A	N/A
	Contact	N/A	N/A	N/A	N/A	N/A	31.39	22.26
Group 2	Inoculated	28.26	28.19	31.27	27.42	N/A	N/A	N/A
	Inoculated	28.36	30.33	33.20	26.4	29.4	N/A	N/A
	Inoculated	28.69	30.37	35.00	26.61	N/A	N/A	N/A
	Contact	35.11	28.68	31.97	24.27	31.2	26.03	N/A
	Contact	N/A	N/A	36.69	25.4	44.63	N/A	N/A
	Contact	36.95	N/A	33.13	24.37	29.28	N/A	N/A
Group 3	Inoculated	29.12	26.03	32.21	30.89	N/A	N/A	N/A
	Inoculated	30.49	26.82	32.39	27.37	N/A	36.54	N/A
	Inoculated	29.04	38.31	43.52	N/A	N/A	N/A	N/A
	Contact	N/A	32.15	29.99	25.74	N/A	28.43	N/A
	Contact	N/A	34.22	33.05	36.73	27.66	N/A	N/A
	Contact	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Figure 4 PRRSV RT-PCR results on individual blood samples. Shaded zones correspond to periods in which individuals were considered viremic. The results are expressed in terms of Ct. dpi: day post infection; nt: not tested; N/A: not amplified; Ct: cycle threshold.

modifies viral fate. The infectious period was longer with PRRSV co-infection: 48.6 days, against 9.7 days with HEV alone, i.e. an increase by a factor of 5 ($p < 0.01$). These results were therefore closer to estimates obtained from field data (27 days (20; 39)) than experimental results obtained with HEV only [26]). In the trial described by Bouwknegt et al., the infectious period was estimated between 13 and 49 days according to replications, showing a high inter-individual variability [20]. Moreover, the origin of the animals included in this study and their status regarding PRRSV were not mentioned.

HEV shedding in inoculated individuals was also significantly increased with PRRSV/HEV co-infection. However, the effect of PRRSV infection on the quantity of shed viral particles was not significant in contact animals. This could be explained by the low number of animals included – especially since one contact animals was lately infected by PRRSV and another did not show any PRRSV viremia during the experiment – and by a large inter-individual variability in contact animals. As a consequence of the longer shedding period and the higher quantity of viral particles shed in feces of co-infected animals, the viral load accumulated in the environment was higher with PRRSV co-infection with more than 10^8 HEV ge/g of feces estimated in the environment, which causes a higher and longer infection pressure on susceptible animals. The direct transmission rate when animals were co-infected was increased by a factor of 4.7 (0.70 versus 0.15 per day with HEV infection only [25]). Thus the direct transmission route played a more important role in HEV transmission when animals

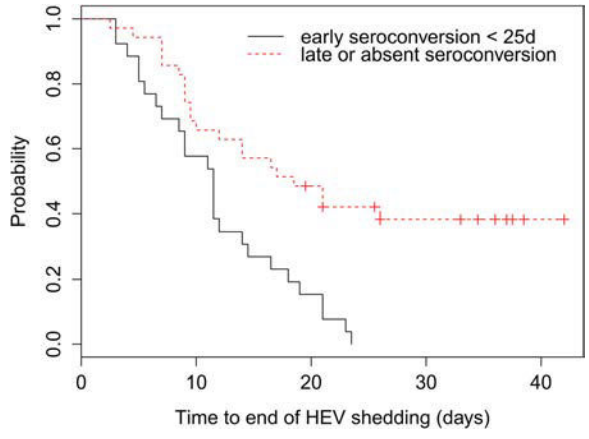

Figure 5 Survival curves of time-to end of HEV shedding according to early or late HEV seroconversion. The black and red survival curves correspond to the duration of the infectious period in pigs having an early seroconversion (less than 25 dpi) or a late or absent seroconversion (more than 25 dpi) respectively.

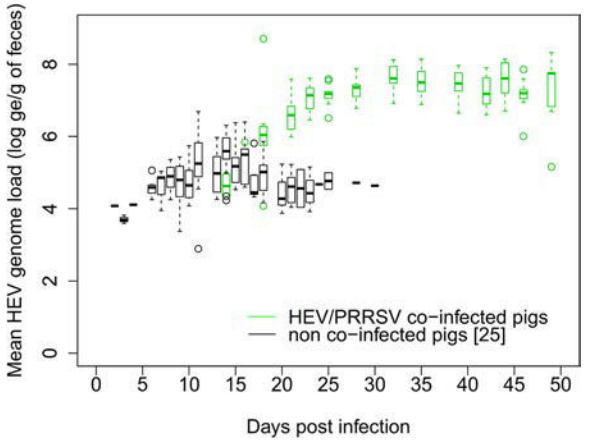

Figure 6 Distribution of the number of HEV genome equivalent (log ge/g feces) shed by individual pigs with time in inoculated animals with or without PRRSV co-infection. Co-infected animals are indicated in green ($n = 9$), only-HEV infected animals [25] are in black ($n = 18$).

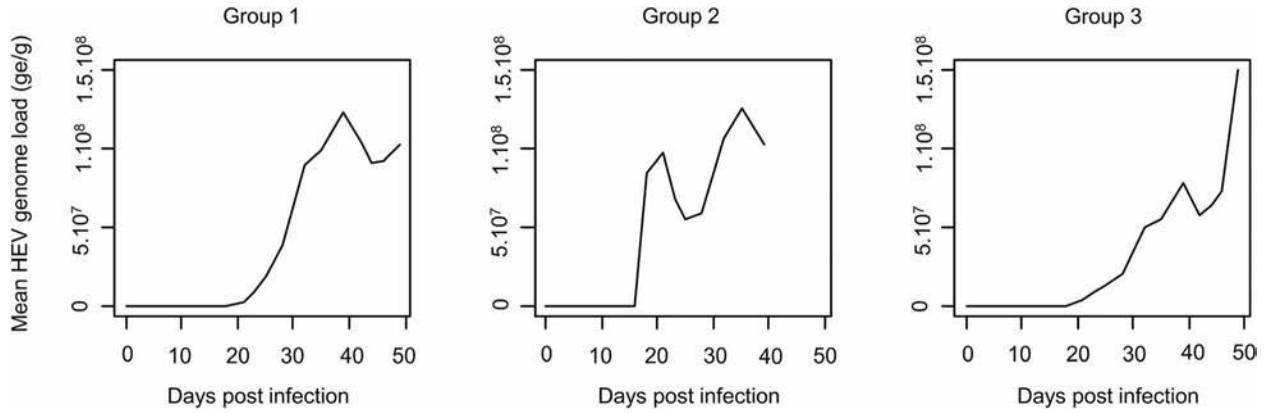

Figure 7 Estimation of HEV environmental accumulation with PRRSV/HEV co-infection. Evolution of the estimated HEV genome load (ge/g) in the environment of each pig group of the PRRSV/HEV co-infection experiment.

were co-infected which was consistent with the larger amount of HEV particles shed individually than in HEV infected pigs only. The indirect transmission rate was 3.3 times higher with co-infection ($6.6{\cdot}10^{-6}$ and $2.0{\cdot}10^{-6}$ g/ge/day respectively [25]). Otherwise stated, 3.3 times less viral particles were required to infect a co-infected animal ($1.5{\cdot}10^{5}$ versus $5.0{\cdot}10^{5}$ ge/g for HEV only infected piglets [25]). Because inoculated and contact animals (except two contact pigs) were infected by PRRSV before HEV shedding, these data suggest a higher HEV susceptibility in PRRSV co-infected pigs. In a model built from an experimental HEV infection by intravenous route, Bouwknegt et al. showed that the HEV oral dose for which the infection probability was equal to 50% would be $1.4{\cdot}10^{6}$ ge/g [22], which was 10 times more than the dose required to infect a PRRSV co-infected pig in our study. These data are consistent with the hypothesis of a higher HEV infection susceptibility in PRRSV co-infected pigs.

The time-to HEV seroconversion was 1.6 times longer in PRRSV co-infected pigs than in HEV only infected pigs (43.1 and 26.3 days respectively [25]). This impaired immune response was significantly associated with a lengthening of the infectious period duration and could thus explain the presence of viral particles in livers when pigs were euthanized more than 49 days post infection for the inoculated ones. However, this study did not aim at investigating the mechanisms leading to a possible immune failure linked to PRRSV infection and the mechanisms causing a chronic HEV infection. In humans, immunopathogenic mechanisms leading to chronic hepatitis E are poorly known. The role of cellular immunity in chronic hepatitis E control has been shown [3,35,36]. A study was led on patients suffering from HIV and chronically infected with HEV [34]. One of them had a low anti-HEV lymphocyte T CD4+ rate, a persistent viremia (longer than 24 months) and a delayed anti-HEV seroconversion. Thus, though immune mechanisms still need to be clarified, literature data suggest that an impaired innate and adaptive immune response could lead to chronic HEV infection in humans. In pigs, the immunopathogenic mechanisms linked to PRRSV infection are not fully understood yet, but PRRSV infection clearly results in a late adaptive immune response [43,44]. Thus the delayed anti-HEV seroconversion and the lengthening of the infectious period duration that we observed in PRRSV co-infected pigs seem consistent with the immunopathogenic mechanisms of chronic hepatitis E that have been described in humans (impaired cellular and humoral immune response) and could be explained by a specific orientation of the immune response linked to PRRSV infection. The increase of the duration of the latent period might be explained by the activation of the innate immune response linked to the PRRSV infection, delaying HEV shedding but this would require further work to assess the underlying mechanisms.

To our knowledge, this work is the first study focusing on the impact of HEV/PRRSV co-infection on hepatitis E epidemiology in pigs. These results show that PRRSV has a major impact on HEV infection dynamics and that HEV/PRRSV co-infection could lead to extended HEV shedding and maybe chronic infection. This chronicity may dramatically increase the risk of pig livers containing HEV at slaughter age. Immunopathogenic mechanisms leading to a chronic HEV infection have to be further investigated. This study shows an important interaction between an animal health concern - PRRSV, which dramatically affects the competitiveness of pig farms, and a zoonotic pathogen - HEV, which has a major impact in human health. These data emphasize the necessity to manage human and animal health globally and the importance of PRRSV eradication programs, which could be a major lever in the control of hepatitis E.

Additional files

Additional file 1: HEV serology results on individual sera samples for only-HEV infected pigs [25]. Optical density (450 nm) values of ELISA test HEV 0.4v per animal at different day post infection. Shaded zones correspond to the period in which individuals were considered HEV seropositive. dpi: days post infection, abs: missing.

Additional file 2: Estimation of transmission parameters by Bayesian inference (MCMC estimation, 3 chains, 110 000 iterations, 10 000 burnin iterations, thinning interval = 10). β_w is the direct transmission rate, defined as the mean number of newly infected pigs generated by a single infectious individual in a fully susceptible population per day. β_E^w represents the within-pen transmission rates related to the environmental component, defined as the mean number of newly infected pigs per viral particle per gram of feces in the environment. δ is the HEV clearance rate, taking into account feces elimination through the metallic flat deck and HEV destruction in the environment. λ_1 to λ_9 are latent periods for contact animals (see text for more details).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MS analyzed the data, participated to the sample analyses and drafted the manuscript, EB analyzed the HEV samples and interpreted the results, MA developed the mathematical model and participated to the data analyses, FE participated in the animal experiment, monitored and pre-treated the samples and participated to the analysis of PRRSV samples, PR and OB analyzed and interpreted the results from PRSSV samples, NP supervised the HEV-related laboratory work and participated in the coordination of the study, NR conceived, coordinated the study and participated in the animal experiment and the data analyses. All co-authors revised the manuscript and approved the final submitted version.

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Short communication

Persistent viremia and presence of hepatitis E virus RNA in pig muscle meat after experimental co-infection with porcine reproductive and respiratory syndrome virus

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ABSTRACT

Although hepatitis E virus (HEV) transmission has been demonstrated after consumption of products containing infected pig liver, human cases can be also associated with other pig meat products, such as sausages. Data on HEV viremia and dissemination in muscle meat of infected animals are still sparse, especially during long-term infection. Previously, we have shown that experimental co-infection of pigs with HEV and porcine reproductive and respiratory syndrome virus (PRRSV) lengthens HEV infection up to 49 days and increases the likelihood of the presence of HEV RNA in the liver of the pig at a later stage of infection. In the present study, we show that during experimental HEV-PRRSV co-infection, prolonged HEV viremia, up to 49 days post-inoculation (dpi), is detected. The long-term viremia observed was statistically associated with the absence of HEV seroconversion. HEV RNA was also frequently detected, at a late stage of infection (49 dpi), in the three different types of muscle tested: femoral biceps, psoas major or diaphragm pillar. The HEV RNA load could reach up to $1 \cdot 10^6$ genome copies per gram of muscle. Detection of HEV in muscle meat was statistically associated with high HEV loads in corresponding liver and fecal samples. The presence of HEV in pig blood, femoral biceps and major psoas, corresponding to ham and tenderloin muscles respectively, is of concern for the food industry. Hence, these results indicate new potential risks for consumers and public health regarding pork products.

1. Introduction

Hepatitis E virus (HEV) is responsible for acute and occasionally chronic hepatitis in humans after enteric transmission. In developed countries, it is mainly of zoonotic origin, with pigs being the major reservoir (Pavio et al., 2017). Confirmed cases of zoonotic transmission have been associated with the consumption of raw or undercooked food products containing infected pig liver (e.g. pig liver sausages) (Colson et al., 2010; Guillois et al., 2016; Renou et al., 2014). More generally, case-control studies have identified the consumption of pig meat products as a major factor associated with HEV infections. Said and colleagues (2017) demonstrated, using epidemiological data collected from confirmed cases, that consuming ham and/or sausages from a given British supermarket brand was statistically associated with a higher risk of having an HEV infection (Said et al., 2017). Faber et al. (2018) collected exposure data from notified hepatitis E cases in Germany, with individually matched population controls, using a semi-standardized telephone interview. They identified ready-to-eat pork products (e.g. raw ham, frankfurter, spreadable sausages made of raw meat, liver sausage or liver pâté) as major sources for autochthonous hepatitis E (Faber et al., 2018). Data on the prevalence of HEV in pork products, other than in pig livers, are still very sparse and few publications report on the detection of HEV RNA in different categories of pig meat (e.g. sausages) (Berto et al., 2012; Di Bartolo et al., 2012;

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Intharasongkroh et al., 2017; Szabo et al., 2015). One study found up to 20% of raw sausages (salami, without liver added) to be positive for HEV RNA (Szabo et al., 2015).

HEV RNA has been frequently detected in liver, bile or fecal samples from slaughtered pigs, (Salines et al., 2017), but until now, few studies have examined the presence of HEV RNA in other organs or tissues of naturally infected pigs at slaughterhouse time. In one report, HEV RNA was amplified in several organs and tissues, such as the bladder (10/43) or tonsils (3/43) of slaughtered pigs (n = 43) (Leblanc et al., 2010). In this study, none of the loin samples tested were HEV-positive (Leblanc et al., 2010). In a second report, HEV RNA was present along the different stages of the pork production chain, from the carcass dissection to liver removal steps and in pig lingual muscle, with an estimated prevalence of 2.7% (n = 112) (Di Bartolo et al., 2012).

HEV replicates in the liver but HEV RNA can be amplified in other pig organs and tissues after experimental infections (Bouwknegt et al., 2009; Williams et al., 2001). The detection of HEV-negative strands (replication intermediate) suggests that HEV can replicate in extra-hepatic sites, such as the small intestine, lymph nodes, and colon (Williams et al., 2001). In another study, positive HEV RNA hybridization signals were also detected in the liver, small and large intestine, tonsil, spleen, and kidney (Choi and Chae, 2003), supporting the presence of HEV in organs other than liver. In the study described by Bouwknegt et al. (2009), where the course of HEV infection was determined in pigs after intravenous inoculation and contact-infection, HEV RNA was detected in the longissimus, biceps femoris and iliopsoas, of both animal categories (Bouwknegt et al., 2009). The authors could not determine whether this was due to intrinsic and/or extrinsic contamination (i.e. cross-contamination with blood during necropsy).

HEV infection in pigs is usually acute, asymptomatic and self-resolving within 3 weeks (Salines et al., 2017). However, like in humans, where chronic cases are observed in solid organ transplant recipients under immunosuppressive treatment (Kamar et al., 2017), experimental HEV infection of pigs under active immune suppression led to chronic HEV shedding, lasting up to 13 weeks (Cao et al., 2017). In natural rearing conditions, pig immune responses can be modulated by frequent intercurrent infection with immune-modulating porcine viruses (e.g. porcine reproductive and respiratory syndrome virus) (Rahe and Murtaugh, 2017). Long-term HEV infection may influence the within-host course and HEV dissemination in organs. No study has addressed the question of the presence of HEV in pig organs during chronic infection, which is important regarding the risk of HEV presence in pig blood or meat at slaughter time. We have previously shown that co-infection with HEV and porcine reproductive and respiratory syndrome virus (PRRSV) affects the HEV time course. PRRSV co-infection with HEV extended HEV fecal shedding by a factor of 5, and increased the frequency of HEV RNA detection in pig livers at late stages of infection (49 days) (Andraud et al., 2013; Salines et al., 2015). These results suggest that HEV pathogenesis and dissemination could be affected by PRRSV co-infection. Thus, the aim of the present study was to assess the presence of HEV in serum and muscle meat of pigs in the context of PRRSV co-infection, after a natural route of inoculation.

2. Materials and methods

2.1. HEV/PRRSV co-infection experiment

Experimental HEV/PRRSV co-infection of Specific-Pathogen-Free (SPF) pigs was previously described (Salines et al., 2015). Briefly, 18 Large-White piglets were randomly allocated to 3 independent pens (3 inoculated and 3 contact piglets per pen). In each pen, the inoculated piglets received: *(i)* orally 10^8 HEV RNA copies in a volume of 10 mL of a genotype 3 HEV suspension (strain FR-SHEV3e, Genbank accession number JQ953665) prepared according to the protocol described in Andraud et al. (2013); and *(ii)* 2.5 mL per nostril of a PRRSV suspension (strain PRRS-FR-2005-29-24-1 “Finistere”, genotype 1, subtype 1, Genbank accession number KY366411) titrating 10^5 $TCID_{50}$/mL. Two negative control pigs were included in a separate room. The protocol was approved by the Anses/ENVA/UPEC Ethics Committee (Approval No. 16 with the French National Committee for Ethics in animal experimentation). Since HEV is a zoonotic agent, biosecurity measures were applied to prevent any transmission from pigs to animal care handlers.

2.2. Sample collection

Blood samples were collected once a week until the end of the study (49 dpi). For euthanasia, anesthesia was carried out with intravenous injection of 1 g/50 kg live weight of Nesdonal® (thiopental-sodium, Merial, Lyon, France). This anesthesia is highly reproducible and has no impact on the quality of bleeding thereafter. Exsanguination was then performed by cutting deeply with a sharp blade into the carotid artery on both sides, with the anesthetized pigs hung by the legs. The carcasses were processed 20 min after exsanguination to ensure the absence of remaining blood flow. Necropsy was performed and liver and muscles samples (femoral biceps, psoas major and diaphragm pillar) were collected and kept frozen until used. To avoid cross-contamination, each muscle sample was handled using single use sterile materials (gloves, clamps, blades, and tips).

2.3. RNA extraction

RNA extractions from serum, fecal or muscle juice samples were performed manually using the QIAamp Viral RNA extraction Mini kit (QIAGEN, Illkirch, France), according to the manufacturer's instructions, except that sample size was 200 µL. Fecal samples were solubilized in a 10% phosphate buffered saline suspension. Muscle juices were recovered after one cycle of freeze and thaw at −20 °C, from 20 g of each muscle (Feurer et al., 2018). Comparison of HEV recovery rate from muscle juice or from muscle homogenate (Fast-prep 24, MP Biomedicals, Illkirch, France), was performed after spiking with a viral suspension of HEV3 (Genbank accession number EF494700), and showed similar results (data not shown).

2.4. HEV RNA quantification

HEV RNA quantification in serum, liver and muscles samples was performed, after RNA extraction, using real-time quantitative RT-PCR targeting HEV ORF3 (Jothikumar et al., 2006). Standard quantification curves were produced by plotting the quantification cycle (Cq) values against the logarithm of the input copy numbers of a standard RNA. Standard RNA was obtained after in vitro transcription of a plasmid pCDNA 3.1 ORF 2–3 HEV, as previously described (Barnaud et al., 2012). Results were expressed in HEV RNA copy number per gram of feces or muscle or per milliliter of serum (RNA copies/g or RNA copies/mL).

2.5. HEV serology

HEV serology was previously determined, and is presented in Fig. 3 of the publication on HEV/PRRSV co-infection of pigs (Salines et al., 2015). Briefly, anti-HEV antibodies were detected using the HEV ELISA 4.0v kit (MP Diagnostics, Illkirch, France), according to the manufacturer's instructions, except the serum quantity used (10 µL instead of 20 µL). Samples were positive when the optical density at 450 nm wavelength was higher than the threshold defined as the mean for negative controls + 0.3.

2.6. Statistical analysis

Time to viremia onset, viremia duration and period between shedding and viremia were estimated using a parametric survival model.

Table 1
HEV RNA quantification in serum, muscle, feces and liver samples from HEV/PRRSV co-infected animals and contact pigs (n = 20).

		Viremia kinetic (RNA copies/mL serum)							HEV RNA in muscle at 49 dpi (RNA copies/g)			HEV RNA in feces at 49 dpi (RNA copies/g)	HEV RNA in liver at 49 dpi (RNA copies/g)
	dpi	7	14	21	28	35	42	49	FB	PM	DP		
	Control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Group 1	Inoculated	nd	nd	nd	$3.56 \cdot 10^3$	$1.43 \cdot 10^4$	$1.91 \cdot 10^4$	$1.70 \cdot 10^3$	nd	nd	nd	$1.49 \cdot 10^7$	$1.46 \cdot 10^6$
	Inoculated	nd	nd	$4.22 \cdot 10^4$	$3.85 \cdot 10^4$	$7.70 \cdot 10^4$	$2.14 \cdot 10^4$	$4.02 \cdot 10^3$	nd	nd	nd	$5.85 \cdot 10^7$	$1.87 \cdot 10^6$
	Inoculated	nd	nd	$1.33 \cdot 10^4$	$2.92 \cdot 10^4$	$7.49 \cdot 10^4$	$5.21 \cdot 10^4$	$2.15 \cdot 10^4$	nd	nd	$1.43 \cdot 10^4$	$6.40 \cdot 10^7$	$1.02 \cdot 10^6$
	Contact	nd	nd	nd	nd	$2.12 \cdot 10^4$	$2.80 \cdot 10^4$	$9.44 \cdot 10^3$	nd	nd	nd	$4.87 \cdot 10^7$	$8.72 \cdot 10^5$
	Contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	$3.49 \cdot 10^5$	nd
	Contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	$1.20 \cdot 10^6$	nd
Group 2	Inoculated	nd	nd	$4.51 \cdot 10^3$	$2.07 \cdot 10^4$	$2.24 \cdot 10^4$	nd	nd	$4.33 \cdot 10^3$	nd	$6.23 \cdot 10^3$	$1.45 \cdot 10^5$	$1.56 \cdot 10^3$
	Inoculated	nd	nd	$2.16 \cdot 10^4$	$2.43 \cdot 10^4$	$2.05 \cdot 10^4$	$1.24 \cdot 10^4$	nd	nd	$2.18 \cdot 10^4$	$2.70 \cdot 10^3$	$6.80 \cdot 10^6$	$2.42 \cdot 10^6$
	Inoculated	nd	nd	$6.31 \cdot 10^4$	$1.69 \cdot 10^4$	$5.55 \cdot 10^4$	$2.80 \cdot 10^4$	nd	$8.14 \cdot 10^3$	$3.55 \cdot 10^3$	$5.40 \cdot 10^3$	$4.86 \cdot 10^6$	$3.59 \cdot 10^5$
	Contact	nd	nd	nd	nd	nd	$2.64 \cdot 10^4$	$7.33 \cdot 10^3$	$5.91 \cdot 10^4$	$6.75 \cdot 10^5$	$6.42 \cdot 10^3$	$5.55 \cdot 10^7$	$1.12 \cdot 10^6$
	Contact	nd	nd	nd	nd	nd	$2.65 \cdot 10^4$	$6.82 \cdot 10^3$	$2.12 \cdot 10^4$	$2.28 \cdot 10^3$	$3.72 \cdot 10^3$	$5.24 \cdot 10^6$	$1.69 \cdot 10^6$
	Contact	nd	nd	nd	nd	$1.76 \cdot 10^4$	$6.64 \cdot 10^4$	$2.94 \cdot 10^3$	$6.92 \cdot 10^3$	nd	nd	$3.07 \cdot 10^8$	$3.44 \cdot 10^6$
Group 3	Inoculated	nd	nd	nd	$4.40 \cdot 10^3$	$2.08 \cdot 10^4$	$2.11 \cdot 10^4$	$1.22 \cdot 10^4$	nd	nd	nd	$2.08 \cdot 10^8$	$3.24 \cdot 10^6$
	Inoculated	nd	nd	nd	nd	$8.68 \cdot 10^3$	$1.72 \cdot 10^4$	$1.92 \cdot 10^4$	nd	$1.59 \cdot 10^4$	$6.17 \cdot 10^3$	$5.75 \cdot 10^7$	$2.04 \cdot 10^4$
	Inoculated	nd	nd	$4.10 \cdot 10^4$	$1.95 \cdot 10^4$	$1.46 \cdot 10^4$	$1.00 \cdot 10^4$	$1.15 \cdot 10^3$	nd	$1.09 \cdot 10^6$	$1.62 \cdot 10^4$	$5.61 \cdot 10^7$	$9.63 \cdot 10^5$
	Contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	$7.39 \cdot 10^5$	nd
	Contact	nd	nd	nd	nd	nd	nd	nd	$5.32 \cdot 10^3$	nd	nd	$1.03 \cdot 10^8$	$5.87 \cdot 10^5$
	Contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	$8.29 \cdot 10^5$	nd

Quantitative RT-PCR results on individual serum samples (HEV RNA copies/mL of serum) at each sampling time and from fecal, liver and muscle samples at necropsy (HEV RNA copies/g). Shaded zones correspond to periods in which infected individuals were viremic and to HEV-positive fecal, liver and muscle samples. dpi: days post infection; nd: not detected; FB: femoral biceps; PM: psoas major; DP: diaphragm pillar.

Two parametric models were tested (lognormal and Weibull distributions of survival times) and compared using Akaike Information Criterion (AIC). Statistical associations between viral RNA quantities in the different matrices were evaluated using Pearson correlation tests. The links between HEV quantities in serum and muscles and the seroconversion as regards HEV (as binary variable) were assessed using Kruskal–Wallis tests. Statistics were analyzed using R software (Ihaka and Gentleman, 1996).

3. Results

The results for HEV RNA quantification in serum, muscle, liver and fecal samples are presented in Table 1. HEV RNA was detected in the serum samples of all inoculated animals at 35 dpi and in 45% of contact pigs at 42 dpi (Table 1, Fig. 1). HEV RNA yields in the sera ranged from $1.1 \cdot 10^3$ to $7.7 \cdot 10^4$ RNA copies/mL. Time-to viremia onset, viremia duration and period between HEV shedding and viremia were fitted to lognormal distributions. On average, HEV viremia, in both inoculated and contact pigs, started at 23.4 dpi [95% confidence interval 21.2–25.7] and lasted 28.8 days [95% CI 18.6–44.8]. The delay between HEV fecal excretion and viremia was on average 7.9 days [95% CI 5.8–11.0], in both inoculated and contact infected pigs. At 49 dpi, HEV RNA quantities in the serum and feces of inoculated pigs were statistically correlated (correlation coefficient CC = 0.83, p-value < 0.01). In contact pigs, significant associations were found between HEV RNA levels in serum and liver (CC = 0.82, p-value < 0.01).

Results on HEV seroconversion of infected pigs have been already published (Salines et al., 2015). Briefly, 4 out of 9 inoculated animals produced anti-HEV antibodies between 35 and 49 dpi, and 7 out of 9 contact individuals seroconverted between 42 and 49 dpi. Statistical analysis indicated that at 49 dpi, in both inoculated and contact pigs, high viral load in serum was significantly associated with the absence of seroconversion during the study period (p-value < 0.01 and p-value < 0.05, respectively).

HEV RNA was detected in the three types of muscles tested: femoral biceps, psoas major and diaphragm pillar (Fig. 2), with quantities ranging from $2.3 \cdot 10^3$ to $1.1 \cdot 10^6$ RNA copies/g. No significant differences in the proportions of positive samples, nor in the mean viral RNA levels, were found between the three types of muscles (p-value > 0.1).

No statistical associations were found between the different parameters tested: HEV RNA yields in serum and in muscle samples at 49 dpi; viremia duration and HEV presence in muscle; time to viremia onset and HEV presence in muscles; HEV presence in muscle and seroconversion; HEV quantities in muscle and seroconversion (p-value > 0.1). In contrast, in contact infected pigs, statistical associations were found between HEV RNA levels in muscle and liver (CC = 0.79, p-value < 0.01), and HEV RNA levels in muscle and feces (CC = 0.68, p-value < 0.05).

PRRSV viremia was detected in all inoculated animals and in all contact infected animals, except one that did not show any detectable PRRSV viremia (results shown in Salines et al., 2015).

4. Discussion and conclusions

Motivated by studies suggesting the presence of HEV in pig blood, muscle or meat products without pig liver (Berto et al., 2012; Di Bartolo et al., 2012; Grierson et al., 2015; Szabo et al., 2015) and by a previous study showing unusually long lasting HEV excretion after PRRSV co-infection (Salines et al., 2015), the presence of HEV RNA was investigated in serum and muscle meat of experimentally HEV/PRRSV co-infected pigs. We found that HEV RNA was frequently detected in both serum and muscles of co-infected pigs. Viremia started 7.9 days after initial fecal shedding and lasted 28.8 days. In a previous study by Bouwknegt et al. (2009), (where HEV transmission and dissemination were studied using a different setting, with different pigs, HEV strains, methods of detection and after intravenous inoculation), HEV contact infected pigs exhibited viremia starting after 13 days of fecal excretion and lasting 11 days (Bouwknegt et al., 2009). In the present study, earlier and longer viremia was observed in the HEV/PRRSV co-infected pigs, which may suggest that PRRSV co-infection modulates HEV physiopathology and length of viremia. The presence of HEV RNA in pig serum has been described in several studies performed at the slaughterhouse (for review Salines et al., 2017); hence the present data

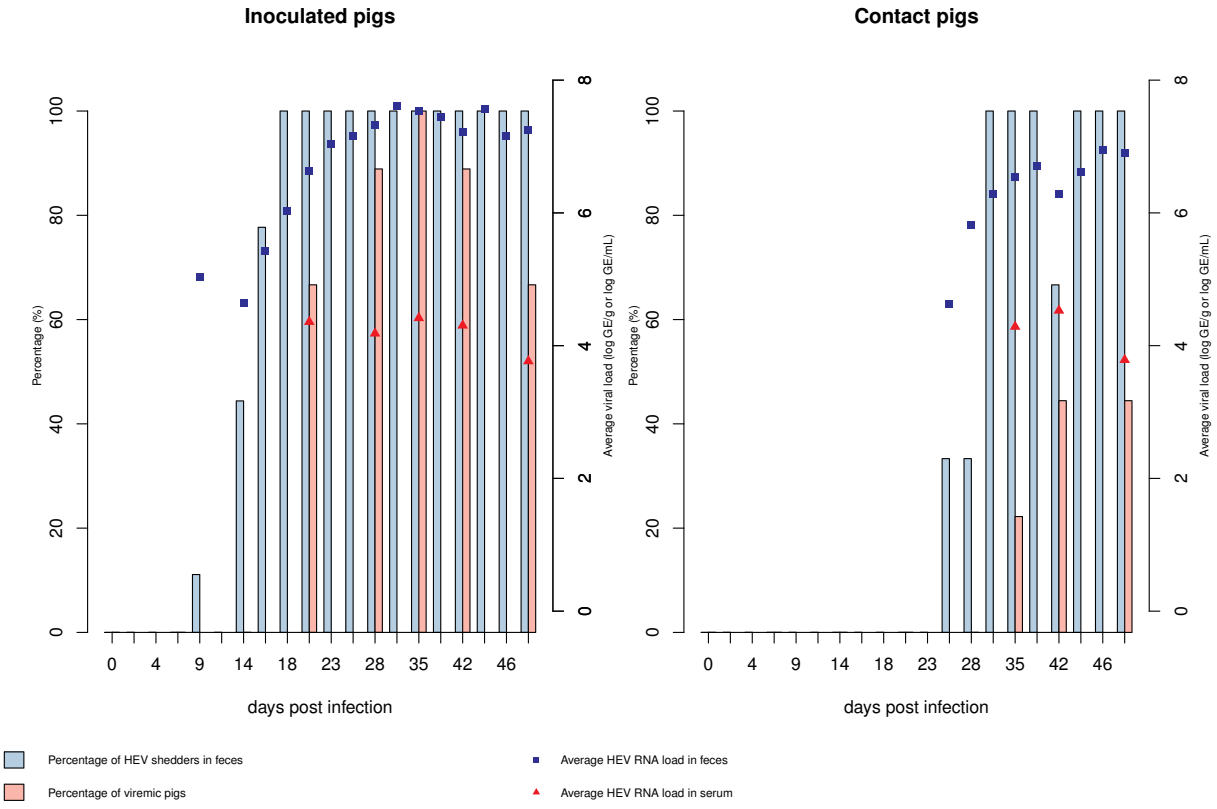

Fig. 1. Percentage of HEV shedding and viremic pigs and average HEV RNA copy numbers in feces and serum (log RNA copies/g or log RNA copies/mL) of HEV/PRRSV co-infected pigs (n = 18).

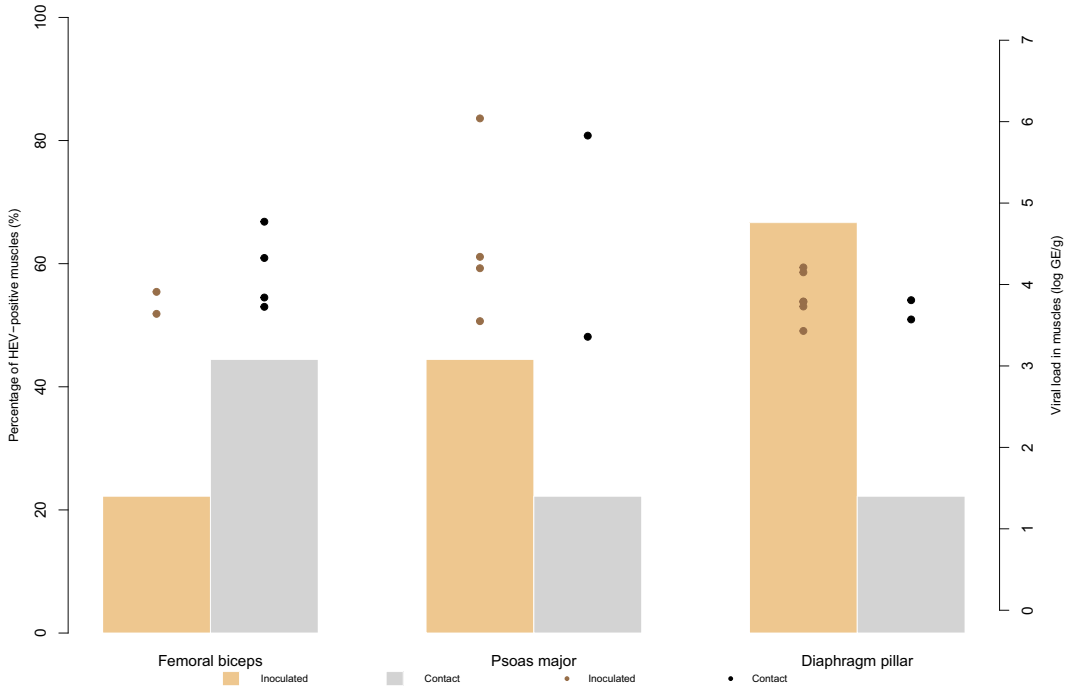

Fig. 2. Percentage of HEV-positive muscle (bars) and HEV RNA copies (log RNA copies/g) in positive muscle (dots) of HEV/PRRSV co-infected pigs (n = 18).

support the possible risk of HEV exposure through any pig blood-derived products insufficiently heated, used in the food industry.

In the present study as well, muscles of both infected and contact pigs were HEV-positive at 49 dpi. Bouwknegt et al. (2009) reported that only a few animals of the HEV-infected group were found HEV-positive in muscle up to 32 days after fecal shedding (Bouwknegt et al., 2009). These findings therefore suggest that muscle from HEV/PRRSV co-infected pigs would be more likely to contain HEV at a later stage than during HEV-only infection.

In our previous results, we have shown that HEV-PRRSV co-infection was associated with delays in HEV seroconversion (Salines et al., 2015). PRRSV infection has an impact on innate immunity that also

affects the development of an effective adaptive immune response, such as production of neutralizing antibodies (Rahe and Murtaugh, 2017). Here, we observed prolonged and high-level viremia in the absence of seroconversion (Salines et al., 2015), in agreement with a lack of virus neutralization. Furthermore, for the 3 inoculated pigs of group 2, HEV RNA was amplified at high levels in muscle, but not detected in serum at 49 days post inoculation (Table 1), which is a rather unique finding. It can be hypothesized that, in some circumstances, induced by the co-infection with PRRSV, HEV may replicate in muscle cells, in spite of the absence of apparent HEV replication in muscle, as shown in one experimental infection (no negative-strand of HEV RNA detected pig muscle) (Williams et al., 2001). Another possible explanation would be that long-term viremia favors the interaction of HEV particles with heparan sulfate expressed at the surface of muscle cells. Indeed, it has been shown that heparan sulfate proteoglycans (HSPGs) are required for cellular binding of the hepatitis E virus ORF2 capsid protein and for viral infection (Kalia et al., 2009). In skeletal muscle, HSPGs are the major proteoglycans (PGs) in the basal lamina and on the cellular surface of myocytes (Sanes et al., 1986). This class of polysaccharides is highly expressed and plays a major role in the functional integrity of skeletal muscle (Jenniskens et al., 2006). In humans, two cases of HEV-associated severe myositis have been described (Del Bello et al., 2012; Mengel et al., 2016). One of them reports on a liver transplant recipient with acute hepatitis E, associated with Guillain-Barre syndrome (Del Bello et al., 2012). The patient developed severe muscle weakness and his condition worsened. HEV viremia was found by RT-PCR, but HEV RNA was undetectable in cerebrospinal fluid. A biopsy of the left biceps showed myopathic changes, with a significant percentage of necrotic muscle fibers (10%), and signs of inflammation. The presence of HEV RNA in the muscle was not investigated in the biopsy. It would be of interest to test for the accumulation of HEV particles in muscle in humans, in cases of severe myositis. The presence of HEV in pig muscle, as observed during the present study, may also have an impact on the understanding of HEV physiopathology.

Although the oral infectious dose of HEV in humans is unknown, in pigs it is estimated to be 10^5 HEV RNA copies (Andraud et al., 2013). Here, up to $6 \cdot 10^5$ HEV RNA copies/g of muscle (psoas major) were quantified. It is therefore possible that these HEV quantities are sufficient to induce an infection in case of consumption of infected raw or undercooked meat. To prevent such exposure of consumers, in the absence of surveillance of HEV in pig meat, consumers should be advised to cook pork products very well.

The present findings highlight that pig meat products such as ham and tenderloin may contain HEV, under specific circumstances. Studies in natural conditions of pig breeding, with multi-pathogen exposure, would provide new insights into HEV dissemination in pigs.

A study conducted on 1034 pig muscles collected in French slaughterhouses did not show any HEV-positive sample, not even in pigs with HEV-positive liver (Feurer et al., 2018). Comparison with the present study is limited since the parts of muscle collected were different (gluteus medius or semi-membranosus), and no indication was provided regarding the pigs' PRRSV status. Hence, based on our study, investigation on the presence of HEV-positive muscles at the slaughterhouse should be conducted with a larger sample, stratified on the farm PRRSV status, and collecting femoral biceps and psoas major muscles.

Ham and tenderloin muscle can be consumed dried or undercooked (rare), respectively. HEV infectivity has not been directly assessed in drying conditions, but HEV remains infectious after 28 days at room temperature (Johne et al., 2016). Sufficient cooking, 20 min at 71 °C, inactivates HEV in food products contaminated artificially (Barnaud et al., 2012).

From our observations, co-infections with swine pathogens impairing the immune response against HEV may increase the risk of contaminated pig meat and products entering the food chain. Further studies are required to investigate whether other intercurrent infections (porcine circovirus-2), exposure to immunomodulatory molecules (toxins), or stress conditions would have an impact on the HEV infection course.

In conclusion, HEV contamination of pig meat and, not only of pig livers, has to be considered when assessing the HEV risk related to the consumption of pork products from a public health perspective, and surveillance plans should be implemented in the pork chain. We found that the presence of HEV in muscle might be predictable from the fecal viral genome load, which would be of great interest for easier detection of infected animals at the slaughterhouse. Testing fecal samples could therefore make it possible to identify pigs at risk of introducing infected meat into the food chain.

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Authors' contributions

MS analyzed the data and drafted the manuscript. AD and GS analyzed the HEV samples and interpreted the results. PR and OB analyzed and interpreted the results from PRRSV samples. MA participated in data analysis. NR designed and coordinated the study, and participated in animal experiments and data analysis. NP supervised the HEV laboratory work and contributed to the coordination of the study and manuscript writing. All co-authors revised the manuscript and approved the final submitted version.

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Short communication

Impact of porcine circovirus type 2 (PCV2) infection on hepatitis E virus (HEV) infection and transmission under experimental conditions

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ABSTRACT

Hepatitis E virus is a zoonotic pathogen for which pigs have been identified as the main reservoir in industrialised countries. HEV infection dynamics in pig herds and pigs are influenced by several factors, including herd practices and possibly co-infection with immunomodulating viruses. This study therefore investigates the impact of porcine circovirus type 2 (PCV2) on HEV infection and transmission through experimental HEV/PCV2 co-infection of specific-pathogen-free pigs. No statistical difference between HEV-only and HEV/PCV2-infected animals was found for either the infectious period or the quantity of HEV shed in faeces. The HEV latency period was shorter for HEV/PCV2 co-infected pigs than for HEV-only infected pigs (11.6 versus 12.3 days). Its direct transmission rate was three times higher in cases of HEV/PCV2 co-infection than in cases of HEV-only infection (0.12 versus 0.04). On the other hand, the HEV transmission rate through environmental accumulation was lower in cases of HEV/PCV2 co-infection ($4.3{\cdot}10^{-6}$ versus $1.5{\cdot}10^{-5}$ g/RNA copies/day for HEV-only infected pigs). The time prior to HEV seroconversion was 1.9 times longer in HEV/PCV2 co-infected pigs (49.4 versus 25.6 days for HEV-only infected pigs). In conclusion, our study shows that PCV2 affects HEV infection and transmission in pigs under experimental conditions.

1. Introduction

Hepatitis E virus is a non-enveloped single-stranded RNA virus that can cause acute hepatitis in humans. Chronic cases have also been described, mainly in immunocompromised patients (Lhomme et al., 2016). Genotypes 3 and 4 affect both humans and other animal species, and are responsible for sporadic autochthonous cases of hepatitis in humans in industrialised countries (Doceul et al., 2016). In particular, genotype 3 is widespread in pig populations (Salines et al., 2017) and a number of autochthonous cases have been linked to the consumption of undercooked pork meat, especially liver products (Colson et al., 2012; Guillois et al., 2016). In order to limit the risk of contaminated products entering the food chain, it is crucial to understand the factors influencing HEV transmission and persistence in pig herds. High variability in HEV infection dynamics has previously been described (Salines et al., 2017) and may be related to husbandry practices in terms of hygiene, biosecurity and rearing conditions (Walachowski et al., 2014; Lopez-Lopez et al., 2018) or to individual characteristics such as protection conferred by maternally-derived antibodies (Andraud et al., 2014). Various factors affecting swine immune response may also influence the course of HEV infection. Notably, in a previous study, we have shown that pigs experimentally co-infected with porcine reproductive and respiratory syndrome virus (PRRSV) exhibited chronic HEV infection with extended latency and infectious periods, increased faecal shedding and transmission, as well as an increased risk of HEV-positive livers at slaughter (Salines et al., 2015). Porcine circovirus type 2 (PCV2) also has immunomodulating characteristics for instance by inhibiting IFN-α production and by increasing the expression of IL-10, an anti-inflammatory cytokine (Darwich et Mateu, 2012). PCV2 may therefore impact HEV infection dynamics. Moreover, as the primary causative

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agent of post-weaning multisystemic wasting syndrome (PMWS) and other porcine circovirus-associated diseases (PCVADs), it can sometimes induce hepatitis in pigs (Rosell et al., 2000). However, to date, only few data report on HEV/PCV2 co-infection (Martin et al., 2007; Hosmillo et al., 2010; Savic et al., 2010; Yang et al., 2015; Jackel et al., 2018). In these studies, PCV2 and HEV were simultaneously detected in pigs but the impact of co-infections on HEV dynamics was not investigated.

Given the lack of data on this specific issue, the present study was designed to investigate how PCV2 infection impacts HEV infection dynamics (in terms of viral shedding duration and quantity, transmission and humoral immune response). A transmission experiment was therefore carried out, with specific-pathogen-free (SPF) pigs infected with HEV or co-infected with HEV and PCV2 at the same time.

2. Material and methods

2.1. Experimental design

The trial was conducted at ANSES's air-filtered level-3 biosecurity facilities. The 44 five-week-old SPF Large White piglets included in the study were HEV- and PCV2-free and with no maternal antibodies against these two viruses at the beginning of the study. These piglets were randomly allocated into eight groups, housed in six rooms (Fig. 1). Two negative control pigs were housed in Room 1. The four piglets housed in Room 2 were only orally inoculated with a PCV2-b genogroup suspension (GenBank accession number AF201311), titrating 10^5 $TCID_{50}$/mL in a volume of 5 mL. In Rooms 5 and 6 (groups 4, 5, 6), three piglets per group were orally inoculated with 10^7 HEV RNA copies of a genotype 3 HEV suspension (strain FR-SHEV3f, GenBank accession number JQ953666) in a volume of 10 mL. In Rooms 3 and 4 (groups 1, 2, 3), three piglets per group were orally inoculated with both HEV and PCV2, following the same inoculation protocols as for the other groups. In each of the six groups (HEV-only and HEV/PCV2), the three inoculated piglets were in contact with three pen mates (contact piglets) from day 1. Individual faecal samples were collected three days before inoculation and three times a week until the end of the experiment at 49 days post inoculation (dpi). Blood samples were collected before inoculation and once a week until the end of the experiment. Clinical examination was also performed (clinical signs, rectal temperature, faeces consistence, weight, food consumption and trough cleanliness were recorded daily). After euthanasia, necropsies were performed and organ and fluid samples collected, among them liver and bile samples. The experiment was performed in accordance with EU and French regulations on animal welfare in experiments. The protocol was approved (referral 17-022) by the ANSES/ENVA/UPEC ethical committee registered under number #16.

2.2. Sample analyses

After performing manual total RNA extraction, HEV RNA in faecal samples was quantified using real-time quantitative RT-PCR as described in Barnaud et al. (2012). Results were expressed in HEV RNA copy number per gram of faeces (RNA copies/g). Since HEV shedding in faeces and presence in serum have been shown to be correlated (Salines et al., 2018), HEV RT-PCR was performed on serum samples of 49 day-old pigs only if their faeces were positive at 46 and/or 49 dpi. Similarly, and as bile is considered as a relevant proxy of liver status (de Deus et al., 2008; Bouwknegt et al., 2009), bile samples of 49-day old pigs having positive faecal samples at 46 and/or 49 dpi were analysed. Anti-HEV antibodies were detected using the HEV ELISA 4.0 V kit (MP Diagnostics, Illkirch, France) according to the manufacturer's instructions, apart from the serum quantity used (10 µL instead of the recommended 20 µL). Samples were considered to be positive when their optical density (OD) at a wavelength of 450 nm was higher than the threshold, which was defined as the mean optical density of negative control pig samples + 0.3. PCV2 DNA was extracted and quantified from the serum using real-time PCR based on TaqMan technology as described in Grasland et al. (2005). Results were expressed in genomic equivalent DNA copies/mL of serum. PCV2-antibodies were detected by PCV2 specific ELISA as already described with a positive cut-off for OD ratios higher than 1.5 (Fablet et al., 2017).

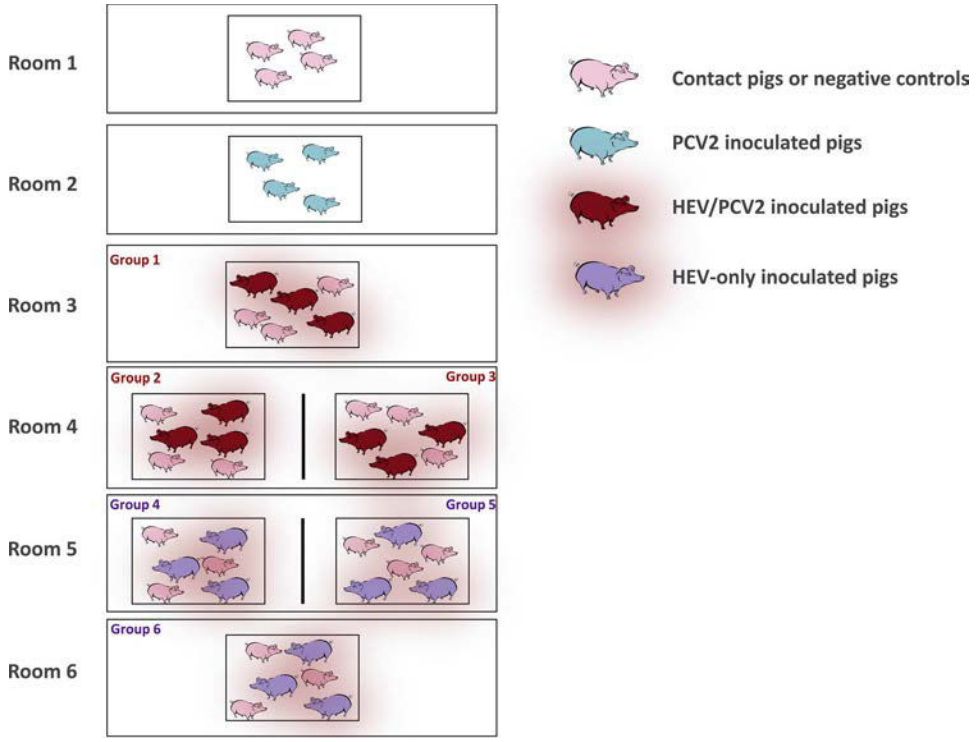

Fig. 1. Experimental design of the HEV/PCV2 co-infection trial.

2.3. Statistical analyses

The infectious period and time prior to HEV seroconversion were estimated using survival analyses. Two parametric models were tested (lognormal and Weibull survival time distributions) and compared using the Akaike Information Criterion (AIC). Cox-proportional hazard models were used to assess the effect of PCV2 co-infection on the lengths of the infectious period and the time prior to HEV seroconversion. The distributions of individual HEV viral loads in faeces were analysed according to time since inoculation (with and without co-infection). A linear mixed model taking into account repeated measurements over time was used for this investigation in order to assess the different quantities of HEV particles shed by co-infected as opposed to HEV-only infected pigs.

The HEV infection dynamics in each group were modelled using a SEIR (Susceptible – Exposed – Infectious – Recovered) model as per the estimation process described in Gallien et al. (2018). Briefly, pigs were considered as "susceptible" during the time window from exposure (day 0 = day of inoculation) to the point at which they actually became infected (t_{Inf}), progressing to the "exposed" state. The time at which individuals were considered to be "infectious" (i.e. began shedding), denoted t_{sh}, was considered to lie between the times of the last HEV-negative PCR sample (t_{neg}) and the first HEV-positive PCR faecal sample (t_{pos}) for each animal ($t_{neg} < t_{sh} < t_{pos}$). The latency period δ_E therefore corresponds to the delay between infection and shedding ($\delta_E = t_{sh} - t_{Inf}$). Pigs were considered "recovered" as soon as they no longer produced HEV-positive PCR samples. Two transmission routes were considered to be involved in this infection process: transmission by direct contact between pen mates and oro-faecal transmission via the environmental compartment. Environmental viral load E_t represents the accumulation of viral particles in the environment through faecal shedding by infected animals. E_t is partially offset by its clearance rate ($\delta = 0.3\, day^{-1}$) and was calculated as described in Andraud et al. (2013) and Salines et al. (2015). Let β_{DC} and β_{Env} denote direct contact and environmental transmission rates, respectively. The force of infection exerted on a typical susceptible individual i located in pen k at time t is defined by:

$$\lambda_k(t) = \beta_{DC}\frac{I_k(t)}{n-1} + \beta_{Env}\frac{E_k(t)}{n},$$

where I and E respectively represent the number of infectious animals and the viral load in pen k at time t, n being the total number of pigs in each pen. With these notations, the probability p_i of individual i getting infected at time $T_{inf}^{(i)}$ is given by

$$p_i = 1 - \exp(-\lambda_k(T_{inf}^{(i)}))$$

while the probability of having escaped infection in time interval $[0,\ t_{inf}^{(i)}[$ is given by

$$q_i = \exp\left(-\int_0^{T_{inf}^{(i)}} \lambda_k(\tau)d\tau\right)$$

An informative gamma prior was used to analyse the duration of the latency period δ_E. Its parameters were fixed using data from previous experiments and from observations of inoculated pigs ($\alpha = 4,\ \kappa = 3$). Very wide normal distributions were initially used as prior for the log-transformed transmission rates ($\log(\beta_{DC}) \sim N(-2,4)$ and $\log(\beta_{Env}) \sim N(-8,4)$). The global likelihood can be written as:

$$\begin{aligned} &L(T_{Neg}, T_{Pos}, I, E|\beta_{DC}, \beta_{Env}, \delta_E, T_{inf}, \alpha, \kappa) \\ &= \prod_{i \in contact-infected} e^{-\int_0^{T_{inf}^{(i)}} \lambda(\tau)d\tau} \times \left(1 - e^{-\lambda(T_{inf}^{(i)})}\right) \times \gamma(\delta_E^{(i)}, \alpha, \kappa) \\ &\quad \times \prod_{\substack{i \in contact- \\ non\ infected}} e^{-\int_0^{max(t_{obs})} \lambda(t)dt} \\ &\quad \times \prod_{i \in inoculated} \gamma(\delta_E^{(i)}, \alpha, \kappa) \end{aligned}$$

The first term of the likelihood denotes the probability of detected infections occurring for an individual i at time $T_{Inf}^{(i)}$; the second term represents the probability of observed infection failure whenever some individual would remain susceptible throughout the experiment; and the third term gives the distribution of the latency period in seeder pigs. Bayesian inference was performed using the Metropolis-Hastings algorithm: ten independent chains of 50,000 iterations were run with a burn-in period of 10%. Initial values were randomly drawn from prior distributions. Convergence was assessed by inspecting parameter outputs visually as well as through conventional diagnostic tests (Heidelberger, Geweke and Gelman-Rubin diagnostics). The impact of PCV2 infection on the HEV latency period and the transmission parameters' distribution were then assessed using a Kruskal-Wallis test. All the analyses were performed using R software (R 3.5.1).

3. Results

3.1. Infection data

No clinical sign related to PCV2 or HEV infection was observed in any infected pig. All PCV2 inoculated pigs and pigs in contact were seropositive at 28 dpi except one that was found seropositive at 45 dpi (Supplementary File 1). Control pigs and HEV-only inoculated pigs remained PCV2 seronegative throughout the study. PCV2 viraemia in contact pigs started between 10 and 28 dpi and lasted until 28–49 dpi. Viral loads ranged between 1.10^3 and 8.10^6 genomic equivalent DNA copies/mL of serum with a viraemia peak around 17 days post-inoculation (Supplementary File 1). HEV infection data are presented in Figs. 2 and 3 for quantitative RT-PCR on faecal samples and serological results respectively. All but two animals (one HEV/PCV2-inoculated pig and one HEV contact pig) shed HEV during the experiment. Inoculated animals started to shed HEV between 11 and 25 dpi, and contact animals between 23 and 46 dpi. Sporadic or intermittent shedding was observed in a few animals (Fig. 2). Of the 36 pigs, 20 produced anti-HEV antibodies: 14 of the 18 HEV-only infected pigs versus just six of the 18 HEV/PCV2 co-infected pigs. Seroconversion occurred between 24 and 49 dpi for inoculated animals, and between 38 and 45 dpi for contact animals (Fig. 3). At the end of the experiment, four out of the 17 analysed pigs (23%) had HEV RNA in their bile and one of them was viraemic (6%), with a viral load of $4.7.10^3$ RNA copies/mL (Fig. 2). These positive pigs were HEV/PCV2 co-infected (both inoculated and contact pigs).

3.2. Estimated durations related to HEV infection dynamics

Latency periods were estimated at 12.3 days [4.4–25.5] in HEV-only pigs and 11.6 days [2–21.6] in HEV/PCV2 co-infected pigs. The latency period was significantly shorter in HEV/PCV2 co-infected pigs than in HEV-only infected pigs ($p < 0.05$).

Survival analysis of the infectious period (lognormal distribution) gave a mean duration of 11.8 days [8.3–16.7] for HEV-only infected animals and 16.6 days [10.7–25.9] for HEV/PCV2 co-infected animals. No statistical difference was found between HEV-only and HEV/PCV2-infected pigs (HR = 0.6 [0.3–1.4], $p > 0.05$).

Survival analysis of the time prior to HEV seroconversion (using the Weibull distribution) gave a mean duration of 25.6 days [19.3–33.8] for HEV-only infection and 49.4 days [40.4–60.4] for HEV/PCV2 co-infection. The time prior to HEV seroconversion was statistically longer in HEV/PCV2- than in HEV-only infected pigs (HR = 0.3 [0.1 − 0.8], $p < 0.05$).

3.3. HEV shedding and environmental accumulation

The distribution of the shed HEV viral load against time (with and without co-infection) is shown in Fig. 4. The linear mixed model accounting for repeated measurements did not show the PCV2 infection to

	ID	dpi	-3	2	4	7	9	11	14	16	18	23	25	28	30	32	36	39	43	46	49	49	49
			Faecal samples																			Bile samples	Serum samples
HEV/PCV2	1	control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	2	control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	3	control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	4	control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	group 1 5	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	1.12E+04	7.09E+03	nd	nd	2.86E+03	1.21E+03	nd	nd	nd	4.95E+03	nd	nd	nd
	6	inoculated	nd	nd	nd	nd	nd	nd	nd	8.42E+03	5.43E+03	nd	4.15E+05	1.90E+06	1.84E+06	8.44E+05	2.45E+06	7.50E+05	1.94E+05	1.76E+03	nd	nd	nd
	7	inoculated	nd	nd	nd	nd	nd	nd	2.72E+04	nd	nd	6.48E+05	1.00E+05	1.85E+06	1.09E+06	1.36E+06	1.36E+06	1.94E+06	3.25E+06	3.72E+06	1.83E+06	1.87E+06	4.71E+03
	8	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.08E+03	nd	1.53E+04	9.94E+03	3.22E+02	2.42E+03	nd	nd
	9	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	4.17E+04	6.59E+03	nd	nd	na	na
	10	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.54E+03	1.92E+03	1.27E+04	6.25E+03	8.62E+01	nd
	group 2 11	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.07E+05	5.77E+04	1.17E+06	8.50E+05	5.85E+05	nd	nd	nd	nd	nd	na	na
	12	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.65E+03	1.57E+04	7.86E+04	nd	nd	nd
	13	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	8.56E+03	4.15E+04	6.68E+04	1.10E+04	4.74E+03	nd	nd	nd	nd	nd	na	na
	14	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.57E+02	7.32E+02	nd	nd
	15	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.30E+03	6.32E+03	3.68E+02	nd
	16	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.34E+04	6.29E+05	3.38E+05	nd	nd
	group 3 17	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.54E+04	7.96E+03	4.62E+04	nd	nd	nd	nd	nd	nd	nd	na	na
	18	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	19	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	9.13E+03	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	20	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	9.41E+04	6.75E+04	7.56E+03	nd
	21	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.95E+05	3.73E+05	3.78E+04	1.18E+05	7.66E+04	1.32E+04	nd	nd	nd	nd	na	na
	22	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
HEV-only	group 4 23	inoculated	nd	nd	nd	nd	nd	nd	9.59E+04	1.60E+05	3.57E+05	4.31E+05	1.91E+04	nd	nd	nd	nd	nd	2.05E+03	3.30E+03	nd	nd	nd
	24	inoculated	nd	nd	nd	nd	nd	nd	1.88E+04	1.72E+04	6.28E+03	2.10E+05	2.26E+05	2.26E+04	nd	nd	5.99E+03	nd	nd	1.24E+04	nd	nd	nd
	25	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.06E+04	7.15E+03	8.93E+04	1.09E+04	1.94E+05	1.53E+06	1.01E+06	1.97E+04	3.19E+03	nd	nd	nd
	26	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	4.49E+03	1.36E+05	nd	nd	2.06E+04	nd	nd	nd
	27	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.91E+04	nd	9.37E+03	3.71E+04	5.82E+04	1.07E+03	1.15E+04	nd	nd	nd
	28	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.84E+05	2.99E+05	5.97E+02	nd	nd
	group 5 29	inoculated	nd	nd	nd	nd	nd	1.20E+04	1.73E+05	8.74E+04	1.14E+06	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	30	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.11E+04	2.10E+03	4.45E+05	1.28E+05	1.27E+06	4.21E+05	nd	nd	nd	nd	na	na
	31	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.87E+03	1.78E+04	1.79E+04	4.69E+05	6.75E+05	5.48E+05	nd	nd	nd	na	na
	32	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	8.81E+04	3.07E+04	2.77E+03	nd	nd	na	na
	33	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.46E+05	1.26E+05	nd	nd	nd	na	na
	34	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.01E+05	nd	nd	nd	nd	na	na
	group 6 35	inoculated	nd	nd	nd	nd	nd	nd	3.87E+03	6.17E+03	6.18E+03	8.93E+04	6.27E+03	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	36	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	1.81E+04	nd	1.13E+04	nd	nd	nd	nd	nd	nd	nd	nd	na	na
	37	inoculated	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.35E+04	9.54E+03	nd	7.02E+03	nd	nd	nd	nd	nd	nd	na	na
	38	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.30E+03	nd	nd	nd	nd	na	na
	39	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.15E+04	nd	nd	nd	nd	na	na
	40	contact	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na

Fig. 2. HEV RNA quantification in faecal, bile and serum samples from HEV-only and HEV/PCV2-infected pigs (inoculated and contact animals, n = 36). In yellow: Quantitative HEV RT-PCR results for individual faecal samples (HEV RNA copies/g of faeces) at each sampling time. Shaded zones correspond to periods during which infected individuals were considered as "infectious", corresponding to the time between the first and final HEV-positive faecal samples for each animal. In blue and red: Quantitative HEV RT-PCR for bile and serum samples respectively (HEV RNA copies/mL) of 49 day-old pigs for which faecal samples were positive at 46 and/or 49 dpi. dpi: days post inoculation; nd: not detected, na: not analysed.

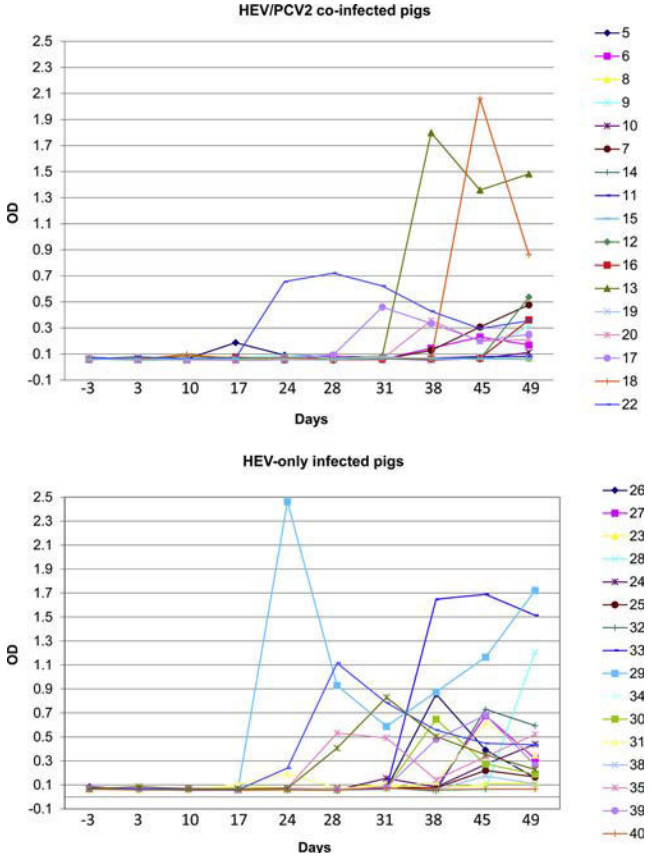

Fig. 3. Kinetic of HEV seroconversion. Results for individual sera samples (in different colours and shape) from HEV/PCV2-infected pigs (upper panel) and HEV-only (lower panel) (inoculated and contact animals, n = 36). OD: optical density; cut off value = 0.3.

have any impact on the quantity of HEV particles shed by inoculated or contact animals ($p > 0.05$). The viral load accumulated in the environment was modelled for each experimental pen. The environment was HEV-free until 15–20 dpi, when the environmental load increased and reached 4.10^5 to 2.10^6 before dropping at the end of the trial (data not shown) when there were no remaining shedders in the pen.

3.4. HEV transmission parameters

In our experimental settings, a single HEV-only infected pig was able to infect 0.04 pigs per day by direct contact (β_{DC} = 0.04 [$2{\cdot}10^{-5}$–0.24]), whereas the direct transmission rate for HEV/PCV2 co-infected pigs was estimated to be significantly higher, with a three-fold difference (0.12 [$5{\cdot}10^{-4}$ - 0.4]; Figs. 5 and 6). The environmental transmission rate β_{Env} can be considered as the average number of animals that a single genome equivalent is able to infect when present in the pen environment. β_{Env} was estimated at $1.5{\cdot}10^{-5}$ g/RNA copies/day [$2{\cdot}10^{-6}$; $4{\cdot}10^{-5}$] when pigs were HEV-only infected versus $4.3{\cdot}10^{-6}$ g/RNA copies/day [$7{\cdot}10^{-8}$; $1.3{\cdot}10^{-5}$] when pigs were HEV/PCV2 co-infected (Figs. 5 and 6). It was statistically lower in cases of HEV/PCV2 co-infection than for HEV-only infected pigs ($p < 0.05$).

4. Discussion

Understanding factors likely to influence HEV infection dynamics on pig farms is a pivotal step in the design of HEV surveillance and control programmes aiming to mitigate the risk of human exposure to HEV. Of those factors, immunomodulating pathogens are suspected to play a key role and PRRSV has previously been shown to strongly influence HEV infection dynamics (Salines et al., 2015). The main aim of the present study was to investigate the potential impact of PCV2 co-infection on HEV infection dynamics under experimental conditions.

PCV2 infection dynamics in our experimental settings did not differ from data in the available literature (Andraud et al., 2008), suggesting that HEV did not impact PCV2 dynamics. Animal follow-up showed high inter-individual variability of HEV infection dynamics, both in HEV-only and HEV/PCV2-infected pigs, with average latency periods of 12.3 and 11.6 days, and infectious periods of 11.8 and 16.6 days respectively. This high variability was already highlighted in previously-

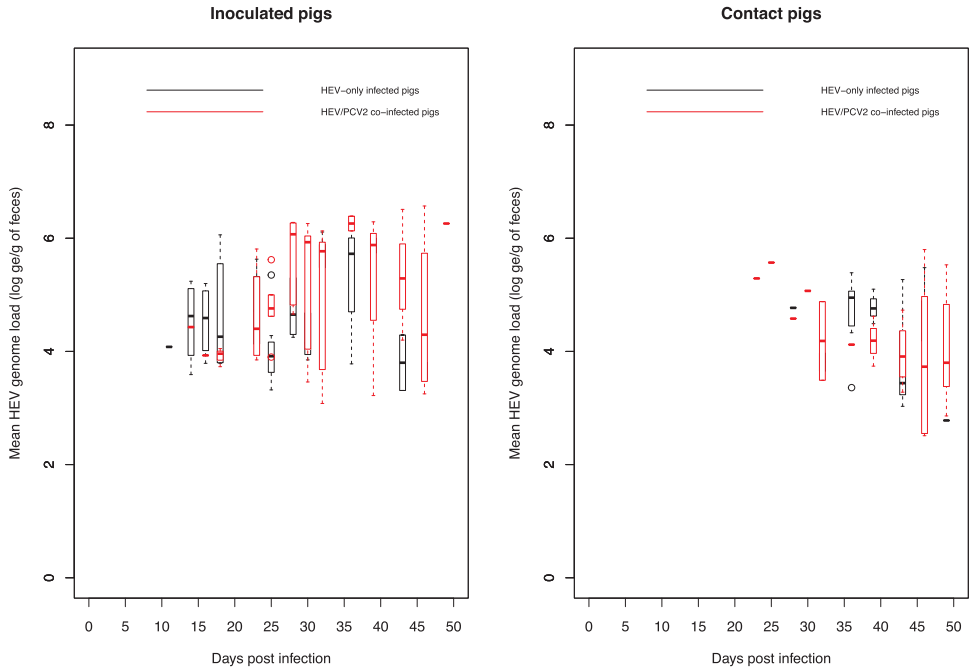

Fig. 4. Distribution of the number of HEV genome equivalents (log RNA copies/g faeces) shed by individual pigs, versus time, in HEV inoculated and contact animals with or without PCV2 co-infection (n = 36).

published studies on the topic, especially in cases of natural infection by the oral route (Bouwknegt et al., 2009; Andraud et al., 2013; Salines et al., 2015). This variability was taken into account for the parameter estimation by taking uninformative or little informative prior distributions; algorithm convergence therefore allows to gain confidence in the obtained results. For the HEV-only infected group, the infection kinetics slightly vary from those described in Andraud et al. (2013), who reported a latency period of 6.9 days [5.8–7.9] and an infectious period of 9.7 days [8.2–11.2]. This gap may be related to the different HEV strain used for inoculation (strain FR-SHEV3e in Andraud et al. (2013), versus strain FR-SHEV3f in the present trial) as well as to the lower inoculation dose (10^7 genomic equivalent in the present experiment versus 10^8 in the HEV/PRRSV experiment). In the trial described by Bouwknegt et al. (2009), the infectious period was estimated at between 13 and 49 days, depending on the replicate block, but their pigs were intravenously inoculated (versus oral inoculation in the present experiment).

From our analyses, no statistical difference was found between HEV-only and HEV/PCV2 groups, either in the infectious period, or in the quantity of HEV shed in faeces. The latency period was found to be less than one day shorter in HEV/PCV2 co-infected pigs than in HEV-only infected pigs which, although statistically significant, is likely to have a limited biological impact on HEV infection dynamics. The direct transmission rate of HEV was found to be three times higher in cases of HEV/PCV2 co-infection than in cases of HEV-only infection (0.12 versus 0.04), meaning that one co-infected pig is likely to infect three times more pigs than a pig infected only with HEV. The environmental transmission rate of HEV was found to be lower in cases of HEV/PCV2 co-infection ($4.3{\cdot}10^{-6}$ versus $1.5{\cdot}10^{-5}$ g/RNA copies/day for HEV-only infected pigs), meaning that three times more HEV particles in the environment are needed in order to infect a pig already carrying PCV2. The lower environmental force of infection in cases of PCV2 infection may delay HEV infections. Short time to slaughter after HEV infection seems to be a key point of liver contamination. Thus, delaying HEV infection is likely to increase the risk of pig livers containing HEV at slaughter time. Regarding immune response, fewer HEV/PCV2-infected pigs than HEV-only infected pigs presented a humoral immune response (6/18 versus 14/18 pigs, respectively). Moreover, the time prior to HEV seroconversion was 1.9 times longer in HEV/PCV2 co-infected pigs than in HEV-only infected pigs (49.4 versus 25.6 days). This could be especially problematic if pig HEV status is screened using serological method: this long time prior to HEV seroconversion would lead to many false negative animals. Although PCV2 did not affect HEV infection dynamics as much as PRRSV did in the trial that we previously conducted (Salines et al., 2015), it cannot be excluded that in combination with other factors, as for PMWS, it may influence HEV infection. This is consistent with the immunomodulating effect of both PCV2 and PRRSV described in literature, where innate immunity is somewhat suppressed due to a reduction in the IFNα response, delaying the onset of the adaptive response (Darwich et Mateu, 2012; Butler et al., 2014). Four out of the 17 tested pigs had HEV RNA in the bile at the end of the experiment, which can be considered as a reliable proxy of the liver contamination. This late-stage positivity illustrates the increased risk of having HEV positive livers entering the food chain when animals were co-infected. Moreover, the detection of one HEV/PCV2 co-infected pig being HEV viraemic at the end of the experiment also raises the question of a potential risk linked to other pork products that is still debated in the literature (Salines et al., 2018). Further analyses would be necessary to assess the level of contamination of pig muscles in cases of PCV2 infection, especially as correlations between HEV RNA levels in muscles, liver and faeces have been shown (Salines et al., 2018). Such analyses could inform on the risk for public health linked to the consumption of undercooked or raw pig meat or other pork products that do not contain liver. Our present results could also be used to feed dynamic models representing HEV spread and persistence on farms in which PCV2 may circulate. Our data, obtained under controlled conditions, can also add supplementary explanations to the previously published field studies in which HEV and PCV2 were detected simultaneously in pigs and in which causal relationship was suspected but not demonstrated (Martin et al., 2007; Hosmillo et al., 2010; Savic et al., 2010; Yang et al., 2015; Jackel et al., 2018). Further work is needed to investigate whether there are other underlying immune mechanisms specific to co-infecting viruses. Moreover, it should be noted that the pigs in the present experiment were simultaneously inoculated with HEV and PCV2; the same kind of study could be reproduced with different inoculation time sequences (e.g. pigs

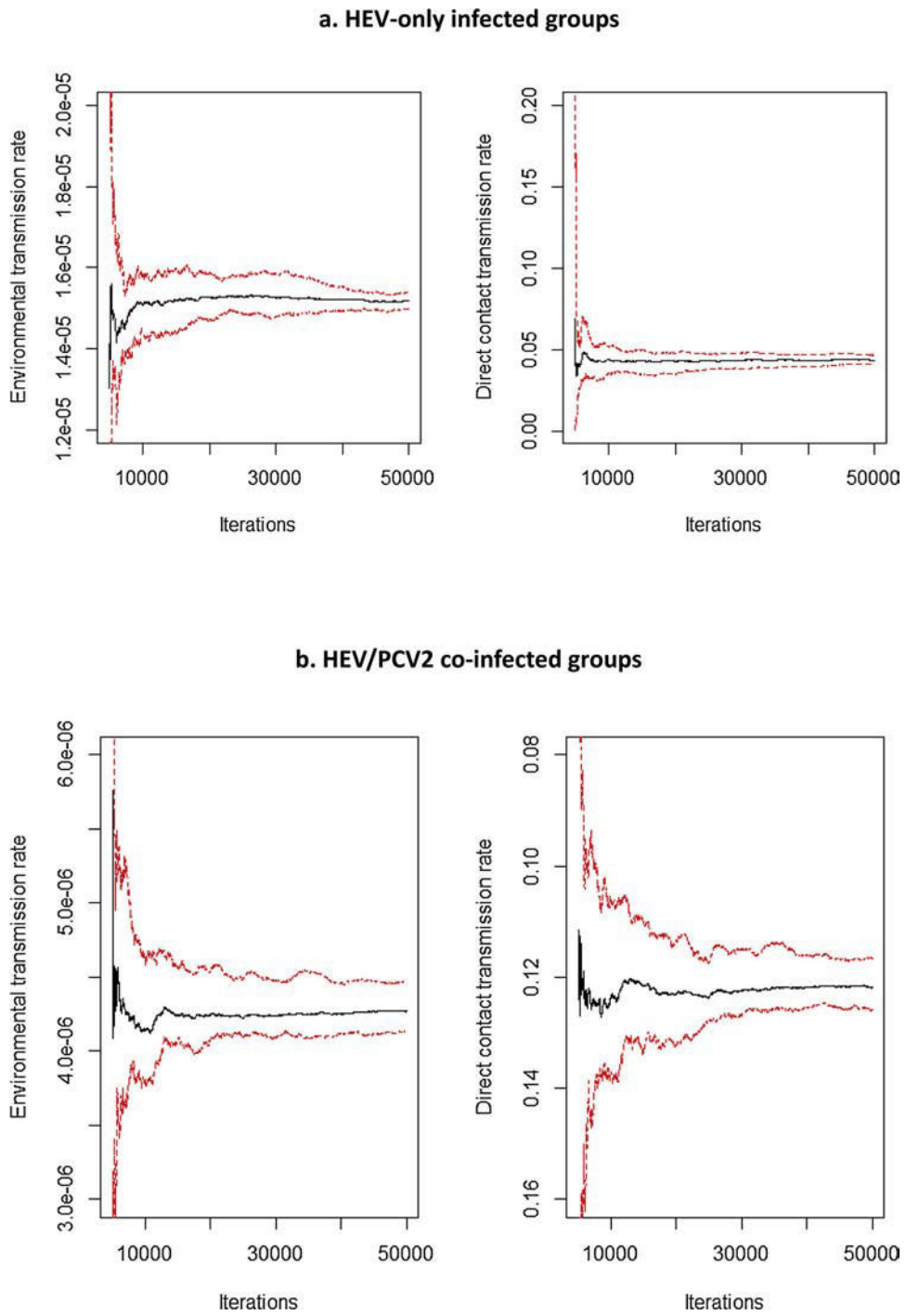

Fig. 5. Running average of transmission parameter estimates from ten independent Monte-Carlo Markov chains for (a) HEV-only and (b) HEV/PCV2-infected groups.

inoculated with PCV2 a week before HEV) and probably with more pigs included to reduce the impact of inter-individual variability in infection dynamics.

To our knowledge, this study is the first to focus on the impact of HEV/PCV2 experimental co-infection on HEV infection and transmission in pigs. Our results show that, in experimental settings, PCV2 co-infection increases the direct transmission of HEV and impairs the humoral immune response towards it. The effect observed in this PCV2/HEV co-infection trial was less marked than previously observed when PRRSV was involved, however, and failed to explain the long-term HEV shedding that has been observed in the field at an individual level. A combination of PCV2 co-infection with other factors may lead to chronic HEV infection. Additional studies (e.g. on-farm intervention studies, other co-infection trials, dynamic modelling approaches) should therefore be conducted to explore the potential synergistic effects of multiple co-infections and devise effective control strategies that would include measures targeting intercurrent pathogens (vaccination, eradication programme).

Authors' contributions

MS and MA developed the mathematical model, analysed the data and drafted the manuscript. MP and CB analysed the HEV and PCV2 samples respectively, and interpreted the results. NP and BG supervised

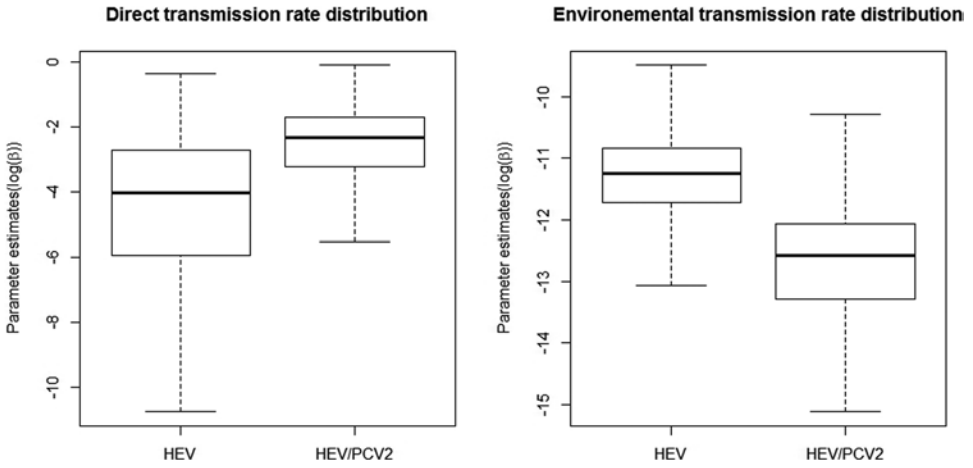

Fig. 6. Distribution of direct and environmental HEV transmission parameters estimated from ten independent Monte-Carlo Markov chains.

the HEV- and PCV2-related laboratory work respectively, and helped coordinate the study. NR conceived and coordinated the study, in addition to participating in the animal experiment and data analyses. All the co-authors revised the manuscript and approved the final submitted version.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.010.

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Ce qu'il faut retenir

Les études réalisées en conditions naturelles et expérimentales ont permis de mettre en évidence et de quantifier le rôle central des co-infections immunomodulatrices dans la dynamique de l'infection par le HEV chez le porc. Ces infections intercurrentes, notamment celle par le virus du SDRP, conduisent à une infection chronique par le HEV, augmentant ainsi significativement le risque de présence du HEV dans le foie lors de l'abattage des porcs. De plus, en situation expérimentale, il a été montré que des porcs co-infectés par le virus du SDRP présentent une virémie persistante et de l'ARN du HEV dans plusieurs de leurs muscles. Les co-infections immunomodulatrices ont ainsi un impact majeur sur le risque pour la santé publique lié au HEV.

Take home message

The studies conducted both under natural and experimental conditions have highlighted and quantified the central role of immunomodulating co-infections in the dynamics of HEV infection in pigs. These intercurrent infections, particularly that caused by PRRSV, lead to chronic HEV infection thus significantly increasing the risk of livers being HEV-positive at slaughter. Moreover, under experimental conditions, it has been shown that PRRSV/HEV co-infected pigs have persistent viraemia as well as HEV RNA in several muscles. Immunomodulating co-infections have therefore a major impact on the public health risk associated with HEV.