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Estimating transmission parameters of F4+ *E. coli* for F4-receptor-positive and -negative piglets: one-to-one transmission experiment

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SUMMARY

F4+ *Escherichia coli* is an important agent of post-weaning diarrhoea in piglets. Piglets that express an adhesion site for F4+ *E. coli* in their small intestine (F4R+) shed higher numbers of F4+ *E. coli* than piglets lacking this site (F4R−). We hypothesized that F4R+ piglets are more infectious and more susceptible for F4+ *E. coli*. This implies that in populations with F4R+ and F4R− piglets, the transmission would be dependent on the frequency of both types of animals. To quantify the difference in infectiousness and susceptibility, a one-to-one transmission experiment was performed with 20 pairs consisting of one inoculated and one contact piglet. Based on the contact infections observed, transmission parameters were estimated with generalized linear models. F4R+ piglets were infectious for other piglets and the reproduction ratio (R_0) for homogeneous F4R+ populations, that is the average number of secondary infections that one F4R+ pig will cause during its entire infectious period in a population of susceptible F4R+ individuals only, was estimated as 7·1. F4R+ piglets were more susceptible than F4R− piglets and reducing the fraction of F4R+ piglets of a population will reduce transmission. It was calculated that in order to prevent major outbreaks of F4+ *E. coli* ($R_0 < 1$), the fraction of F4R+ piglets must be lower than 0·14.

INTRODUCTION

Enterotoxigenic *Escherichia coli* serotypes with adhesin F4 (or K88) are frequently found to be causative agents of post-weaning diarrhoea (PWD) in piglets [1–3]. PWD causes diminished animal health and also causes economic losses for the farmer, due to increased mortality and growth retardation. Therefore intervention measures should be developed to reduce the symptoms or to prevent the spread of the bacteria.

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One of the factors that has an influence on the clinical signs is the presence of an adhesion site in the small intestine, which is usually referred to as the F4 receptor (F4R) or K88 receptor [4–6]. This adhesion site is a genetically inherited dominant characteristic and its presence can be shown by *in vitro* adhesion assays [7, 8]. Based on this test, pigs can be classified as F4R-positive (F4R+, adhesive brush borders) or F4R-negative (F4R−, non-adhesive brush borders). A previous study showed that F4R has an effect on the level of bacterial shedding of *E. coli* serotype O149:K91:F4ac (Geenen et al., unpublished observations), which in turn, might be an indication for infectivity. Whether this higher infectivity also affects

transmission, however, could not be determined from those experiments.

Selection of F4R− pigs may be one way to reduce the PWD problem [9]. Whether the infection will spread depends not only on the susceptibility of the as yet uninfected pigs but also on the infectivity of the infected pigs. The question is whether the F4R determined either variable.

Transmission can be studied under experimental conditions [10–14]. These experiments have the advantage that the effect of infectivity as well as susceptibility on transmission are combined.

Group experiments are less useful here, because the groups will probably be mixed populations of F4R+ and F4R− piglets which are expected to differ in infectiousness and susceptibility. Therefore, a more suitable experiment is a one-to-one experiment, in which one infectious pig is housed with one susceptible pig. This experimental design has the advantage that within a pair of piglets it is clear who infected whom [15]. In these experiments, the transmission from either type of pig to a contact pig can be quantified.

METHODS

Experimental design

On the day of weaning (day 0), 40 male, castrated piglets (age 21–30 days) from 20 different litters were brought from a commercial farm to the Animal Sciences Group. Rectal swabs were taken on arrival and were checked for haemolytic *E. coli*.

Pairs of piglets were housed in separate pens with four pens per stable. All pens were placed on grid floors and had a window made of perspex in one wall so that piglets in adjacent pens had visual but not physical contact. Density of the piglets was one piglet per 0·45 m² floor surface and the mean temperature of the stables was 25 °C with a 16-h light/8-h dark cycle.

Piglets were assigned randomly to the pairs with restriction that littermates were not housed together and that the piglets within a pair were of comparable weight (weights 5·5–9·7 kg). The mean weights of the pairs were equally distributed over the five stables. During the experiment the pens were not cleaned to ensure a maximum infectivity in the pen. Special care was taken during sampling, feeding, etc. to prevent faeces being transmitted from one pen to another.

All piglets were fasted on days 0 and 1 with water available *ad libitum*. From day 2, piglets were fed *ad libitum* with standard feed for weaned piglets (Hope Farms bv, Woerden, The Netherlands). At day 4, all piglets were orally infected with rotavirus. At day 5, 20 randomly chosen piglets, one from each pair, were brought to a separate stable and were orally inoculated with 5 ml 10^9 c.f.u. F4+ *E. coli*/ml. Four hours p.i., a rectal faecal sample was taken of the inoculated piglets and they were returned to their pen mates (contact piglets). At day 6 rectal faecal samples were taken from all piglets at 24 and 28 h p.i. From day 7 rectal faecal samples were taken once daily. The number of F4+ *E. coli*/g faeces was determined for all samples following excretion by the inoculated piglets, to see whether transmission to the contact piglets had occurred. At the daily sampling, faeces were observed and a 4-point scoring scale (0 = normal, 1 = shapeless, 2 = diarrhoea, 3 = liquid) was used to describe the consistency. Also the percentage dry matter of the faeces was determined and all piglets were checked daily for their health. On day 19 the remaining piglets were euthanized, bled and necropsied. A 5–10 cm jejunal sample was taken for determination of the F4R status by brush border adhesion assay (BBA). The local Ethics Committee for Animal Experiments approved the experimental protocols.

Inoculation

Rotavirus strain RV277 is maintained at the facilities of the Animal Sciences Group and was originally isolated from piglets with rotaviral neonatal diarrhoea. The average virus concentration, determined by negative stain electron microscopy, was $1{\cdot}0 \times 10^6$ particles/ml.

E. coli serotype O149K91F4ac (LT+, STb+), strain CVI-1000 (Animal Sciences Group, Lelystad, The Netherlands) [16], was isolated from a pig farm with PWD. As a negative control in the BBA, *E. coli* strain CVI-1084 (Animal Sciences Group, The Netherlands) was used. This strain is identical to CVI-1000 but without fimbrial expression of F4ac. The strains were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA), pelleted by centrifugation, resuspended in phosphate buffer solution (PBS) pH 7·2 (Biotrading, Mijdrecht, The Netherlands), to an absorption value of 1·050 at 600 nm which corresponds to a suspension of 10^9 c.f.u/ml.

Inoculation efficacy was calculated as the fraction of the inoculated piglets that had become infectious

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according to our infectiousness measure (see later). Inoculation efficacies of F4R+ and F4R− piglets were studied using Fisher's exact test for association.

Analysis of faeces

Determination of percentage dry matter

Faecal samples (0·8–4·3 g) were weighed into aluminium trays. Samples were desiccated for 22 h in an incubator at 80 °C, and weighed again to determine water loss.

Determination of F4+ E. coli*/g faeces*

Ten-fold dilutions of faeces homogenized in saline (Biotrading, The Netherlands) were plated on selective His-agar plates containing 5% sheep blood, 50 μg/ml streptomycin, 25 μg/ml tetracycline and 50 μg/ml vancomycin (Biotrading, The Netherlands). Haemolytic colonies of F4+ *E. coli* were counted with a lower limit of 100 c.f.u. F4+ *E. coli*/g faeces. In cases of uncertainty regarding the colony morphology, identity was confirmed by slide agglutination with pig sera (Animal Sciences Group, The Netherlands) to establish the *E. coli* OK type.

Determination of F4R status

At necropsy, 5–10 cm of jejunal mucosa was scraped off and epithelial brush borders were prepared to determine the F4R status of the piglets. The method was essentially that of Sellwood et al. [7]. Mucosal scrapings were put in PBS containing 0·005 M EDTA (Merck, Darmstadt, Germany) at 4 °C. Tissue was disrupted and dispersed by Ultrathorax, followed by filtration through a 100-μm mesh gauze. This filtrate was centrifuged for 10 min at 500 ***g*** to collect the cells. Cells were resuspended in PBS containing 0·05% D(+) mannose (Merck, Germany) and a CVI-1000 suspension of 0·25 ml containing 10^9 bacteria/ml PBS was added to 0·25 ml of the cell suspension. A second 0·25 ml cell suspension with a 0·25 ml CVI-1084 (F4−) suspension (10^9 bacteria/ml PBS) was added and served as a negative control. The samples were gently mixed at room temperature for 45 min. A small aliquot was put on a slide under a coverslip, and bacterial adherence was determined by phase contrast microscopy (magnification ×400). Only cells with well-defined brush borders were studied. Animals with no or an average of 1–2 bacteria/brush border were consi dered F4R− ; samples exceeding this were judged F4R+. In case of ambiguity, the test was repeated.

Determination of clinical parameters

To classify piglets as having diarrhoea or having normal faeces, a principal component analysis (PCA) on faecal dry matter data (% DM) was performed in an earlier study (Geenen et al., unpublished observations). Unfortunately this did not result in a measure that could distinguish two significantly different groups. Therefore, we made a second attempt on the dataset of the former study in which we truncated all % DM values >25% to 25%, the mean % DM of normal faeces. Truncation was performed because we were interested in the effect that F4+ *E. coli* toxins would have on % DM and these toxins mainly cause fluctuations in % DM below 25%. Fluctuations above 25% were regarded as having other causes.

After truncation was applied, PCA was performed again on this dataset. The Maximum Likelihood Discriminant Rule [17] was applied on the first principal component resulting from the PCA, and it was concluded that by using this measure based on the truncated % DM data we can distinguish two significantly different groups ($P=0{\cdot}00$). The fractions of piglets in groups 1 and 2 (0·482 and 0·518) and means and variances of the underlying distributions were estimated by maximum likelihood with the program EMMIX [18, 19]. The boundary value with the most optimal allocation of the error over the two types of error terms found was $-5{\cdot}16$. Piglets of which Σ coefficient $\%\,\mathrm{DM1}_k * (\%\,\mathrm{DM}_k - \mu\,\%\,\mathrm{DM}_k) > -5{\cdot}16$ ($k=1, 2, \ldots, 8$) were classified as having diarrhoea, in which coefficient $\%\,\mathrm{DM1}_k$ is the truncated % DM of an individual piglet at day k and coefficient $\%\,\mathrm{DM1}_k$ and $\mu\,\%\,\mathrm{DM}_k$ are the coefficients and means obtained from the PCA. For the inoculated piglets day 1 ($k=1$) is the first day after inoculation and for the contact piglets day 1 is the first day a positive F4+ *E. coli* sample was found. When no F4+ *E. coli*-positive samples were found, k was varied from 1 to 7 and for each individual the most frequently found outcome (diarrhoea or normal) was taken as the result. The association between piglets with and without diarrhoea and their F4R status and classification in high and low shedders was studied using Fisher's exact test for association.

The major objection to using this measure is that the truncated % DM data does not follow a normal

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distribution. Therefore we also used an alternative test and the agreement in outcome of both tests has been quantified using the kappa value [20].

To see whether piglets were suffering from diarrhoea during the experiment, their faeces were observed daily and a 4-point scale (0 = normal, 1 = shapeless, 2 = diarrhoea, 3 = liquid) was used to describe the consistency. In this second test, only piglets with one or more samples with a score of 3 were considered to have severe clinical symptoms. The association between these piglets and their F4R status and classification in high and low shedders was studied using Fisher's exact test for association.

Weight gain of the piglets was calculated as the mean weight gain over 19 days (g/day). It was tested whether high shedders and piglets with severe diarrhoea had a lower weight gain using the Mann–Whitney U test. Fisher's exact test and Mann–Whitney U test were performed with GenStat [21].

Determination of transmission parameters

Calculations of the transmission parameters were based on the stochastic SIR model [22]. In this model individuals are susceptible (S), infectious (I) or recovered and immune (R). The rate at which new infections occur is $(\beta \cdot S \cdot I)/N$, where β is the infection rate parameter and N the total number of individuals (here $N=2$). The probability of a susceptible animal to become infected within an interval Δt, is $1-e^{-\beta \cdot \Delta t \cdot (I/N)}$. From the data of the transmission experiment it is known between which subsequent samplings the contact piglets start excreting F4+ *E. coli*. We assumed that infection of the contact piglet (a case) occurred 1 day before the first F4+ *E. coli*-positive sample was found. This assumption was based on findings that after inoculation with F4+ *E. coli* most piglets started shedding F4+ *E. coli* 1 day after infection. As we were interested in following the infection chain, we defined a contact infection as an individual that had picked up the infection and was infectious for others. Therefore, in our definition a contact infected piglet was a piglet that shed a sufficient amount of F4+ *E. coli* to be infectious for others (for definitions of infectiousness, see below). The number of cases (C) in a period Δt follows a binomial distribution with parameter $1-e^{-\beta \cdot \Delta t \cdot (I/N)}$ and index S, the number of susceptible individuals at the start of the period. Thus the relation between the expected number of cases per unit of time E(C) and I, N, S and β is $E(C) = S \cdot (1-e^{-\beta \cdot I/N})$. Since S, I, N and C were known from the transmission experiment, β was estimated using a generalized linear model (GLM) [23]. For each of the F4R status combinations one β was estimated; β_{pp}, β_{pn}, β_{np} and β_{nn}, in which the first letter in the subscript is the F4R status of the contact piglet and the second letter is the F4R status of the inoculated piglet (p = positive, n = negative). A GLM with a complementary log-log link function and log (I/2) as offset variable was used [24]. GLMs were performed with GenStat [21].

An important transmission parameter is the reproduction ratio (R_0) which is defined as the average number of secondary infections that one typical infectious individual will cause during its entire infectious period in a population of susceptible individuals only. R_0 for this model is $R_0 = \beta \cdot T$, where β is the infection rate parameter and T is the average infectious period. T was calculated as the number of days from the first until the last F4+ *E. coli*-positive sample. It was hypothesized that F4R+ and F4R− piglets differed in susceptibility and in infectiousness. Therefore R_0 for heterogeneous populations was calculated depending on the fraction of F4R+ piglets (f) in the population, which is the dominant eigenvalue of matrix **K**

$$\mathbf{K} = \begin{pmatrix} f \cdot \beta_{pp} \cdot T_p & f \cdot \beta_{pn} \cdot T_n \\ (1-f) \cdot \beta_{np} \cdot T_p & (1-f) \cdot \beta_{nn} \cdot T_n \end{pmatrix}.$$

From this it follows that $R_0(f) = \frac{1}{2}(k_{11}+k_{22}+\sqrt{(k_{11}+k_{22})^2-4(k_{11}k_{22}-k_{12}k_{21})})$ [25]. The maximum fraction of F4R+ piglets with which major outbreaks of F4+ *E. coli* can be prevented was calculated by assigning $R_0 = 1$ and assigning the estimated values to the βs and T.

To determine whether piglets are infectious or not we assumed that (1) high shedding piglets were infectious, or as an alternative (2) every piglet with one or more F4+ *E. coli*-positive samples was infectious (independent of the number of *E. coli*/g).

All piglets of which the sum: Σ coefficient $1_k * (\ln \text{cfu}_k - \mu \ln \text{cfu}_k)$, with $k = 1, 2, \ldots, 8$, was smaller than 1·96 were high shedders (Geenen et al., unpublished observations). ln cfu_k are the log-transformed numbers of F4+ *E. coli*/g +1 found in the faecal samples of the inoculated piglets for days 1–8. For the contact piglets we determined day 1 to be the first day an F4+ *E. coli*-positive sample was found. For missing values a value of 0 was given. The values of the coefficient ln $\text{cfu}1_k$ and $\mu \ln \text{cfu}_k$ were obtained from an earlier study (Geenen et al., unpublished observations) and are given in Table 1.

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Table 1. *Coefficient and mean obtained from an earlier study* (*Geenen et al., unpublished observations*), *for classification of high- and low-shedding piglets*

k	Coefficient ln cfu1$_k$	μ ln cfu$_k$
1	−0·1792	7·031
2	−0·34811	7·212
3	−0·39279	6·634
4	−0·44959	6·664
5	−0·43543	5·844
6	−0·38253	4·757
7	−0·33518	3·827
8	−0·205	2·57

RESULTS

Mortality and F4R status

Two piglets were found dead during the experiment; one inoculated piglet (6160) died of severe dehydration due to PWD on day 6 and one contact piglet (6177) of another pair died on day 11 and had clinical signs of sepsis at post-mortem. F4R status of these two piglets could not be determined. Of the remaining 38 piglets, 18 were determined F4R+ and 20 F4R−.

Bacteriological examination and determination of shedding type

No haemolytic *E. coli* were found on the rectal swabs upon arrival. Table 2 shows the results of the determination of F4+ *E. coli*/g faeces of all faecal samples, sorted on F4R status combination. Two out of 19 faecal samples that were taken 4 h after inoculation were F4+ *E. coli*-positive. Data of these samples were not taken into account for the calculation of high and low shedders nor for the calculation of transmission parameters.

All four combinations of contact/inoculated pigs: F4R+/F4R+ (5); F4R−/F4R+ (3); F4R+/F4R− (5); F4R−/F4R− (5) were present. From five contact piglets F4+ *E. coli*-positive samples were found, four in F4R+/F4R+ pairs and one in an F4R−/unknown pair. The F4R status of the inoculated piglet in this last pair could not be determined as it died due to severe diarrhoea. The last column of Table 2 shows whether the piglet was determined a high or low shedder based on classification by its temporal shedding profile.

We determined that of three inoculated piglets all faecal samples were negative for F4+ *E. coli* until day 8. As it is unlikely that pigs will start shedding this many days after inoculation, they were euthanized together with their contact piglets on day 9.

Inoculation efficacies of the F4R+ and F4R− piglets were 0·67 (6/9) and 0·0 (0/11) respectively. Association of receptor status and shedding type after inoculation is highly significant ($P<0·01$, Fisher's exact test). Thus, F4R+ piglets were more susceptible for F4+ *E. coli* than F4R− piglets.

All contact piglets that had *E. coli*-positive faecal samples were also high shedders with the exception of piglet 6161 (Table 2). The shaded parts show the F4+ *E. coli*-positive samples and the number of F4+ *E. coli*/g. The inoculated infectious F4R+ piglets shed F4+ *E. coli* for a longer period (mean 11·4 days) than the contact-infected F4R+ piglets (mean 7·0 days). This difference was significant ($P<0·01$, Student's t test).

Clinical parameters

PCA measure on truncated % DM data

Using the measure derived from the PCA on the truncated % DM data, 26 piglets (65·0%) had diarrhoea. Thirteen of these diarrhoeic piglets were F4R+, eleven were F4R− and two were unknown. Nine of the diarrhoeic piglets were high shedders and 17 were low shedders. Association calculated on the 2×2 table using Fisher's exact test resulted in no significant association with F4R status ($P=0·22$) and no significant association with high and low shedding ($P=0·06$). Three out of four contact infected piglets had diarrhoea.

Clinical scores

In total, 589 faecal samples were collected of which 35 samples were given a score of '3' (severe diarrhoea). The mean % DM of these samples was 8·6 (S.D. = 2·8). These 35 samples were taken from 17 piglets (34%) of which eight were high shedders and nine low shedders. Eleven piglets with a score of '3' were F4R+, four were F4R− and two were unknown. Association of shedding with severe diarrhoea resulted in $P=0·01$ and association of receptor status and severe diarrhoea resulted in $P=0·01$ (Fisher's exact test). Thus classification into high and low shedding and receptor status were both significantly associated with the occurrence of severe diarrhoea. Three out of four cases had severe diarrhoea for 1 or more days. Not all samples scoring '3' could be assigned to high numbers of F4+ *E. coli* in the faeces. Only 16 samples (45·7%)

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Table 2. *Number of F4+* E. coli/*g faeces*

Stable pen	pig no.[a]	Time after inoculation: 4 h	1 d	28 h	2 d	3 d	4 d	5 d	6 d	7 d	8 d	9 d	10 d	11 d	12 d	13 d	14 d	Weight gain[b]	Shedding type[c]
F4R+ /F4R+[d]																			
1·2	6158i		$1·9\times10^{4}$	$3·2\times10^{4}$	$1·1\times10^{5}$	$4·4\times10^{5}$	$4·6\times10^{8}$	$1·9\times10^{10}$	$1·9\times10^{8}$	$1·4\times10^{8}$	$2·9\times10^{7}$	$1·7\times10^{5}$		$7·0\times10^{3}$			$1·0\times10^{3}$	142·11	High
	6159c	n.d.					$1·6\times10^{6}$	$1·4\times10^{7}$	$6·6\times10^{6}$	$5·6\times10^{5}$	$1·3\times10^{7}$	$1·2\times10^{5}$	$7·3\times10^{2}$	$6·0\times10^{2}$				57·89	High
2·1	6164i	$4·2\times10^{4}$																126·32	Low
	6165c	n.d.																242·11	Low
4·1	6181i		$8·2\times10^{4}$	$3·6\times10^{4}$	$2·4\times10^{6}$	$1·5\times10^{6}$	$7·0\times10^{4}$	$6·5\times10^{4}$	$4·3\times10^{3}$	$1·0\times10^{2}$	$3·1\times10^{3}$	$9·7\times10^{3}$	$9·9\times10^{3}$	$3·7\times10^{3}$	$1·6\times10^{3}$			142·11	High
	6180c	n.d.					$5·8\times10^{4}$	$1·4\times10^{4}$	$2·5\times10^{4}$	$6·7\times10^{5}$	$3·3\times10^{7}$	$2·9\times10^{8}$	$1·7\times10^{6}$	$1·6\times10^{4}$	$1·0\times10^{2}$			57·89	High
4·4	6186i		$5·0\times10^{4}$	$8·9\times10^{3}$	$2·7\times10^{4}$	$1·2\times10^{5}$	$1·3\times10^{6}$	$3·0\times10^{7}$	$6·0\times10^{6}$	$2·6\times10^{3}$	$2·0\times10^{2}$		$3·0\times10^{2}$					173·68	High
	6187c	n.d.										$1·1\times10^{5}$	$1·7\times10^{4}$	$1·1\times10^{4}$	$6·0\times10^{4}$			152·63	High
5·4	6195i		$1·7\times10^{7}$	$1·1\times10^{8}$	$2·5\times10^{9}$	$4·9\times10^{9}$	$2·0\times10^{9}$	$1·3\times10^{9}$	$2·4\times10^{9}$	$4·4\times10^{7}$	$8·0\times10^{6}$	$5·2\times10^{3}$	$2·4\times10^{3}$		$1·1\times10^{2}$			84·21	High
	6194c	n.d.			$5·4\times10^{5}$	$7·8\times10^{5}$	$1·5\times10^{4}$	$3·0\times10^{4}$	$1·0\times10^{3}$	$8·0\times10^{2}$	$2·0\times10^{3}$							200·00	High
F4R− /F4R−																			
1·1	6157i		$1·0\times10^{3}$	$9·9\times10^{4}$														63·16	Low
	6156c	n.d.																36·84	Low
1·4	6163i			n.d.			$5·4\times10^{4}$	$1·0\times10^{4}$										252·63	Low
	6162c	n.d.																236·84	Low
2·2	6167i		$2·5\times10^{5}$	$1·1\times10^{4}$														178·95	Low
	6166c	n.d.																231·58	Low
5·1	6188i				$3·0\times10^{4}$	$1·1\times10^{4}$												42·11	Low
	6189c	n.d.																189·47	Low
5·2	6191i		$2·7\times10^{5}$	$1·1\times10^{5}$														200·00	Low
	6190c	n.d.																226·32	Low
F4R− /F4R+																			
2·3	6169i												//////////	//////////	//////////	//////////	//////////	n.d.	Low
	6168c	n.d.									n.d.		//////////	//////////	//////////	//////////	//////////	n.d.	Low
2·4	6171i		$2·3\times10^{5}$															173·68	Low
	6170c	n.d.																205·26	Low
5·3	6192i		$7·0\times10^{2}$	$1·6\times10^{3}$		$1·0\times10^{2}$	$1·5\times10^{5}$	$3·0\times10^{4}$	$2·9\times10^{6}$	$1·5\times10^{6}$	$2·2\times10^{6}$	$1·4\times10^{5}$						242·11	High
	6193c	n.d.																252·63	Low
F4R+ /F4R−																			
3·1	6173i												//////////	//////////	//////////	//////////	//////////	n.d.	Low
	6172c	n.d.											//////////	//////////	//////////	//////////	//////////	n.d.	Low
3·2	6175i	n.d.	$4·0\times10^{2}$			$2·4\times10^{2}$												184·21	Low
	6174c	n.d.		n.d.														57·89	Low

3·4	6178i										////////	////////	////////	////////	////////	n.d.	Low
	6179c	n.d.									////////	////////	////////	////////	////////	n.d.	Low
4·2	6182i		$2{\cdot}0\times10^{3}$	$9{\cdot}0\times10^{2}$												5·26	Low
	6183c	n.d.														178·95	Low
4·3	6185i		$1{\cdot}8\times10^{4}$	$2{\cdot}0\times10^{3}$						$1{\cdot}0\times10^{8}$						168·42	Low
	6184c	n.d.														215·79	Low
F4R−/?																	
1·3	6160i		$5{\cdot}0\times10^{5}$	$8{\cdot}0\times10^{6}$	$9{\cdot}9\times10^{6}$	$4{\cdot}9\times10^{7}$	$2{\cdot}4\times10^{8}$	$3{\cdot}1\times10^{8}$	$1{\cdot}1\times10^{10}$	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	n.d.	High
	6161c	n.d.							$3{\cdot}5\times10^{3}$		$7{\cdot}0\times10^{8}$					131·58	Low
? /F4R−																	
3·3	6176i															210·53	Low
	6177c	n.d.										XXXX	XXXX	XXXX	XXXX	n.d.	Low

[a] A pig no. followed by 'i' is an inoculated piglet, and followed by 'c' a contact piglet.
[b] Weight gain over 19 days (g/day).
[c] Classification into high shedders and low shedders based on temporal *E. coli* F4 shedding profiles.
[d] Receptor status combination: status contact piglet followed by status inoculated piglet.
n.d., Not done.
XXXX, Dead.
////////, Killed early, no *E. coli* F4-positive samples found.

taken from nine piglets were found positive for F4+ *E. coli* on the same day.

To compare the two tests, the PCA measure and the clinical scores, the agreement in results were expressed as the kappa value [20]. Both tests assigned 14 piglets to the diarrhoea group and 17 piglets to the normal faeces group. The remaining nine piglets were assigned by the 'score 3' test to the normal group whereas the % DM measure assigned them as having diarrhoea. The agreement in results, expressed as the kappa value, was 0·57. This is regarded as an acceptable level of agreement between the two tests [20].

Weight gain

Weight gain of the individual piglets is shown in Table 2. The mean weight gain of the high-shedding piglets was 165·7 g/day (S.D. = 75·4) and 139·2 g/day (S.D. = 63·2) for the low-shedding piglets which was not significantly different, $P = 0{\cdot}20$, Mann–Whitney U test. The mean weight gains for diarrhoeic and non-diarrhoeic piglets classified by the % DM measure were 163·7 g/day (S.D. = 72·6) and 147·8 g/day (S.D. = 73·7). For the diarrhoeic and non-diarrhoeic piglets classified by 'score 3', the mean weight gains were 119·9 g/day (S.D. = 68·0) and 188·0 g/day (S.D. = 55·2) respectively. Only with the 'score 3' classification did diarrhoeic pigs have a significantly lower weight gain than piglets with normal faeces, $P < 0{\cdot}01$ (% DM measure, $P = 0{\cdot}25$) based on the Mann–Whitney U test.

Transmission parameters

Transmission parameters β were estimated (1) under the assumption that a 'high shedder' is infectious and alternatively (2) that a piglet with '$\geqslant 1$ positive sample' is infectious. The estimated βs and the matching 95 % confidence intervals (CIs) are shown in Table 3. Since piglet 6160 shed a very high number of F4+ *E. coli* and suffered from severe PWD, we assumed that it was a F4R+ piglet. Unfortunately this inoculated piglet died soon after the moment its contact piglet 6161 picked up the infection. The contact piglet alone was not able to sufficiently replicate F4+ *E. coli* to become a case according to the measure of 'high shedder'. We do not rule out the possibility that it would have become infectious if the inoculated piglet had remained alive and had shed F4+ *E. coli* for some more days. We took the data of this pair into account to calculate β_{np} as a worst-case scenario and this result is also shown in Table 3.

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Table 3. *Estimates of the transmission parameters β and their 95% confidence interval (CI) of the four type of pairs using two different measures of infectiousness*

Measure of infectiousness	Estimate of β	95% CI
'High shedder'	$\beta_{pp} = 0{\cdot}62$	0·19–2·06
	$\beta_{np} = 0{\cdot}00$*	0·00–1·98
	$\beta_{np} = 0{\cdot}16$†	0·03–0·75
'$\geqslant$1 F4+ *E. coli*-positive sample'	$\beta_{pp} = 0{\cdot}58$	0·19–1·75
	$\beta_{np} = 0{\cdot}15$	0·03–0·66

* Excluding pair 6160/6161 as a case.
† Including pair 6160/6161 as a case.

As no infectious piglets and no contact infections were observed in the F4R+/F4R− and F4R−/F4R− pairs we could not estimate transmission parameters β_{pn} and β_{nn}. In the F4R−/F4R+ pairs only one infectious piglet but not contact infections were observed, thus, β_{np} is estimated as 0. The upper limit of the confidence interval was calculated assuming that all three inoculated piglets of the F4R−/F4R+ pairs were infectious. Assuming this, the upper limit (β_{upper}) of the 95% CI can be calculated by: $\beta_{upper} = 2 \cdot \ln(1 - P)$, $\Pr(C = 0|P) = (1 - P)^n = 0{\cdot}05$; C is the number of cases and n is the number of pairs.

To evaluate whether 'high shedder' and '$\geqslant$1 F4+ *E. coli*-positive sample' were good measures for infectiousness, the association of the inoculated piglets being 'high shedder' or having '$\geqslant$1 F4+ *E. coli*-positive sample' with the number of their contact piglets that became 'high shedder' or had '$\geqslant$1 F4+ *E. coli*-positive sample' was tested with Fisher's exact test. For 'high shedder' a P value $<0{\cdot}01$ was found (both with and without piglet 6161 as a case) and for '$\geqslant$1 F4+ *E. coli*-positive sample sample' $P = 0{\cdot}53$.

The reproduction ratio calculated for homogeneous F4R+ piglet populations was estimated 7·1 ($T = 11{\cdot}4$) with 95% CI (2·3–21·9). R_0 for homogeneous F4R− piglet populations could not be calculated, as there were no cases observed in the F4R−/F4R− pairs.

To calculate $R_0(f)$ we assumed that $\beta_{nn} = 0$, $R_0(f) = f \cdot \beta_{pp} \cdot T_p$. Thus, R_0 is at unity $f = 1/(\beta_{pp} \cdot T_p)$. In order to make $R_0(f) < 1$, the fraction of F4R+ piglets must be lower than 0·14.

DISCUSSION

In this study we have shown that F4R+ piglets were more susceptible than F4R− piglets and that F4R+ piglets were able to infect other piglets. This study is inconclusive as to whether F4R+ piglets are also more infectious than F4R− piglets as none of the inoculated F4R− piglets became infectious.

We evaluated the measures 'high shedder' and '$\geqslant$1 positive sample' as measures for infectiousness. We concluded that 'high shedder' is a useful measure for infectiousness as it has a high association with the cases found in this study. It is a better measure for infectiousness than '$\geqslant$1 positive sample' which had a very low association with the cases found. Using the measure 'high shedder', we found that although F4R− piglets did shed some F4+ *E. coli*, replication within the intestine was not sufficient for the piglets to become infectious after inoculation or after picking up the infection from the environment.

Considering the range of expected responses and receptor status combinations studied, 40 piglets might have been insufficient to estimate all parameters. However, from earlier studies it was known that the percentage of F4R+ piglets in the herd was approximately 50%, which made it very likely that all receptor-status combinations would be present in this experiment. As this was the first transmission study on F4+ *E. coli*, it was not possible to calculate the minimum number of pigs needed to estimate all transmission parameters. Due to practical constraints, we restricted the number of piglets to 40.

We have calculated that with the estimated transmission parameters from our study, the fraction of F4R− piglets in the population must be higher than $1 - (1/\beta_{pp} \cdot T_p)$ to eradicate F4+ *E. coli* from this population. This result is similar to the critical proportion of the population that needs to be successfully immunized to eradicate a microparasite [26] and almost similar to the findings on the proportion of homozygous pigs for a fictive major disease resistance gene to bring R_0 below 1, assuming an underlying pig farm structure [27].

The main feature of this result is that it is not necessary for the entire population to be F4R− to bring R_0 under unity. Whether indeed the F4R− piglets indirectly protect the F4R+ piglets by a weaker force of infection we cannot tell from this experiment as none of the inoculated F4R− piglets was infectious and consequently we could not estimate transmission parameters β, for these pairs.

The infection pressure within a one-to-one experiment might be considerably lower than in a group of piglets. Therefore, we could have underestimated the role of F4R− piglets in transmission, as they might need higher infection pressure to become infectious

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themselves. In case $\beta_{nn} > 0$ the fraction of F4R+ piglets in the population should be even lower than the 0·14 calculated in this study.

The longer average infectious period of F4+ *E. coli* excretion in high-shedding inoculated F4R+ piglets compared to high-shedding contact F4R+ piglets might have several causes. The rapid physiological changes and flora shifts that occur after weaning could have made the contact piglets, which are one or more days older at the moment of infection, less susceptible. Also the way in which infection is acquired (inoculum or environment), the dose and the vehicle (PBS or faeces) might influence the outcome of infection. Also reinfection of the inoculated piglet by an infectious contact piglet might cause extended excretion periods of the inoculated piglet. This will all lead to overestimation of R_0. This can be prevented by setting up a so-called extended transmission experiment in which, as soon as the majority of the contact piglets pick up the contact infection, the inoculated piglets are replaced by new contact piglets [28]. However, differences in age between the infectious contact piglet and the new contact piglet and the resulting behavioural differences might affect the contact pattern and amount of stress. Furthermore, determining the right moment of replacement of the inoculated piglets is complicated, as we have seen there can be large differences in the moment of infection.

To study the clinical symptoms we have used and compared two different classifications based on the severity of diarrhoea and we have studied the weight gain of individual piglets. The two classifications, one based on the PCA measure obtained from truncated %DM data and the other on one or more faecal samples with 'score 3' (visual observation of liquid faeces), have an acceptable agreement and, thus, both can be used. The PCA measure has the advantage that it is better repeatable than the more subjective measure 'score 3'. The fact that only 45·7% of the 'score 3' samples were positive for F4+ *E. coli* on the same day and that nine low-shedding piglets were classified as diarrhoeic means that besides F4+ *E. coli* other diarrhoeagenic agents and causes, e.g. rotavirus could have provoked diarrhoea.

Although the role of rotavirus in the aetiology of PWD is not clear, it is likely that rotavirus, by damaging the epithelium and thereby changing the small intestinal environment in favour of F4+ *E. coli*, is a predisposing factor in outbreaks of PWD [29]. It is unknown whether interference of rotavirus with the intestinal mucosa integrity affects F4R detection. In this study we did not find any indication that this was the case.

The heterogeneity in infectiousness and susceptibility to F4+ *E. coli* found in this study raises the question whether selection on non-adherent F4R pigs is a good option as a PWD control strategy. Feasibility of this option depends on the available tests and the possible function and significance of this receptor for the pig. Until now it has been unknown, which gene or genes are responsible for expression of the F4R and only adhesion tests are available. High costs, laboriousness and the fact that pigs have to be slaughtered and, therefore, cannot be used for breeding purposes are serious drawbacks for the common adhesion tests to be used on large scale, as is also discussed for selection on *E. coli*-F18 resistance [30, 31]. Moreover, it is debatable whether it is advisable to breed out a trait that might have an unknown beneficial function [32, 33] or that will change the selection pressure on pathogenic *E. coli*. We calculated that, assuming that the transmission from F4R− piglets to other piglets is 0, the maximum fraction of F4R+ piglets should be 0·14 to prevent large outbreaks of F4+ *E. coli*. Whether this is sufficient and feasible to reduce outbreaks in the field has to be studied further.

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