F4+ *Escherichia coli* in piglets: effect of host characteristics on population dynamics

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Proefschrift

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Stellingen

1. De mate van transmissie van F4+ *E. coli* wordt voornamelijk bepaald door de fractie F4R+ biggen in de populatie; door het beperken van deze fractie zullen grote uitbraken van F4+ *E. coli* worden voorkomen.
   (Dit proefschrift)

2. Uit interventiestudies van F4+ *E.coli*, zowel challenge- als transmissie studies, kunnen alleen juiste conclusies worden getrokken, wanneer bij de analyse van de experimenten de F4 receptor status van de biggen wordt meegenomen als co-variabele.
   (Dit proefschrift)

3. Gezien het positieve effect van groene thee op de oxidatieve gevolgen van alcohol, is het wachten op mixdrankjes met groene thee.
   Dobrzynska et al., Chem. Biol. Interact. (2005), in press.

4. Tijdens het verplicht ophokken wegens AI, zouden pluimveehouders van kippen met uitloop en handelaren in producten van dergelijke kippen, een lagere prijs voor hun producten moeten vragen, om te laten zien dat zij de uitloop van kippen belangrijk vinden voor het dierenwelzijn.

5. Pas wanneer we in onze slaapkamers weer wakker liggen van de malariamug, krijgt de malariabestrijding de aandacht die het nu al verdient.

6. Vrienden maken het leven niet alleen mooier maar ook langer.
   Giles et al., J. Epidemiol. Community Health (2005) 59; 574-579.

Stellingen behorende bij het proefschrift van Petra Geenen:

F4+ *Escherichia coli* in piglets:
effect of host characteristics on population dynamics

Wageningen, 3 oktober 2005

Abstract

Post-weaning diarrhoea (PWD) is a multifactorial disease of newly-weaned piglets that occurs in the first two weeks after weaning. PWD causes growth retardation and increased mortality, resulting in reduced animal welfare and economical damage. The main causative agent of PWD is enterotoxigenic *Escherichia coli* expressing F4 fimbriae (F4+ *E. coli*). Intervention measures for F4+ *E. coli* can be aimed at preventing diarrhoea or at preventing transmission and should be evaluated in challenge or transmission studies. In this thesis, transmission of F4+ *E. coli* was studied and quantified. Factors affecting the population dynamics of this bacterium were investigated in individual piglets and at population level. Special interest was taken in the F4 receptor (F4R), a specific adhesion site for F4+ *E. coli* in the small intestine. Not all pigs express F4R; pigs with adhesive (F4R+) and non-adhesive brush borders (F4R–) can be distinguished by *in vitro* adhesion tests.

The set up and analysis of transmission experiments with F4+ *E. coli* are described in this thesis and a definition of the infectious state based on the shedding patterns of individual animals was developed. No indication was found that reinfection affected the population dynamics of F4+ *E. coli*.

F4R+ piglets were found to be more susceptible to become infectious than F4R– piglets. It was concluded that the F4R status of the pig has a strong effect on the population dynamics of F4+ *E. coli*. The F4R status should therefore be used as a co-variate in the statistical analysis of F4+ *E. coli* intervention studies, both in challenge and transmission studies. The level of transmission is mainly dependent on the fraction of F4R+ piglets in the population. By reducing the fraction of F4R+ piglets in the population to <0.09 large outbreaks of F4+ *E. coli* will be prevented.

The heterogeneity in susceptibility may serve as a point of departure to control F4+ *E. coli* by selective breeding for F4R– pigs. With a simple discrete model, it was illustrated that selective breeding for F4R– piglets by using F4R– boars, is an effective way to reduce the fraction of F4R+ piglets in the population, given the presence of a sensitive F4R status test.

Wat mij belangt ik oordeel van mijn selven datter sooveel menschen niet leven in onze vereenigde Nederlanden, als ick heden levende dieren in mijn mond draag. ... ik imagineerde mij wel 1000 levende dierkens te sien in een quantiteit materie, die niet grooter was als een honderste part van een sand groote.

- Antoni van Leeuwenhoek (1632-1723)
Brief aan de Royal Society, 17 September 1683

Voor mijn moeder

Voorwoord

Vol trots presenteer ik jullie mijn proefschrift. Laat ik beginnen met kort uit te leggen, waarom ik heb gekozen voor de afbeelding op de voorkant. Onderzoek doen aan een bacterie die diarree veroorzaakt, is niet bepaald reukloos. Niet alleen ik, maar ook de omgeving van de diverse labs waar ik heb gewerkt hebben mee mogen genieten van de luchtjes die mijn onderzoek verspreidde. Vandaar dat de voorkant van dit proefschrift is geïnspireerd op het schilderij 'De reuk' uit de reeks 'De zintuigen' van Jan Miense Molenaer (Haarlem, ca. 1610-1668). Op het originele schilderij staat een tafereel afgebeeld waarin een moeder haar kind verschoont middenin een café.

Ik ben heel dankbaar voor de ondersteuning die ik van velen heb mogen ontvangen tijdens dit onderzoek, zowel op het werk als van familie en vrienden. Allereerst wil ik graag de drie mensen bedanken die vanaf het eerste begin (of bijna eerste begin) tot aan het eind zeer betrokken zijn geweest bij dit project: Annemarie Bouma, Jan van der Meulen en mijn promotor Mart de Jong. Annemarie, Jan en Mart, enorm bedankt voor alle samenwerking, goede adviezen en het lezen en corrigeren van alle stukken. Ik heb heel veel van jullie geleerd! Naast deze 'kleine stuurgroep', hebben heel veel mensen voor langere of kortere tijd meegedacht over de wetenschappelijke en praktische invulling van dit project; Arjan Stegeman, Marius Nabuurs, Jos Verheijden, Fred van Zijderveld, Norbert Stockhofe, Aline de Koeijer, Hans Heesterbeek, Dörte Döpfer en Gonnie Nodelijk. Heel erg bedankt voor jullie interesse en inzet voor dit project. Daarnaast heb ik uiteraard met vele anderen mensen op het ASG van gedachten mogen wisselen over *E. coli* en speendiarree, ik wil met name Theo Niewold en Michiel Harmsen noemen. Bedankt voor jullie interesse.

Tijdens de uitvoering van mijn experimenten heb ik meerdere labs mogen 'bewonen' en ik wil iedereen die zich over mij, mijn samples en de biggen hebben ontfermd graag bedanken voor de plezierige samenwerking. Ten eerste de dierverzorgers en de stagiaires van dierverzorging; hartelijk dank voor jullie goede verzorging van de biggen en het nemen van de samples. Ank van Zijderveld, hartelijk dank dat je mij de nodige microbiologische technieken hebt geleerd en dat het inoculum altijd weer stipt op tijd klaar stond. Ad & Ad: de sectiezaal was niet mijn favoriete stekkie, maar met jullie erbij was het altijd interessant. Esther van Andel, Arie Hoogendoorn en Gert Jan de Graaf; onzettend bedankt voor jullie inzet bij de diverse analyses van de samples. Bij het uitplaten en tellen van de coli's van de transmissie-experimenten heb ik veel ondersteuning gehad van Henk Wisselink, Hilde Smith, Frans Putirulan en Albert de Boer. Hartelijk dank dat jullie met mij deze berg werk hebben verzet! Na de lab-analyses volgden de statistische analyses; Joop, Bas en Willem, hartelijk dank voor jullie uitleg en hulp.

Naast werk was er ook voldoende afleiding in Lelystad. Met name van het samen thee drinken, de 'leermomentjes', de uitjes en sportdagen heb ik enorm

genoten. Herman, Aline, Dörte, Elly, Michiel, Joop, Jan, Bas, Willem, Thomas, Gonnie, Gert Jan, Don, Annet en natuurlijk mijn kamergenote Liesbeth, bedankt voor alle gezelligheid! Liesbeth dankjewel dat je mijn paranimf wil zijn, binnenkort vieren we vast ook jouw feestje! Ook het aio-overleg zorgde voor de nodige in- en ontspanning naast het gewone werk. Ik heb jullie steun en enthousiasme enorm gewaardeerd en ik ben trots dat we samen zoveel voor elkaar hebben gekregen. Petra, ik vond het heel bijzonder dat we samen van Wageningen naar Lelystad zijn gegaan om aio te worden en ik ben erg blij dat ook jij mijn paranimf wil zijn. Jouw boekje komt ook vast binnenkort af! Mede door mijn schilderlessen en muziekgroepje heb ik het enorm naar mijn zin gehad in Lelystad, ik hoop dat ik in Nijmegen binnenkort weer bij net zulke gezellige mede-schilders en -muzikanten zal kunnen aansluiten.

Vanwege dit proefschrift, mijn nieuwe baan in Utrecht en de verhuizing naar Nijmegen waren de afgelopen twee jaar wel bijzonder hectisch, maar daarom zeker niet minder leuk. Een beetje eng vond ik het in het begin wel als bioloog tussen de informatici, informatiekundigen en wiskundigen. Maar de goede sfeer van de DSS groep maakt dat ik me er helemaal thuis voel en met veel plezier iedere keer weer de reis naar Utrecht onderneem. Linda, dankjewel voor je enorme steun en vertrouwen de afgelopen twee jaar. Mădălina, Janneke, Silja, Dirk, Peter, Theodore, Eveline, Arjan, Martijn en oud-collega's Danielle, Edwin en Steven, bedankt voor jullie belangstelling en de prettige werksfeer!

Beste vrienden en vriendinnen, we hebben elkaar de afgelopen jaren niet veel gezien. Veel van jullie besloten ook te promoveren of hebben drukke banen elders gekregen. Hopelijk zien we elkaar binnenkort weer veel vaker! Arno, de eerste tijd in Lelystad was voor mij niet bepaald het makkelijkste; heel erg bedankt voor je steun. Zonder jouw enorme hulp met mijn huis had ik me in Lelystad nooit zo thuis gevoeld.

Lieve familie, het is af!!! Ik weet dat dit niet alleen voor mij, maar ook voor jullie een grote opluchting zal zijn. Bedankt dat jullie zo hebben meegeleefd. Hopelijk kunnen we elkaar nu weer wat vaker zien.

Tenslotte, lieve Bart, wil ik jou graag bedanken. Toen je me net kende, gaf je me een 'gemanipuleerde' wekker, die alleen uitkon als ik de batterij eruit wist te halen, omdat ik altijd zo'n moeite had met vroeg opstaan tijdens de dierproeven. Die wekker heb ik al heel snel laten 'verdwijnen', maar gelukkig ben jij gebleven. Jouw betrokkenheid en enorme vertrouwen dat het allemaal goed zou komen was een enorme steun voor mij de afgelopen jaren. Laten we er samen wat moois van maken in Nijmegen!

Contents

1

General introduction

General introduction

Gastro-intestinal bacterial infections are common in animal husbandry and may cause animal welfare problems as well as economical damage. Prevention and control of these diseases are major topics in veterinary research. So far, most research focussed on the reduction of clinical signs in animals. However, since these diseases are contagious, also processes at population level contribute to the development of disease in groups of animals. As a consequence, intervention measures can also be aimed at the prevention of transmission of bacteria within the population. Therefore, it is necessary to gain knowledge about the population dynamics of the bacteria by studying the interaction between individual hosts in a population and by quantifying parameters characterizing the transmission process.

The level of transmission is determined by the infectiousness of infected animals and the susceptibility of contact-exposed animals, and the contacts between these two types of animals. It is important to know that an infectious animal is not necessarily diseased and that a susceptible animal is susceptible to become infectious rather than diseased. Susceptibility and infectiousness will be affected by certain characteristics of the individual animals (host characteristics). Heterogeneity in host characteristics and their effect on transmission between animals should therefore be investigated.

An example of a gastro-intestinal bacterial infection is post-weaning diarrhoea (PWD) in newly-weaned piglets. PWD is a multi-factorial disease and one of the causative agents of this disease is enterotoxigenic *Escherichia coli* (ETEC) expressing F4 fimbriae (F4+ *E. coli*). The pathogen characteristics of F4+ *E. coli* and the disease caused by this bacterium in piglets have been studied intensively e.g. (Cox *et al.*, 1991; Hampson *et al.*, 1985; Madec *et al.*, 2000; Melin *et al.*, 2004; Nabuurs *et al.*, 1993). Most studies on PWD were aimed to understand how certain factors lead to the development of clinical signs in individual piglets. However, as mentioned before, in order to understand how PWD can be controlled by reducing transmission of F4+ *E. coli*, it is also necessary to study the interaction between individual piglets in a population and to quantify transmission parameters. Host characteristics that are likely to affect the susceptibility and infectiousness of piglets should be investigated and their effect on the level of F4+ *E. coli* transmission between piglets should be quantified.

In the following sections a brief description of PWD and the role of ETEC in the pathogenesis of PWD is given, followed by a description of F4+ *E. coli,* its virulence factors and adhesion site. Finally, the aim and outline of this thesis are presented.

Post-weaning diarrhoea

PWD is defined as diarrhoea occurring in the first two weeks after weaning without severe changes of the intestinal mucosa and with recovery of large numbers of haemolytic *E. coli* from the small intestine (Hampson, 1994). PWD causes increased mortality and growth retardation of newly-weaned piglets resulting in animal welfare problems and economic losses for the farmer. Various factors are involved of which infection with ETEC is only one, but essential factor.

ETEC can adhere to the microvilli of the small intestinal enterocytes without inducing morphological lesions. The bacteria produce enterotoxins that act locally on the enterocytes resulting in increased secretion of fluid and $NaHCO_3$ which can lead to diarrhoea, dehydration and acidosis (Nagy and Fekete, 1999).

A major risk factor for PWD is the process of weaning itself (Nagy and Fekete, 1999). First, piglets are abruptly deprived of the passive protection by immunoglobulins in the milk. Second, weaning is a stressful event and the change of diet from easily digested milk to solid feed has several physiological and immunological consequences whereby piglets are predisposed to develop PWD. The height of the villi is reduced whereas crypt depth increases and enzyme activity in the brush borders is not optimal. This results in a reduction in digestive and absorptive function of the small intestine and leads to more unabsorbed feed in the gastrointestinal tract that can function as a substrate for ETEC (Hampson, 1994; Van Beers-Schreurs, 1992).

From field studies and experimental studies various other risk factors for PWD have been identified e.g. diarrhoea during the suckling period, litter size, weaning age, herd size, low creep feed intake, and low temperatures (Madec *et al.*, 1998; Skirrow *et al.*, 1997; Svensmark *et al.*, 1989; Wathes *et al.*, 1989). As many predisposing factors of PWD were found, it is clear that PWD is a multifactorial disease.

Enterotoxigenic F4+ *Escherichia coli*

Enterotoxigenic *E. coli* (ETEC) is a collective term for *E. coli* strains producing at least one heat-stable (ST) or heat-labile (LT) enterotoxin. Besides the enterotoxins, important virulence factors of diarrhoeagenic *E. coli* strains are the specific fimbrial antigens that allow adherence to the small intestinal mucosa (Nataro and Kaper, 1998). Only a limited range of serotypes are involved in the aetiology of PWD in piglets of which *E. coli* O149:F4 seems to be the prevailing serotype in many countries (Frydendahl, 2002; Nagy *et al.*, 1990; Wittig *et al.*, 1992).

Enterotoxins

Enterotoxins are extracellular proteins that are plasmid regulated. Two types can be distinguished: the antigenic heat-labile toxin and the non-antigenic heat-stable toxins STa and STb. Porcine F4+ *E. coli* strains produce either one of these toxins or a combination of these, but are most often associated with LT and STb (Dubreuil, 1997, Moon *et al.*, 1986).

The binding of LT to the GM1 ganglioside receptor in the mucosal cell membrane can lead to fluid and electrolyte secretion, decreased absorption and transepithelial ion transport disorders. STa can lead to an increased secretion of chloride ions and water in crypt cells and reduced absorption of water and electrolytes by villus tips (Nagy and Fekete, 1999). STb activates a G protein, which mediates the release of serotonin which is associated with fluid secretion in the intestine (Harville and Dreyfus, 1996).

The F4 fimbrial antigen and the F4 receptor

For extensive reviews on the features of the F4 (or K88) fimbrial antigen and its adhesion site, the F4 receptor (F4R), I would like to refer to the papers by Van den Broeck *et al.* (2000) and Jin and Zhao (2000). In this section, the features of the F4 fimbriae and F4R that are relevant for the population dynamics of F4+ *E. coli* will be summarized.

F4 fimbriae are long surface proteins of F4+ *E. coli* of which three different antigenic types have been described: F4ab, F4ac (Ørskov *et al.*, 1964) and F4ad (Guinée and Jansen, 1979). With these fimbriae, F4+ *E. coli* are able to adhere to F4 receptors that can be present on the brush borders of the small intestinal enterocytes in the pig. The attachment of F4+ *E. coli* is a first and crucial step in the pathogenesis whereby the bacteria can withstand the continual clearance of the small intestine by normal passage of the intestinal contents and are thus able to colonize the small intestine (Nagy and Fekete, 1999).

It was shown by *in vitro* adhesion assay that F4+ *E. coli* did not adhere to the brush borders of all piglets; some piglets had adhesive brush borders (F4R+), whereas others had non-adhesive brush borders (F4R–) (Sellwood *et al.*, 1975). This difference in expression appears to be genetic in origin and is inherited in a Mendelian way with adherence being the dominant characteristic (Sellwood and Kearns, 1979). In this thesis, the host characteristic that comprises the F4R+ and F4R– phenotype is referred to as F4R status of the piglets.

Six phenotypes of pigs have been described with regard to the attachment of the three different antigenic types (Baker *et al.*, 1997; Bijlsma *et al.*, 1982) and thus, most likely, different receptors must be involved. There have been many attempts to characterize the F4 receptors and to localize the genes involved as summarized by Van den Broeck *et al.* (2000). The genetics are not completely

elucidated and it is not clear whether the F4 receptors are linked to production traits. The function of the receptors is unknown; it has been suggested that they might be involved in antigen presentation in the small intestine (Newby and Stokes, 1984). As there are no gene-markers available yet, only *in vitro* adhesion assays on small intestinal enterocytes are available for determination of the F4 receptor phenotypes. In the Figure, examples of enterocytes with an adhesive and non-adhesive brush border in the adhesion assay are shown.

In the experiments described in this thesis, *E. coli* serotype O149:K91:F4ac was used to infect the piglets and to determine the F4R status of the piglets. Hence, in this thesis, F4R+ and F4R– refer to the F4acR+ and F4acR– pig phenotypes and F4+ *E. coli* refers to F4ac+ *E. coli*.

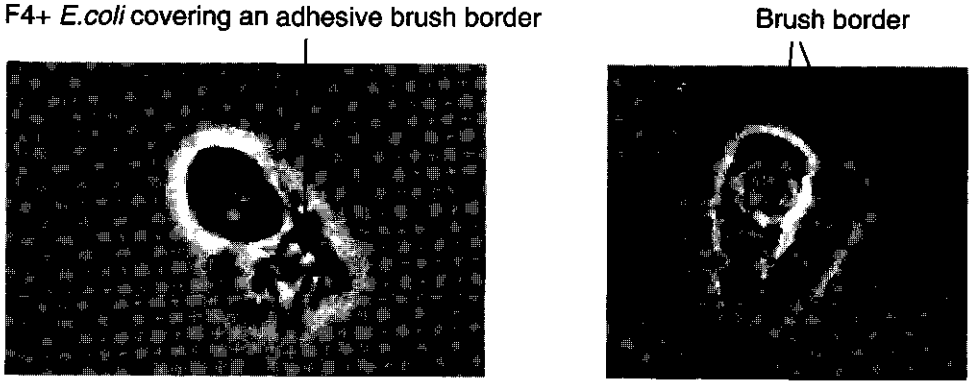

Fig. Examples of enterocytes with an adhesive brush border (left, F4R+ piglet) and non-adhesive brush border (right, F4R– piglet) as seen in the brush border adhesion assay. The brush border of the F4R+ piglet is completely covered with F4+ *E. coli*; the brush borders of the F4R– piglet can clearly be seen on top of the enterocytes, which are surrounded by free-floating F4+ *E.coli*.

Aim and outline of this thesis

The main objective of this thesis was to gain more insight in the population dynamics of enterotoxigenic F4+ *E. coli*. Processes within individual piglets and transmission between piglets contribute to the development of PWD. The approach, therefore, was to study the F4+ *E. coli* dynamics at two different integration levels: in the individual piglet and at population level (between pair-housed piglets and between group-housed piglets). Host characteristics that were likely to affect the susceptibility and infectiousness of individual piglets were investigated at both levels, and their effect on F4+ *E. coli* transmission between piglets was quantified.

(1) *The individual piglet*

The main goal of studying F4+ *E .coli* infection on individual level is to determine which variables affect the population dynamics of F4+ *E. coli* and to develop a definition of the infectious state of piglets.

The amount of F4+ *E. coli* shed in the faeces of an individual piglet as a function of time might be one of the measures of infectiousness. Temporal profiles of F4+ *E. coli* shedding were analyzed to elucidate the main determinants of the variation in shedding at the individual level. This resulted in a measure that distinguished between high and low shedding piglets, with 'high shedder' as a possible definition of the infectious state. One of the host characteristics that are likely to affect the susceptibility and infectiousness of individual piglets is the presence of the F4 receptor (F4R). The effect of this receptor on shedding of F4+ *E. coli* in faeces and on diarrhoea should be determined. This was investigated by studying the association of the presence of F4R with 'high shedding' and with the occurrence of severe diarrhoea (Chapter 2).

Since piglets are housed in an *E. coli*-contaminated environment, it should be determined whether reinfection of piglets affects the shedding patterns of individual piglets. Individually-housed piglets were challenged with F4+ *E. coli* and subsequently, the shedding patterns of piglets that were artificially prohibited to ingest their own faeces, were compared with those of piglets that could ingest their faeces (Chapter 3).

(2) *Pair-housed piglets*

The association between individual shedding patterns and infectiousness has to be determined, as well as the effect of F4R on susceptibility and infectiousness. The definition of infectious state, 'high shedder', obtained from the individual shedding patterns, could only be evaluated in a set-up where transmission between individuals was possible. In transmission experiments with pair-housed piglets it is clear who infects whom and therefore this set-up was used to evaluate 'high shedder' as a definition for infectiousness (Chapter 4). Furthermore, it was tested whether F4R+ and F4R– piglets differed in susceptibility and infectiousness.

(3) *Group-housed piglets*

In practice, newly-weaned piglets are housed in groups of 10-50 piglets. The interaction between group-housed piglets and the fraction of F4R+ piglets may affect the transmission of F4+ *E. coli* in groups. Therefore, transmission parameters and the effect of the presence of F4R were quantified in a transmission experiment with group-housed piglets (Chapter 5). Results of the transmission in pair-housed and group-housed piglets were compared. With a simple discrete

model, the effect of selective breeding for F4R– piglets on the fraction of F4R+ piglets in the population was illustrated (Supplement to Chapter 5). Finally, in Chapter 6, the results from the studies are integrated and implications of the findings of this thesis are discussed.

2 Classification of temporal profiles of F4+ *E.coli* shedding and faecal dry matter in experimental post-weaning diarrhoea of pigs

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submitted

Abstract

Enterotoxigenic F4+ *Escherichia coli* can colonize the intestine of pigs and cause diarrhoea. Our primary goal was to find a discriminant rule to discriminate between F4+ *E. coli* shedding profiles as this may reflect differences in the infectiousness of pigs. Our secondary goal was to find a discriminant rule to discriminate between diarrhoeic and non-diarrhoeic pigs. Repeated measurements (bacterial shedding and percentage dry matter of faeces) were taken of 74 weaned pigs that were infected experimentally with F4+ *E. coli*. These measurements were summarized into two new variables by means of a principal components analysis. Discriminant rules were derived based on these summary variables by fitting a mixture of normal distributions. Finally, the association between the classifications (as derived from the discriminant rules) and the F4 receptor, an adhesion site for F4+ *E. coli*, was studied.

We found that only the classification based on bacterial shedding allowed us to distinguish two significantly different groups of pigs (high and low shedders). Presence of the F4-receptor was associated strongly with pigs being high shedders.

Introduction

Enterotoxigenic *Escherichia coli* strains possessing fimbriae with adhesin F4 (F4+ *E. coli*) are an important cause of post-weaning diarrhoea (PWD) (Frydendahl, 2002; Nagy *et al.*, 1990; Wittig *et al.*, 1995). PWD is an infectious disease of newly weaned pigs and causes substantial weight loss and mortality.

Clinical symptoms after infection with F4+ *E. coli* have been studied well, but transmission of F4+ *E. coli* has not. Because an important characteristic of PWD is its contagiousness (Amass *et al.*, 2003), the transmission characteristics of this infection also should be determined to support the development of proper control measures. However, before we were able to study transmission and diarrhoea among pigs, we needed to find a measure to discriminate between infectious and non-infectious pigs and a measure to discriminate between diarrhoeic and non-diarrhoeic pigs. Discrimination is far from straightforward since the colonization and replication of F4+ *E. coli* within the intestine (Jones and Rutter, 1972) and clinical symptoms vary considerably (Hampson *et al.*, 1985; Madec *et al.*, 2000; Rutter *et al.*, 1975; Wijeratne *et al.*, 1970). The number of F4+ *E. coli* shed in the faeces as a function of time might be one of the measures of infectiousness of the pigs, which is of importance with respect to transmission. The degree of diarrhoea also might influence the spread of F4+ *E. coli* because it might produce aerosols (although oral uptake might be less).

In vitro tests have shown that F4+ *E. coli* does not adhere to the brush borders of all pigs (Sellwood *et al.*, 1975). This difference is explained by the absence or presence of an F4 receptor (F4R), but the structure of F4R or the genes involved have not been characterised yet. F4R promotes adherence of F4+ *E. coli* to the brush border of small-intestinal enterocytes and is thought to be important for the colonization and replication of F4+ *E. coli* in the small intestine, which is followed by production of enterotoxins (resulting in diarrhoea) (Jones and Rutter, 1972). Pigs can be classified upon slaughter by in vitro adhesion assay (Sellwood *et al.*, 1975) into F4R+ (adherent brush borders) and F4R– (non-adherent brush borders).

Three different antigenic types of F4 fimbriae have been described: F4ab, F4ac and F4ad. With regard to the attachment of these different antigenic types, six phenotypes of pigs have been found (Bijlsma *et al.*, 1982; Baker *et al.*, 1997).

F4+ *E. coli* was expected to replicate more within the intestine of F4R+ pigs than of F4R– pigs. This would lead to shedding of higher numbers of F4+ *E. coli* by F4R+ pigs. Consequently, F4R+ pigs could be more susceptible because entering the intestine would lead more often to colonization. F4R+ pigs also could be more infectious because they are expected to shed more bacteria and to have a longer excretion period. This is important for the course of transmission on the level of the population.

In this paper, our objective was to find a measure to discriminate between infectious and non-infectious pigs and a measure to discriminate between diarrhoeic and non-diarrhoeic pigs. Therefore, two discriminant rules were

developed: one that discriminates between F4+ *E. coli* shedding profiles of individual pigs (regardless of their F4R status), and one that discriminates between the faecal dry matter profiles of individual pigs, because this is related to seriousness of diarrhoea and (possibly) to infectiousness.

Subsequently, we investigated the association between F4R status and both classifications as derived from the discriminant rules.

Methods

Experimental design

We used data obtained from five experiments (Table 1) in which all piglets were inoculated with the same strain of F4+ *E. coli* [Animal Sciences Group (ASG), Lelystad, The Netherlands]. For our experiments, an F4ac strain was used and thus, in this paper F4+ *E. coli* refers to F4ac+ *E. coli* and F4R+ and F4R– refer to the F4acR+ and F4acR– pig phenotypes. For each piglet the number of colony forming units of F4+ *E. coli*/g faeces at days 1-8 p.i., the percentage faecal dry matter at days 1-8 p.i. and the F4R status was known. All experiments were carried out at the experimental facilities of the Animal Sciences Group.

All animals were purchased from a commercial piggery in The Netherlands and transported to the facilities on the day of weaning. All pigs were in good health, no haemolytic *E. coli* were found on rectal swabs taken upon arrival and none of the pigs had diarrhoea at weaning. The set-up of the experiments is summarized below.

Experiment 1: Ten male, castrated pigs were housed individually. At day 5 they were infected orally with 5 ml 10^8 c.f.u. F4+ *E. coli*/ml. Faeces were collected by colostomy pouches that were attached around the anus and the number of c.f.u. of F4+ *E. coli*/g faeces (denoted by CFU) and percentage faecal dry matter (denoted by %DM) were determined daily. At day 21, all pigs were anaesthetised, bled and necropsied and F4R status of the pigs was determined by brush border adhesion assay (BBA) (Sellwood *et al.*, 1975).

Experiment 2: Ten male, castrated pigs were housed individually. At day 4, pigs were infected orally with rotavirus [a predisposing factor for F4+ *E. coli* infection (Nabuurs *et al.*, 1993)] followed by oral infection with 5 ml 10^9 c.f.u. F4+ *E. coli*/ml on day 5. CFU and %DM were determined daily in faeces collected by colostomy pouches. F4R status was determined by BBA after euthanasia at day 19.

Experiments 3 and 4: Ten and twelve castrated pigs, respectively, were housed individually. Faecal samples were taken directly from the rectum, daily and from all pigs. Oral infection of rotavirus and F4+ *E. coli* was done as described for experiment 2. CFU and %DM were determined on rectal faecal samples and F4R status was determined by BBA after euthanasia at day 19.

Experiment 5: Thirty-two pigs (17 male and 15 female) were housed in four groups of eight pigs. Pigs were assigned randomly to the groups with restriction that littermates were not housed in the same group and that the weight and sex of the pigs were equally distributed over the groups. At day 4, all pigs were infected orally with rotavirus, followed by oral infection with 5 ml, 10^9 c.f.u. F4+ *E. coli*/ml on day 5, which was repeated on day 6. CFU and %DM were determined on rectal faecal samples and F4R status was determined by BBA after euthanasia at day 14.

All experiments were performed with permission of the institutional local ethics committee for animal experiments. Differences in the design of the experiments have led to variation in the day of euthanasia. This has not affected the F4R status of the pigs, because presence of the receptor in the brush border fraction is independent of age (Willemsen and De Graaf, 1992).

Table 1. Summary of the five experiments in which 74 newly weaned pigs were inoculated with *E. coli* serotype O149:K91:F4ac (LT+, STb+), ASG, The Netherlands, (April 2000 - September 2001).

Experiment	No. of pigs	No. F4R+	Housing	Inoculation[a]	Feed[b]	Colostomy pouch	Mean weight at weaning (kg), (SD)
1	10	3	Individual	1	A	yes	6.4 (1.4)
2	10 (9)[c]	5 (4)[c]	Individual	2	B	yes	6.9 (1.4)
3	10	3	Individual	2	B	5 yes, 5 no	6.8 (1.0)
4	12	4	Individual	2	B	6 yes, 6 no	8.1 (1.6)
5	32 (28)[c]	11 (10)[c]	Groups of 8	3	C	no	6.9 (0.8)

[a] (1) $5x10^8$ c.f.u. F4+ *E. coli* on day 5;
(2) fasting on day 0 and 1, rotavirus on day 4 and $5x10^9$ c.f.u. F4+ *E. coli* on day 5;
(3) fasting on day 0 and 1, colistin sulphate in water day 0-4, rotavirus on day 4 and $5x10^9$ c.f.u. F4+ *E. coli* on day 5 and 6.

[b] (A) '5110p Superkorrel', 16.4% crude protein (Hendrix UTD, Boxmeer, The Netherlands);
(B) '2740 biggenbatterij 4 mm', 18.9% crude protein (Hope Farms bv, Woerden, The Netherlands);
(C) Weanling diet, 17.6 % crude protein (Cehave, Veghel, The Netherlands).

[c] Between brackets: the number of pigs used in the statistical analysis when different from those given.

Inoculation

E. coli serotype O149:K91:F4ac (LT+, STb+) was isolated from a pig in a farm with post-weaning diarrhoea and designated CVI-1000 (ASG, Lelystad) (Nabuurs *et al.*, 1996). As a negative control in the BBA *E. coli* strain CVI-1084 (ASG, Lelystad) was used. This is also an O149:K91 strain (LT+, STa+) but without fimbrial expression of F4ac. The strains were grown overnight in brain-heart

infusion broth (Difco Laboratories, Detroit, USA) and pelleted by centrifugation. The pellets were 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.

Rotavirus strain RV277 originally was isolated from pigs with rotaviral neonatal diarrhoea and is maintained in the laboratory of the Animal Sciences Group. The average virus concentration (determined by negative-stain electron microscopy) was $1.0x10^6$ particles/ml. Inoculation with rotavirus in addition to inoculation with F4+ *E. coli* (except in experiment 1) was chosen because in our experience, rotavirus facilitates infection with F4+ *E. coli* and often is found as a co-infection in the field (Nabuurs *et al.*, 1993).

Analysis of faeces

Determination of %DM

Faeces (0.5-3.0 g) were weighed into aluminium trays. Samples were desiccated for 22 h in an incubator at 80 °C, and weighed again to determine lost water.

Determination of CFU

Ten-fold dilutions of faeces homogenised in saline (Biotrading, The Netherlands) were made. Of each dilution, 10 µl was plated manually on selective His-agar plates containing 5% sheep blood, streptomycin (50 µg/ml), tetracycline (25 µg/ml) and vancomycin (50 µg/ml) (Biotrading, The Netherlands). Plates with < 200 haemolytic F4+ *E. coli* colonies were counted and the number of c.f.u./g faeces was calculated (lower limit 100 c.f.u./g faeces). In cases of uncertainty on the colony morphology, identity was confirmed by slide agglutination to establish the *E. coli* OK type (ASG, Lelystad).

Determination of F4R status

Determination of F4R status by the BBA (Sellwood *et al.*, 1975) was performed by lab technicians without prior knowledge about the performance of the pigs during the experiment, the bacterial counts or any information that possibly could be related to F4R status.

At necropsy, 5 to 10 cm of jejunal mucosa was scraped off and 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. The filtrate was centrifuged for 10 min at 500 *g* to

harvest the cells. Cells were resuspended in PBS containing 0.05% D(+) mannose (Merck, Germany). A CVI-1000 suspension (F4ac+) of 0.25 ml containing 10^9 bacteria/ml PBS was added to 0.25 ml of the cell suspension. The same was done with strain CVI-1084 (F4ac–), as a negative control. The samples were mixed gently 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 x400). Only cells with well-defined brush borders were studied. Animals with no or an average of 1 to 2 bacteria of strain CVI-1000 per brush border were considered F4R–; samples exceeding this were judged F4R+. In case of ambiguity, the test was repeated.

Statistical evaluation

Principal components analysis (PCA) was used in the analysis of the CFU and %DM measurements to summarize individual profiles in time. PCA is a statistical technique to derive a limited set of new (independent) variables (called principal components) that capture as much as possible of the variation in the original (dependent) variables. The principal components (pc's) are ordered with respect to decreasing variance. The percentage variance explained by, say, the first two pc's (pc_1 and pc_2) is equal to (variance of pc_1 + variance of pc_2) / (sum of the variances of the original variables) * 100%. All components together explain 100% of the data. A pc, say pc_1, is a linear combination of the original variables (y_1, y_2, ...): $pc_1 = c_{11} \cdot y_1 + c_{12} \cdot y_2 + \ldots$, where the coefficients c_{11}, c_{12}, ... (the loadings) are the elements of the eigenvector with the largest eigenvalue of the covariance matrix of the original variables.

When the original variables are associated with time points, the loadings can be used to derive "eigenfunctions". These eigenfunctions (Engel *et al.*, 2003; Kingsolver *et al.*, 2001; Kirkpatrick and Heckman, 1989) reflect important characteristics to describe variation between individual profiles in time (e.g. increase over time or curvature). A profile (p) can be decomposed into a linear combination of the eigenfunctions (f_1, f_2, ...): $p = pc_1 \cdot f_1 + pc_2 \cdot f_2 + \ldots$, where the coefficients are the values of the pc's of an individual piglet. The aim is to describe the variation between the profiles with a small number of eigenfunctions, i.e. focus on the most important characteristics of the profiles. A pc indicates how strong such a characteristic is expressed in a profile of an individual piglet.

Missing values cannot be handled by PCA and were replaced by the average of the two neighbouring time points. An observation was considered to be a missing value when no faeces or insufficient faeces (< 0.3 g) was collected to determine %DM and/or CFU. Data from animals that died during the experiments and data from animals with more than 3 missing values in CFU or %DM were considered unreliable and excluded from the analysis. In the event of observed absence of F4+ *E. coli* c.f.u./g faeces, CFU was set to 0.

To obtain a more normal distribution in the statistical analysis, CFU data were log-transformed; lnCFU = ln(CFU+1).

The analysis of the CFU and %DM profiles consisted of three steps.

Step 1

A separate PCA was performed for the CFU and %DM data. The first principal components of lnCFU and %DM, denoted by CFU PC_1 and %DM PC_1 respectively, were retained as a summary of the profiles. A biological interpretation of these pc's was inferred from the shape of the associated eigenfunctions (as visualized by plots of the loadings against time). These pc's will be used in subsequent steps for clustering and discrimination between pigs.

Step 2

Pigs were clustered into two groups on the basis of CFU PC_1 and %DM PC_1. CFU PC_1 and %DM PC_1 were assumed jointly to follow a mixture of two (bi-variate) normal distributions. Each pig was assumed to follow one of the two normal distributions, under the assumption that there are two different shedding types and two different diarrhoea types of pigs. The proportions of pigs corresponding to the two distributions are the so-called mixture probabilities. These proportions, together with the means and variances of the underlying normal distributions, were estimated by maximum likelihood with the program EMMIX (Peel and McLachlan, 1999; McLachlan and Peel, 2000). Discrimination was based on the classical maximum likelihood discriminant rule (Mardia *et al.*, 1979) that allocates an animal to the category where its observations have the largest likelihood to appear. To test whether the assumption of a mixture was tenable, the model consisting of a mixture of two normal distributions was compared with a model with a single common normal distribution by the likelihood ratio test [GenStat, (GenStat Committee, 2000)].

Step 3

In this final step, the association between F4R status and the result of clustering of step 2 was studied in a 2x2-table, employing Fisher's exact test [GenStat, (GenStat Committee, 2000)].

Results

Of the 74 pigs, five pigs were excluded from analysis; one pig died and four pigs had more than three missing values. Of the remaining 69 pigs, 45 pigs were determined F4R– and 24 F4R+. Nine pigs showed no F4+ *E. coli*-positive

samples in the first eight days after inoculation (two F4R+ and seven F4R–). In Table 2, the median number of F4+ *E. coli*-positive samples, the median number of F4+ *E. coli* in the positive samples and the mean %DM are shown for the F4R+, the F4R– and of all pigs.

Table 2. The median number of F4+ *E.coli* positive samples, the median number of F4+ *E.coli* in the positive samples and the mean percentage faecal dry matter of the F4R+ pigs, the F4R– pigs and all pigs.

	F4R+ pigs (n = 24)	F4R– pigs (n = 45)	All pigs (n = 69)
Median (min – max) number of F4+ *E.coli*-positive samples	6 (0 - 8)	2 (0 - 8)	3 (0 - 8)
Median (min – max) number of F4+ *E.coli* in positive samples (c.f.u.)	1.10^6 ($1.10^2 - 1.10^{10}$)	7.10^3 ($1.10^2 - 1.10^9$)	7.10^4 ($1.10^2 - 1.10^{10}$)
Mean % faecal dry matter (SD)	21.7 (9.3)	25.9 (6.1)	24.5 (7.6)

The first two PCs of lnCFU (lnCFU PC_1 and lnCFU PC_2) explained 76.2 and 9.3% of the variation in lnCFU, respectively; in Fig 1 the associated eigenfunctions are shown.

%DM PC_1 and %DM PC_2 explained 57.6 and 19.5% of the variation in %DM, respectively; their eigenfunctions are shown in Fig 2. LnCFU PC_1 and %DM PC_1 explained the major part of the variation between the individual profiles; all other PCs explained only a relatively small amount of variation. The shape of the eigenfunctions associated with lnCFU PC_1 and %DM PC_1 (eigenfunction 1 in Fig 1 and 2) is basically constant, apart from a slight increase at the extremes. This suggests that major differences between the individual profiles of the animals are reflected by their overall level of lnCFU and their overall level of %DM. The eigenfunctions associated with lnCFU PC_2 and %DM PC_2 (eigenfunction 2 in Fig 1 and 2), show a contrast between the first and last days of the sampling period and largely represent a trend in time.

Classification results based on the PCs of lnCFU and %DM are summarized in Table 3. As can be seen from this table, only lnCFU PC_1 and %DM PC_2 could significantly discriminate between two types of pigs ($p < 0.05$). Although, discrimination between pigs with high and low %DM summarized by %DM PC_1 was not significant ($P = 0.06$), there was a strong indication that the pigs in group 2

Fig 1. Coefficients of the first and second eigenfunction of the log-transformed number of F4+ *E. coli*/g faeces data (lnCFU) plotted against time.

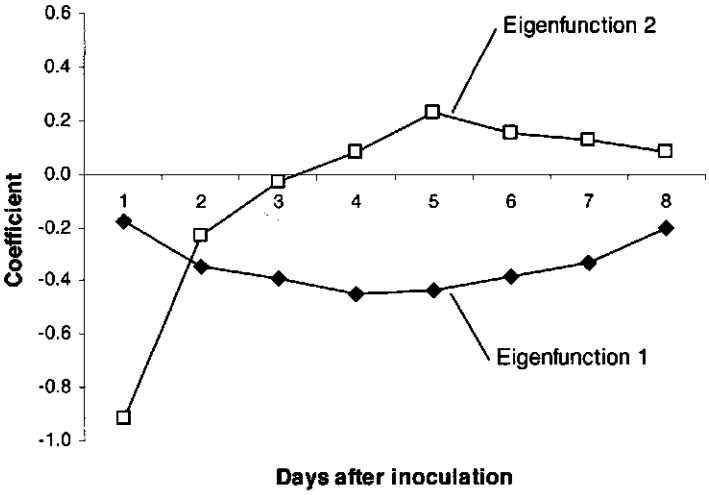
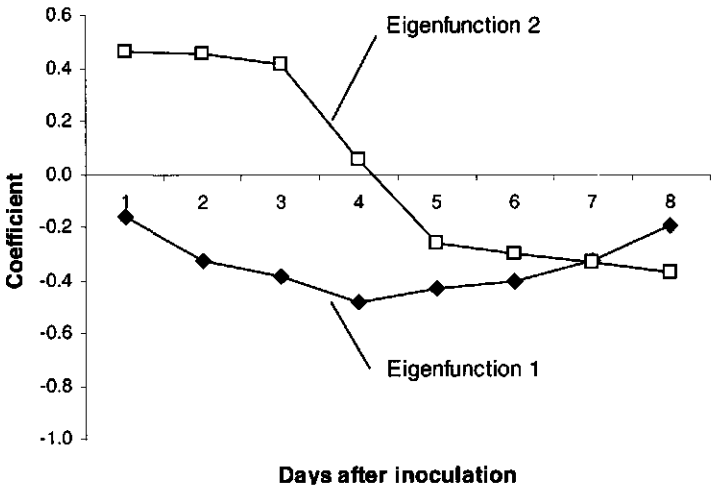


Fig. 2. Coefficients of the first and second eigenfunction of the percentage dry matter of faeces data (%DM) plotted against time.



(Table 3) suffered from diarrhoea. Classification based on %DM PC_2 was significant, but the amount of variation explained by %DM PC_2 was low (19.5%). Moreover, %DM PC_2 distinguished one group consisting of only four piglets with diarrhoea at days 5 to 8 p.i., and one group with all other 65 piglets. Therefore, %DM PC_2 was considered not to be a biological meaningful measure to discriminate between diarrhoeic and non-diarrhoeic piglets and was not evaluated further.

The major difference between the piglets was their overall level of bacteria shed at log scale, which was summarized by lnCFU PC_1. This means that it is possible to discriminate between pigs that shed high numbers of F4+ *E. coli*/g faeces at log scale (group 2, Table 3) and pigs that shed low numbers at log scale (group 1, Table 3). These two shedding types will therefore be referred to as high and low shedders.

Table 3. Classification results of the 69 pigs using the first or second principal component (PC_1 or PC_2) derived from PCA on the log-transformed number of F4+ *E.coli*/g faeces (lnCFU) or on the percentage dry matter of faeces (%DM).

Summary variable	Proportion of pigs[a]		Mean[b]		Variance[b]		Likelyhood ratio test P-value[c]	Cut-off value[d]
	group 1	group 2	group 1	group 2	group 1	group 2		
lnCFU PC_1	0.64	0.36	11.1	-18.6	0.00	16.2	0.00	1.96
lnCFU PC_2	0.52	0.48	-4.4	5.1	0.07	13.8	0.07	0.95
%DM PC_1	0.83	0.17	-5.7	27.3	0.06	113.0	0.06	11.65
%DM PC_2	0.94	0.06	-1.6	27.1	0.00	45.9	0.00	13.64

[a] Group with the highest proportion of pigs is referred to as group 1 and other as group 2.

[b] Means and variances of group 1 and 2 estimated by maximum likelihood.

[c] Probability that pigs can be classified into two groups vs one group.

[d] Cut-off values between groups are calculated by the Maximum Likelihood Discriminant rule.

Cut-off values for the different groups calculated by the maximum likelihood discriminant rule are also shown in Table 3. For example, pigs with values of lnCFU PC_1 smaller than the boundary value 1.96 were classified as high shedders. Thus, the discriminant rule found to classify piglets as high shedder is:

$$\sum_k \text{coeffcient}_k \cdot (\ln \text{CFU}_k - \mu \ln \text{CFU}_k) < 1.96, \text{ with } k = 1,2,.. 8.$$

$\ln CFU_k$ are the log-transformed numbers of F4+ *E. coli*/g +1 found in the faecal samples of individual piglets on days 1 to 8 after inoculation, $\mu \ln CFU_k$ are the mean number of lnCFU shed at days 1 to 8 of all 69 piglets and coefficient_k are the coefficients (or loadings) of the first eigenfunction at days 1 to 8 (see Fig 1).

In the last step of the analysis, it was investigated how the F4R+ and F4R– piglets were distributed over the shedding and diarrhoea types. In Fig 3 and 4, the proportion of the F4R– and F4R+ pigs from all five experiments on intervals of lnCFU PC_1 and %DM PC_1 are shown. In Fig 3, a decreasing value at the x-axis of the lnCFU PC_1 plot means an increasing number of F4+ *E. coli* shed. As can be seen, the distributions of the F4R– and F4R+ pigs differed considerably along lnCFU PC1; F4R+ pigs had a more equal distribution from low to high values whereas F4R– pigs were more concentrated on lower values of lnCFU PC1 and

thus on a lower number of bacteria shed. Only 8 (17%) of the F4R– pigs were in the group of high shedders whereas of the F4R+ pigs 17 (71%) were in this group. Fisher's exact test for a 2x2-table results in $P < 0.001$ thus showing that F4R status was highly associated with the classification into high and low shedders.

In Fig 4 an increasing value at the x-axis of the %DM PC_1 plot means a decreasing overall mean and thus a lower %DM. As can be seen, there was considerable overlap between F4R+ and F4R– pigs, but F4R– pigs were more concentrated on a higher %DM. Applying Fisher's exact test on these results in a 2x2-table resulted in $P = 0.002$. So, although discrimination based on %DM PC_1 between the two diarrhoea types was not significant, the association of the F4R status with classification into high and low %DM clearly was significant.

The majority of the pigs (61%) were assigned to both the low shedders and low %DM groups. Ten pigs (14%) were high shedders and in the low %DM group. Two pigs (3%) shed low numbers of F4+ *E. coli*, but were still classified into the low %DM group. Fifteen pigs were assigned to both the high shedding group and the high %DM group. Analysing these results in a 2x2-table with Fisher's exact test, showed that lnCFU and %DM were significantly associated ($P < 0.001$).

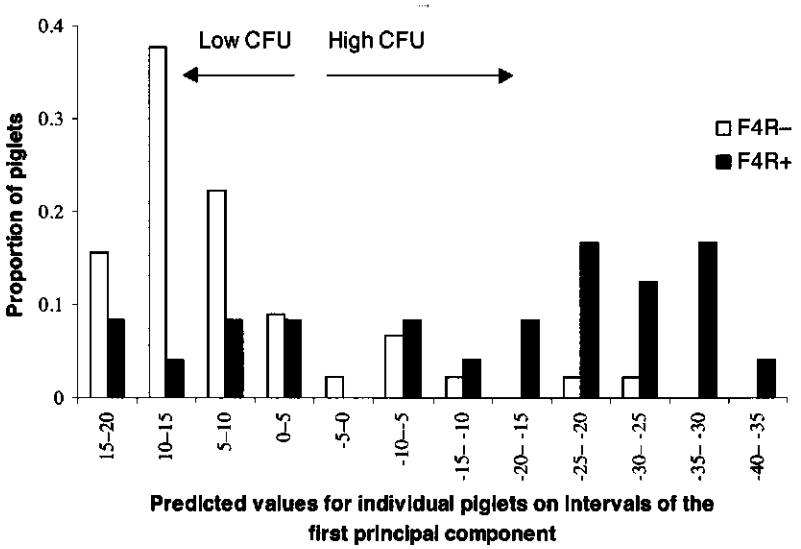


Fig 3. Proportions of F4R+ (n=24) and F4R– pigs (n=45) (all experiments combined) on the predicted values of the first principal component of the log-transformed number of F4+ E.coli/g faeces (lnCFU PC1). Piglets with a predicted value < 1.96 were defined to be high shedders.

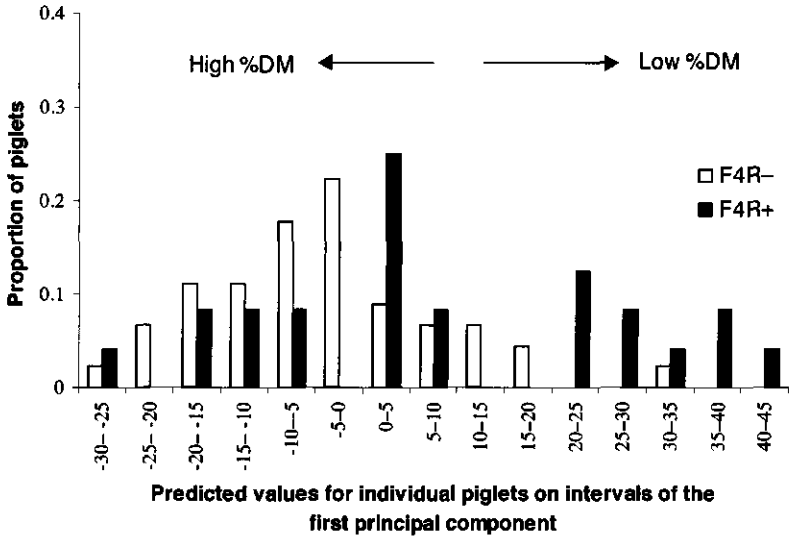


Fig 4. Proportions of F4R+ (n=24) and F4R– pigs (n=45) (all experiments combined) on the predicted values of the first principal component of the percentage dry matter of faeces (%DM PC1). Piglets with a predicted value > 11.65 were defined to have low %DM.

Discussion

Studies about PWD caused by F4+ *E. coli* have mainly focused on clinical symptoms of pigs (Cox *et al.*, 1991; Madec *et al.*, 2000) and not on the transmission of F4+ *E. coli*. However, for understanding the epidemiology of PWD and for development of control measures, information on the infectiousness and susceptibility of the individuals to F4+ *E. coli* within a population is also needed. Shedding of F4+ *E. coli* in faeces may reflect differences in colonization and replication between pigs, and accordingly, differences in infectiousness. In this paper, we showed that by performing a principal components analysis on the (log-transformed) F4+ *E. coli* shedding profiles of individual piglets, an objective linear discrimination measure could be derived that was able to classify these piglets into two significantly different groups. The shape of the eigenfunctions resulting from the principal components analysis, indicated that the main difference in shedding profiles of these two groups of piglets, was the total amount of bacteria shed at log-scale. We therefore referred to these groups as high and low shedding piglets.

Whether the classification into high and low shedders reflects a classification into high and low infectiousness can only be studied in pigs housed together, because transmission is a process at the population level. Therefore, this measure for infectiousness was evaluated in a transmission experiment (Chapter 4).

Besides the level of bacteria shed, other aspects like acid tolerance (Merrell and Camilli, 2002), survival of *E. coli* in faeces (Kudva *et al.*, 1998) and behaviour of the diseased pig (Krsnik *et al.*, 1999) are likely to affect infectiousness. The degree of diarrhoea might also be a vector for spread of F4+ *E. coli*, since diarrhoeal faeces may form aerosols. Therefore, also the faecal percentage dry matter profiles of the pigs were studied to find a measure to discriminate between diarrhoeic and non-diarrhoeic piglets. Large variation in percentage dry matter profiles was found between pigs, but the separation of the profiles into two distinct groups was not significant.

One of the factors that causes high variability in colonization and replication and thus in shedding and diarrhoea, is the presence or absence of the F4 receptor (F4R). Presence of F4R was found to be associated significantly with a low percentage dry matter. We also showed that presence of F4R was significantly associated with high shedding of F4+ *E. coli* at log scale. This indicates that mixed F4R+/F4R– populations cannot be considered homogeneous with respect to shedding of F4+ *E. coli*. Therefore, F4R status of the pigs and its effect in populations will have to be taken into account in the evaluation of F4+ *E. coli* infection models or when testing F4+ *E. coli* intervention measures. Although the distributions of the F4R+ and F4R– pigs on the level of F4+ *E. coli* shedding at log scale had some overlap (Fig 3), F4R seemed to be the major factor affecting the shedding profiles.

High shedders were classified more often in the group with a low percentage dry matter and low shedders in the group with a high percentage dry matter. However, 22% of the pigs (8 F4R+ and 7 F4R–) were both in the high shedding and the high percentage dry matter group. This indicates that compensatory mechanisms must be active to avoid fluid loss due to bacterial toxins both with F4R+ and F4R– pigs. Only two pigs were found in both the low percentage dry matter and low shedding group. These pigs might have suffered from rotavirus diarrhoea or other diarrhoeagenic causes.

In this study we wanted to find general measures to discriminate between infectious and non-infectious and between diarrhoeic and non-diarrhoeic piglets. We expected that the range in shedding profiles and percentage dry matter profiles of the five experiments together would best resemble the range in common pig practice. Therefore, we ignored the experiments and differences in experimental design as factors in the statistical analysis. The results gave no reason to think that differences in experimental design had major effects, except in experiment 5, where seven out of eleven F4R– pigs housed with F4R+ pigs were found in the high shedding group. These pigs accounted for the majority of high shedding F4R– pigs found in all experiments. This indicated that F4R+ pigs might have had an effect on the F4+ *E. coli* shedding of F4R– pigs. In this analysis we regarded the data from the pigs as independent data, but for the aforementioned pigs of experiment 5 this was not necessarily true. However, the relatively simple summary statistics of shedding and percentage dry matter derived in this paper gave robust results and showed the main differences between the individual profiles.

Acknowledgements

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3

F4+ *Escherichia coli* shedding pattern of newly weaned piglets is mainly determined by F4-receptor status and not by reinfection

P.L. Geenen, J. van der Meulen, A. Bouma, M.C.M. de Jong

Abstract

In oral-faecal transmitted gastrointestinal pathogens, reinfection from the environment could possibly affect the shedding patterns of the infected host and as a consequence influence its infectiousness. Whether reinfection plays a role in the population dynamics of F4+ *Escherichia coli* (F4+ *E. coli*), an important causative agent of post-weaning diarrhoea, is unknown. In this study, 36 piglets with and without the F4-receptor (F4R), were challenged with F4+ *E. coli*. During two weeks after challenge, half of the piglets were prevented to reinfect themselves using pouches by which all faeces were collected and contamination of the pen with faeces were prevented. Shedding of F4+ *E. coli* (intermittence, the number of positive samples and the amount of bacteria shed) was determined during 14 days after inoculation. The effects of the factors pouch and F4R status on the shedding patterns were tested using a generalized linear model and Fisher's exact tests.

Only F4R status was found to affect the shedding patterns. No indications were found that the F4+ *E. coli* shedding patterns of piglets (without pouches) that could reinfect themselves were different from those of piglets with pouches. It was concluded that the shedding patterns are mainly determined by F4R status and not by reinfection.

Introduction

When studying the epidemiology of an infectious gastrointestinal pathogen, it is important to understand its population dynamics from characteristics of individual hosts. Important host characteristics of an oral-faecal transmitted gastrointestinal pathogen are the pathogen uptake from the environment, colonization and replication of the pathogen within the host, and the infectiousness of the host which is related to pathogen shedding, contact behaviour of the host and pathogen survival in the environment.

After ingestion of the pathogen from the environment, successful colonization and replication within the gastrointestinal system will result in an infectious host that will shed the pathogen with its faeces and hereby increasing the infectious load in the environment. This will lead to a higher probability that a susceptible host ingests the pathogen, becomes infectious too and increases the infectious load in the environment even more, etc. This chain of infections can be described by simple mathematical models, for example the stochastic SIR-model (Bailey, 1975).

For some infectious diseases, it was found that reinfection plays an important role in the long term transmission of the pathogen, for example Tuberculosis (Gomes *et al.*, 2004) or helminth infections (Paterson *et al.*, 2000), by causing recurrent infections or superinfection. In this paper, we are mainly interested in the effects of reinfection during or soon after primary infection. Therefore, we would like to define reinfection as the uptake of the pathogen from the environment (an infectious host or other infectious source) that will lead to successful colonization of a host that is still infected or has only recently stopped shedding. This may lead to longer periods of shedding and possibly higher numbers of the pathogen shed, resulting in increased infectiousness.

Reinfection during primary infection has, as far as the authors know, never been quantified for gastrointestinal bacteria. The question addressed in this paper is whether reinfection affects the shedding patterns of enterotoxigenic F4+ *Escherichia coli* (F4+ *E. coli*) and thereby affects its population dynamics. F4+ *E. coli* is an important causative agent of post-weaning diarrhoea (PWD) in newly-weaned piglets (Frydendahl, 2002; Nagy and Fekete, 1999; Wittig *et al.*, 1995), in which the presence of an adhesion factor for F4+ *E. coli* (F4-receptor; F4R) in the small intestine is generally thought to be an important factor in the attachment of F4+ *E. coli* to the small intestine.

The number of positive samples, the number of bacteria shed and the occurrence of intermittent shedding patterns were analysed for piglets with and without pouches and with and without F4R. By quantifying reinfection of F4+ *E. coli* in individual piglets, more insight in the population dynamics of this pathogen is gained.

Materials and methods

Experimental design

Data were obtained from three experiments (A, B and C). A total number of 36 (3 x 12) male, castrated piglets, 3-4 weeks old, were brought from a commercial farm in the Netherlands to the animal facilities of the Animal Sciences Group (ASG) in Lelystad, The Netherlands at the day of weaning (day 0). The piglets were obtained from different litters to prevent litter effects as much as possible. At arrival, piglets were weighed and rectal swabs were taken and checked for haemolytic *E. coli*. Piglets were individually housed in pens that were positioned in two different stables per experiment, with the weights of the piglets equally distributed over the two stables. The six pens per stable were put in a row and had closed wooden partitions that were placed on grid floors, with one piglet per 0.51 m^2 floor surface. The mean temperature of the stables was 25°C with a 16-hr light, 8-hr dark scheme.

On day 0 and 1, piglets were fasted, with water available *ad libitum*. From day 2 on, piglets were fed *ad libitum* a standard commercial piglet feed (Hope Farms bv, Woerden, The Netherlands).

At day 2, a 45 mm flange belonging to a human two-piece ostomy system (ConvaTec, Uxbridge, UK) was placed around the anus of all six piglets in one stable and fixed with medical adhesive (Hollister, Libertyville, USA) and Leukoplast® (5 cm, Beiersdorf AG, Hamburg, Germany). Closed pouches were attached to the flange and fixed with Leukoplast® (2.5 cm, Beiersdorf AG, Hamburg, Germany). The pouches were changed twice daily, by which all faeces were collected. During the experiment, the pens were not cleaned to ensure a maximum infectivity in the pens of the piglets without pouches.

On day 4, all piglets were inoculated orally with 2 ml of a rotavirus strain suspension (strain RV277, ASG, Lelystad, The Netherlands). Inoculation with rotavirus preceding F4+ *E. coli* inoculation was chosen, because rotavirus may act as a predisposing factor for *E. coli* infections (Melin *et al.*, 2004) and is often found as a co infection in the field (Hampson *et al.*, 1994).

On day 5, all piglets were orally inoculated with 5 ml 10^9 c.f.u./ml F4+ *E. coli* (ASG, Lelystad, The Netherlands) with exception of two piglets in experiment A (one with pouch and one without pouch). These two piglets served as sentinels to check for transmission over a distance from pen to pen. Rectal faecal samples were collected daily and the number of F4+ *E. coli*/g faeces and the percentage dry matter of these samples were determined.

During the experiment, feed intake and the health of the piglets were recorded daily, to observe any deviations in the wellbeing of the piglets. Strict hygienic measures were taken to prevent transmission between pens; changing of boots, clothing and hair nets before entering the stables, handling the piglets without stepping into the pen and not cleaning the pens, as this would have increased the risk of spread of contaminated faeces from pen to pen.

On day 19, the piglets were weighed, euthanized, bled and necropsied. A 5-10 cm jejunal sample was taken for determination of the F4R status by Brush Border Adhesion assay (BBA), (Sellwood *et al.*, 1975). The local Ethics Committee for Animal Experiments approved the experimental protocols.

Inoculation

E. coli serotype O149;K91;F4ac (LT+, STb+), isolated from pigs with post-weaning diarrhoea (strain CVI-1000, ASG, Lelystad), was grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, USA) at 37°C, pelleted by centrifugation, and resuspended in PBS (pH 7.2) to an optical density of 1.050 at 600 nm which corresponds to a suspension of 10^9 c.f.u./ml.

Rotavirus was originally isolated from piglets with rotaviral neonatal diarrhoea (strain RV277, ASG, Lelystad, The Netherlands). The average virus concentration, determined by negative stain electron microscopy, was 10^6 particles/ml.

Analysis of faeces

Determination of c.f.u. F4+ E. coli/g faeces

Ten-fold dilutions of faeces homogenized in saline (Biotrading, Mijdrecht, The Netherlands) were plated on selective agar plates containing 5% sheep blood, streptomycin 50 µg/ml, tetracycline 25 µg/ml and vancomycin 50 µg/ml (Biotrading, Mijdrecht, 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 on the colony morphology, identity was confirmed by slide agglutination with pig sera (ASG, Lelystad, The Netherlands) to establish the *E. coli* OK type.

Faecal dry matter and diarrhoea score

A small amount of the faecal sample was weighed into an aluminium tray. Samples were desiccated for approximately 22 h in an incubator at 80 °C, and weighed again to determine water loss.

At sampling, the appearance of each faecal sample was examined and a 4-point score 0 = normal, 1 = unformed or loose consistency, 2 = pasty diarrhoea, 3 = liquid, was given to describe the consistency. For each set of faecal samples with a particular score, the mean percentage dry matter and a 95% confidence interval (CI) was calculated. Only piglets with one or more samples with a percentage dry matter below the upper limit of the 95% CI of score 3 were classified as severely diarrhoeic. The association between absence or presence of

severe diarrhoea and the F4R status of the piglets was studied with Fisher's exact test for association using GenStat (GenStat Committee, 2000).

F4+ *E. coli* contamination of pens

In experiment A, swabs were taken of the pens to investigate whether F4+ *E. coli* could be recovered in the piglets' environment. At day 4, 6, 8 and 13 swabs were taken in the pens of all six piglets without pouches. Three 25 cm^2 spots, two at the grid floor and one at the partition close to the grid floor were swabbed, as were the trough, nipple drinker and dunging area which, for most piglets, was the area underneath the nipple drinker. On day 13 the grid floor of three piglets with pouches (including the sentinel piglet) were swabbed.

Determination of the 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 in the BBA modified after Sellwood *et al.* (1975) as described before (Chapter 4). 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.

A minimum of 10 enterocytes were examined for the adhesion of F4+ *E. coli*. Animals with no bacteria or just 1-2 bacteria adhering to a maximum of 10% of the brush borders were considered F4R–. In case of ambiguity, the sample was compared to the negative control.

Statistical analysis of the shedding patterns

A faecal sample was considered positive when at least one colony of F4+ *E. coli* was found on the plate with the lowest dilution, which corresponds to 100 c.f.u. F4+ *E. coli*/g faeces. The median of the number of positive samples per pig, the median of the sum of F4+ *E. coli* in the samples of each pig and the median of the number of bacteria shed per F4+ *E. coli*-positive sample were calculated for the F4R+ and F4R– piglets, with and without pouches. It was tested whether the medians for the piglets with and without pouches were different, and whether the medians were different between piglets with and without F4R. This was done by counting the number of piglets or samples above and below the combined median (the median for all piglets) of the different groups and compare these numbers with Fisher's exact test using GenStat (GenStat Committee, 2000).

We also studied the intermittence of the shedding patterns. An intermittent shedding pattern was defined as an F4+ *E. coli* shedding pattern of positive faecal

samples (1) interrupted by at least one F4+ *E. coli* negative faecal sample (0), for example: (0, 1, 1, 1, 0, 0, 0, 1, 1). It was assumed that piglets with pouches do not contaminate their own environment with F4+ *E. coli* and thus cannot reinfect themselves. There might be other causes within the piglet's gastrointestinal tract that cause intermittent shedding and there might be sampling artefacts, but we assume that these will happen equally often in piglets with and without pouches.

Reinfection may give rise to the event that a negative sample is followed by a positive sample (0, 1), given that it is preceded by at least one positive sample at some point in the shedding pattern. This 'reinfection transition' (0, 1) and the three other transitions that may occur in the shedding patterns ((0, 0), (1, 1) and (1, 0)) were assumed to occur with fixed probabilities λ, $1-\lambda$, $1-\mu$ and μ respectively. The transitions were assumed to be dependent on the result of the last sample in the preceding transition only.

The likelihood kernel of the shedding pattern of each individual piglet was obtained by multiplying the conditional probabilities of the sequence of transitions found from the first positive sample onwards, e.g. (0, 1, 1, 1, 0, 0, 0, 1, 1) resulted in $(1-\lambda)^2.\lambda.\mu.(1-\mu)^3$. Clearly, maximisation of the likelihood amounts to the same procedure as in the case of binomial sampling and can be performed for λ and μ separately. Thus, the maximum likelihood estimator for λ is the number of (0, 1) transitions observed, divided by the total number of transitions starting with 0, that is (0, 1) and (0, 0). Given this result, the data of the transitions rates were analysed using a generalized linear model with binomial distribution and a logit link function. It was tested whether the factors presence of a pouch (yes or no) and F4R status (F4R+ or F4R–) had a significant effect on λ, the rate at which (0, 1) transitions occurred. The GLMs were performed using GenStat (GenStat Committee, 2000).

Results

Mortality and F4R status

Two piglets in experiment C had to be killed for ethical reasons at day 6 and 7 p.i. of the study period. The post mortem examination showed that they had suffered from polyserositis and arthritis; these diseases were considered not to be related to the treatment. F4R status of these piglets was not determined and their data were excluded from analysis. The F4R status of the two sentinel piglets was F4R– (sentinel with pouch) and F4R+ (sentinel without pouch). Of the remaining 32 piglets, 20 were F4R– and 12 were F4R+.

Bacteriological examination

No haemolytic *E. coli* were found on the rectal swabs of day 0. The two sentinel piglets in experiment A had normal faeces and remained F4+ *E. coli*

negative throughout the experiment, thus indicating that transmission from the other pens to these piglets had not taken place. Table 1 shows the results of the 32 F4+ *E. coli* inoculated piglets of experiments A, B and C.

Of all the swabs taken of the pens in experiment A, only the dunging areas of two piglets without pouches, piglet 4647 (day 6 and 8) and piglet 4649 (day 6, 8 and 13), were found to be F4+ *E. coli* positive. On all other places in the pens of the piglets without pouches, the faeces were observed to be very dry. Apparently, F4+ *E. coli* could not survive here for a long period or was only present in undetectable numbers. The swabs taken of the floor area of the three piglets with pouches (n = 3, including one sentinel) on day 13, were found to be negative, indicating that their pens were not contaminated or at the most contaminated with undetectable numbers of F4+ *E. coli*.

Analysis of shedding patterns

In Table 2, the median of the number of positive samples per pig, the median of the sum of F4+ *E. coli* in the 14 samples of each pig and the median of the number of bacteria shed per F4+ *E. coli*-positive sample are shown.
The median number of positive samples were not significantly different between piglets with and without pouches ($p > 0.05$). For the F4R+ piglets, the number of positive samples was higher than for the F4R– piglets ($p < 0.05$). Also the median of the total amount of bacteria shed for F4R+ piglets was higher than for F4R– piglets ($p < 0.05$), but did not differ between piglets with and without pouches ($p > 0.05$). The median of the number of bacteria shed per F4+ *E. coli*-positive sample for piglets without pouches was higher than for piglets without pouches ($p < 0.05$), but after correction for the F4R status, the medians were found not to be significantly different ($p > 0.05$ for F4R+ and F4R–). F4R+ piglets had a significantly higher number of F4+ *E. coli* per piglet than the F4R– piglets ($p < 0.05$).

In total, 13 transitions from negative to positive samples (0, 1) were observed: seven in piglets with pouches (4 F4R– and 3 F4R+) and six in piglets without pouches (3 F4R– and 3 F4R+). Using a GLM, the factor 'presence of a pouch' did not have a significant effect on λ, the probability of (0, 1) transitions, but the factor 'F4R status' did. For F4R+ piglets, λ was estimated 0.15 [0.11 - 0.29], and for F4R– piglets, λ was estimated 0.04 [0.01 - 0.08].

Table 1. F4+ *E. coli*/g faeces days 1-14 p.i., F4R and shedding status of piglets with and without pouches in experiments A, B and C.

Experiment	F4R	Pig no	Pouch present	1 d	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
A	–	4638	yes	$1.1 \cdot 10^6$	$5.7 \cdot 10^4$	$1.0 \cdot 10^2$								$3.0 \cdot 10^2$			mv*
A	+	4639	yes	mv	$6.4 \cdot 10^5$	$4.5 \cdot 10^4$	$1.1 \cdot 10^4$		$5.8 \cdot 10^3$	$1.5 \cdot 10^5$	$1.2 \cdot 10^3$	$1.0 \cdot 10^4$					mv
A	–	4641	yes		$1.7 \cdot 10^6$	$3.4 \cdot 10^7$	$9.1 \cdot 10^7$	$1.7 \cdot 10^7$	$7.1 \cdot 10^4$	$7.5 \cdot 10^3$	$8.0 \cdot 10^2$	$8.7 \cdot 10^3$	$2.9 \cdot 10^3$	$1.1 \cdot 10^3$		$1.0 \cdot 10^2$	mv
A	+	4642	yes	$1.2 \cdot 10^7$	$8.7 \cdot 10^7$	$2.2 \cdot 10^8$	$2.6 \cdot 10^8$	$1.5 \cdot 10^9$	$1.3 \cdot 10^7$	$8.1 \cdot 10^5$	$4.0 \cdot 10^2$			$1.0 \cdot 10^2$			mv
A	–	4643	yes	$3.0 \cdot 10^3$													mv
A	–	4644	no	$5.7 \cdot 10^3$													mv
A	–	4645	no				$1.0 \cdot 10^2$				$1.0 \cdot 10^3$						mv
A	–	4647	no	$7.0 \cdot 10^4$								$4.0 \cdot 10^3$	$1.1 \cdot 10^4$	$2.6 \cdot 10^4$	$6.0 \cdot 10^3$		mv
A	–	4648	no	$4.0 \cdot 10^5$													mv
A	+	4649	no	$4.8 \cdot 10^6$	$5.6 \cdot 10^5$	$4.6 \cdot 10^9$	$1.3 \cdot 10^9$	$3.0 \cdot 10^8$	$1.8 \cdot 10^7$	$1.6 \cdot 10^5$	$1.4 \cdot 10^3$			$1.0 \cdot 10^2$			mv
B	+	5602	no	$5.9 \cdot 10^5$	$4.1 \cdot 10^8$	$7.5 \cdot 10^7$	$2.0 \cdot 10^7$	$1.0 \cdot 10^6$	$3.5 \cdot 10^4$	$2.1 \cdot 10^3$	$2.0 \cdot 10^2$	$3.5 \cdot 10^4$	$1.0 \cdot 10^2$				
B	–	5603	no														
B	–	5604	no	$1.5 \cdot 10^3$	$1.0 \cdot 10^2$												
B	–	5605	no														
B	–	5606	no	$4.6 \cdot 10^4$	$2.0 \cdot 10^3$		$1.0 \cdot 10^2$										
B	+	5607	no	$1.1 \cdot 10^7$	$2.5 \cdot 10^5$	$2.6 \cdot 10^5$									$2.0 \cdot 10^2$	$1.0 \cdot 10^2$	$1.8 \cdot 10^5$
B	+	5608	yes	$5.0 \cdot 10^2$		$2.0 \cdot 10^2$							$1.0 \cdot 10^2$	$3.0 \cdot 10^2$			
B	+	5609	yes					$1.2 \cdot 10^4$	$4.0 \cdot 10^2$	$1.8 \cdot 10^3$	$9.0 \cdot 10^2$						
B	–	5610	yes	$7.0 \cdot 10^6$	$2.6 \cdot 10^4$												
B	–	5611	yes	$7.4 \cdot 10^4$	$2.0 \cdot 10^2$												
B	–	5612	yes	$3.6 \cdot 10^3$													
B	–	5613	yes	$8.6 \cdot 10^3$	$3.0 \cdot 10^2$		$2.0 \cdot 10^2$										
C	–	6580	no	$9.0 \cdot 10^5$													
C	–	6581	no	$8.7 \cdot 10^5$	$1.4 \cdot 10^3$												
C	+	6582	no	$8.0 \cdot 10^8$	$1.8 \cdot 10^9$	$4.5 \cdot 10^9$	$2.6 \cdot 10^9$	$3.0 \cdot 10^9$	$7.8 \cdot 10^7$	$7.8 \cdot 10^6$	$1.3 \cdot 10^5$	$1.6 \cdot 10^4$	$6.4 \cdot 10^3$	$3.7 \cdot 10^4$		$1.0 \cdot 10^3$	
C	+	6583	no	$7.4 \cdot 10^8$	$2.1 \cdot 10^8$	$7.9 \cdot 10^8$	$2.4 \cdot 10^8$	$2.9 \cdot 10^9$	$3.7 \cdot 10^7$	$6.3 \cdot 10^5$	$9.7 \cdot 10^7$						
C	+	6584	no														
C	+	6585	no	$1.7 \cdot 10^6$	$6.3 \cdot 10^6$	$5.5 \cdot 10^7$	$4.0 \cdot 10^7$	$5.4 \cdot 10^7$	$4.3 \cdot 10^9$	$5.3 \cdot 10^8$	$9.8 \cdot 10^7$	$1.2 \cdot 10^8$	$7.9 \cdot 10^7$	$1.1 \cdot 10^6$			
C	–	6587	yes														
C	+	6588	yes	$2.6 \cdot 10^9$	$5.7 \cdot 10^9$	$1.8 \cdot 10^9$	$1.3 \cdot 10^9$	$6.0 \cdot 10^9$	$3.0 \cdot 10^8$	$3.0 \cdot 10^8$	$4.5 \cdot 10^5$	$4.1 \cdot 10^5$	$9.0 \cdot 10^2$				
C	–	6589	yes	$7.8 \cdot 10^4$	$7.0 \cdot 10^5$	$2.1 \cdot 10^6$	$1.4 \cdot 10^4$	$5.6 \cdot 10^5$									
C	–	6591	yes														

*mv, missing value

Table 2. The median of the number of F4+ *E. coli*-positive samples per pig, the median of the sum of F4+ *E. coli* (c.f.u.) in the 14 samples of each pig, and the median for the number of c.f.u. F4+ *E. coli* in the positive samples, for F4R+ and F4R– piglets, with and without pouches.

	F4R+ pouch present	F4R+ no pouch	F4R- pouch present	F4R- no pouch
Median (min-max) of the number of positive samples	7 (4-10)	9 (0-12)	2 (0-11)	1.5 (0-5)
Median (min-max) of the sum of F4+ *E.coli* shed in the 14 samples per pig	$8.6 \cdot 10^5$ ($1.1 \cdot 10^3$-$1.8 \cdot 10^{10}$)	$5.0 \cdot 10^9$ (0-$1.3 \cdot 10^{10}$)	$4.2 \cdot 10^4$ (0-$1.4 \cdot 10^8$)	$2.7 \cdot 10^4$ (0-$9.0 \cdot 10^5$)
Median (min-max) of the number of c.f.u. F4+ *E.coli* in positive samples	$2.8 \cdot 10^5$ ($1.0 \cdot 10^2$-$6.0 \cdot 10^9$)	$1.5 \cdot 10^7$ ($1.0 \cdot 10^2$-$4.6 \cdot 10^9$)	$1.4 \cdot 10^4$ ($1.0 \cdot 10^2$-$9.1 \cdot 10^7$)	$5.7 \cdot 10^3$ ($1.0 \cdot 10^2$-$9.0 \cdot 10^5$)

Diarrhoea

Of the 448 faecal samples collected, 305 were scored as normal (0), 104 as unformed or loose consistency (1), 15 as pasty diarrhoea (2) and 23 as liquid diarrhoea (3). The mean percentage dry matter of the samples with score 0-3 and their 95% CI are shown in Table 3. The upper limit of the 95% CI of score 3 was 12.6%. Ten piglets had one or more samples with a percentage dry matter ≤12.6%, and were therefore classified as severely diarrhoeic. Of these ten piglets, six were F4R+ and four were F4R–. Association of F4R status and severe diarrhoea resulted in $p = 0.08$. Thus, in this experiment, classification into F4R+ and F4R– was not significantly associated with the occurrence of severe diarrhoea. Sixteen out of 23 samples of liquid diarrhoea, were found positive for F4+ *E. coli*, indicating that liquid diarrhoea was linked to F4+ *E. coli* excretion.

Table 3. Mean % dry matter and 95% CI of faecal samples with scores 0-3 indicating the consistency of the faeces.

Score	Mean	95% CI
0 = normal	28.9	28.4 – 29.4
1 = unformed or loose consistency	23.0	22.1 – 23.9
2 = pasty diarrhoea	15.5	13.1 – 18.0
3 = liquid	10.1	7.6 – 12.6

Discussion

In this study, the effect of reinfection on the shedding patterns of individually housed piglets challenged with F4+ *E. coli* was quantified, thus, strictly speaking, self-reinfection was measured. This was done in order to determine whether reinfection during primary infection can be considered an important factor in the population dynamics of F4+ *E. coli* between piglets. Prevention from reinfection by collecting all faeces in pouches did neither affect the number of positive samples nor the amount of bacteria shed. Moreover, the pouches did not affect the probability that a negative faecal sample was followed by a positive sample (λ), which characterises intermittent shedding patterns. The observed intermittent shedding patterns mainly concerned low numbers of F4+ *E. coli*, and may therefore be related to failure of detection.

Applying an infectiousness measure obtained from F4+ *E. coli* transmission studies (Chapter 4) to the shedding patterns of the piglets in the current experiment, determined 10 piglets to be infectious, five with pouches and five without pouches. Reinfection and infectiousness were not associated ($p > 0.05$, Fisher's exact test). It was therefore concluded that we did not find any indication that reinfection contributed to the shedding patterns and hence to the infectiousness of the piglets in the first two weeks after infection. After experimental inoculation, colonization and the resulting shedding seem to be an autonomous process that is not or only little affected by the uptake of new bacteria from the environment. Moreover, at the end of the shedding period, local immunity may have been developed by which the piglets were (partly) protected from reinfection with F4+ *E. coli*. Van den Broeck *et al.* (1999) showed that F4R+ piglets orally challenged with F4+ *E. coli* developed an immune reaction eight days after infection. F4R+ piglets orally immunized with only the F4 fimbrial adhesin FaeG monomers also showed an immune response (Verdonck *et al.*, 2004). In the latter study, the mean number of faecal F4+ *E. coli* shed by these piglets after challenge at 24 days after first immunization was significantly lower than in piglets that were not immunized, indicating that some weeks after immunization protection from reinfection occurred. It is not clear whether, soon after experimental infection with F4+ *E. coli*, the displayed immune responses would have been sufficient to protect the piglets from reinfection.

In contrast to reinfection, presence or absence of the adhesion site for F4+ *E. coli* was found to affect the shedding patterns. The reinfection probability λ was significantly different for F4R+ and F4R– piglets. This was mainly caused by the long periods of negative samples, that is many (0,0)-transitions, in the F4R– piglets, which decreased λ significantly. F4R+ piglets shed a higher total amount of F4+ *E. coli* and had more positive samples.

Eight F4R+ piglets and two F4R– piglets were classified to be infectious by the aforementioned infectiousness measure; F4R status and infectiousness were significantly associated ($p < 0.05$).

In this paper, we mainly focussed on pathogen shedding. However, reinfection of the host is also related to the contact behaviour of the host and the pathogen survival in the environment. The survival of F4+ *E. coli* in the dunging area of the pens seemed to be sufficiently long for reinfection to occur. In earlier studies, young piglets have been reported to spend a considerable time rooting in faeces (Newberry and Wood-Gush, 1988). Moreover, it has been reported that young piglets were estimated to ingest 8.5-12.3 g faeces/day (De Passillé *et al.*, 1989; Gleed and Sansom, 1982). We therefore assumed that there was sufficient contact of the piglets with the infected faeces.

In this study more insight was gained in the population dynamics of F4+ *E. coli*. No indications were found that reinfection with F4+ *E. coli* from the environment affected the shedding patterns of individually housed, inoculated piglets. The F4R status on the contrary, was found to be an important factor in F4+ *E. coli* dynamics.

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

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Abstract

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 causative agents of post-weaning diarrhoea (PWD) in piglets (Frydendahl, 2002; Nagy *et al.*, 1990; Wittig *et al.*, 1995). 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 spread of the bacteria.

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 (Hampson *et al.*, 1985; Madec *et al.*, 2000; Rutter *et al.*, 1975). This adhesion site is a genetically inherited dominant characteristic and its presence can be shown by *in vitro* adhesion assays (Sellwood *et al.*, 1975; Sellwood *et al.*, 1979). 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 (Chapter 2), 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 (Edfors-Lilja, 1991). Whether the infection will spread depends not only on the susceptibility 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 (Bouma *et al.*, 1995; Bouma *et al.*, 1996; Bouma *et al.*, 2000; De Jong and Kimman, 1994; Nodelijk *et al.*, 2001). 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 (Velthuis *et al.*, 2002). 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 of adjacent pens had visual but not physical contact. Density of the piglets was one piglet per 0.45m^2 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 to follow the excretion of the inoculated piglets and 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 and 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 1.0x10^6 particles/ml.

E. coli serotype O149K91F4ac (LT+, STb+), strain CVI-1000 (Animal Sciences Group, The Netherlands) (Nabuurs *et al.*, 1996), 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 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 homogenised 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.* (1975). 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 x400). Only cells with well-defined brush borders were studied. Animals with no or an average of 1-2 bacteria/brush border were studied 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 (Chapter 2). 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 (Mardia *et al.*, 1979) was applied on the first principal component resulting from the PCA, and it was concluded that using this measure based on the truncated %DM data we can distinguish two significantly different groups ($P = 0.00$). The fractions of piglets in groups 1 and 2 (0.482 en 0.518) and means and variances of the underlying distributions were estimated by maximum likelihood with the program EMMIX (Peel and McLachlan, 1999; McLachlan and Peel., 2000). The boundary value with the most optimal allocation of the error over the two types of error terms found was –5.16. Piglets of which $\Sigma\text{coeffecient}\%DM1_k * (\%DM_k - \mu\%DM_k) > -5.16$ ($k = 1, 2,..8$) were classified as having diarrhoea, in which $\%DM_k$ is the truncated %DM of an individual piglet at day k and $\text{coefficient}\%DM_k$ and $\mu\%DMk$ 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 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.

Most important objection to using this measure is that the truncated %DM data does not follow a normal distribution. Therefore we have also used an alternative test and the agreement in outcome of both tests has been quantified using the kappa value (Noordhuizen *et al.*, 1997).

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 and 3 = liquid) was given 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 (GenStat Committee, 2000).

Determination of transmission parameters

Calculations of the transmission parameters were based on the stochastic SIR model (Bailey, 1975). 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 started 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 $E(C) = S \cdot (1 - e^{-\beta \cdot \Delta t \cdot (I/N)})$. Since S, I, N and C were known from the transmission experiment β was estimated using a generalized linear model (GLM) (Becker, 1989). 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 (McCullagh and Nelder, 1989). GLMs were performed with GenStat (GenStat Committee, 2000).

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

$$R_0(f) = \tfrac{1}{2}\left(k_{11} + k_{22} + \sqrt{(k_{11} + k_{22})^2 - 4 \cdot (k_{11}k_{22} - k_{12}k_{21})}\right),$$

(Diekmann and Heesterbeek, 2000). 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: Σcoeffecient$\ln cfu_k \cdot (\ln cfu_k - \mu \ln cfu_k)$, with k = day 1, 2,..., 8 was smaller than 1.96 were high shedders (Chapter 2). $\ln cfu_k$ are the log-transformed numbers of (F4+ *E. coli*/g +1) found in the faecal samples 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 $\ln cfu_k$ and $\mu \ln cfu_k$ were obtained from an earlier study (Chapter 2) and are given in Table 1.

Table 1. Coefficient and mean obtained from an earlier study (Chapter 2), for classification of high- and low-shedding piglets.

k	Coefficient $\ln cfu_k$	$\mu \ln cfu_k$
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 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 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).

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		$1.9 \cdot 10^4$	$3.2 \cdot 10^4$	$1.1 \cdot 10^5$	$4.4 \cdot 10^5$	$4.6 \cdot 10^9$	$1.9 \cdot 10^{10}$	$1.9 \cdot 10^9$	$1.4 \cdot 10^9$	$2.9 \cdot 10^7$	$1.7 \cdot 10^5$		$7.0 \cdot 10^3$			$1.0 \cdot 10^5$	142.11	high
	6159c	n.d.					$1.6 \cdot 10^5$	$1.4 \cdot 10^7$	$6.6 \cdot 10^6$	$5.6 \cdot 10^4$	$1.3 \cdot 10^7$	$1.2 \cdot 10^5$	$7.3 \cdot 10^2$	$6.0 \cdot 10^2$				57.89	high
2.1	6164i	$4.2 \cdot 10^4$																126.32	low
	6165c	n.d.																242.11	low
4.1	6181i		$8.2 \cdot 10^4$	$3.6 \cdot 10^4$	$2.4 \cdot 10^5$	$1.5 \cdot 10^5$	$7.0 \cdot 10^4$	$6.5 \cdot 10^4$	$4.3 \cdot 10^3$	$1.0 \cdot 10^5$	$3.1 \cdot 10^5$	$9.7 \cdot 10^5$	$9.9 \cdot 10^3$	$3.7 \cdot 10^4$	$1.8 \cdot 10^5$			142.11	high
	6180c	n.d.					$5.8 \cdot 10^4$	$1.4 \cdot 10^4$	$2.5 \cdot 10^5$	$6.7 \cdot 10^3$	$3.3 \cdot 10^7$	$2.9 \cdot 10^5$	$1.7 \cdot 10^5$	$1.6 \cdot 10^4$	$1.0 \cdot 10^5$			57.89	high
4.4	6186i		$5.0 \cdot 10^4$	$8.9 \cdot 10^3$	$2.7 \cdot 10^4$	$1.2 \cdot 10^5$	$1.3 \cdot 10^5$	$3.0 \cdot 10^7$	$6.0 \cdot 10^5$	$2.6 \cdot 10^5$	$2.0 \cdot 10^5$		$3.0 \cdot 10^5$					173.68	high
	6187c	n.d.										$1.1 \cdot 10^5$	$1.7 \cdot 10^4$	$1.1 \cdot 10^4$	$6.0 \cdot 10^4$			152.63	high
5.4	6195i		$1.7 \cdot 10^7$	$1.1 \cdot 10^5$	$2.5 \cdot 10^5$	$4.9 \cdot 10^5$	$2.0 \cdot 10^5$	$1.3 \cdot 10^5$	$2.4 \cdot 10^5$	$4.4 \cdot 10^7$	$8.0 \cdot 10^5$	$5.2 \cdot 10^4$	$2.4 \cdot 10^5$		$1.1 \cdot 10^5$			84.21	high
	6194c	n.d.			$5.4 \cdot 10^5$	$7.8 \cdot 10^5$	$1.5 \cdot 10^4$	$3.0 \cdot 10^4$	$1.0 \cdot 10^3$	$8.0 \cdot 10^5$	$2.0 \cdot 10^4$							200.00	high
F4R−/F4R−																			
1.1	6157i		$1.0 \cdot 10^5$	$9.9 \cdot 10^4$														63.16	low
	6156c	n.d.																36.84	low
1.4	6163i			n.d.			$5.4 \cdot 10^4$	$1.0 \cdot 10^5$										252.63	low
	6162c	n.d.																236.84	low
2.2	6167i		$2.5 \cdot 10^5$	$1.1 \cdot 10^5$														178.95	low
	6166c	n.d.																231.58	low
5.1	6188i				$3.0 \cdot 10^4$	$1.1 \cdot 10^4$												42.11	low
	6189c	n.d.																189.47	low
5.2	6191i		$2.7 \cdot 10^5$	$1.1 \cdot 10^5$														200.00	low
	6190c	n.d.																226.32	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 F4+ *E.coli* shedding profiles.

[d] Receptor status combination: status contact piglet followed by status inoculated piglet.

n.d., Not determined.

xxxx, Dead.

////////, Killed early, no F4+ *E.coli*-positive samples.

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

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]																			
2.3	6169i												////////	////////	////////	////////	////////	n.d.	low
	6168c	n.d.											////////	////////	////////	////////	////////	n.d.	low
2.4	6171i		$2.3 \cdot 10^6$															173.68	low
	6170c	n.d.																205.26	low
5.3	6192i		$7.0 \cdot 10^6$	$1.6 \cdot 10^3$		$1.0 \cdot 10^6$	$1.5 \cdot 10^6$	$3.0 \cdot 10^6$	$2.9 \cdot 10^6$	$1.5 \cdot 10^6$	$2.2 \cdot 10^6$	$1.4 \cdot 10^6$						242.11	high
	6193c	n.d.																252.63	low
F4R+/F4R–																			
3.1	6173i												////////	////////	////////	////////	////////	n.d.	low
	6172c	n.d.									n.d.		////////	////////	////////	////////	////////	n.d.	low
3.2	6175i	n.d.	$4.0 \cdot 10^6$			$2.4 \cdot 10^6$												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		$2.0 \cdot 10^5$	$9.0 \cdot 10^2$														5.26	low
	6183c	n.d.																178.95	low
4.3	6185i		$1.6 \cdot 10^4$	$2.0 \cdot 10^3$								$1.0 \cdot 10^6$						168.42	low
	6184c	n.d.																215.79	low
F4R–/ ?																			
1.3	6160i	$5.0 \cdot 10^5$	$8.0 \cdot 10^6$	$9.9 \cdot 10^6$	$4.9 \cdot 10^7$	$2.4 \cdot 10^8$	$3.1 \cdot 10^8$	$1.1 \cdot 10^9$	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	n.d.	high
	6161c	n.d.						$3.5 \cdot 10^6$		$7.0 \cdot 10^2$								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 F4+ *E.coli* shedding profiles.
[d] Receptor status combination: status contact piglet followed by status inoculated piglet

n.d., Not determined.
xxxx, Dead.
////////, Killed early, no F4+ *E.coli*-positive samples.

Clinical parameters

PCA measure on truncated %DM data

Using the measure derived from the principal component analysis 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 2x2-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%) 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 (Noordhuizen *et al.*, 1997). 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 (Noordhuizen *et al.*, 1997).

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, $P = 0.20$, Mann Whitney U test. The mean weight gain for diarrhoeic and non-

diarrhoeic piglets classified by the %DM measure were 163.7 g day –1 (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 diarrhoeic pigs had a significantly lower weight gain than piglets with normal faeces, $P < 0.01$, (% DM measure, $P = 0.25$) 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 '≥ 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 post-weaning diarrhoea, 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 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.

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'	$\beta_{pp} = 0.62$	0.19 - 2.06
	$\beta_{np} = 0.00$ [a]	0.00 - 1.98
	$\beta_{np} = 0.16$ [b]	0.03 - 0.75
'≥ 1 F4+ *E. coli*-positive sample'	$\beta_{pp} = 0.58$	0.19 - 1.75
	$\beta_{np} = 0.15$	0.03 - 0.66

[a] Excluding pair 6160/6161 as a case.

[b] 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: $\beta_{upper} = 2 \cdot \ln(1-P)$, $Pr(C=0|P) = (1-P)^n = 0.05$; C is the number of cases and n is the number of pairs.

To evaluate whether 'high shedder' and '≥ 1 F4+ *E. coli*-positive sample' were good measures for infectiousness, the association of the inoculated piglets being 'high shedder' or having '≥ 1 F4+ *E. coli*-positive sample' with the number of their contact piglets that became 'high shedder' or had '≥ 1 F4+ *E. coli*-positive sample' was tested with Fisher's exact test. For 'high shedder' a P value < 0.01 was found (both with and without piglet 6161 as a case) and for '≥ 1 F4+ *E. coli* pos sample sample' $P = 0.53$. The reproduction ratio (R_0) calculated for homogeneous F4R+ piglet populations was estimated 7.1 ($T=11.4$) with 95% confidence interval [2.3-21.9]. R_0 for homogeneous F4R– piglet populations could not be calculated, as there were not 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 '≥ 1 positive sample' as measures for infectiousness. We conclude 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 '≥ 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 immunised to eradicate a microparasite (Anderson and May, 1991) and almost similar to the findings on the proportion of homozygous pigs

for a fictive major disease resistance gene to bring R_0 below one, assuming an underlying pig farm structure (MacKenzie and Bishop, 1999). 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 themselves. In case $\beta_{nn} > 0$ the fraction of F4R+ piglets in the population should be even lower than the 0.14 calculated from 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 (Velthuis *et al.*, 2003). 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 (Lecce *et al.*, 1982). It is unknown whether interference of rotavirus with the intestinal mucosa integrity affects F4-receptor 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 F4-receptor 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 F18+ *E. coli* resistance (Vögeli *et al.*, 1996; Frydendahl *et al.*, 2003). Moreover, it is debatable whether it is advisable to breed out a trait that might have an unknown beneficial function (Edfors-Lilja *et al.*, 1986; Baker *et al.*, 1997) 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 fraction of F4R+ piglets at most 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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5

Transmission of F4+ *E. coli* in groups of early weaned piglets

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Abstract

The aim of this study was to estimate transmission parameters of enterotoxigenic F4+ *Escherichia coli* (F4+ *E. coli*) in groups of early weaned piglets with F4-receptor-positive (F4R+) and F4-receptor-negative piglets (F4R–). Transmission of F4+ *E. coli* was quantified in four heterogeneous groups of F4R+ and F4R– piglets. Infectiousness was determined by the number of F4+ *E. coli*/g faeces shed during 8 days. Transmission parameters were estimated using generalized linear models assuming a stochastic SIR- model.

F4R+ piglets were found to be more susceptible than F4R– piglets, but F4R+ and F4R– piglets were not different in infectiousness. The reproduction ratios for homogeneous F4R+ and F4R– populations were estimated as 6.37 (95% CI 1.89 - 21.48) and 0.02 (95% CI 0.00 - 1.13), respectively.

The implication of these results is that in order to prevent major outbreaks, the fraction of F4R+ piglets should be small (approximately 10% or less). Therefore, selective breeding programs could contribute to reducing F4+ *E. coli* related diarrhoea and transmission.

Introduction

In 2001, an evaluation of the importance of several diseases for the Dutch pig farming was undertaken, and it was reported that post-weaning diarrhoea (PWD) is one of the diseases which causes substantial damage (Smits and Merks, 2001). PWD is associated with the colonization and mass proliferation of enterotoxigenic *Escherichia coli* strains in the small intestine of newly weaned piglets (Van Beers-Schreurs *et al.*, 1992).

At the moment there are no effective vaccines available (Van den Broeck *et al.*, 1999; Snoeck *et al.*, 2003) and it is feared that the present more restrictive use of anti-microbial growth promotors in the European Union and a complete ban of these growth promotors by 2006 will lead to an increase in diarrhoea incidence after weaning (Casewell *et al.*, 2003). Therefore other measures have to be investigated that either prevent clinical symptoms by directly interfering on the individual level or by preventing transmission of the bacterium between piglets.

Adherence of *E. coli* to the small intestine is an important step in the colonization and massive replication of *E. coli* (Beachy, 1981). Several adherence factors have been described and enterotoxigenic *E. coli* strains with adhesin F4 (or K88) have been reported as the main agents of PWD in several countries (Frydendahl, 2002; Nagy *et al.*, 1990; Wittig *et al.*, 1995).

It was shown that F4+ *E. coli* do not adhere to the small intestine of all piglets, some piglets do not express the adherence site, F4-receptor (F4R) (Sellwood *et al.*, 1975). The adherence strategy of F4+ *E. coli* and the differences in expression of the receptor offers opportunities to prevent F4+ *E. coli* infections for example by F4-based oral vaccines (Van den Broeck *et al.*, 1999; Snoeck *et al.*, 2003) and genetic selection for F4R– piglets (Edfors-Lilja and Wallgren, 2000). Selection for F4R– piglets could be a good option to interfere in both F4+ *E. coli* diarrhoea and transmission. To what extent this measure will affect transmission can be quantified in transmission experiments.

One-to-one transmission experiments, in which one infectious pig is housed with one susceptible pig, have the advantage that, within a pair of piglets it is clear who infected whom. Results of a one-to-one transmission experiment with F4+ *E. coli* showed that F4R– piglets were less susceptible for F4+ *E. coli* infection than F4R+ piglets (Chapter 4). Results of this experiment were inconclusive on whether F4R+ and F4R– piglets differed in infectiousness, as none of the inoculated F4R– piglets was infectious, probably due to a low infection pressure. The implications of these findings are that the fraction of F4R+ piglets in a population will have influence on F4+ *E. coli* transmission.

To further investigate the effect of the fraction of F4R+ piglets in a population we estimated transmission parameters in groups of early weaned piglets. The group experiments provide new data in addition to the data from the one-to-one transmission experiment. The new data is important for a more

accurate estimation of the transmission parameters and for insight in the interaction of piglets with different shedding patterns and different receptor status.

Methods

Experimental Design

Forty-eight castrated male piglets, age 3-4 weeks, were brought from a commercial farm in the Netherlands to the Animal Sciences Group at the day of weaning (day 0).

The piglets were obtained from 14 litters of 1-5-parity sows. Upon arrival piglets were weighed (weights 4.3-11.3 kg) and rectal swabs were taken and were checked for haemolytic *E. coli*. Piglets were housed in four groups of 12 piglets, one group per stable. They were assigned randomly to the groups with restriction that littermates were not housed in the same group and that the weights of the piglets were equally distributed over the groups. All pens consisted of wire-mesh partitions that were placed on grid floors, with one piglet per 0.45 m^2 floor surface. The mean temperature of the stables was 25°C with a 16-h light/8-h dark cycle. During the experiment the pens were not cleaned to ensure a maximum infectivity in the pen.

On days 0 and 1, piglets were fasted with water available *ad libitum*, from day 2 piglets were fed *ad libitum* with standard commercial piglet feed containing 18.9% crude protein (Hope Farms bv, Woerden, The Netherlands). This diet did not contain any antibiotics.

On day 4, all piglets were inoculated orally with 2 ml of a rotavirus strain RV277 suspension. On day 5, six randomly chosen piglets per group were orally inoculated with 5 ml of 10^9 c.f.u./ml F4+ *E. coli* suspension [Animal Sciences Group (ASG), Lelystad, The Netherlands]. Rectal faecal samples were collected daily of both inoculated and contact piglets and the number of F4+ *E. coli*/g faeces and the percentage dry matter of these samples was determined. Faeces were also examined and a 4-point scoring scale (0 = normal, 1 = unformed or loose consistency, 2 = pasty diarrhoea and 3 = liquid) was used by the animal caretakers to describe the consistency. During the experiment the health of the piglets was recorded daily. On day 19 piglets were weighed, euthanized, bled and necropsied. A 5-10 cm jejunal sample was taken for determination of the F4R status by brush border adhesion assay (BBA), (Sellwood *et al.*, 1975).

For the record, we would like to point out that biopsies were taken of the small intestine of the piglets in group 4 on day 1 for future research possibilities. Before the biopsies were taken, piglets were sedated with azaperone (2.0 mg/kg; Stresnil®, Janssen-Cilag, Tilburg, The Netherlands) and were anaesthetized using 3% sevoflurane (Sevorance®, Abbott, Zwolle, The Netherlands), (1 l O_2 and 0.8 l N_2O), no medication was given. As far as we could ascertain, this did not affect the

health of the individual piglets and could not have interfered with our study. The local Ethics Committee for Animal Experiments approved the experimental protocols.

Inoculation

Rotavirus strain RV277 is maintained at the laboratory 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 $1.0x10^6$ particles/ml. Inoculation with rotavirus preceding F4+ *E. coli* inoculation was chosen, because in our experience, rotavirus is a predisposing factor for F4+ *E. coli* infections and is often found as a co-infection in the field.

E. coli serotype O149:K91:F4ac (LT+, STb+), strain CVI-1000 (ASG, The Netherlands) (Nabuurs *et al.*,1996), was isolated from a pig farm with PWD. This strain was found to be resistant to a combination of streptomycin, tetracycline and vancomycine, which was therefore added to selective His-agar to prevent overgrowth by other bacteria (see also 'Determination of c.f.u. F4+ *E.coli*/g faeces' section below). As a negative control in the BBA, *E. coli* strain CVI-1084 (ASG, The Netherlands) was used. This is also an O149:K91 (LT+, STa+) strain, but without fimbrial expression of F4. The strains were grown overnight in brain-heart infusion broth (Difco Laboratories, Detroit, MI, USA) at 37°C, pelleted by centrifugation, resuspended in PBS at pH 7.2, (Biotrading, Mijdrecht, The Netherlands), to an optical density value of 1.050 at 600 nm which corresponds to a suspension of 10^9 c.f.u./ml.

Faecal dry matter and faecal scores

Faeces (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 lost water.

Determination of c.f.u. F4+ *E.coli*/g faeces

Of ten-fold dilutions of faeces homogenized in saline (Biotrading, The Netherlands), 100 µl was plated on selective His-agar plates containing 5% sheep blood, streptomycin 50 µg/ml, tetracycline 25 µg/ml and 50µg/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 on the

colony morphology, identity was confirmed by slide agglutination to establish the *E. coli* OK type (ASG, The Netherlands).

Determination of F4R status

Receptor status of the duodenal biopsies taken from the piglets in group 4 at day 1 were determined as described below.

At necropsy, 5-10 cm of jejunal mucosa was scraped off of all piglets including the piglets in group 4. Epithelial brush borders were prepared to determine the F4R status of the piglets modified after Sellwood *et al.* (1975). Mucosal scrapings were placed in PBS containing 0.005 M EDTA (Merck, 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) added, 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 cover slip, and bacterial adherence was determined by phase contrast microscopy (magnification 400x). Only cells with well-defined brush borders were studied. Animals with no or just 1-2 bacteria per brush border were considered F4R–; samples exceeding this were judged F4R+. In case of ambiguity, bacterial adherence of a new sample of the cell suspension with bacteria was determined.

Determination of shedding type

Piglets were classified in high and low shedders according to their F4+ *E. coli* shedding patterns of days 1-8 after inoculation. In Chapter 2, a classification rule was determined by principal components analysis (PCA) to distinguish between high and low shedders. High shedding is associated with piglets being infectious (Chapter 4). All piglets of which the sum: $\sum$ coeffecient $\ln cfu_k \cdot (\ln cfu_k - \mu \ln cfu_k)$, with k = day 1, 2,..., 8 is smaller than 1.96 are high shedders. $\ln cfu_k$ are the log-transformed numbers of (F4+ *E. coli*/g +1) found in the faecal samples of days 1-8 after inoculation in the current study. For the contact piglets, day 1 was defined as the first day an F4+ *E. coli*-positive sample was found. For missing values the mean of the two surrounding values was filled in. The values of coefficient $\ln cfu_k$ (the coefficients of the first eigenfunction resulting from principal component analysis) and $\mu \ln cfu_k$ [the mean of the log-trans-formed numbers of (F4+ *E. coli*/g +1) in the population] were obtained from Chapter 2 and are given in Table 1.

Table 1. The coefficients of the first eigenfunction resulting from principal components analysis (coefficient $\ln cfu_k$) and the population mean ($\mu \ln cfu_k$) of log-transformed F4$^+$ *E.coli*/g data (days 1-8 after inoculation, n=69) obtained from Chapter 2, which were needed for the classification of high- and low-shedding pigs.

K	coefficient ln cfu_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

*Coefficients of the first eigenfunction resulting from principal components analysis.

†Population mean of the log-transformed number of F4+ *E.coli*/g faeces.

Determination of diarrhoea and weight gain

For each set of faecal samples with a particular score (0-3), the mean percentage dry matter and 95% confidence intervals (CI) was calculated. Only piglets with one or more samples with a percentage dry matter below the upper limit of score 3 were considered to have severe diarrhoea. The association between absence or presence of severe diarrhoea and F4R status and between absence or presence of severe diarrhoea and classification as high and low shedders was studied using Fisher's exact test for association.

Weight gain of the piglets was calculated as the mean weight over 19 days (g/day). It was tested whether F4R+ piglets or 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 (GenStat Committee, 2000).

Determination of transmission parameters

Infection rate parameter

For the calculations of the transmission parameters we assumed a stochastic SIR model (Bailey, 1975). In this model individuals can either be susceptible (S), infectious (I) or recovered and immune (R). New infections are assumed to occur at the rate $(\beta \cdot S \cdot I)/N$, where β is the infection rate parameter and N the total number of individuals.

The probability of one susceptible animal to become infected (a case) within an interval Δt is $(1-e^{-\beta \cdot \Delta t \cdot (I/N)})$. 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 $E(C) = S \cdot (1-e^{-\beta \cdot \Delta t \cdot (I/N)})$.

As F4R+ and F4R– piglets are thought to differ in susceptibility and infectivity, we distinguished between S_p and S_n, I_p and I_n, R_p and R_n individuals in which the subscript p stands for F4R positive and subscript n for F4R negative. This resulted in four different infection rate parameters; β_{pp}, β_{pn}, β_{np} and β_{nn}. Here the first character in the subscript is the F4R status of the contact piglet and the second is the F4R status of the infectious piglet (p = positive, n = negative). Thus,

for F4R+ cases it applies that: $E(C_p) = S_p \cdot (1\text{-}\exp\{-(\beta_{pp} \cdot I_p \cdot \beta_{pn} \cdot I_n)\Delta t / N\}$,

and for F4R–: $E(C_n) = S_n \cdot (1\text{-}\exp\{-(\beta_{np} \cdot I_p \cdot \beta_{nn} \cdot I_n)\Delta t / N\}$.

From the experiment it is known between which subsequent samplings the contact piglets started excreting F4+ *E. coli*. It was assumed that a contact infection occurs one day before the contact piglets were found to start excreting F4+ *E. coli*.

Furthermore, it was assumed that in order to become a case, a piglet sheds a sufficient amount of F4+ *E. coli* to be infectious. 'High shedders' (see also 'Determination of shedding type' section above) were assumed to be infectious as this was found to be highly associated with the occurrence of cases in a one-to-one transmission experiment Chapter 4). Since S, I, N and C were measured daily from the transmission experiment, we may drop Δt, being the unit of time, from the equation above, without loss of generality. With these measurements, the four βs were estimated using a generalized linear model (GLM) (Becker, 1989) with a complementary log-log link function and $Log\left(\frac{I_p + I_n}{N}\right)$ as offset variable (McCullagh and Nelder, 1989). For F4R+ cases it applies that:

$$Log(-Log(1-\frac{E(C_p)}{S_p})) = Log\beta + Log\left(\frac{I_p + I_n}{N}\right) = a + b \cdot q + Log\left(\frac{I_p + I_n}{N}\right)$$

and for F4R– cases:

$$Log(-Log(1-\frac{E(C_n)}{S_n})) = Log\beta + Log\left(\frac{I_p + I_n}{N}\right) = c + d \cdot q + Log\left(\frac{I_p + I_n}{N}\right)$$

with $q = \frac{I_p}{I_p + I_n}$ and $\beta_{pn} = e^a, \beta_{pp} = e^{a+b}, \beta_{np} = e^{c+d}, \beta_{nn} = e^c$.

GLMs were performed with GenStat (GenStat Committee, 2000).

Reproduction ratio (R_0)

An insightful way to express the transmission parameters is by the 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 (Diekmann *et al.*, 1990). R_0 for the model described above is $R_0=\beta \cdot T$ where T is the average infectious period. T is estimated as the number of days from the first till the last F4+ *E. coli*-positive sample, which is consistent with the infectious period for the estimation of the infection rate parameters. R_0 for heterogeneous populations (that is populations with F4R+ and F4R–) was calculated depending on the fraction of F4R+ piglets (f) in the population. R_0 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:

$$R_0(f) = \tfrac{1}{2}\left(k_{11} + k_{22} + \sqrt{(k_{11} + k_{22})^2 - 4 \cdot (k_{11}k_{22} - k_{12}k_{21})}\right),$$ (Diekmann *et al.*, 1990) and $R_0(f)$ for the estimated βs and Ts is shown in the Figure.

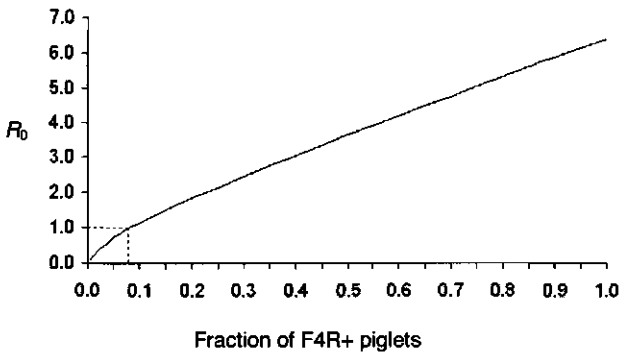

Fig. R_0 plotted as a function of the fraction of F4R+ piglets in a population, with transmission rate parameters β_{pn} = 1.42, β_{pp} = 0.77, β_{np} = 0.05, β_{nn} = 0.00 and the average infectious periods T_n = 10.8 and T_p = 8.3.

Results

Mortality and F4R status

Two piglets had to be killed for ethical reasons and were removed from the groups before F4+ *E. coli* inoculation. Therefore, groups 2 and 4 consisted of five inoculated and six contact piglets. During the experiment, one piglet (6760) with symptoms of severe PWD was found dead at day 3 after F4+ *E. coli* inoculation. Receptor status of this piglet could not be determined, but because of the severity of the diarrhoea and the high number of F4+ *E. coli* shed, it was assumed to be an F4R+ piglet. Of the other 45 piglets, 21 were F4R+ and 24 were F4R–. Distribution of F4R+ and F4R– over the groups is shown in Table 2.

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 the number of F4+ *E. coli*/g faeces (the results shown are in c.f.u./g, not log-transformed) in the faecal samples collected after inoculation. Only six out of 46 piglets had no F4+ *E. coli*-positive samples during the experiment. They were all F4R–, five piglets were housed in group 3. Shedding status of piglet 6760 could not be determined since only samples of day 1 and 2 p.i. were obtained. For the estimation of the transmission parameters it was assumed to be a high shedder and thus infectious, considering the high number of F4+ *E. coli* in the first faecal samples. Of the 40 piglets with at least one F4+ *E. coli*-positive sample or more, 26 were determined to be high shedders and 14 low shedders. Association of receptor status and shedding type of the inoculated piglets was highly significant ($P < 0.01$, Fisher's exact test).

Clinical parameters

Clinical scores

Of the 644 faecal samples collected, 361 were scored normal, 187 with unformed or loose consistency, 57 with pasty diarrhoea and 39 with liquid diarrhoea. The mean percentage dry matter of the samples with scores 0-3 and the 95% CIs are shown in Table 3. Nine piglets (3 inoculated and 6 contact piglets) had one or more samples with a percentage dry matter ≤ 10.6%, the upper limit of the 95%-confidence interval of score 3, and were classified as severely diarrhoeic. All nine piglets were F4R+ and high shedders. Both receptor and shedding status were highly associated with the occurrence of liquid stools ($P < 0.01$, Fisher's exact

Table 2. Number of F4+ *E.coli*/g faeces (c.f.u./g, not log-transformed), receptor status and shedding type of piglets in groups 1-4

Group	Receptor	Pig no.[a]	1 d	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	Shedding type
1	–	6741i[b]						$1.0 \cdot 10^3$	$1.0 \cdot 10^4$	$2.0 \cdot 10^5$	$3.2 \cdot 10^4$	$7.0 \cdot 10^4$	$1.0 \cdot 10^2$				high
1	–	6742i	$1.8 \cdot 10^6$	$6.0 \cdot 10^2$			$2.0 \cdot 10^2$	$5.0 \cdot 10^3$	$1.0 \cdot 10^5$	$2.0 \cdot 10^4$	$1.6 \cdot 10^4$			$1.0 \cdot 10^4$			high
1	+	6744i	$3.1 \cdot 10^5$	$1.7 \cdot 10^6$	$4.4 \cdot 10^6$	$1.6 \cdot 10^6$	$2.7 \cdot 10^6$	$1.6 \cdot 10^6$							$8.0 \cdot 10^2$		high
1	+	6745i	$4.9 \cdot 10^6$	$8.1 \cdot 10^6$	$5.5 \cdot 10^5$	$1.2 \cdot 10^6$	$1.3 \cdot 10^5$	$3.6 \cdot 10^5$	$5.6 \cdot 10^5$	$7.8 \cdot 10^5$	$3.9 \cdot 10^3$						high
1	+	6750i	$3.0 \cdot 10^4$	$1.2 \cdot 10^5$		$5.0 \cdot 10^2$		$3.8 \cdot 10^5$	$4.2 \cdot 10^5$								high
1	–	6752i								$3.7 \cdot 10^3$	$1.1 \cdot 10^4$						low
1	+	6743c			$5.5 \cdot 10^4$	$3.5 \cdot 10^6$	$2.6 \cdot 10^7$	$2.4 \cdot 10^7$	$2.3 \cdot 10^6$	$2.5 \cdot 10^6$	$1.5 \cdot 10^5$	$5.0 \cdot 10^3$	$4.6 \cdot 10^3$	$1.7 \cdot 10^3$			high
1	–	6746c			$1.2 \cdot 10^3$				$6.2 \cdot 10^2$	$2.0 \cdot 10^2$	$1.8 \cdot 10^3$		$3.0 \cdot 10^2$				low
1	+	6747c				$7.7 \cdot 10^4$	$4.0 \cdot 10^5$	$1.9 \cdot 10^5$	$1.8 \cdot 10^5$	$1.6 \cdot 10^5$	$1.7 \cdot 10^5$						high
1	+	6748c		$1.5 \cdot 10^4$	$9.2 \cdot 10^6$	$6.3 \cdot 10^7$	$4.0 \cdot 10^7$	$1.5 \cdot 10^6$	$3.6 \cdot 10^5$	$4.2 \cdot 10^5$	$1.5 \cdot 10^5$	$4.6 \cdot 10^4$	$8.0 \cdot 10^2$	$1.0 \cdot 10^3$	$4.0 \cdot 10^3$		high
1	+	6749c	$4.6 \cdot 10^2$		$3.8 \cdot 10^4$	$1.8 \cdot 10^6$	$8.7 \cdot 10^6$	$7.5 \cdot 10^4$	$2.2 \cdot 10^4$	$9.1 \cdot 10^5$	$3.6 \cdot 10^4$	$1.1 \cdot 10^4$	$7.2 \cdot 10^5$	$7.8 \cdot 10^5$	$1.2 \cdot 10^5$		high
1	+	6751c		$1.0 \cdot 10^4$	$2.0 \cdot 10^5$	$6.8 \cdot 10^5$	$1.5 \cdot 10^6$	$1.6 \cdot 10^6$	$5.2 \cdot 10^5$	$2.5 \cdot 10^4$	$2.8 \cdot 10^5$	$2.2 \cdot 10^2$	$7.4 \cdot 10^5$		mv		high
2	–	6753i	$1.2 \cdot 10^5$	$7.7 \cdot 10^5$		$3.2 \cdot 10^6$	$7.1 \cdot 10^6$	$9.0 \cdot 10^6$	$5.5 \cdot 10^5$	$2.4 \cdot 10^4$							high
2	+	6754i	$2.2 \cdot 10^5$	$1.5 \cdot 10^6$	$1.5 \cdot 10^6$	$6.5 \cdot 10^6$	$4.2 \cdot 10^6$	$1.4 \cdot 10^6$	$1.2 \cdot 10^4$	$6.0 \cdot 10^2$		$2.0 \cdot 10^3$					high
2	–	6758i	$2.3 \cdot 10^5$	$1.0 \cdot 10^2$		$1.0 \cdot 10^3$	$1.5 \cdot 10^3$	$6.0 \cdot 10^2$	$1.6 \cdot 10^2$	$2.6 \cdot 10^4$	$1.3 \cdot 10^2$	$1.8 \cdot 10^2$		$1.0 \cdot 10^2$			high
2	–	6759i						$1.5 \cdot 10^5$									low
2	?	6760i	$3.9 \cdot 10^5$	$8.3 \cdot 10^5$	#	#	#	#	#	#	#	#	#	#	#	#	?
2	–	6755c		$2.0 \cdot 10^4$				$7.0 \cdot 10^3$	$2.4 \cdot 10^3$					$1.0 \cdot 10^3$			low
2	+	6756c		$4.4 \cdot 10^6$	$2.6 \cdot 10^6$	$1.6 \cdot 10^7$	$1.0 \cdot 10^6$	$4.8 \cdot 10^6$	$1.8 \cdot 10^6$								high
2	+	6757c		$2.7 \cdot 10^6$	$6.3 \cdot 10^7$	$4.8 \cdot 10^7$	$2.2 \cdot 10^6$	$1.2 \cdot 10^6$	$2.8 \cdot 10^6$	$4.0 \cdot 10^6$	$3.8 \cdot 10^5$						high
2	+	6761c					$1.2 \cdot 10^5$	$1.3 \cdot 10^4$	$2.4 \cdot 10^5$	$4.5 \cdot 10^6$	$1.9 \cdot 10^6$	$9.0 \cdot 10^5$	$2.3 \cdot 10^5$	$7.0 \cdot 10^4$			high
2	–	6762c															low
2	–	6764c							$2.6 \cdot 10^4$								low

[a] A pig no. Followed by i is an inoculated piglet, followed by c a contact piglet.
[b] For calculation of the transmission parameters this piglet was assumed to be contact-infected.
The shaded areas show the infectious periods of the individual piglets.
mv, Missing value
#, Dead

Table 2. (continued)
Number of F4+ *E.coli*/g faeces (c.f.u./g, not log-transformed), receptor status and shedding type of piglets in groups 1-4

Group	Receptor	Pig no.[a]	1 d	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	Shedding type
3	–	6765i															low
3	–	6766i													$1.0 \cdot 10^2$		low
3	–	6768i															low
3	+	6769i	$2.5 \cdot 10^6$	$1.9 \cdot 10^5$	$5.0 \cdot 10^4$		$4.2 \cdot 10^5$	$5.2 \cdot 10^6$	$3.1 \cdot 10^6$	$8.0 \cdot 10^5$	$9.0 \cdot 10^6$		$1.5 \cdot 10^6$				high
3	–	6773i															low
3	–	6774i	$3.0 \cdot 10^4$														low
3	–	6767c															low
3	+	6770c			$1.0 \cdot 10^4$		$9.2 \cdot 10^4$	$3.5 \cdot 10^5$	$1.4 \cdot 10^6$	$1.1 \cdot 10^7$	$2.9 \cdot 10^7$	$1.1 \cdot 10^6$	$4.9 \cdot 10^6$				high
3	+	6771c						$6.0 \cdot 10^2$				$1.0 \cdot 10^3$	$1.3 \cdot 10^3$				low
3	–	6772c										$2.0 \cdot 10^4$	$3.8 \cdot 10^4$				low
3	–	6775c				$1.0 \cdot 10^3$											low
3	–	6776c															low
4	–	6778i	$1.0 \cdot 10^5$	$1.0 \cdot 10^7$		$8.0 \cdot 10^7$	$1.5 \cdot 10^6$	$2.6 \cdot 10^6$		$1.0 \cdot 10^7$		$2.0 \cdot 10^7$	$4.1 \cdot 10^6$				high
4	–	6781i					$3.1 \cdot 10^4$		$2.4 \cdot 10^3$								low
4	+	6785i	$4.5 \cdot 10^5$	$1.1 \cdot 10^7$	$5.0 \cdot 10^4$	$1.9 \cdot 10^5$	$1.9 \cdot 10^6$	$6.0 \cdot 10^5$	$1.0 \cdot 10^5$								high
4	+	6786i	$1.5 \cdot 10^5$	$2.6 \cdot 10^6$	$5.2 \cdot 10^7$	$5.0 \cdot 10^7$	$7.0 \cdot 10^7$	$1.7 \cdot 10^6$		$2.6 \cdot 10^4$	$2.9 \cdot 10^6$						high
4	+	6788i	$2.4 \cdot 10^5$	$7.0 \cdot 10^5$	$1.1 \cdot 10^6$	$2.8 \cdot 10^7$	$6.3 \cdot 10^6$	$4.0 \cdot 10^4$	$7.1 \cdot 10^6$								high
4	+	6777c		$3.7 \cdot 10^4$		$2.7 \cdot 10^3$	$2.0 \cdot 10^3$		$1.4 \cdot 10^4$								low
4	–	6779c				$4.0 \cdot 10^2$	$1.4 \cdot 10^5$	$2.4 \cdot 10^3$									low
4	+	6782c		$7.4 \cdot 10^6$	$1.1 \cdot 10^7$	$8.0 \cdot 10^6$	$5.5 \cdot 10^6$	$3.7 \cdot 10^6$	$6.3 \cdot 10^6$	$6.7 \cdot 10^6$	$9.0 \cdot 10^6$	$9.5 \cdot 10^6$		$3.0 \cdot 10^6$	$1.0 \cdot 10^6$		high
4	–	6783c			$2.4 \cdot 10^6$	$9.3 \cdot 10^6$	$4.7 \cdot 10^6$	$9.4 \cdot 10^5$	$8.9 \cdot 10^6$	$3.2 \cdot 10^6$							high
4	+	6784c		$9.1 \cdot 10^6$	$2.8 \cdot 10^6$	$1.3 \cdot 10^6$	$1.7 \cdot 10^6$	$2.7 \cdot 10^7$	$4.9 \cdot 10^7$	$7.4 \cdot 10^6$	$1.0 \cdot 10^7$						high
4	–	6787c					$1.0 \cdot 10^2$	$9.0 \cdot 10^2$									low

[a] A pig no. Followed by i is an inoculated piglet, followed by c a contact piglet.

The shaded areas show the infectious periods of the individual piglets.

mv, Missing value

#, Dead

test). Only four out of 39 samples of liquid stools were found negative for F4+ *E. coli* on the same day. The time of onset of the diarrhoea (soon after inoculation), the high numbers of F4+ *E. coli* shed by most diarrhoeic piglets and the length of the excretion period indicate that F4+ *E. coli* successfully colonized the small intestine of most diarrhoeic piglets.

Table 3. Scores of the consistency of the faeces samples and the matching mean percentage faecal dry matter and 95% confidence interval (CI).

Score: consistency of faeces	Mean percentage faecal dry matter (95% CI)
0, normal	23.3 (22.9 – 23.7)
1, unformed or loose consistency	19.5 (18.9 – 20.1)
2, pasty diarrhoea	15.3 (14.1 – 16.5)
3, fluid	8.7 (6.8 – 10.6)

Weight gain

The mean weight gain of the F4R+ piglets was 230.3 g/day (S.D. = 106.3) and 231.1 g/day (S.D. = 83.9) for the F4R– piglets which is not significantly different, P = 0.41 (Mann-Whitney U test). For severely diarrhoeic piglets, the mean weight gain was 157.9 g/day (S.D. = 82.4) and for piglets with no or milder forms of diarrhoea the mean weight gain was 246.5 g/day (S.D .= 89.6) respectively, which is significantly different (P = 0.02, Mann- Whitney U test).

Transmission parameters

Piglet 6741 (F4R–, group 1) was considered to be contact-infected instead of being infectious due to inoculation, as its first F4+ *E. coli*-positive sample was not found until day 6 after inoculation. Including piglet 6741 a total number of 13 contact infections were observed, two F4R– and eleven F4R+.The estimated βs and the matching 95% CIs are shown in Table 4.

When we compare the intervals of β_{pp} and β_{np} or the intervals of β_{pn} and β_{nn} we see that they do not overlap or only slightly overlap and therefore it can be concluded that F4R+ and F4R– piglets differ in susceptibility. The intervals of β_{pn} and β_{pp} or β_{np} and β_{nn} do overlap almost entirely, therefore we may not conclude that F4R+ and F4R– piglets also differ in infectiousness.

The periods in which we assumed piglets to be infectious are shown in Table 2. For the inoculated piglets, this resulted in T_p=8.3 and T_n=10.8 days, which were used for the calculation of the reproduction ratios.

The reproduction ratio calculated for homogeneous F4R+ piglet populations based on the four βs, was estimated 6.37 (95% CI 1.89-21.48). R_0 for homogeneous F4R– piglet populations was much smaller, 0.02 (95% CI 0.00-1.13). In order to achieve $R_0(f) < 1$, the fraction of F4R+ piglets in the population must be lower than 0.08.

Table 4. Estimates of the four transmission parameters and their 95% confidence intervals (CI).

Transmission parameter*	Estimate (95% CI)
β_{pn}	1.42 (0.06 -34.81)
β_{pp}	0.77 (0.23 - 2.58)
β_{np}	0.05 (0.02 - 0.14)
β_{nn}	0.00 (0.00 - 0.10)

*The first character in the subscript is the F4R status of the contact piglet and the second is the F4R status of the infectious piglet (p, positive; n, negative)

Discussion

It this study, transmission parameters were estimated in randomly mixed groups of F4R+ and F4R– piglets and it was investigated whether F4R+ and F4R– piglets differed in infectiousness and susceptibility. F4R+ piglets were more susceptible to F4+ *E. coli* infection than F4R– piglets, but were not more infectious. This confirms and completes the findings of a one-to-one transmission experiment (Chapter 4) in which susceptibility was found to differ, but which was inconclusive on the infectiousness as none of the inoculated F4R– piglets became infectious. In the current study, six F4R– piglets did become infectious, four inoculated and two contact piglets. A higher infection pressure in groups compared to pairs and consequently more ingestion of bacteria from the environment in combination with non-specific binding of F4+ *E. coli* to the intestinal wall might have been the underlying cause. The results of the group transmission experiment has led to a more accurate estimation of the transmission parameters than the results of the one-to-one experiment. Moreover, transmission between group-housed piglets is more representative for the practical situation.

Whereas 51% of the F4+ *E. coli*-positive samples of infectious F4R+ reach numbers of 10^6 c.f.u./g or higher, only 2% (1 sample) of the infectious F4R– piglets reaches this level. This indicates that the colonization of the infectious F4R– piglets was less effective than the colonization of the infectious F4R+ piglets, which is also

reflected in the number of F4R+ and F4R– piglets with diarrhoea. Despite this difference in the level of shedding, infectiousness did not differ between F4R+ and F4R– piglets. Probably, the power of the experiment was too small as only four inoculated F4R– piglets were infectious.

In an earlier study, we developed an objective measure to distinguish between diarrhoeic and non-diarrhoeic piglets using PCA on the percentage dry matter data of F4+ *E. coli* inoculated piglets (Chapter 2). In this study we did not succeed as there was too much overlap in the data. Therefore, in the current study visual observation of fluid diarrhoea by the animal caretakers was linked to the percentage dry matter determined on the same samples. It was determined that piglets with one or more samples with a percentage dry matter ≤ 10.6% were severely diarrhoeic, which was valid for nine piglets. Using the same methods in a study with individually housed piglets, a threshold value of 12.6 % dry matter was found. Using this threshold value in the current study, 11 piglets with severe diarrhoea would have been found, but the conclusions on the association with receptor and shedding status and conclusion on weight gain would have remained the same.

In this study, transmission was studied in groups of piglets of which the individual piglets differ in susceptibility that is mainly determined by the absence or presence of an adhesion site. From the estimated transmission parameters it was calculated that it is only possible to prevent major outbreaks when the fraction of F4R+ piglets in the population is lower than 0.08. This is a slightly lower but more accurate estimate than the fraction of 0.14 calculated from the data in the aforementioned one-to-one transmission experiment. The main conclusion remains that in order to reduce transmission sufficiently, the fraction of F4R+ piglets has to be small.

Although the intestinal receptor locus for F4+ *E. coli* has been mapped on porcine chromosome 13 (Edfors-Lilja *et al.*, 1986), it has been shown that the expression of the receptor genes in not always complete and is influenced by epistatic inhibitor genes (Baker *et al.*, 1997) making breeding programs difficult. Moreover, selection on F4R– piglets could have some disadvantages like the change in selection pressure on pathogenic *E. coli* strains or negative effects on production and welfare as the function of the genes involved are still unknown (Bijlsma and Bouw, 1987; Python *et al.*, 2002). Furthermore, different serological variants of F4 with different receptors exist. Other intervention measures could be more beneficial and more feasible than a programme for selective breeding and their effect on clinical symptoms and transmission should be quantified.

In the future, it is necessary that genetic research on the F4R genes should be executed not only to find a gene marker that makes it possible to test pigs on their receptor status in a sensitive and non-invasive way but also to give more insight into the possible side effects of selection on F4R– pigs. Furthermore a cost-benefit analysis should be done and the long-term effects of the change in selection pressure on pathogenic *E. coli* should be monitored.

PWD is a multifactorial problem and post weaning proliferation of enterotoxigenic *E. coli* is an important step in a complex process (Madec *et al.*, 2000). The problem will not be solved by removing the receptor gene(s) alone. In addition to genetic selection, conditions under which piglets are reared will have to be evaluated thoroughly.

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Selective breeding for F4R– piglets

Supplement to Chapter 5

Introduction

Selective breeding for disease resistance is receiving more and more attention from livestock breeders as it may improve animal health, welfare and productivity. Several examples have shown that enhanced disease resistance is stable under natural selection and it is argued that artificial selection for disease resistance should also be stable and sustainable (Stear *et al.*, 2001). Moreover, enhanced disease resistance could result in reduced transmission between individuals or between herds (Bishop and Stear, 1997).

In Chapter 5, it was calculated that major outbreaks of enterotoxigenic F4+ *E. coli* among newly-weaned piglets can occur (the reproduction ratio, R_0, is >1 in homogeneous F4R+ populations) and that R_0 is dependent on the fraction of piglets in the population that express the adhesion site receptor F4 (F4R). This heterogeneity in the pig population may serve as a point of departure to control F4+ *E. coli* by breeding for genetic resistance, that is to select for F4R– pigs, as was suggested by several authors (Sellwood, 1979; Bijlsma *et al.*, 1985; Edfors-Lilja, 1991). It was calculated that selective breeding can prevent major outbreaks of F4+ *E. coli* (R_0<1) when the fraction of F4R+ piglets in the population is reduced to <0.08 (Chapter 5). The question is whether such a reduction is feasible for the Dutch pig population.

A simple discrete model: breeding with F4R– boars

Breeding with F4R– boars could be an effective strategy to select for F4R– piglets. To calculate the time (in generations) that is needed to sufficiently reduce the fraction of F4R+ piglets to prevent major outbreaks, a simple discrete model can be used. As the genetics are not completely elucidated and in this thesis only transmission of an F4ac+ *E. coli* strain was estimated, the model described below will be limited to selective breeding of F4acR– piglets, assuming one locus with two alleles S (adhesion) and s (no adhesion) and assuming no intermediate phenotypes (Gibbons *et al.*, 1977; Sellwood *et al.*, 1979).

It is likely that the prevalence of the F4acR– phenotype will vary between regions, pig breeds etc. For example, the Swiss pig population was estimated to consist of approximately 18% F4acR– pigs (Python *et al.*, 2005). There is no recent information on the prevalences of the F4acR– phenotype in the Netherlands. For the calculations it was therefore assumed that the mean fraction of F4acR– pigs in the Dutch pig population was 0.6 as reported by Bijlsma *et al.* (1985). According to the Hardy-Weinberg equilibrium, the genotype distribution within the population of sows will then be 0.05, 0.35 and 0.60 for SS, Ss and ss respectively (generation 0).

It was assumed that it is possible to select a boar population that consists of 100% F4acR– phenotype to breed with. This will accelerate the selection for

disease resistance. After each generation n, the sow population was fully replaced with sows randomly chosen from their progeny, assuming a very large population. Each generation is approximately one year. In case of 100% F4acR– phenotype boars, the genotypes of the sows for each generation will then be:

$$SS_{n+1} = 0$$
$$Ss_{n+1} = 1 - ss_{n+1}$$
$$ss_{n+1} = ss_n + 0.5 \cdot Ss_n$$

As stated above, the maximum fraction of F4R+ piglets in the population (f) at which $R_0 < 1$, was calculated to be 0.08. The minimum number of generations needed to reach this threshold value were calculated and the results of the calculations are shown in the Figure. The number of F4acR+ sows decreased rapidly and $R_0(f) < 1$ was already reached at the third generation. Thus, under these assumptions, it would take only a few generations to select a sow population with a sufficiently low number of F4acR+ individuals. If only 45% of the sows was replaced per year, which is a more common percentage in pig husbandry, then $R_0(f) < 1$ was reached at the seventh generation (see Figure).

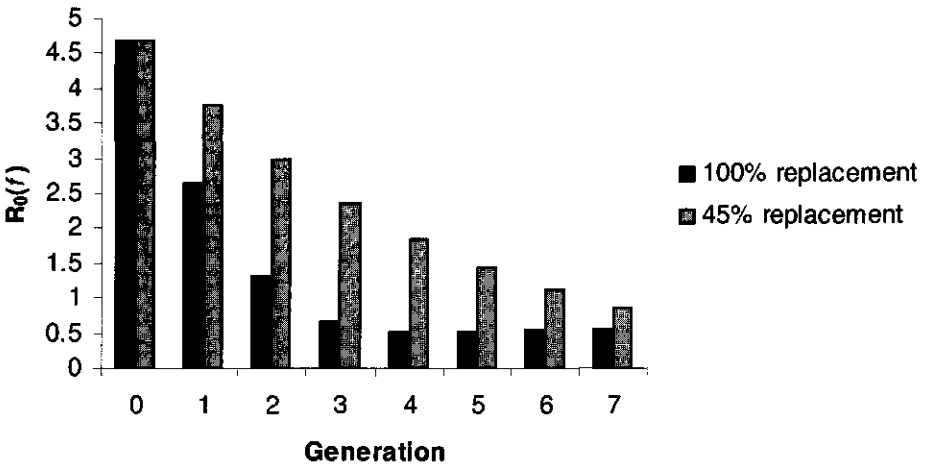

Fig. Decrease in $R_0(f)$ within the piglet population by breeding with F4acR– boars and replacement of 100% or 45% of the sows per year, assuming a perfect test and transmission parameters as found in Chapter 5.

The F4acR status of the boars currently has to be tested by *in vitro* adhesion assay (Sellwood *et al.*, 1975) or by related ELISA or enzyme immunoassays (Chandler *et al.*, 1986; Valpotic *et al.*, 1989). One of the main obstacles for selective breeding against F4+ *E. coli* at this moment, is the absence of a cheap and non-invasive test method to identify the receptor status of individual

piglets. Several laboratories are trying to identify the gene coding for F4R. In the near future, this will hopefully make direct typing of breeding animals possible in a sensitive and non-invasive way and will allow distinction between heterozygous and homozygous animals (Edfors-Lilja and Wallgren, 2000).

Since no test is perfect, the sensitivity of the test by which the F4acR status is determined will affect the time that is needed to reach the threshold value. In this case, a positive test result means that a boar is determined to be F4acR–. Assuming that false-negative results are distributed as 1:7 over the SS and Ss genotypes (Hardy-Weinberg equilibrium), the highest percentage of false-negative results acceptable is 7% (sensitivity of 93%) and the number of generations needed to bring $R_0(f)$ below unity (assuming a 100% replacement of the sows) is eight. Assuming a 45% replacement of the sows, the highest percentage of false-negative results acceptable is also 7%, but it will then take 20 generations before $R_0(f) < 1$. If the percentage of false-negative results >7% the threshold value $f = 0.08$ will not be reached.

Summarizing the results; given a sensitive test to determine the F4acR status of the boars, and given that the assumptions concerning the genetics are valid, breeding with F4R– boars could be an effective strategy to select for F4R– pigs within a reasonable number of generations. Reduced transmission as a result of enhanced F4+ *E. coli* resistance has not yet been verified in combined breeding and transmission studies, but would be an interesting subject for future research.

More complex genetics: the F4ab, ac and ad receptors

Although F4ac is the predominant F4 variant associated with diarrhoea (Westerman *et al.*, 1988), in the field two other variants of the F4 antigen are present, F4ab and F4ad, and in total six corresponding phenotypes of pigs are found (Baker *et al.*, 1997), see also the Table below. This makes breeding for F4 resistance more complex (Ollivier and Renjifo, 1991).

Table. Phenotype designations A–F according to Baker *et al.*, 1997

F4 phenotype	ab	ac	ad
A	+	+	+
B	+	+	–
C	+	–	+
D	–	–	+
E	–	–	–
F	+	–	–

Recently, studies investigating the genetic resistance against all three variants of F4+ *E. coli* have given more insight into the different receptors involved (Python *et al.*, 2002, 2005). It was found that F4ac+ and F4ab+ *E. coli* most likely attach to the same strong receptor F4bcR that is controlled by one gene localized on chromosome 13 (Python *et al.*, 2002). Consequently, F4ac+ and F4ab+ resistance can be enhanced simultaneously by selecting for F4bcR– piglets. A weak receptor for F4ab+ *E. coli* F4abR^{w} was found in pigs devoid of F4ac adhesion, but the inheritance of this receptor is still unclear. It would be interesting to investigate whether reducing the fraction of F4bcR+ piglets to <0.08 will also be sufficient to prevent major outbreaks of F4ab+ *E. coli* and to investigate the role of F4abR^{w} in the transmission of this bacterium. Highly variable strength of adhesion of F4ad fimbriae was observed; the segregation analysis indicated a dominant inheritance of F4adR (Python *et al*, 2005).

It has been suggested that the expression of the receptor genes is not always complete and is influenced by epistatic inhibitor genes (Baker *et al.*, 1997) or by inhibition or modification of the receptor expression (Bijlsma and Bouw, 1987), which may result in a weak-adhesive phenotype for F4ac (Sellwood, 1980). In the breeding study by Python *et al.* (2005), 14 pigs out of 299 offspring were found to be adhesive (9 weak and 5 strong adhesive) despite their F4acR– parents. No explanation could be found for the 5 strong adhesive pigs that were all from one litter. The 9 weak adhesive piglets originated from 8 different litters, but inheritance of a weak receptor for F4acR could not be demonstrated. It is hypothesized that enterocytes showing strong-adhesion to F4ad+ *E. coli* coincidently show weak-adhesion to F4ac, but this has to be substantiated (P. Vögeli, personal communication). Weak-adhesion of F4ac+ *E. coli* is important for the definition of the phenotypes and genotypes, but was found to be irrelevant to the occurrence of diarrhoea after inoculation of 8 pigs with F4ac+ *E. coli* (Sellwood, 1984; Bijlsma and Bouw, 1987). Since these piglets were found to shed the strain in low numbers for 3 days only, it is unlikely that piglets with this phenotype will be infectious for other piglets. Whether this phenotype would only play a minor role in the transmission chain like was shown for the F4acR– piglets, will need to be investigated. Probably, for selective breeding of F4R– piglets two genes need to be taken into account.

The pros and cons of selective breeding

In this paper, it was illustrated that using F4R– boars for breeding is an effective means to reduce the fraction of F4R+ pigs in the population. In several studies it was investigated whether selection for F4R– piglets had any negative effects on production and welfare, as the function of the gene(s) involved are still unknown (Edfors-Lilja *et al.*, 1986; Engel *et al.*, 1998; Gibbons *et al.*, 1977). Until now, no clear answer to this question can be given; it was suggested that pig

populations not selected for growth have a low frequency of the F4R– receptor (Edfors-Lilja and Wallgren, 2000) and also co-selection with litter size cannot be excluded (Engel *et al.*, 1998). Moreover, it is unknown what the effect of selective breeding will be on other pathogenic *E. coli* strains.

Before implementing a breeding programme for F4+ *E. coli* resistance, first methods need to be developed that make direct typing of breeding animals possible. Effects on production and welfare need to be investigated more thoroughly and the long-term effects of the change in selection pressure on pathogenic *E. coli* should be monitored.

The advantages of selective breeding for disease resistance against F4+ *E. coli* are clear; animal health will very likely be improved. Moreover, if the fraction of F4acR+ piglets in the population will be brought below the threshold value of 0.08, the pigs will be prevented from major outbreaks of this bacterium.

6

General discussion

General Discussion

Enterotoxigenic *Escherichia coli* expressing F4 fimbriae (F4+ *E. coli*) is an important causative agent of post-weaning diarrhoea (PWD) in early-weaned piglets, a disease that causes substantial damage to animal welfare and the pig industry e.g. (Smits and Merks, 2001). PWD is a multifactorial disease and an essential characteristic of PWD is a contagious factor, i.e. certain strains of *E. coli* (Amass *et al.*, 2003).

PWD intervention measures can be evaluated in challenge and in transmission experiments. Two different designs of transmission experiments that can be used to quantify the reduction of F4+ *E. coli* spread by intervention measures were described in this thesis: a one-to-one transmission experiment with pair-housed piglets (Chapter 4) and a transmission experiment with group-housed piglets (Chapter 5).

To understand how transmission of F4+ *E. coli* can be prevented, more insight in the population dynamics of this bacterium is needed, which should be gained at both the individual and the population level. In this thesis, F4+ *E. coli* dynamics were studied at these two integration levels. In this chapter, the results of the studies in individual piglets, between pair-housed piglets and between group-housed piglets and their consequences for the population dynamics are discussed. This chapter ends with a discussion on the implications of the results for intervention studies and the conclusions.

The dynamics of F4+ *E. coli* in the individual piglet

After ingestion of *F4+ E. coli* from the environment, successful colonisation and replication within the gastrointestinal system may result in an infectious piglet that will shed the bacterium with its faeces and hereby increases the infectious load in the environment. This may lead to a higher probability that a susceptible piglet ingests the pathogen and will become infectious too. Therefore it was assumed that an important characteristic in the population dynamics of F4+ *E. coli* is the amount of bacteria shed in time (the temporal shedding pattern) of individual piglets.

To study F4+ *E. coli* shedding at the individual level, a number of factors were taken into account that could affect F4+ *E. coli* shedding patterns and the infectiousness of piglets: (1) presence or absence of the F4 receptor (F4R), (2) reinfection by taking up F4+ *E. coli* from the contaminated environment and (3) diarrhoea. These factors are discussed for each integration level where applicable.

Before these factors could be studied, however, a definition of the infectious state had to be developed. It was assumed that the infectious state of the piglets could be defined based on the shedding patterns of individual piglets after inoculation. Therefore, a principal component analysis (PCA) was performed on the shedding patterns of 69 piglets to find a linear discrimination measure to

classify the piglets into shedding types (Chapter 2). With the first principal component, two significantly different groups of piglets could be distinguished. The main difference between these two groups was the overall level of shedding at log scale and therefore these shedding types were referred to as 'high' and 'low shedders'. However, whether 'high shedding' is a useful definition of the infectious state could only be evaluated in a transmission study (see 'F4+ *E. coli* dynamics between pair-housed piglets').

F4 receptor

The F4R status of the piglet was found to be highly associated with the shedding type. After challenge, 57% (8/14) of the individually housed F4R+ piglets was found to be a high shedder, for F4R– piglets this percentage was much lower, 4% (1/27) (Chapter 2). Presence of F4R in the small intestine of the piglet enables F4+ *E. coli* to adhere to the enterocytes and thereby withstand the continual clearance of the small intestine. This may result in colonisation of the small intestine by F4+ *E. coli* and massive replication of the bacteria (Beachy, 1981).

This colonization is temporary. After experimental inoculation, the piglets stopped shedding F4+ *E. coli* within two weeks (Chapter 2). Cessation of shedding may be caused by the development of immunity against F4+ *E. coli,* increasing age of the piglet or competition of strains in the small intestine (Nabuurs *et al.*, 1993). F4R+ piglets infected with this bacterium are found to develop an immune reaction eight days after infection (Van den Broeck *et al.*, 1999). The development of an immune response was found to be dependent on the F4R status; F4R– piglets did not show any immune response after oral challenge (Van den Broeck *et al.*, 1999). However, it is not clear whether, soon after experimental infection with F4+ *E. coli,* the displayed immune responses are sufficient to prevent newly-weaned piglets from a new infection with F4+ *E. coli.*

Reinfection

If shedding patterns and infectiousness are affected by reinfection, then this process is an important factor in the population dynamics of F4+ *E. coli.*

Main interest is taken in the effects of reinfection during or soon after primary infection. Therefore, reinfection was defined in Chapter 3 as the uptake of the pathogen from the environment that will lead to successful colonisation of a host that is still infected or has only recently stopped shedding. This may lead to longer periods of shedding and possibly higher numbers of the pathogen shed, resulting in increased infectiousness.

Reinfection was studied by comparing the shedding patterns of individually housed piglets that were prevented from reinfecting themselves by collecting all faeces in pouches, with the shedding patterns of piglets without pouches that could

come into contact with their own contaminated faeces. So strictly speaking self-reinfection was studied.

The shedding patterns were quite different between F4R+ and F4R– piglets. All F4R– piglets without pouches shed only a low amount of bacteria in their faeces (they were all 'low shedders'), possibly too low for reinfection (Chapter 3). This would imply that reinfection can only occur in F4R+ piglets. No significant differences were found between the shedding patterns of the F4R+ piglets with and without pouches. Based on the results of this experiment, no indication was found that reinfection affected the shedding patterns of the piglets and thus no indication was found that reinfection was an important factor in the population dynamics of F4+ *E. coli*.

Diarrhoea

In this thesis, the consistency of faeces was measured by the percentage dry matter of faeces samples and by classification (scoring) of the visual observation of the consistency of faeces samples in four classes. The first method used was an objective and repeatable method, whereas the other method, visual observation, is more dependent on the observer but is often used by other authors (Nabuurs *et al.*, 1993; Madec *et al.*, 2000). Based on these observations, measures to distinguish between diarrhoeaic and non-diarrhoeaic piglets were developed.

Using a combination of scoring and percentage dry matter of the samples collected after challenge, resulted in four classes describing the consistency of faeces that were significantly different in the matching percentage dry matter (Chapter 3). Most interest was taken in the class of liquid diarrhoea, the most severe type of diarrhoea, since it usually contains 10^9-10^{10} c.f.u. *E. coli*/g faeces and may promote transmission, but oral uptake might be less. Moreover, piglets with liquid diarrhoea were found to have a lower feed intake and consequently a significantly lower weight gain. All piglets with at least one sample of liquid diarrhoea were classified as diarrhoeaic. Classification into diarrhoeaic and non-diarrhoeaic piglets was significantly associated with F4R status of the piglets in the majority of experiments.

F4+ *E. coli* dynamics between pair-housed piglets

Transmission studies at population level are essential to get insight in the relation between the measurements at the individual level and spread of F4+ *E. coli*. The smallest possible transmission experiment was performed; one inoculated piglet housed together with one susceptible contact piglet, a one-to-one transmission experiment (Chapter 4). The composition of the pairs may differ in the F4R status and shedding type. In each replicate of the experiment (each pair) it is clear which piglet infected the contact piglet. Therefore it seemed a good design to evaluate 'high shedder' as a definition of the infectious state.

'High shedder' was found to be a good measure to distinguish between infectious and non-infectious piglets based on the association with the number of contact infections (cases) found and because none of the 'low shedders' was found to infect another piglet. This classification was better than a classification in which it was assumed that a piglet was infectious when at least one F4+ *E. coli* positive sample was found. From this point on, high shedder will be used to classify piglets as infectious.

One of the main questions in this thesis was whether F4R+ and F4R– pigs differ in infectiousness and susceptibility and how this will affect transmission. For this purpose, the one-to-one transmission experiment is a good design as well.

However, only F4R+ piglets were found to be high shedders, which was interpreted as being infectious (see above). Consequently, only information about transmission between F4R+ piglets and from F4R+ piglets to F4R– piglets was obtained. It was estimated that R_0 in a homogeneous F4R+ population is 7.1 with 95% CI [2.3 – 21.9]. To calculate R_0 for other compositions of the population, assumptions had to be made on the transmission parameters.

Based on the high association of the F4R status and the infectious state of the inoculated piglets, it was concluded that F4R+ piglets were more susceptible (to become infectious) than F4R– piglets, but the study was inconclusive regarding infectiousness, since none of the F4R– piglets became infectious.

The F4R– piglets could not infect other piglets and could not be infected themselves. This reinforces the impression that it is unlikely that self-reinfection will affect shedding patterns of F4R– piglets.

All the high shedding inoculated piglets that developed liquid diarrhoea did so after their contact piglet was infected, which makes a direct effect of liquid diarrhoea on transmission not very likely.

Dynamics of F4+ *E. coli* between group-housed piglets

Transmission of F4+ *E. coli* among group-housed piglets is more representative for the practical situation than transmission among pair-housed piglets. As in the pair-experiment, F4R+ piglets were found to be more susceptible than F4R– piglets. There were large differences in the amount of bacteria shed between the infectious F4R+ and F4R– piglets, but despite these differences, the F4R+ and F4R– piglets were found to be equally infectious.

Given the estimated transmission parameters, there was no indication that the transmission process was different between pairs and groups; all differences could be explained within the statistical model. Therefore, overall estimates of the R_0's were computed based on the results of the pair- and group-housed piglets and assuming that F4R+ and F4R– piglets were equally infectious. R_0 for homogeneous F4R+ populations was estimated to be 7.97 with 95% CI [4.67-13.60], and for homogeneous F4R– populations 0.28 with 95% CI [0.15-0.52]. The

fraction of F4R+ piglets in the population for which the infection will fade out was calculated to be 0.09.

Although statistically no differences were found between the pairs and the groups, one marked difference was observed. In contrast to the one-to-one transmission experiment, some inoculated and some contact F4R– piglets became infectious in the groups. This was also observed in the group-housed piglets in experiment 5, Chapter 2. Possibly, if a higher number of infectious piglets is present in groups than in pair-housed piglets, F4R– piglets pick up the bacteria from the environment more frequently and may become infectious. The percentage of high shedding inoculated F4R+ piglets was also higher in the groups (≥90%) than in pairs.

Like before, both receptor and shedding status were highly associated with the occurrence of liquid stools in the group-housed piglets. Several high shedding contact piglets, all F4R+, had one or more samples classified as liquid diarrhoea on one or more days. Thus, the experimental model used in this thesis cannot only be used for studying the effect of preventive measures on transmission, but on F4+ *E. coli* related diarrhoea as well.

F4+ *E. coli*: from individual to population level

It was shown that the temporal shedding patterns of individual piglets can be used to define which piglets are infectious and which are not. The F4R status was associated with the shedding status of individual piglets after challenge with F4+ *E. coli*, which indicated that this host characteristic affects the susceptibility of the piglets to become infectious. The level of transmission in the population was calculated to be mainly dependent on the fraction of F4R+ piglets; F4R– piglets only play a minor role in the transmission chain.

Within the F4R+ piglets, high shedding was significantly associated with the occurrence of severe diarrhoea (Chapter 2). The fraction of F4R– piglets with diarrhoea was low among individually housed piglets, but was somewhat higher in piglets housed in pairs or groups. Overall, the fraction of F4R+ piglets with liquid diarrhoea was much higher than the fraction of F4R– piglets with liquid diarrhoea.

The insight gained from the studies performed in this thesis have implications for the performance of transmission studies to evaluate preventive measures to control F4+ *E. coli*. Moreover, the importance of the F4R status for the transmission of F4+ *E. coli*, makes reducing the fraction of F4R+ piglets in the population by breeding a possible strategy to control PWD (Supplement to Chapter 5). The implication of the results for the performance of intervention studies will be discussed in the next section.

Implications for intervention studies

For PWD caused by F4+ *E. coli*, there are several possible intervention measures. For example, adherence and colonization of F4+ *E. coli* can be prevented by proteases, receptor analogues, probiotic bacteria, spray-dried porcine plasma and egg-yolk antibodies with or without anti-microbial agents like carbadox, zinc oxide and fumaric acid (Jin and Zhao, 2000; Owusu-Asiedu *et al.*, 2003), or by changing the diet of the weanling piglets (Pluske *et al.*, 2002). F4-based oral vaccines for weaned piglets are currently under development and are promising intervention measures for the future (Snoeck *et al.*, 2003; Verdonck *et al.*, 2004).

Intervention measures can be aimed at preventing the clinical symptoms directly or indirectly by preventing transmission between piglets. In this thesis, two different designs of transmission experiments that can be used to quantify the reduction of F4+ *E. coli* transmission by preventive measures were described.

Given the heterogeneity in susceptibility in the pig population, the F4R status of the piglets should always be taken into account as a co-variate when analysing the results of an intervention study. The generalized linear models used in Chapters 4 and 5 can deal with heterogeneity and are therefore preferred over final size methods when analysing F4+ *E. coli* transmission experiments.

Because of the importance of the F4R status, controlled composition of the experimental groups in intervention studies is sometimes desirable and may reduce the number of piglets needed. BBA on intestinal biopsies of the piglets before challenge (Snodgrass *et al.*, 1981), allows for controlled composition of the experimental groups, but is not always practically feasible because of the facilities and equipment needed and the time needed for the pigs to recover before challenge. It was also experienced, that the amount of tissue collected by biopsy is sometimes too small, which makes correct classification, compared to BBA on samples taken at post-mortem, more difficult (unpublished results).

The results of an intervention studies cannot simply be extrapolated to the field, because in the field, many external factors that will affect PWD cannot be controlled and may interfere with the effect of the preventive measure tested. Intervention studies are useful tools to select promising intervention measures for PWD, but should always be evaluated in the field.

Conclusions

The F4R status of the pig is an important host characteristic that affects the shedding patterns of individual pigs and their susceptibility for F4+ *E. coli* infection. By reducing the fraction of F4R+ piglets in the population to <0.09, it was calculated that major outbreaks of F4+ *E. coli* will be prevented (R_0<1).

Intervention measures for F4+ *E. coli* can be aimed at preventing diarrhoea or at preventing transmission and should be evaluated in challenge or transmission

studies. The set up and analysis of transmission experiments with F4+ *E. coli* are described in this thesis and a definition of the infectious state based on the shedding patterns of individual animals was developed. F4R status should be used as a co-variate in the statistical analysis of intervention studies.

A third, more drastic way of intervention, is to breed for genetic resistance against this bacterium, that is to select for F4R– pigs. With a simple discrete model, it was illustrated that selective breeding for F4R– piglets by using F4R– boars, is an effective way to reduce the fraction of F4R+ piglets in the population.

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Summary

Post-weaning diarrhoea (PWD) is a multifactorial disease of piglets that is characterized by diarrhoea in the first two weeks after weaning. PWD results in growth retardation and increased mortality, resulting in reduced animal welfare and economical damage. The main causative agent of PWD is enterotoxigenic *Escherichia coli* expressing F4 fimbriae (F4+ *E. coli*).

Until recently, most studies on PWD were aimed to understand how certain factors contribute to the development of diarrhoea, which has led to several intervention measures to reduce the clinical signs of PWD. However, intervention measures for PWD can also be aimed at reducing the transmission of F4+ *E. coli* between piglets. In this thesis, transmission of F4+ *E. coli* was investigated. Two types of experiments that can be used to evaluate F4+ *E. coli* intervention measures were performed and parameters characterizing the transmission process were quantified.

Before transmission could be studied and quantified, factors that may affect transmission of F4+ *E. coli* needed to be investigated. These factors can be characteristics of the bacteria, the individual piglets and the piglet population and thus more insight in the population dynamics was required. The dynamics were investigated in individual piglets and at population level. Special interest was taken in an adhesion site on the brush borders of the small intestinal enterocytes of the piglets, the F4 receptor (F4R). The F4 fimbriae enable F4+ *E. coli* to adhere to this site, which may result in colonization of the small intestine. Not all pigs express F4R; pigs with adhesive (F4R+) and non-adhesive brush borders (F4R–) can be distinguished by *in vitro* adhesion tests.

After challenge, the number of F4+ *E. coli* shed in the faeces as a function of time varies considerably between piglets, and might be a measure of infectiousness. It was found that two shedding types, 'high shedders' and 'low shedders', could be distinguished based on the (log-transformed) number of bacteria shed per gram faeces by individual piglets during eight days after challenge. 'High shedders' shed more F4+ *E. coli* in time than 'low shedders'; 'high shedder' was therefore proposed as a definition of the infectious state, which is of importance with respect to transmission. The shedding type of the piglets was found to be highly associated with their F4R status and it was found that F4R+ piglets are more susceptible to become infectious due to inoculation than F4R– piglets.

If shedding patterns and infectiousness are affected by reinfection, then this process is an important factor in the population dynamics of F4+ *E. coli*. Reinfection was studied by comparing the shedding patterns of individually housed piglets that were prevented from reinfecting themselves by collecting all faeces in pouches, with the shedding patterns of piglets without pouches that could come into contact with their own contaminated faeces. No indication was found that reinfection affected the

shedding patterns of the piglets and thus no indication was found that reinfection was an important factor in the population dynamics of F4+ *E. coli*.

Concerning diarrhoea, most interest was taken in the most severe class of diarrhoea, liquid diarrhoea, since it usually contains high numbers of F4+ *E. coli*/g faeces and may promote transmission, but oral uptake might be less. Overall, more F4R+ piglets were classified as diarrhoeic than F4R– piglets, and within the group of F4R+ piglets the shedding status was significantly associated with the occurrence of liquid diarrhoea. Liquid diarrhoea did not seem to have a direct effect on F4+ *E. coli* transmission.

Two kind of transmission experiments were performed: an experiment with piglets housed in pairs, and a transmission experiment with group-housed piglets. In the pairs, one inoculated piglet was housed with one susceptible contact piglet. In the groups 11 or 12 pigs were housed together of which 6 piglets were inoculated and the others were contact piglets. The definition of the infectious state developed at the individual level, 'high shedder', was evaluated in the pair-experiment, because in the pairs it is clear which piglet infected the contact piglet. 'High shedder' was found to be a good measure to distinguish between infectious and non-infectious piglets and was used in the analysis of both transmission experiments.

To estimate the transmission parameters in both experiments, the stochastic Susceptible-Infectious-Removed model was used. An insightful way to express the transmission parameters is by the reproduction ratio, R_0, which is defined as the average number of secondary infections that one typical infectious individual will cause during its infectious period in a completely susceptible population. When $R_0<1$, the infection will fade out, and when $R_0>1$, the infection may spread in the population and major outbreaks may occur.

F4R+ piglets were found to be more susceptible to become infectious than the F4R– piglets. Despite the differences in the amount of bacteria shed, no differences were found in the infectiousness of the high shedding F4R+ and F4R– piglets.

Statistically, no indication was found that the transmission process was different between pairs and groups. Therefore, overall estimates of the R_0's were computed based on the results of the pair- and group-housed piglets. The R_0 of homogeneous F4R+ populations was found to be significantly >1, whereas the R_0 of homogeneous F4R– populations was significantly <1. R_0 in heterogeneous populations was found to be dependent on the fraction of F4R+ piglets in the population. It was calculated that the fraction of F4R+ piglets in the population for which the infection will fade out is 0.09.

This heterogeneity in susceptibility of individual piglets in the population may serve as a point of departure to control F4+ *E. coli* by selective breeding for F4R– pigs. With a simple discrete model, it was illustrated that selective breeding is an effective way to reduce the fraction of F4R+ piglets in the population, given that the test to determine the F4R status has a high sensitivity. However, since the

gene(s) and the function of the receptor(s) involved are still unknown, effects of selective breeding on production and welfare need to be investigated more thoroughly, and methods that make direct typing of breeding animals possible, need to be developed.

It was concluded that the F4R status of the pig is an important host characteristic that affects the population dynamics of F4+ *E. coli.* The F4R status should be used as a co-variate in the statistical analysis of F4+ *E. coli* intervention studies, both in challenge and transmission studies. The level of transmission is dependent on the fraction of F4R+ piglets; F4R– piglets only play a minor role in the transmission chain. By reducing the fraction of F4R+ piglets in the population to <0.09, e.g. by selective breeding for F4R– piglets, large outbreaks of F4+ *E. coli* will be prevented.

Samenvatting

In de eerste twee weken na spenen komt het regelmatig voor dat biggen een ernstige mate van diarree ontwikkelen, die speendiarree wordt genoemd. Speendiarree zorgt ervoor dat biggen achterblijven in hun groei en leidt tot een verhoogde sterfte onder deze dieren. Dit leidt tot een verminderd welzijn van de varkens en tot economische schade voor de varkenshouderij. De belangrijkste ziekteverwekker, die betrokken is bij het ontstaan van speendiarree, is een enterotoxine-producerende *Escherichia coli* bacterie, die zogenaamde F4 fimbriae (aanhechtingsdraden) heeft (F4+ *E. coli*).

Om speendiarree te voorkomen of te verminderen kunnen verschillende interventiemaatregelen worden toegepast. Deze interventiemaatregelen kunnen hun doel op twee manieren bereiken: door het verminderen van de klinische symptomen bij biggen of door het beperken van de transmissie (overdracht) van F4+ *E. coli* tussen biggen. Er zijn al vele studies gedaan naar de factoren, die van invloed zijn op het ontstaan van diarree en hieruit zijn verscheidene interventiemaatregelen ontwikkeld. In dit proefschrift wordt het onderzoek beschreven, dat is uitgevoerd om meer inzicht te verkrijgen in de transmissie van de bacterie F4+ *E. coli* tussen biggen. Hiervoor zijn o.a. twee transmissieproeven met een verschillende opzet uitgevoerd, die gebruikt kunnen worden om het effect van interventiemaatregelen op de transmissie van F4+ *E. coli* te kunnen kwantificeren.

Voordat transmissie kon worden bestudeerd en gekwantificeerd was er meer inzicht nodig in de factoren die de transmissie van F4+ *E. coli* beïnvloeden. Dit kunnen kenmerken zijn van de bacterie zelf, van het individuele varken of van de populatie varkens. Kortom, er was meer inzicht nodig in de populatiedynamica van deze bacterie. Hiervoor zijn experimenten uitgevoerd op zowel individu- als op populatieniveau. Met name één gastheerkenmerk heeft veel aandacht gekregen in dit onderzoek: de zogenaamde F4 receptor status van de varkens. Hiermee bedoelt men de aan- of afwezigheid van een specifieke aanhechtingsplaats, de F4 receptor (F4R), op de borstelzones van de epitheelcellen in de dunne darm. F4+ *E. coli* kan met zijn F4 fimbriae aanhechten op deze receptor en kan op deze manier de dunne darm koloniseren. Niet alle varkens hebben deze receptor. In *in vitro* aanhechtingsstudies is gebleken dat er receptor-positieve varkens zijn met borstelzones waaraan de bacterie kan hechten (F4R+) en receptor-negatieve varkens met borstelzones waar de bacterie niet aanhecht (F4R–).

Biggen die zijn geïnoculeerd met de bacterie, vertonen een grote verscheidenheid in het aantal F4+ *E. coli* dat wordt uitgescheiden in de mest in de tijd. Hieruit zou een maat voor de infectieusiteit van de biggen kunnen worden afgeleid. In de experimenten op individuniveau konden inderdaad twee verschillende uitscheidingstypen, hoge en lage uitscheiders, worden onderscheiden op grond van het aantal F4+ *E. coli* bacteriën per gram mest (na

log-transformatie) in de eerste 8 dagen na inoculatie. 'Hoge uitscheider' is daarmee een potentiële definitie voor het infectieus zijn van biggen, zodat infectieuze en niet-infectieuze biggen kunnen worden onderscheiden. Dit is erg belangrijk om de overdracht van deze bacterie te kunnen bestuderen en kwantificeren. Er werd een significante associatie tussen het uitscheidingstype van de individuele biggen en hun F4R status gevonden en er werd gevonden dat de F4R+ biggen gevoeliger zijn om infectieus te worden na inoculatie dan F4R– biggen.

Herinfectie zou van belang kunnen zijn voor de populatiedynamica van F4+ *E. coli* indien herinfectie zou leiden tot veranderde uitscheidingspatronen van de bacterie in de mest en dus de infectieusiteit van de biggen zou beïnvloeden. De mogelijke effecten van herinfectie werden bestudeerd in een challenge experiment met individueel gehuisveste biggen, waarin de uitscheidingspatronen van biggen met stomazakjes, waarmee alle mest werd opgevangen, is vergeleken met de uitscheidingspatronen van biggen zonder stomazakjes. Biggen met stomazakjes konden niet in contact komen met hun eigen infectieuze mest; biggen zonder stomazakjes konden dit wel. Er werd geen indicatie gevonden dat herinfectie een belangrijke factor is in de populatiedynamica van F4+ *E. coli*.

Diarree, die ontstond na de inoculatie van de biggen, werd ook bestudeerd en dan met name de meest ernstige klasse van diarree die kan optreden: de zogenaamde waterdunne diarree. Deze diarree bevat hoge aantallen F4+ *E. coli*, wat de transmissie van F4+ *E. coli* naar gevoelige biggen zou kunnen bevorderen, alhoewel de opname van zulke dunne diarree misschien wel minder is. Over het algemeen hadden met name F4R+ biggen diarree en binnen de groep van F4R+ biggen was het uitscheidingstype sterk geassocieerd met het optreden van waterdunne diarree. In de transmissie-experimenten is er geen direct effect gevonden van waterdunne diarree op de verspreiding van de bacterie.

Er zijn twee verschillende soorten transmissie-experimenten uitgevoerd, een paartjesproef en een groepstransmissieproef. In de paartjesproef waren de biggen gehuisvest in tweetallen bestaande uit één geïnoculeerde big en één gevoelig contactdier. In de groepsexperimenten waren 11 of 12 biggen samen gehuisvest waarvan 6 dieren waren geïnoculeerd en de overige biggen contactdieren waren. De infectieusiteitsmaat 'hoge uitscheider', die eerder was bepaald in individuele dieren, werd getest in de paartjesproef omdat bij deze proefopzet, in tegenstelling tot transmissie in groepen, altijd duidelijk is welk dier het contactdier heeft geïnfecteerd. Hoge uitscheider bleek een goede maat te zijn voor infectieusiteit en is als zodanig gebruikt in de analyse van beide transmissie-experimenten.

Voor de analyse van de tranmissie-experimenten is gebruik gemaakt van het SIR-model (S, susceptible oftewel gevoelig; I, infectieus; R, resistent). Een inzichtelijke manier om de met het model geschatte transmissie-parameters te beschrijven, is de basis reproductie ratio, R_0. R_0 is gedefinieerd als het gemiddeld aantal secundaire infecties dat wordt veroorzaakt door één infectieus dier gedurende zijn gehele infectieuze periode in een gevoelige populatie. Als R_0 groter

is dan 1, dan kan de infectie spreiden in de populatie en kunnen er grote uitbraken plaatsvinden. Wanneer R_0 kleiner is dan 1, dan zal een infectieus dier gemiddeld minder dan één gevoelig dier infecteren en zal de infectie uitdoven in de populatie.

In de transmissie-experimenten werd aangetoond dat de F4R+ biggen gevoeliger waren om infectieus te worden dan de F4R– biggen. Er werden geen verschillen gevonden in de infectieusiteit van de hoog-uitscheidende F4R+ en F4R– biggen, ondanks het grote verschil in het aantal bacteriën dat door deze twee typen varkens werd uitgescheiden.

Statistisch gezien zijn er geen verschillen gevonden in de transmissie-parameters van de paartjes- en de groepsexperimenten. Daarom zijn de resultaten van beide proeven gecombineerd en zijn de R_0's hiermee berekend. Er is berekend dat de R_0 van een homogene F4R+ populatie significant groter dan 1 is en dat de R_0 van een homogene F4R– populatie significant kleiner dan 1 is. R_0 van een heterogene populatie (met zowel F4R+ als F4R– biggen) is afhankelijk van de fractie F4R+ biggen. Als de fractie van F4R+ biggen in de populatie kleiner wordt dan 0.09, dan wordt R_0 kleiner dan 1 en zullen er geen grote uitbraken van F4+ *E. coli* plaatsvinden.

De gevonden heterogeneniteit in gevoeligheid van de biggen kan worden gebruikt als uitgangspunt voor een F4+ *E.coli* beheersingsmaatregel, nl. voor het selectief fokken van F4R– varkens. Met behulp van een eenvoudig discreet model werd getoond dat selectief fokken met F4R– beren een effectieve manier is om het aantal F4R+ dieren in de populatie te reduceren, mits de gebruikte receptor status test een voldoende hoge sensitiviteit bezit. Aangezien het gen of de genen die betrokken zijn bij de expressie van de receptor status nog steeds onbekend zijn evenals de functie(s) van de receptor(en), is eerst meer onderzoek nodig naar de effecten van selectief fokken op het welzijn en de productie. Bovendien moeten er methoden worden ontwikkeld, die het direct typeren van de fokvarkens mogelijk maken.

Concluderend kan worden gesteld, dat de F4R status van het varken de populatiedynamica van F4+ *E. coli* sterk beïnvloedt. De F4R status moet daarom worden meegenomen als covariabele in de statistische analyse van F4+ *E. coli* interventiestudies, zowel in challenge- als in transmissie-studies. De mate van transmissie van de bacterie hangt sterk af van de fractie F4R+ biggen in de populatie; de F4R– biggen spelen slechts een zeer beperkte rol in de transmissie-keten. Door het beperken van de fractie F4R+ biggen in de populatie tot minder dan 0.09, bijvoorbeeld door selectief te fokken op F4R–, zullen grote uitbraken van F4+ *E. coli* kunnen worden voorkomen.

List of publications

Full papers (refereed)

Geenen, P.L., Bresciani, J., Boes, J., Pedersen, A., Eriksen, L., Fagerholm, H.P., Nansen, P., 1999. The morphogenesis of *Ascaris suum* to the infective third-stage larvae within the egg. J. Parasitol. 85: 616-622.

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Geenen, P.L., Van der Gaag, L.C., Loeffen, W.L.A., Elbers, A.R.W., 2005. Naive Bayesian classifiers for the clinical diagnosis of Classical Swine Fever. In: D.J. Mellor, A.M. Russell, J.L.N. Wood (Eds.), Proceedings of the 2005 meeting of the Society for Veterinary Epidemiology and Preventive Medicine, 169-176. Nairn, Inverness, Scotland.

Curriculum vitae

Pieternella Laura Geenen (Petra) werd op 18 maart 1975 geboren te Papendrecht als jongste dochter van Gerrit en Ria Geenen. Na het behalen van haar VWO-diploma in 1993, verruilde zij de Zuid-Hollandse polders voor de Gelderse heuvels om te beginnen met de studie Biologie aan de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit). Zij verdiepte zich hier in de taxonomie van blackflies uit Equatoriaal Guinea en de transmissie van de ziekte rivierblindheid door dit insect. Vervolgens deed zij veldonderzoek aan de transmissie van de ziekten malaria en elefantiasis door muskieten in Sulawesi (Indonesië). Tenslotte deed zij infectieproeven met de varkensspoelworm en onderzocht de diverse larvenstadia van deze parasiet aan de Koninklijke Veterinaire en Landbouwuniversiteit in Frederiksberg (Kopenhagen, Denemarken). In september 1998 studeerde zij met lof af. In mei 1999 verruilde zij de Gelderse heuvels weer voor de polder, dit maal in Flevoland, om te beginnen met het in dit proefschrift beschreven promotieonderzoek. Zij was hiervoor aangesteld bij de leerstoelgroep Kwantitatieve Veterinaire Epidemiologie van Wageningen Universiteit en gedetacheerd bij de gelijknamige groep aan het Instituut voor Dierhouderij en Diergezondheid, ID-Lelystad (nu Animal Sciences Group van Wageningen UR). Sinds oktober 2003 werkt zij als post-doc in de Decision Support Systems groep van de Faculteit der Bèta-wetenschappen van de Universiteit Utrecht. Zij houdt zich hier bezig met het onderzoek aan en de ontwikkeling van een Bayesiaans netwerk voor de vroege detectie van klassieke varkenspest op basis van klinische symptomen, in samenwerking met het Centraal Instituut voor Dierziekte Controle (CIDC-Lelystad). Zij is momenteel weer woonachtig in Gelderland, dit maal in het mooie Nijmegen.

Training and Supervision Plan Graduate School WIAS

EDUCATION AND TRAINING

The Basic Package	year	cp*
Wias Common Course	1999	2.0
Course on philosophy of science and ethics	2000	1.0

Scientific Exposure

International conferences

International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), Copenhagen, Denmark	1999	1.0
Conference of the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM), Noordwijkerhout, The Netherlands	2001	0.6
International Symposium for Veterinary Epidemiology and Economics (ISVEE), Vina del Mar, Chile	2003	1.0

Seminars and workshops

Nederlandse Darmendag	99/02	0.4
Groep Geneeskunde Varken	1999	0.1
Voorjaarsvergadering NVVM/MM/NVP	2001	0.2
Trypanosomes: host parasite interaction in the genomics era (WIAS plus)	2002	0.3
Gezondheidsfokkerij varkens (WIAS)	2002	0.2
3rd GSAH-students meeting *'All models are wrong but some are useful'*	2000	0.2
Dutch Society for Veterinary Epidemiology and Economics (VEEC)	'99/'00/'03	0.6
WIAS Science Day	'00/'02/'03	0.6

Presentations

International Conference of the World Association for the Advancement of Veterinary Parasitology (poster)	1999	0.5
Conference of the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM), Noordwijkerhout, The Netherlands (poster)	2001	0.5
Voorjaarsvergadering NVVM/MM/NVP (oral)	2001	0.5
WIAS Science Day (oral)	2002	0.5
Nederlandse Darmendag (oral)	2002	0.5
Dutch Society for Veterinary Epidemiology and Economics (poster)	2003	0.5
International Symposium for Veterinary Epidemiology and Economics	2003	0.5

* One credit point (cp) equals a studyload of approximately 40 hours

In-Depth Studies

Disciplinary and interdisciplinary courses	year	cp*
Populatiedynamica van infectieziekten, ID-Lelystad, Lelystad	1999	0.8
3rd Winterschool on population dynamics, Woudschoten	2000	1.0
Biology underpinning animal sciences: broaden your HORIZON, WIAS	2001	0.8
3rd International summer school on infectious disease epidemiology, University of Bielefeld, Germany	2001	1.0
Summerschool on mathematical biology, University of Lisbon, Portugal	2002	1.0
Advanced statistics courses		
ID-Lelystad Course on statistics, J. de Bree, Lelystad	1999	1.0
Professional Skills Support Courses		
ID-Lelystad Course Techniques for scientific writing, Lelystad	2001	1.0
Use of laboratory animals	1999	3.0
Algemene inleiding Mathematica, CANdiensten, Amsterdam	1999	0.4
Course 'Hoorcollege geven', Onderwijsondersteuning, Wageningen	2003	1.0
Project management, Dutch Institute for Biology (NIBI)	2000	0.5
Inleiding S-plus, CANdiensten, Amsterdam	2001	0.2
Research Skills Training		
Preparing own PhD research proposal	1999	1.0
Didactic Skills Training		
Introductievak gezondheidsleer en reproductie	'01/'02	0.5
Management Skills Training		
Organisation of seminars and courses:		
Organisation of PhD retreat (WIAS, GSAH en ID-Lelystad)	2002	1.0
Membership of boards and committees:		
Chair of the ID-Lelystad PhD-board	'02/'03	2.0
Education and Training Total		**27.9**

* One credit point (cp) equals a studyload of approximately 40 hours

Notes

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