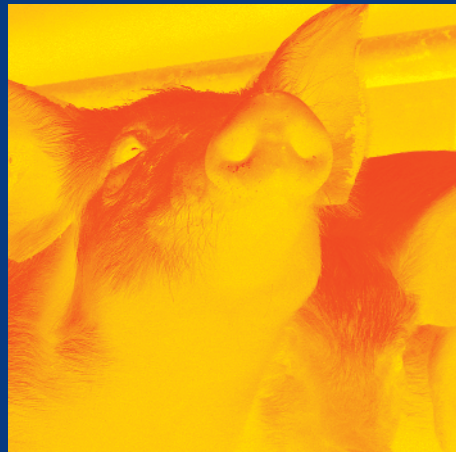# QUANTIFICATION OF
Actinobacillus pleuropneumoniae
TRANSMISSION

Annet Velthuis

QUANTIFICATION OF

Actinobacillus pleuropneumoniae

TRANSMISSION

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QUANTIFICATION OF

Actinobacillus pleuropneumoniae

TRANSMISSION

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Quantification of *Actinobacillus pleuropneumoniae* transmission

Kwantificering van *Actinobacillus pleuropneumoniae* transmissie

More insight into the transmission dynamics of bacteria between animals is gained with help of transmission experiments. In a transmission experiment various aspects of transmission can be studied. For example, more insight into the transmission dynamics can be gained, transmission can be quantified, or the effect of interventions on transmission can be quantified so that better-directed intervention strategies can be devised. The main goal of the research described in this thesis was the development of methods to quantify bacterial transmission in an experimental setting. We restricted the research to the transmission of one specific bacterium, i.e. *Actinobacillus pleuropneumoniae* serotype 9 in pigs. *A. pleuropneumoniae* is regarded as a primary pathogen that causes pleuropneumonia in pigs and brings considerable economic losses about world-wide. Direct transmission from pig to pig is believed to be the most important transmission route of this bacterium, therefore, prevention or reduction of transmission in direct animal to animal contact should in principle lead to eradication. By conducting several transmission experiments we got a better understanding of the transmission dynamics of the bacterium. It was concluded that an infectious state is related to an *A. pleuropneumoniae* positive tonsil at necropsy. Another conclusion was that the infectivity of a pig is a tenfold higher on days where more than ten *A. pleuropneumoniae* bacteria were isolated from the nasal swab than on the other days. Furthermore, new statistical and mathematical methods were developed to estimate or test hypothesis about the level of transmission. Statistical methods were based on the transient state (TS) algorithm. The TS algorithm is based on the stochastic susceptible-infectious-removed (SIR) model and provides a time-dependent probability distribution over the number of infected individuals during an epidemic. TS methods are difficult to calculate due to numerical limitations. Therefore, one would probably resort to the easily applicable but less appropriate final size (FS) methods. So, we investigated the error made when FS methods are used instead of TS methods. This error was generally not substantial. Furthermore, a new method to find a difference in transmission between two treatment groups (*MaxDiff* test) has been developed and compared to tests based on FS and TS algorithms and a test based on a Generalised Linear Model (GLM). The GLM test was most powerful in finding a difference in transmission. Next were the TS test and the *MaxDiff* test, which were approximately equally powerful, but more powerful than the FS test especially when the R_0 in both treatment groups are larger than 1. At the end, we tested the effect of vaccination on the transmission of *A. pleuropneumoniae* in the newly developed experimental design. The effect of vaccination was quantified with a method based on a generalised linear model, which appeared to be most appropriate for the quantification of *A. pleuropneumoniae* transmission. The effect of vaccination on the susceptibility could not been demonstrated.

Aan mijn ouders: Johan en Tiny

1

General Introduction

A. G. J. Velthuis

1. MOTIVATION

Eradication of infectious agents is an important issue in modern livestock sector. Governments are allowed to impose trade restrictions based on the health status of farm animals to ensure food safety and animal health protection. Also, farmers with a certified high health status herd may have increased production levels, will save on costs for treatment of diseased animals, and may get better prices for their products.

To eradicate an infectious agent from a population the transmission among individuals has to be reduced the extent that persistence of the infectious agent becomes impossible. Two types of transmission can be distinguished: vertical and horizontal. Vertical transmission means that the infection is transmitted from parent to offspring during gestation and birth, and horizontal transmission is any other form of transmission between individuals.

Transmission (vertical or horizontal) is often quantified by the reproduction ratio (R_0) which is defined as 'the expected number of secondary cases produced by a typical infected individual during its entire period of infectiousness in a completely susceptible population' (Diekmann *et al.* 1990). Thus R_0 is a measure of the degree to which an infectious agent spreads at the start of an epidemic. On average, for an infection to progress, each infected animal must infect more than one susceptible animal. If R_0 exceeds the threshold value 1 the infectious agent can spread and major outbreaks may occur. If R_0 is smaller than 1, the infectious agent may spread a little but it cannot persist in the population (Diekmann *et al.* 1990; Nasell 1995). So, a successful eradication strategy should bring R_0 below the threshold value 1. To investigate whether intervention strategies can reduce the transmission so that R_0 becomes smaller than 1, we need to have quantitative knowledge about the transmission of the infectious agent between individuals in a population.

One area of epidemiology encompasses studies resulting in associative models that attempt to quantify the relationship between risk factors and the occurrence (or presence) of disease. These models do not consider the transmission dynamics of the infectious agent and the interactions among the individuals in the population, which are both important factors for transmission (Koopman and Longini 1994). Another area of epidemiology concerns methodologies to study the transmission dynamics and to quantify transmission parameters of an infectious agent in a population of individuals (Becker 1989; Diekmann and Heesterbeek 2000). One of these methodologies is being used to develop mathematical models that mimic the transmission dynamics of infectious agents within populations (Bailey 1975; Diekmann and Heesterbeek 2000). Based on these mathematical models statistical methods have been developed to quantify transmission parameters from observed data (Becker 1989). Transmission data can be obtained from transmission experiments or field studies. By

reflecting the observed data to the mathematical models, the most appropriate statistical method can be chosen and the transmission can be quantified (Kroese and De Jong 2001).

With help of experiments it is possible to study and quantify transmission in a controlled environment and subsequently to test the effect of interventions on transmission. A transmission experiment often consists of several replicated transmission trials in which the horizontal spread of an infectious agent is followed. In each trial a number of infectious animals and a number of susceptible animals are housed together in a controlled environment (e.g. an isolation unit). To closely monitor the infection chain, all individuals are sampled regularly and, sometimes, scored on clinical symptoms to diagnose the establishment of an infection. The classification of the animals in the different infection classes – assumed in the mathematical model on which the analysis will be based – is crucial. Note, in transmission studies it is important to identify infected animals rather than diseased ones, as processes that cause disease are not always relevant for the spread of the infectious agent (Anderson 1991; De Jong and Bouma 2001). So, infected and infectious animals are by no means automatically diseased, while susceptible animals are susceptible to become infected rather than diseased. This distinction is important to bear in mind when translating the observations and measurements to infection events.

The success of a transmission experiment depends on its design, the quality of the technical implementation and the availability of an appropriate statistical analysis. Transmission experiments can be analysed by fitting a mathematical epidemiological model to the data. A simple epidemic model that is often used in the epidemiology of directly transmitted diseases is the stochastic susceptible-infectious-removed (SIR) model (Bailey 1975; Bartlett 1949). The SIR model incorporates two processes: infection and recovery (Table 1.1). If the model is appropriate, it can be used to estimate biologically interpretable transmission parameters, like R_0, to get an impression of the degree of transmission. Another use of the model is to test hypotheses. A possible null hypothesis to test is 'there is no difference in transmission between control and treatment group' against its alternative 'there is a difference'. If the null hypothesis is rejected it can be concluded that the treatment (or intervention) has an effect on the transmission. Besides comparing treatments it is also possible to test the null hypothesis 'R_0 is equal or larger than 1' against its alternative 'R_0 is smaller than 1'. If this null hypothesis is rejected than it can be concluded that R_0 is significantly less than 1, which implies that the infection will fade out under these circumstances (De Jong *et al.* 1995). Conversely, we could test the null hypothesis 'R_0 is equal or smaller than 1' against its alternative 'R_0 is larger than 1'. If this null hypothesis is rejected it can be concluded that R_0 exceeds 1, implying that these are two possible scenarios. First, the infection spreads and a substantial part of the population will be infected (major

outbreak) or, second, only a few individuals will be infected and the infection will fade out right after its onset (minor outbreak).

Table 1.1.   Description of the stochastic SIR model.

Event	Symbolic representation [a]	Rate
Infection	$(S,I) \rightarrow (S-1,I+1)$	$\beta SI/N$
Recovery	$(S,I) \rightarrow (S,I-1)$	αI

[a] S = the number of susceptible individuals; I = the number of infectious individuals; N = the total number of individuals; β = the infection rate parameter; α = the recovery rate parameter.

Compared to field studies, an important advantage of transmission experiments is that they offer a controlled environment in which the influence of a single factor (i.e. an intervention) on the transmission can be investigated, while variation caused by other factors is minimised. This implies that more insight can be obtained into causative mechanisms underlying the transmission dynamics of a specific infectious agent. Furthermore, experiments can be less expensive and less time-consuming than field studies, so more interventions can be tested, even those that are not yet actually used or those that cannot be studied under field conditions. Examples are studies on exotic diseases or vaccines that have not yet officially been approved. A disadvantage of transmission experiments is that extrapolation to the field situation might be questionable. On the other hand, it has been shown with pseudorabies virus that the experimental results corresponded well to the results observed in the field (Stegeman *et al.* 1995). Still, it is advisable to first study the effect of different interventions on the transmission of an infectious agent in transmission experiments, followed by field studies on the interventions proven to substantially reduce experimental transmission.

All of the many 'qualitative' transmission experiments that have been published (Jobert *et al.* 2000; Lechtenberg *et al.* 1994; Torremorell *et al.* 1997) have failed to address two crucial questions. First, to what extent does the infectious agent spread? Second, are the observed differences in transmission significant? To our knowledge, the first quantitative transmission experiments were published by Greenwood *et al.* (1936). The authors used experiments to quantify the transmission of several bacterial and viral infections among mice using endemic equilibria as a measure for transmission. Kermack and McKendrick (1936; 1939) estimated transmission from the data of the Greenwood experiments using a specific deterministic mathematical model. Later, Anderson and May (1979) published a paper about the Greenwood experiments in which they fitted a simple deterministic mathematical model to

the data. Their model assumes that transmission depends on population size. De Jong *et al.* (1995) showed that the observed transmission in the Greenwood experiments could just as well be explained with a model that assumes that transmission does not depend on population size. In transmission experiments using pseudorabies virus among pigs Bouma *et al.* (1995) demonstrated that, given a constant density, transmission did not depend on population size.

De Jong and Kimman (1994) published the first paper about a transmission experiment in which the effect of an intervention was tested and quantified, namely, the effect of vaccination on the transmission of pseudorabies virus among pigs. Several other studies using transmission experiments in which viral transmission was quantified have subsequently been published (Table 1.2). Thus far, methodological development of transmission experiments has been based on viral infections and, as yet, no quantitative bacterial transmission experiments have been published.

Table 1.2:   An overview of published studies on transmission experiments in which transmission has been quantified with help of mathematical models.

Year	Reference	Infectious agent	Host	D/St[a]
1936	Greenwood *et al.* 1936	*Bacterium aertrycke*	Mice	D
1936	Greenwood *et al.* 1936	Virus disease mouse ectromelia	Mice	D
1936	Kermack and Mckendrick 1936	Virus disease mouse ectromelia	Mice	D
1939	Kermack and Mckendrick 1939	*Bacterium aertrycke*	Mice	D
1994	De Jong and Kimman 1994	Pseudorabies	Pigs	St
1995	De Jong *et al.* 1995	*Bacterium aertrycke*	Mice	St
1995	Bouma *et al.* 1995	Pseudorabies virus	Pigs	St
1996	Bouma *et al.* 1996	Pseudorabies virus	Pigs	St
1997	Bouma *et al.* 1997a	Pseudorabies virus	Pigs	St
1997	Bouma *et al.* 1997b	Pseudorabies virus	Pigs	St
1998	Wit *et al.* 1998	Infectious bronchitis virus	Chickens	St
1998	Laevens *et al.* 1998	Classical swine fever virus	Pigs	St
2000	Bouma *et al.* 2000	Classical swine fever virus	Pigs	St
2000	Mars *et al.* 2000 a	Bovine herpes virus	Cattle	St
2000	Mars *et al.* 2000 b	Bovine herpes virus	Cattle	St
2000	Mars *et al.* 2000 c	Bovine herpes virus	Cattle	St
2000	Nodelijk *et al.* 2001	Porcine reproductive and respiratory syndrome virus	Pigs	St
2001	Van Nes *et al.* 2001	Pseudorabies virus	Pigs	St
2001	Dewulf *et al.* 2001	Classical swine fever virus	Pigs	St
2002	Klinkenberg *et al.* 2002	Classical swine fever virus	Pigs	St
2002	Velthuis *et al.* Accepted a	*A. pleuropneumoniae*	Pigs	St
2002	Velthuis *et al.* Accepted b	*A. pleuropneumoniae*	Pigs	St
2002	Maurice *et al.* Accepted	Encephalomyocarditis virus	Pigs	St

[a] A deterministic (D) or stochastic (St) model was used for the quantification of transmissions

To quantify the transmission of a specific bacterial infection among individuals a suitable experimental design and an appropriate statistical method are required. One aspect of finding the optimal design is the choice of experimental procedures appropriate to the host-agent biology, which includes the best infection route and dose to produce infectious individuals, and the definition of infected and infectious individuals. An appropriate mathematical or statistical model needs to be selected for the quantification of the transmission. Experience with addressing such biological and mathematical issues regarding viral transmission experiments have been published (Bouma 1997), but as yet there has been no experience with bacterial transmission experiments. This led us to the main goal of the research described in this thesis:

'Development of methods to quantify bacterial transmission in an experimental setting'

In order to define a more workable aim we confined the research to the study of the transmission of one specific bacterium, i.e. *Actinobacillus pleuropneumoniae* serotype 9 in pigs. We chose *A. pleuropneumoniae* because this bacterium is of great concern to the swine industry worldwide and its transmission dynamics is only poorly understood. Furthermore, the Institute of Animal Science and Health in Lelystad, where the research was conducted, had already acquired much experience with this organism (Cruijsen 1995; Jansen *et al.* 1995; Kamp *et al.* 1991; Kamp *et al.* 1997; Kamp and Van Leengoed 1989; Kamp *et al.* 1994; Van Leengoed 1988).

2. *ACTINOBACILLUS PLEUROPNEUMONIAE*

A. pleuropneumoniae causes porcine pleuropneumonia, a disease that has a serious impact on the economy in most pig-rearing countries. Acute clinical outbreaks of pleuropneumonia are characterised by a hemorrhagic necrotising pneumonia and fibrinous pleuritis resulting in high morbidity and mortality (Nicolet 1992; Cruijsen *et al.* 1995; Cruijsen 1996). The disease is most prevalent in fattening pigs at approximately 3 months of age (Cruijsen *et al.* 1995). In an endemic infected breeding herd, most piglets seem to be protected against the disease by maternally derived immunity. However, in endemic-infected fattening herds, the bacterium causes a decreased weight gain and a sub-optimal feed conversion (Straw *et al.* 1989; Straw 1991). Studies done in slaughterhouses have shown that many pigs had old pleuropneumonia lesions in their lungs (Elbers *et al.* 1992), and that many pigs carried *A. pleuropneumoniae* in or on their tonsils (Mousing 1990; Møller *et al.* 1993). Preceding infections with for instance pseudorabies virus and *Mycoplasma hyopneumoniae* may predispose pigs to pleuropneumonia (Yagihashi 1984; Elbers *et al.* 1992; Sakano *et al.* 1993; Loeffen *et al.*

1999), while a preceding infection with porcine reproductive and respiratory syndrome virus does not enhance the severity of an infection with *A. pleuropneumoniae* (Pol *et al.* 1997).

A. pleuropneumoniae can be divided into two biotypes: biotype 1 is nicotinamide adenine dinucleotide (NAD) dependent whereas biotype 2 is not. Until now, 15 serotypes (13 for biotype 1 and 2 for biotype 2) have been described based on the basis of antigenic diversity of the capsular polysaccharides and lipopolysaccharides (Nicolet 1992; Blackall *et al.* 2002). All *A. pleuropneumoniae* serotypes vary in virulence, but they can all cause severe disease and death in pigs.

The pathogenesis of pleuropneumonia is not fully understood. Many virulence factors have been described that enable the bacterium to survive *in vivo* and contribute to the pathogenesis of the disease. The capsular polysaccharide (CPS) protects the bacterium against the defence mechanism of the host. CPS does not cause pulmonary lesions when instilled endobrochially. It does not activate complement and has no toxic activity (Fenwick *et al.* 1986; Inzana *et al.* 1988; Inzana 1991) but encapsulated *A. pleuropneumoniae* strains are resistant to killing by antibody and complement and to phagocytosis by PMNs (Inzana 1991; Rycroft *et al.* 1990; Ward 1994). However, after opsonisation, PMNs can phagocytise and kill *A. pleuropneumoniae* but alveolar macrophages can not (Cruijsen *et al.* 1992, Van Leengoed *et al.* 1992). Non encapsulated mutants of *A. pleuropneumonia* have been shown to be considerably less virulent than their encapsulated parent strains (Rosendal and MacInnes 1990; Inzana *et al.* 1993).

Purified lipopolysaccharide (LPS) from *A. pleuropneumoniae* induced lung lesions when given intratracheally. Depending on the dose, the lesions varied from a mild interstitial pneumonia to a consolidating lobular pneumonia with inflammatory cell infiltration (Fenwick and Osburn 1986; Udeze *et al.* 1987). These lesions are similar to those of an infection with *A. pleuropneumoniae* except that the haemorrhagic necrosis, which is typical for porcine pleuropneumonia is missing (Fenwick and Osburn 1986; Udeze *et al.* 1987). Furthermore, smooth LPS, in particular the high molecular weight O antigen has been shown to play a role in *in vitro* adherence of *A. pleuropneumoniae* to mucus, tracheal rings and frozen lung sections (Belanger *et al.* 1990; Paradis *et al.* 1994; Belanger *et al.* 1994). LPS is not involved in adherence to cultured porcine alveolar epithelial cells (Boekema, to be published). Recently, intact fimbriae and fimbrial subunits have been purified from some serotypes of *A. pleuropneumoniae* (Zhang *et al.* 2000).

Three extracellular pore-forming RTX toxins have been described in *A. pleuropneumonia*. ApxI is strongly haemolytic and strongly cytotoxic, ApxII is weakly haemolytic and moderately cytotoxic, and ApxIII is not haemolytic but strongly cytotoxic (Kamp *et al.* 1991). Strains of serotypes 1, 5a, 5b, 9, and 11 produce ApxI and ApxII, strains

of serotypes 2, 3, 4, 6, and 8 produce ApxII and ApxIII, strains of serotypes 7 and 12 produce ApxII only and strains of serotype 10 produce ApxI only (Kamp *et al.* 1994). The toxins are toxic for alveolar macrophages, neutrophils and a variety of other cells (Bendixen *et al.* 1981; Van Leengoed and Kamp 1989; Kamp *et al.* 1991). They stimulate neutrophils and macrophages at low doses but kill these cells at higher doses (Dom *et al.* 1992a; Dom *et al.* 1992b). *In vivo*, the activities of the toxins could interfere with the pulmonary macrophage and neutrophil function allowing the bacterium to multiply instead of being cleared. Furthermore, lysed neutrophils may release lysosomal contents and oxigen radicals that could contribute to the massive inflamation and necrosis in typical lesions. The genes encoding the three toxins have been sequenced (Jansen *et al.* 1992, Jansen *et al.* 1993a, Jansen *et al.* 1993b). The Apx toxins of *A. pleuropneumoniae* are essential virulence factors. Mutants that do not produce ApxI and ApxII do not produce pleuropneumonia (Tascon *et al.* 1994). Furthermore, purified recombinant Apx I, ApxII and ApxIII, when instilled edobrochially, trigger the development of clinical symptoms and lunglesions typical for porcine pleuropneumonia (Kamp *et al.* 1997). Recently a fourth Apx-toxin, ApxIV, was described (Schaller *et al.* 1999). This toxin seems to be expressed *in vivo* only and its role in the pathogenesis needs to be established.

Antibiotic therapy is effective in clinical outbreaks. Prevention of clinical outbreaks can be accomplished using continuous or intermittent antimicrobial therapy (Ueda *et al.* 1995; Moore *et al.* 1996).

Pigs, which have been infected with *A. pleuropneumoniae* are immune to infections with the homologous serotype and at least partly resistant to challenge with heterologous serotypes (Nielsen 1974, Nielsen 1984, Cruijsen *et al.* 1995). In contrast, immunisation with killed bacteria induces only partial protection against the homologous serotype and no protection against heterologous serotypes (Nielsen 1984). Second generation vaccines containing the RTX-toxins induce good protection against clinical disease caused by any serotype (Van Leengoed *et al.* 1988, Kamp *et al.* 1992, Fedorka-Cray *et al.* 1993, Van den Bosch *et al.* 1992). However, these vaccines do not prevent the animals from becoming carriers through subclinical infections (Beskow *et al.* 1993; Hensel *et al.* 2000; Van Overbeke *et al.* 2001).

Improvement of management and housing conditions can contribute to a reduction of clinical outbreaks and, consequently, economic losses (Beskow *et al.* 1998).

Direct transmission of *A. pleuropneumoniae* from pig to pig is believed to be the predominant infection route, since the bacterium does not survive for long in the environment (Nicolet 1992). Subclinically infected carrier pigs are currently believed to be the most important cause of the spread of the pathogen within and between herds (Møller *et al.* 1993; Sidibe *et al.* 1993; Chiers *et al.* 2001). Cruijsen (1995) showed that almost all one-week-old

piglets of three endemically infected breeding to finishing herds had high detectable haemolysin and cytotoxic neutralising antibodies. The antibody titres generally decreased during the first weeks of life, indicating the presence of antibodies of maternal origin. In 10% of the pigs the Apx-neutralising antibody titres increased again before the age of 9 weeks, suggesting that these pigs might carry the bacterium subclinically. This implies that young subclinically infected carrier piglets, which are often transported from breeding to finishing herds, might introduce *A. pleuropneumoniae* into finishing herds. Furthermore, from about the age of 12 weeks onwards the neutralising antibody titres of most pigs increased again, while none of these pigs had shown clinical signs of pleuropneumonia indicating that *A. pleuropneumoniae* was circulating (Cruijsen *et al.* 1995).

Given that subclinically infected carriers transmit the bacterium, prevention or a sufficient reduction of direct transmission from carriers to susceptible pigs may lead to eradication of *A. pleuropneumoniae* from a population. Knowledge about the transmission dynamics of *A. pleuropneumoniae* from subclinically infected carriers to susceptible animals is needed for a proper design of a control and/or eradication program.

Many infection models for *A. pleuropneumoniae* to study the pathogenesis of porcine pleuropneumonia and to test the effect of interventions on clinical disease have been developed (Liggett *et al.* 1987; Van Leengoed and Kamp 1989; Hensel *et al.* 1995a). However, thus far, no experimental transmission model has been developed in order to study the transmission dynamics and to quantify the effect of interventions on the transmission of *A. pleuropneumoniae* from sub-clinically infected carriers to susceptible pigs.

3. AIM AND OUTLINE OF THE THESIS

We designed an experimental method to study the transmission of *A. pleuropneumoniae* among pigs in a controlled environment in order to:
- gain a better understanding of the transmission dynamics of A. pleuropneumoniae;
- test the effect of interventions on the transmission of A. pleuropneumoniae;
- develop new statistical or mathematical methods to quantify (bacterial) transmission.

Before this could be achieved we had several difficulties to overcome. For instance, in the first couple of transmission trials the observed numbers of contact infections was found to vary considerably, which needed investigation before we could continue. After exploring the data of the trials, we concluded that the variation in observed transmission could be attributed to two reasons. First, the intranasal inoculation method did not lead to carriers in a repeatable and consistent way (Chapter 2). Second, the infectivity of *A. pleuropneumoniae* appeared to be very variable and was closely related to nasal excretion (Chapters 2 and 5). We then tried a different inoculation method. Carriers were created by exposing susceptible pigs to heavily

infected, endobronchially inoculated pigs. Next, the endobronchially-inoculated pigs were removed and replaced by a second batch of susceptible pigs. This second batch of susceptible pigs was thus exposed to the first batch of sub-clinically infected pigs (Chapter 5). Thus, after a first infection chain between endobronchially-inoculated pigs and susceptible pigs to create carrier pigs, the experiment was extended to start the infection chain between subclinically infected carrier pigs and susceptible pigs. This design is also called an extended transmission experiment (Chapter 5).

Other difficulties occurred when analysing data of the *A. pleuropneumoniae* transmission experiments. First, we had to determine which pig was considered to be infectious, and to do this we needed to have a good definition of the infectious state for *A. pleuropneumoniae*. With help of a Principal Component Analysis a definition of the infectious state was determined, concluding that pigs from which *A. pleuropneumoniae* was isolated from the tonsil (at necropsy) had been infectious in the experiment (Chapter 2).

Another problem that cropped up when analysing the data was that the traditional Final Size (FS) methods developed for viral infections are based on the assumption that the infection chain has reached a final size situation before the experiment was terminated. In other words, at the end of the experiment there should be no possibilities for new infections to occur as there would be no infectious individuals or no susceptible individuals left. Reaching a final size situation with *A. pleuropneumoniae* experiments was not always feasible. So, other statistical methods not dependent on the final size assumption had to be developed, which are presented in this thesis (Chapters 3 and 4). Some of these methods are based on the Transient State (TS) algorithm (Chapters 3 and 4), but it turned out that the TS algorithm is not easily applicable. Therefore, we investigated the error of using the FS method knowing that the final size situation has not been reached in all trials. At first, we investigated the error analytically based on one-to-one experiments (Chapter 3), and in a later stage we did this numerically for five-to-five experiments (Chapter 4). It was concluded that the error was acceptable in both types of transmission experiments.

The last problem we tackled was the difference in the number of contact infections between two treatment groups that was observed during the course of the experiment, which faded away at the end of the experiment. Here, both the FS method and the TS method would not reject the null hypothesis 'there is no difference in transmission between the two treatment groups'. This phenomenon is likely to occur when the R_0 is different in both treatment groups, and greater than 1 in both cases. Therefore, a third method, the *MaxDiff* method, was developed. The *MaxDiff* method can detect a difference in the number of contact infections in the course of the experiment (Chapter 4). The *MaxDiff* method is based on a probability distribution that can be derived numerically using the Markov Chain Monte Carlo technique.

We compared the effectiveness of the *MaxDiff* method to other testing methods based on the FS and TS algorithms, and to the existing method that is based on a Generalised Linear Model (Chapter 4). Chapter 6 discusses several aspects of the methodological approaches that have been presented in this thesis.

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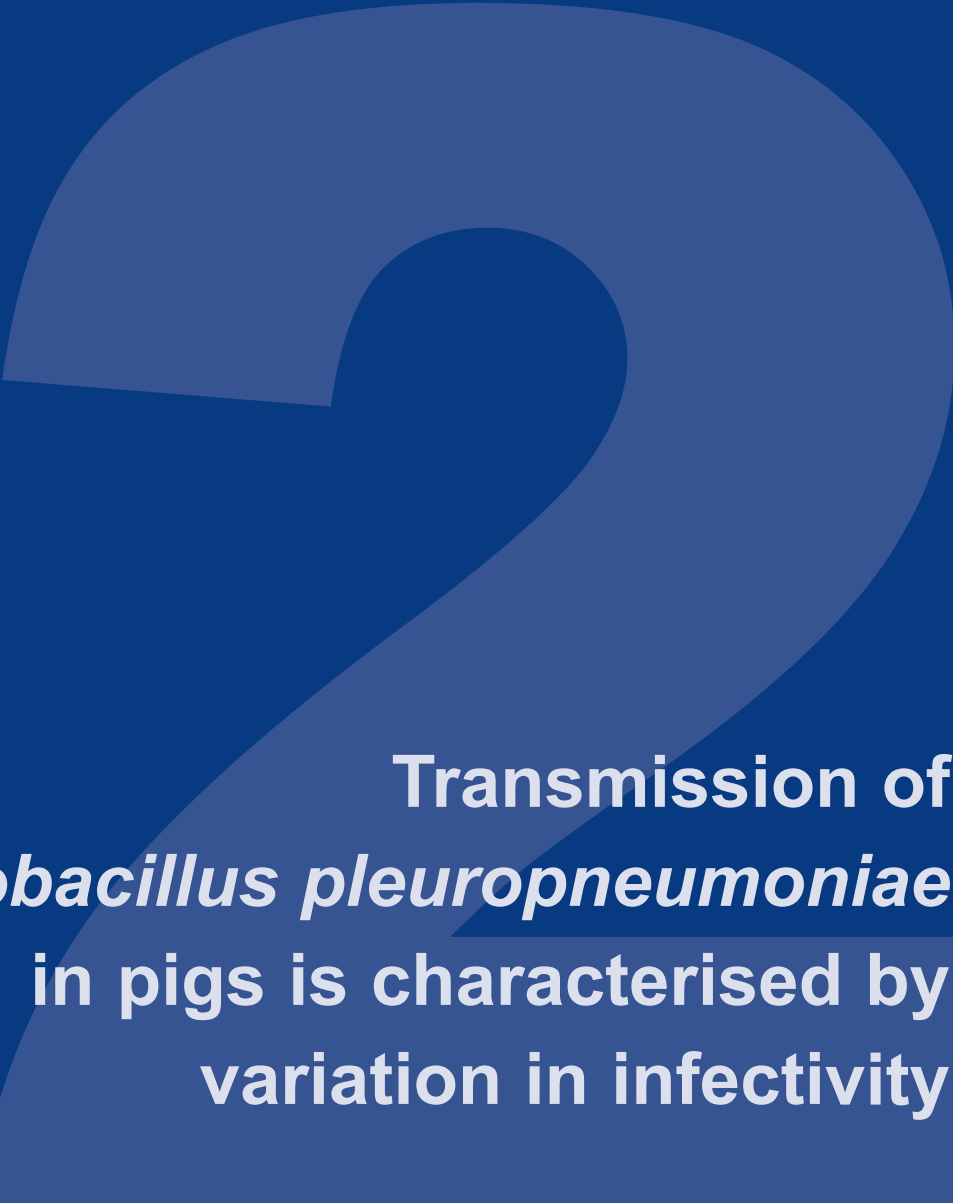
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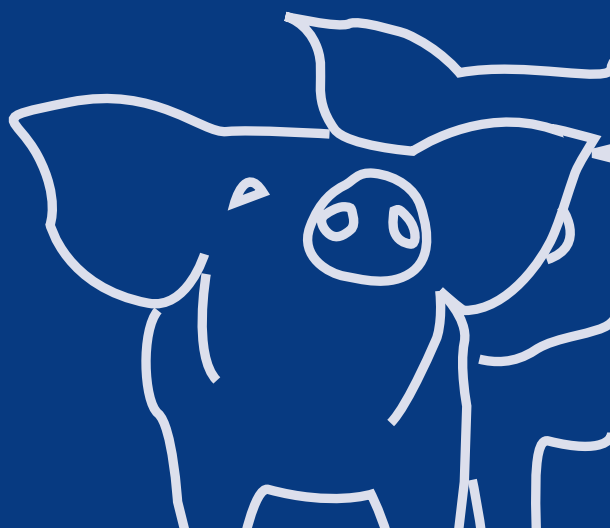
Transmission of *Actinobacillus pleuropneumoniae* in pigs is characterised by variation in infectivity

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SUMMARY

Ten transmission trials with *Actinobacillus pleuropneumoniae* were carried out. The observed transmission was highly variable, which was surprising since the design of the trials was very similar. We investigated whether the variable transmission could be explained by variation in infectivity of *A. pleuropneumoniae* infected pigs. We looked for measurable characteristics, which could be indicative for infectious pigs or for the level of infectivity. The characteristic that appeared to be most indicative for a pig being infectious was *A. pleuropneumoniae* positive tonsils at necropsy. The characteristic that was correlated to the level of infectivity was the number of *A. pleuropneumoniae* colonies isolated from the nasal swab, i.e. the probability for an infectious pig to infect a susceptible pig was tenfold higher on days where at least ten colonies were isolated. In this study it is shown that it is possible to measure the bacterial transmission of *A. pleuropneumoniae* under controlled circumstances if variation in infectivity is taken into account.

1. INTRODUCTION

To eradicate successfully a bacterium from a population, it is important to reduce the transmission to such an extent that it is impossible for the bacterium to maintain itself in the population. Knowledge about the transmission mechanism of the bacterium is needed to know how to reduce its transmission.

Laboratory transmission experiments combined with mathematical models can provide information about transmission mechanisms of infectious agents and about the effect of control measures on transmission (De Jong and Kimman 1994). A transmission experiment often consists of several transmission trials. In a transmission trial infectious and susceptible animals are housed together in an isolation unit and the infection chain is followed by taking samples and by clinical inspection of the animals during the experimental period. The transmission mechanism can be studied when fitting a mathematical model, which describes the infection chain theoretically, to the experimental data. Important transmission parameters can be estimated or effects of control measures on these transmission parameters can be tested.

Viral transmission experiments were developed for Pseudorabies Virus (De Jong and Kimman 1994; Bouma *et al.* 1995; Bouma *et al.* 1996; Bouma *et al.* 1997a; Bouma *et al.* 1997b), Classical Swine Fever Virus (Bouma *et al.* 2000), and Bovine Herpes Virus (Mars *et al.* 2000a; Mars *et al.* 2000b; Mars *et al.* 2000c). The vaccination scheme used in the Dutch eradication program for Pseudorabies Virus was developed with knowledge about the transmission obtained from transmission experiments combined with mathematical modelling (Stegeman *et al.* 1997).

To get more knowledge about the transmission of bacterial infections, ten transmission trials were carried out with the bacterium *A. pleuropneumoniae* in pigs. *A. pleuropneumoniae* is regarded as a primary pathogen that causes pleuropneumonia in pigs and brings great economic losses about world-wide (Hunneman 1986). Direct transmission from pig to pig is believed to be the most important transmission route, since *A. pleuropneumoniae* does not survive in the environment for prolonged periods of time (Nicolet 1992). Therefore, prevention or reduction of transmission in direct animal to animal contact should in principle lead to eradication of the disease.

In contrast to transmission trials with viral infections, the observed transmission in the *A. pleuropneumoniae* trials was highly variable. This was surprising since the design of the trials was chosen so that the variation in outcome would be as small as possible and would be very similar in each trial (De Jong and Kimman 1994).

The observed transmission varied too much to be explained by the general stochastic SIR model, which was used for analysing transmission experiments with viral infections (De Jong

and Kimman 1994). In this study we investigated which part of the general SIR model should be adjusted to have a better fit with the observed transmission of *A. pleuropneumoniae* in our trials.

The hypothesis investigated was whether variation in infectivity could cause the variable transmission. To do this, measurable characteristics of the individual animals that could be indicative for being infectious or for the level of infectivity present in the population were selected from the available data. These included quantitative bacteriology of swabs and of post mortem tissues, antibody titres against toxins in sera, and rectal temperatures.

In the different analysis described in this paper the characteristic which indicated infectious pigs was determined, the intranasal inoculation was evaluated and the variation in individual infectivity was studied.

2. MATERIAL AND METHODS

2.1. Pigs

Hundred and twenty two five-week-old Dutch Landrace pigs from the Specific Pathogen Free herd of the Institute of Animal Science and Health were used in the transmission trials. Both females and uncastrated males were used.

2.2. Experimental design

Ten transmission trials within six experiments were carried out. To create infectious animals, pigs were intranasally inoculated in eight trials and endobronchially inoculated in two trials. One trial included only three (intranasally) inoculated pigs and four contact pigs, whereas the others included five inoculated pigs and five contact pigs.

Seven days post inoculation, the pigs were already housed together in order to get used to each other. The inoculation took place on day zero, while the contact pigs were temporary housed in another isolation unit. One day later, the contact pigs were housed with the inoculated pigs again to minimise the possibility that the contact pigs would come in contact with the inoculum. All pigs were sampled almost every other day to check whether the contact pigs became infected and whether the inoculated pigs were excreting *A. pleuropneumoniae*.

Four trials were extended transmission trials: the inoculated pigs were replaced by new group of contact pigs, C_2-pigs, when a defined number of contact infections was observed in the first group of contact pigs, C_1-pigs (Figure 2.1). In the extended trials the transmission

between naturally infected C_1-pigs and C_2-pigs was measured, which is more similar to the transmission in the field. One of the extended trials was actually a double extended trial; the C_1-pigs were once more exchanged for five susceptible pigs (C_3-pigs) when the majority of the C_2-pigs got infected. The experimental periods of the ten trials and the replacement days of the extended trials are shown in Figure 2.1.

Trial	Exp.	Length			
1	A	28 d	$5\ I_n + 5\ C_1$		
2	A	28 d	$5\ I_n + 5\ C_1$		
3	B	21 d	$5\ I_n + 5\ C_1$		
4	C	28 d	$3\ I_n + 4\ C_1$		
5	D	35 d	$5\ I_n + 5\ C_1$		
6	D	37 d	$5\ I_n + 5\ C_1$ →15 d→ $5\ C_1 + 5\ C_2$		
7	E	35 d	$5\ I_n + 5\ C_1$		
8	E	36 d	$5\ I_n + 5\ C_1$ →15 d→ $5\ C_1 + 5\ C_2$ →25 d→ $5\ C_2 + 5\ C_3$		
9	F	35 d	$5\ I_b + 5\ C_1$ →4 d→ $5\ C_1 + 5\ C_2$		
10	F	36 d	$5\ I_b + 5\ C_1$ →7 d→ $5\ C_1 + 5\ C_2$		

D_1 D_2

Legend:
D_1 first replacement day
D_2 second replacement day
I_n intranasally inoculated pigs
I_b endobronchially inoculated pigs
C_1 first group contact pigs
C_2 second group contact pigs
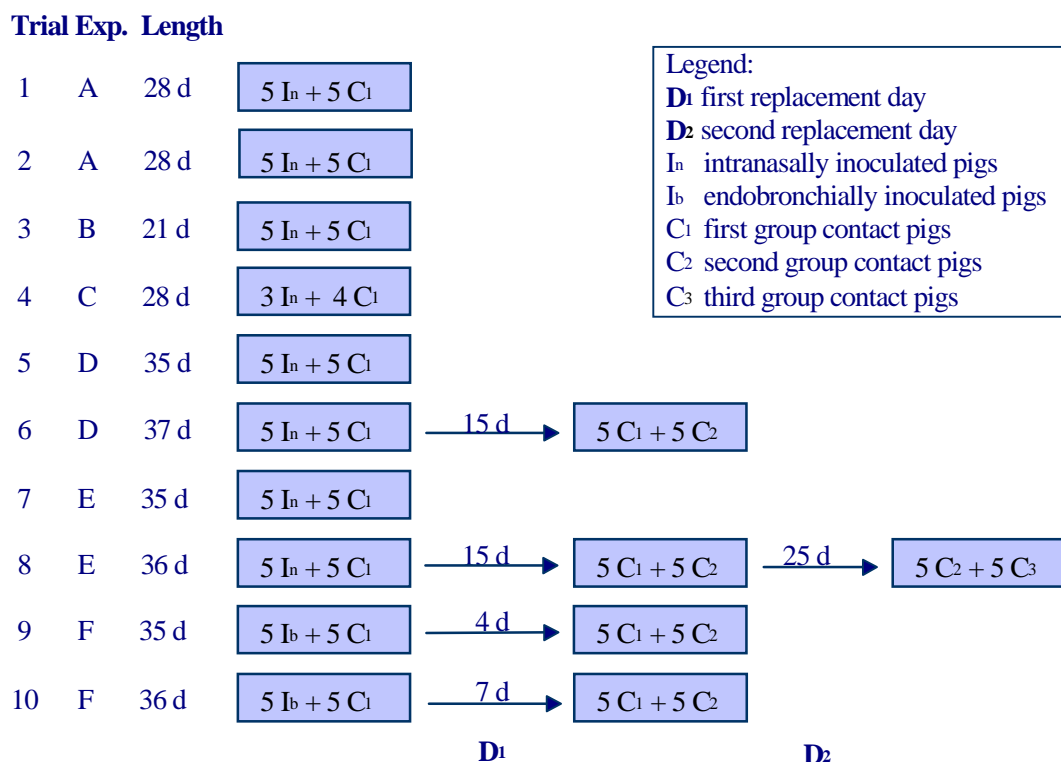C_3 third group contact pigs

Figure 2.1. Design of the ten transmission trials carried out with *A. pleuropneumoniae*. Four of the ten trials were extended, and one of those four was double extended. See text for details.

In all trials, the pigs were housed in isolation units at about 25°C, with *ad libitum* feeding and drinking. The density at which the pigs were kept was one pig per 0.85 m^2, which is similar to field conditions.

Nasal and tonsillar swabs were taken of all pigs almost every other day. Blood samples were taken weekly starting on day –7 and body temperatures were recorded almost every other day starting on day 0. At the end of the experiment the pigs were examined at necropsy and bronchial lymph nodes, palatine tonsils, nasal lymph node, and if present pneumonic parts of the lungs (lesions) were collected for bacteriological examination.

2.3. Inoculation

A. pleuropneumoniae strain 13261, the reference strain for serotype 9, was used for the inoculum. An early passage of this strain was stored in aliquots at -70 °C. For each experiment one vial was thawed and the suspension was inoculated on Heart Infusion agar (Difco, Sparks, MA) supplemented with 5% defibrinated sheep blood and 0.1% nicotinamide adenine dinucleotide (Calbiochem, La Jolla, CA) (HISV-plate). This plate was incubated overnight at 37°C in an atmosphere of 5% CO_2. Next morning colonies were transferred to two HISV-plates and after incubation for 6 hours at 37°C and 5% CO_2 the plates were rinsed with 5 ml Eagles minimal essential medium (EMEM) per plate. The number of colony forming units (CFU) in the suspension was determined by plating tenfold dilutions on HISV-plates, which were incubated overnight at 37°C and 5% CO_2. The suspension was stored overnight at 4°C. Next morning the number of CFU was counted and the inoculum was prepared. For intranasal inoculation the suspension was diluted in EMEM to 10^4 CFU/ml and for endobronchial inoculation to 10^2 CFU/ml. The number of CFU in the inoculum was determined by plating tenfold dilutions on HISV-plates just before and right after the performed inoculations. The number of CFUs was counted the next morning. For both inoculation methods the pigs were anaesthetised shortly with halothane gas and were held in upright position. The pigs were intranasally inoculated by dripping 1 ml of the inoculum into both nostrils when the pigs were visibly inhaling. The pigs were endobronchially inoculated by slowly injecting 10 ml of the inoculum deep into the lungs using a catheter (Nicolet 1992).

2.4. Bacteriological examination

The collected swabs were streaked out directly on a specific plate that contains Heart Infusion Agar supplemented with 5% sheep blood, 0.2% Nicotinamide Adenine Dinucleotide, 0.75 µg/ml Clindamycin-HCl, 0.75 µg/ml Gentamycin, 4 µg/ml Vancomycin-HCl, 35 µg/ml Amphothericine B (Sigma, St. Louis, MO) (CGVA-plate). The sample was diluted with a loop using the quadrant streaking method. Thereafter the swab was suspended in 3-ml saline solution and of this suspension a 1:100 dilution was made. 0.1 ml of both solutions was inoculated on CGVA-plates by using a spiral plater (Salm en Kipp BV, Breukelen, The Netherlands). The plates were incubated overnight at 37 °C and 5% CO_2. The number of suspected colonies, i.e. small white/grey colonies surrounded by a large β haemolytic zone, was counted and if there were too many, the number of colonies was estimated.

To confirm that the colonies were *A. pleuropneumoniae* serotype 9, one typical colony per plate was tested for satellite growth and agglutination in a specific anti serotype 9 serum. We concluded that the sample contained *A. pleuropneumoniae* serotype 9 bacteria if both

above-mentioned tests were positive. The average number of suspected colonies counted on the directly swabbed plate and the plate with the 3-ml saline suspension was recorded.

The collected tissue specimens at necropsy were dipped into boiling water for three seconds to disinfect possible contamination of the surface. Thereafter the sample was cut into pieces and added to 3-ml saline solution. This suspension was mingled with a stomacher (Salm en Kipp BV, Breukelen, The Netherlands) and different dilutions of the suspension were inoculated on CGVA-plates by using a spiral plater. The number of suspected colonies was counted and if there were too many, this number was estimated. The confirmation of *A. pleuropneumoniae* serotype 9 was the same as described earlier for the swabs.

2.5. Haemolysin neutralisation assay

The ability of the sera to neutralise haemolytic culture supernatant fluids of *A. pleuropneumoniae* serotype 9 was determined, using sheep erythrocytes (Kamp and Van Leengoed 1989). Twofold dilutions of 50-µl volumes of the sera in GVS (gelatine veronal saline buffer) were incubated for 30 minutes at 37°C with an equal volume of culture filtrate adjusted to a haemolysin titre of 16. Then, 50-µl of a 1% suspension of sheep red blood cells in GVS was added to each well, incubated for 2 hours at 37°C and the extent of haemolysis was determined. Titres are expressed as the reciprocal of the highest dilution of serum that showed less than 50% haemolysis.

2.6. SIR model

The transmission of *A. pleuropneumoniae* in an experimental population has to be described by a stochastic model, because in small populations chance processes may play an important role. The stochastic general SIR model from Becker (Becker 1989) was used as a starting point for the analysis. In this model, two events can occur when infectious individuals I and susceptible individuals S are present in a population: an infection event $(S,I) \rightarrow (S-1,I+1)$ and a recovery event $(S,I) \rightarrow (S,I-1)$. The formulations of the rates at which the events occur are formulated according to the true mass-action theory (De Jong *et al.* 1995). The rate, at which an infection event occurs, depends on the density of S-pigs, the number of I-pigs present and an infection rate parameter β (Figure 2.2). In this parameter β, the contact rate and the probability per contact to infect another individual are included. The rate, at which a recovery event occurs, depends on the number of I-pigs and the recovery rate parameter α. Assuming that at one moment only one of both events can occur, transmission parameters can be estimated according to the generalised linear model method of Becker (1989).
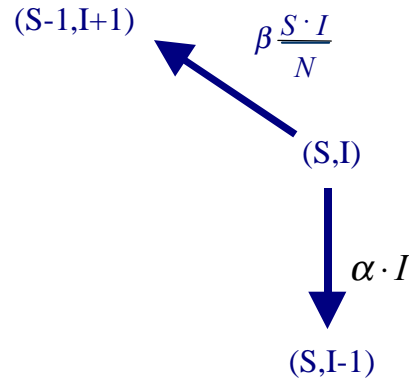
$$(S\text{-}1,I\text{+}1) \qquad \beta\frac{S \cdot I}{N}$$

$$(S,I)$$

$$\alpha \cdot I$$

$$(S,I\text{-}1)$$

Figure 2.2. The transition probabilities from state (S,I) to the two following states due to an infection (S-1,I+1) or recovery event (S,I-1).

De Jong and Kimman (1994) described an algorithm for calculating the probability distribution of the final size of the outbreak given transmission parameters and start condition. An important insight derived from this algorithm is that the probability distribution depends only on R_0, when $R_0 = \beta/\alpha$. Thus, R_0 is a suitable parameter to characterise the transmission. This R_0 is also called the basic reproduction ratio and is biological defined as the average number of secondary cases caused by one typical infectious pig (Diekmann *et al.* 1990).

2.7. Statistic to test variance in transmission between trials

We used the following statistic to test whether the variability in transmission between the trials was too high to be explained with the transmission mechanism described in the SIR model:

$$V = \sum_{i=1}^{n} \frac{X_i - \varepsilon X_i}{\text{var}(X_i)}$$

In which X_i is the number of contact infections observed in trial i, εX_i the expected value of X_i in trial i, and $var(X_i)$ its variance. To calculate the expected value and the variance, an estimate of the basic reproduction ratio R_0 was used. This estimate was based on the maximum likelihood estimator where the likelihood function for the observed number of cases is achieved from probability distribution of the final size of the outbreak (De Jong and Kimman 1994). So, we tested whether $H_0{:}R_{0,exp\ 1}{=}R_{0,exp\ 2}{=}\ldots{=}R_{0,exp\ n}{=}R_0$, assuming that the

estimation of R_0 according to the general SIR model was correct. Finally the probability of having the estimated V or more extreme values was calculated. The null-hypothesis was rejected if this probability is having a low value (<0.05).

2.8. Principal component analysis

To examine which of the possible indicators for individual infectivity have a high variation, we did a principal component analysis (PCA). A PCA is a multivariate technique in which a number of related variables are transformed to a set of uncorrelated variables that summarises the variability in the data (Chatfield 1980). In a PCA, principal components are created which are linear functions of the original variables, with each variable multiplied by a loading that corresponds to its correlation with the principal component vector. The first principal component accounts for as much as possible of the variation in the original data. The loading of each variable in a principal component has a value between -1 and 1. For interpretation purposes, the retained principal components was rotated in such a way that the rotated components have high loadings on a small set of variables, and zero or near zero loadings on the remaining variables. Most variance in the original data is explained by the set of variables with the highest absolute value of the loadings in the first principal component.

2.9. Estimating variation in individual infectivity

Variability in individual infectivity is characterised by variability in the infection rate β of the SIR model. In the SIR model it is assumed that β is constant during the whole infectious period (De Jong and Kimman 1994), but this could be an invalid assumption for the transmission of *A. pleuropneumoniae*. To investigate this, the infection rate parameter β was estimated from the experimental data.

For this estimation the following statistics of each period between subsequent samplings were computed: the number of susceptible pigs at the start and at the end of each period, S_1 and S_2, the average number of infectious pigs I_2 and the total number of pigs present N. The number of cases C is defined as the average number of pigs that became infected per day during that period. We assumed that the new cases in each period were caused by the infectious pigs of the previous period I_1. C is binomially distributed and every susceptible pig at the start of each period has a probability q to become infected, where q is a function of β, I_1 and N:

$$\Pr ob[C|S_1,q] = Bin(S_1,q), \text{ where } q = 1 - e^{-\beta \frac{I_1}{N}}.$$

The above mentioned statistics are sufficient to estimate the infection rate parameter β using a generalised linear model (GLM) with a complementary-log-log link, a binomial error term, and *ln(I$_1$/N)* as the offset (Mccullagh and Nelder 1989).

To determine whether there is variation in individual infectivity, the relation between *C* and the number of bacteria isolated from the swabs was tested by expanding the model with other variables. These variables indicated the number of bacteria isolated in that period and were supposed to be indicative for the level of the infectivity present in the population. They describe the fraction of I_1-pigs that had either no colonies or more than x colonies isolated from the nasal or tonsillar swab as an average of the two samplings at the start and end of each period. The minimal number of the isolated colonies *x* was 1, 10, 20, 40, and 100 for the different variables. For example variable H_{10} is the fraction of I_1-pigs from which on average of two subsequent samples more than ten colonies *A. pleuropneumoniae* were isolated from the nasal swab.

To be sure that the effect of each variable would independently be estimated from the constant, the variables were made orthogonal to the constant. We fitted all variables individually in a univariate model, and a variable was added to the model if significantly more variation in *C* was explained than in the model without this variable. In the next step we expanded the model with another variable to see if even more variation in *C* could be explained.

The infection rate parameter β was calculated with the estimates of the variables in the fitted model according to the following formula:

$$\beta = e^{b_0 + \sum_1^i b_i \cdot x_i}$$

The lower and upper limit of the 95% confidence interval for the estimated β was calculated with the estimates and the accompanying covariance matrix.

3. RESULTS

In Table 2.1 an overview of the results of the ten transmission trials is shown. It is clear that the observed transmission is highly variable when looking at the results of the bacteriological examination of the specimens of the C_1-pigs.

Table 2.1. Overview of the results of the ten transmission trials

		Number of pigs		A. pleuropneumoniae isolated from						HN-positive	
Trial	I/C[1]	Total	Found death	Tonsil	Lesion[2]	T_1[3]	T_{10}[4]	N_1[5]	N_{10}[6]	Start[7]	End[8]
1	I_n	5	0	5	1	5	4	4	3	0	$0_{(28)}$
	C_1	5	0	5	0	5	3	3	2	0	$0_{(28)}$
2	I_n	5	0	5	0	5	4	2	2	0	$0_{(28)}$
	C_1	5	0	5	0	5	4	4	3	0	$0_{(28)}$
3	I_n	5	0	1	2	2	2	4	2	0	$5_{(21)}$
	C_1	5	0	0	1	2	1	4	3	0	$5_{(21)}$
4	I_n	3	2	3	3	2	2	2	2	0	$1_{(28)}$
	C_1	4	0	4	0	4	4	4	4	0	$3_{(28)}$
5	I_n	5	0	1	0	0	0	1	1	0	$3_{(35)}$
	C_1	5	0	0	0	0	0	0	0	0	$0_{(35)}$
6	I_n	5	0	5	4	5	5	5	4	0	$4_{(14)}$
	C_1	5	0	5	0	5	5	5	2	0	$5_{(35)}$
	C_2	5	0	1	0	0	0	0	0	0	$0_{(35)}$
7	I_n	5	0	0	0	4	4	1	1	0	$4_{(35)}$
	C_1	5	0	0	0	0	0	0	0	0	$2_{(35)}$
8	I_n	5	0	4	1	4	4	2	2	0	$1_{(14)}$
	C_1	5	0	5	1	5	5	4	3	0	$5_{(28)}$
	C_2	5	0	5	0	5	5	4	3	0	$5_{(35)}$
	C_3	5	0	5	0	5	5	0	0	0	$3_{(35)}$
9	I_b	5	3	3	5	4	3	3	3	0	$0_{(4)}$
	C_1	5	0	3	0	4	3	3	1	0	$3_{(35)}$
	C_2	5	0	0	0	0	0	0	0	0	$0_{(35}$
10	I_b	5	3	5	5	5	5	2	2	0	$0_{(4)}$
	C_1	5	0	5	0	5	5	5	3	0	$5_{(35)}$
	C_2	5	0	5	2	5	5	5	4	0	$5_{(35)}$

[1] intranasally or endobronchially inoculated (I_n or I_b) or first, second or third contact pig (C_1, C_2 or C_3)
[2] lung lesion
[3] ≥ 1 colony isolated from a tonsillar swab
[4] ≥ 10 colonies isolated from a tonsillar swab
[5] ≥ 1 colony isolated from a nasal swab
[6] ≥ 10 colonies isolated from a nasal swab
[7] Number of pigs with raised titre (with respect to 1/8) at the start of the trial (day 0)
[8] End of the trial: between brackets the day number

3.1. Variation in transmission between trials

We assumed that the transmission mechanism of *A. pleuropneumoniae* is described by the stochastic general SIR model of Becker (1989). Which means that a typical susceptible pig will become infectious for other pigs after a typical infectious contact, and that a typical infectious pig can be removed from the population due to recovery or to death. Whether a pig recovers from an *A. pleuropneumoniae* infection and stops being infectious is not known, but we assume that in the time frame of the experiments a pig stays infectious until the end of the experiment.

In contrast to what was expected from the general SIR model, the observed transmission between the inoculated and the contact pigs in the ten *A. pleuropneumoniae* transmission trials appeared to be highly variable. There were two trials with no contact infection at all, six trials where all C_1-pigs got infected, and two trials where some but not all C_1-pigs seemed infected (Table 2.1).

The variability in transmission observed between the trials was tested, using test statistic V. This test statistic was estimated to be 29.3, and the probability of having $V=29.3$ or even more extreme outcomes was 0.014. Thus, the null-hypothesis was rejected which means that or the basic reproduction ratio R_0 was different in the different trials or that the general SIR model is not the right model to describe the transmission mechanism of *A. pleuropneumoniae*.

3.2. Indicators for infectious pigs

Data of the inoculated pigs were used to investigate which variable or combination of variables indicate whether a pig is infectious or not, since those animals were most likely to become infectious. Only the data of the intranasally inoculated pigs were included to exclude extra variation due to another inoculation route.

A whole range of measurements of the individual pigs, collected during the experimental period, was available. Because, it is very likely that a single measurement is not indicative for infectious animals, new variables were created which are possibly more indicative. These variables are listed in Table 2.2.

A variable that is indicative for the infectivity of animals, should be related to the level of transmission that took place within the experimental population, since the level of transmission and the number of infectious animals present are assumed to be directly related. Also, possible indicator variables should have enough variation to explain the variation in the level of transmission. To examine which combination of the variables explains a lot of the variation in the data, we did a principal component analysis (PCA).

The first principal component accounted for 42% of the total variance in the data (Table 2). The variables in this principal component are ranked in order of the absolute size of their loading. The ones with the highest absolute size of the loading caused most of the variation in the data. The first four variables were (i) having an *A. pleuropneumoniae* positive tonsil at necropsy, (ii) the number of nasal swabs with *A. pleuropneumoniae*, (iii) the percentage of nasal swabs with *A. pleuropneumoniae*, and (iv) the successive number of nasal swabs with *A. pleuropneumoniae* (Table 2.2).

The next step was to determine whether these variables could explain the variable transmission that was observed. This was done by ranking the 38 intranasally inoculated

animals, using the above four variables. The ranking of the animals appeared to correspond to the amount of transmission observed in the trials where they were in. In fact, the variable with the highest loading alone was enough to explain the different levels of transmission. This indicated that a pig was infectious for *A. pleuropneumoniae* if the bacterium was isolated from its tonsils at necropsy.

Table 2.2. The variables, the descriptions and their loadings in the first principal component.

Variable	Description	Loading[1]
Tonsil	Having an *A. pleuropneumoniae* positive tonsil at necropsy	-0.468
NS-#	Number of A pleuropneumoniae positive nasal swabs	-0.458
NS-%	Percentage of *A. pleuropneumoniae* positive nasal swabs	-0.396
NS-succ	Successive number of *A. pleuropneumoniae* positive nasal swabs	-0.347
NS-per	Length of period with successive *A. pleuropneumoniae* positive nasal swabs	-0.251
N-ln	Nasal tonsil with *A. pleuropneumoniae* at necropsy	0.195
TS-%	Percentage of *A. pleuropneumoniae* positive tonsillar swabs	-0.171
Lesion	Lung lesion with *A. pleuropneumoniae* at necropsy	-0.093
TS-#	Number of A pleuropneumoniae positive tonsillar swabs	-0.079
Temp-max	Maximal body temperature	0.074
Temp-avg	Average body temperature	-0.070
HN-max	Maximal titre found in the HN-test	-0.069
Abscess	Number of abscesses found in the lung at necropsy	-0.041
TS-period	Length of period with successive *A. pleuropneumoniae* positive tonsillar swabs	-0.030
TS-succ	Successive number of *A. pleuropneumoniae* positive tonsillar swabs	-0.020
Br-ln	Bronchial lymph nodes with *A. pleuropneumoniae* at necropsy	0.000

[1] The loading of each variable in a principal component has a value between –1 and 1. The set of variables with the highest absolute value of the loading explains the most variance in the original data.

3.3. Efficacy of intranasal inoculation

Now that we have determined which pigs were infectious due to *A. pleuropneumoniae* in the trials, it was possible to evaluate the effectiveness of the intranasal inoculation route. The effectiveness of the endobronchial inoculation could not be evaluated because we had not enough data.

Assuming that the number of successful inoculations is binomially distributed, it was possible to estimate the probability of becoming infectious due to intranasal inoculation in two different ways. The first estimate p_1 was determined by calculating the proportion of intranasally inoculated pigs from which *A. pleuropneumoniae* was isolated from their tonsils at necropsy, which is 24/38=0.63. The second estimate p_2 was determined by backward calculation. In one of seven similar trials, in which five animals were intranasally inoculated,

none of the inoculated pigs became infectious (exp. 7, Table 2.1). Therefore, a good estimate for the probability that none of five inoculations were successful is 1/7=0.143. The number of successful inoculations is binomially distributed and knowing that the probability of having zero successes out of five inoculations is 0.143, p_2 was estimated to be 0.32.

The plausibility of the estimates was tested as follows. With estimates, p_1 and p_2, we calculated the expected frequency of the seven trials over the number of successful inoculations. These are plotted in Figure 2.3 together with the actual observed frequency. The estimated frequencies differed very much from the observed frequency, especially for the number of trials in which many pigs became infectious. A biological explanation for this may be that not all inoculated pigs became infectious due to the inoculation but got infected due to infectious contacts. Considering this, the second estimate p_2 is more reliable than the first estimate p_1. Because in the estimation of p_1 all inoculated pigs with a *A. pleuropneumoniae* positive tonsil were regarded as being infectious due to inoculations, but some of them may have become infectious due to contact infections. So, p_2 is a better estimate for the probability of having a successful intranasal inoculation than p_1.
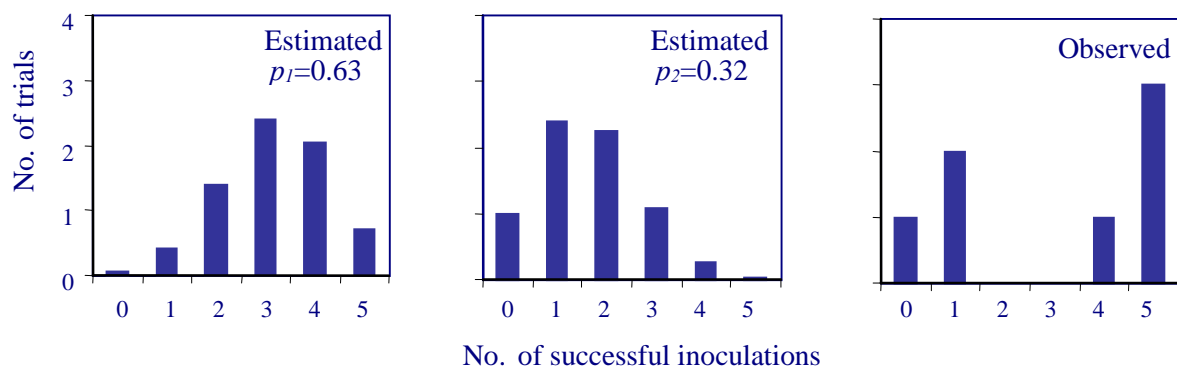

Figure 2.3.   The estimated and observed frequencies of seven trials over the number of successful intranasal inoculations *x*, when executing five intranasal inoculations per trial. The left panel shows the estimated frequency that was based on the success probability p_1, the middle panel gives the frequency that was based on p_2, and the right panel represents the observed frequency of trials over the number of successful intranasal inoculations.

3.4. Variation in individual infectivity

The variation in infectivity can be caused by assuming a wrong number of infectious animals present at the start of the trial and by variation in individual infectivity. The first

explanation was supported by the conclusion that the intranasal inoculation was less successful than expected and hence the number of infectious pigs at the start of each trial was smaller than the number of inoculated pigs.

To investigate whether there exists variability in individual infectivity, a GLM was carried out. For this analysis the number of susceptible and infectious animals on each sampling day had to be determined. This was possible, because we knew that infectious pigs had *A. pleuropneumoniae* positive tonsils at necropsy. Furthermore we assumed that an infectious animal became infectious in the period before the first sampling day where at least one colony was isolated from a swab. Until then the animal was called susceptible.

First only the constant b_0 was fitted on the data and was estimated to be –1.7974. To check if β is higher in periods where more bacteria were isolated, the relation between created variables and the number of cases was investigated. Variable H_{10}, the fraction of infectious pigs from which more than ten colonies were isolated from the nasal swab, gave the highest reduction in deviance (10.8) in the univariate model compared to the others and was added to the model. In the next step of model building only the variables related to the tonsillar swab were added to the model individually, but none of those expanded models could explain significantly more variation of C than the model with only the constant and variable H_{10}. The estimated constant (b_0) was –1.95 with standard error 0.24 and the estimate for H_{10} (b_1) was 2.36 with standard error 0.63. Thus, the relation between β and the fraction infectious pigs with more than ten *A. pleuropneumoniae* colonies isolated from the nasal swab can be expressed as follows:

$$\beta = e^{-1.95+2.36 \cdot H_{10}}$$

So, the infection rate parameters β increases with the fraction of infectious pigs in which on average more than ten *A. pleuropneumoniae* colonies were isolated from two subsequent samplings. When this fraction is zero, β equals 0.15 with confidence interval (0.00; 0.61), and when this fraction equals one β equals 1.54 with confidence interval (1.04; 1.98). In other words, an infectious pig is about ten times more infectious on days were more than ten colonies were isolated from the nasal swab than on the other days.

4. DISCUSSION

Ten transmission trials with *A. pleuropneumoniae* were carried out to study the transmission of this bacterium in pigs. These trials had a similar design, but the observed variation in transmission was very high. The general SIR model could not explain the

variation in transmission, but when the model was adjusted by taking variation in infectivity in the population into account the variation in transmission could be explained. It is possible to measure the transmission of *A. pleuropneumoniae* under experimental conditions when using models incorporating variation in infectivity. Consequently, the effect of control measures on the transmission can be tested.

The goal of this study was to test if variation in infectivity could explain the variation in transmission observed in the ten trials, and to find measures that are indicative for infectivity. To be able to count the number of infectious pigs, one should be able to distinguish infectious pigs from non-infectious ones. A conclusion of this paper is that if a pig carries *A. pleuropneumoniae* in its tonsils at necropsy it was infectious for *A. pleuropneumoniae* during the trial. It was quite surprising that an eventual carrier status in the tonsils was enough to explain the main variability in transmission between the transmission trials. In other studies about bacterial infections an animal is often called infected if the bacteria was isolated from more than two successive samples, e.g. *Staphylococcus aureus* intramammary infections (Lam *et al.* 1996). If this definition was applied to the tonsillar swabs taken in the trials a lot more pigs would be called infectious than there actually were. If this definition was applied to the nasals swabs taken in the trials a lot less pigs were called infectious than there actually were. Thus, having *A. pleuropneumoniae* positive tonsils is a better indicator for infectious pigs than successive swabs positive for *A. pleuropneumoniae*. Unfortunately the status of the tonsils can only be measured at necropsy or maybe earlier when taking tonsil biopsies, which was not tested in this study.

The importance of the colonisation of *A. pleuropneumoniae* in the tonsils is also mentioned in studies from Nicolet (1992), Møller *et al.* (1993) and Chiers *et al.* (1999). Møller *et al.* (1993) compared the results of bacteriological examination of tonsils and nasal swabs for *A. pleuropneumoniae* with serological (complement fixation test) and pathological findings, measured in 303 slaughterhouse pigs. In accordance with our study, the tonsil was most often positive for *A. pleuropneumoniae* (42%) followed by a positive test in CFT (20%) and a positive nasal swab (1.3%).

Detecting *A. pleuropneumoniae* in the tonsil via bacteriological culturing could improve the diagnosis of infectious pigs, but according to Gram *et al.* (1996) this method is not as sensitive as using PCR. A high sensitivity is supposed to be an important feature of a diagnostic test, but an even more important feature is to know at which level of detection the infection can really maintain it self or multiply in the animal. For example a PCR is very sensitive in detecting a few bacteria (dead or alive), but this does not automatically mean that the bacterium did really colonised in the animal or even more important that the animal was infectious for other pigs.

An *A. pleuropneumoniae* positive tonsil appeared to be related to being infectious. It would be interesting to know if this relation is causal. If so, it should be considered that colonisation of *A. pleuropneumoniae* in the tonsils and also excretion of *A. pleuropneumoniae* from the tonsils is very important for the transmission of this agent. Maybe the focus of future treatments to prevent transmission should be upon the interaction between *A. pleuropneumoniae* and the tonsils. Little is known about this interaction. Chiers *et al.* (1999) looked at early interactions of *A. pleuropneumoniae* with the tonsils of 1-week-old gnotobiotic pigs that were inoculated onto their tonsils. He showed that in a few hours the bacteria attached on the tonsillar epithelial cells and that within 24 h after inoculation the bacteria were found closely associated to the crypt-walls together with detached cells in the crypts. He concluded that attachment of *A. pleuropneumoniae* to tonsillar epithelial cells was probably the first step in establishing bacteria in this body site.

Even, if the correlation between a positive *A. pleuropneumoniae* tonsil at necropsy and the transmission does not have biological relevance, the detection of *A. pleuropneumoniae* in the tonsils could be a good diagnostic tool to detect carrier pigs within a population. Savoye *et al.* (2000) developed a PCR test that could be applied directly on samples of tonsil biopsies and tracheobronchial lavage fluids, without a culture step. He concluded that this was a good tool to detect healthy carrier pigs, but further validation of his PCR assay is still in progress. Further research to optimise the sampling strategy of the tonsils of pigs when still alive and to detect *A. pleuropneumoniae* from this sample is desirable.

We expected that all inoculated animals would become infectious due to the inoculation in order to start an infection chain. In contrast, it was concluded in this study was that intranasal inoculation with 10^4 CFU *A. pleuropneumoniae* does not always lead to infectious pigs. The probability of becoming infectious due to this inoculation that was estimated to be 0.32.

There are several reasons why the intranasal inoculation is probably not a good inoculation strategy to induce infectious animals. (i) The inoculum is dripped into the nostrils in the hope that the whole dose enters the respiratory tract. This is not certain, since the pig can swallow (a part of) the inoculum. (ii) The inoculum is only given once, which is probably unnatural. We believe that repeated inoculation might lead to more infectious animals. (iii) The bacteria within the inoculum was an early passage of a field isolate. To prepare the inoculum it was at least three times cultivated on blood agar, which is the minimal number of passages to culture a standard dose inoculum. The bacteria could have adjusted to the circumstances of the laboratory conditions and were maybe not fit enough to achieve an optimal colonisation within the pig.

Thus, when using an inoculation method in an experiment, one should be aware that inoculation not always leads to infectious animals, and on the other hand when inoculating with a very high dose or another route the individual infectivity could not be comparable with that in the field situation. The reliability of the results of transmission experiments can depend for a great deal on the inoculation method used. For the ultimate design of our *A. pleuropneumoniae* transmission experiment, we choose to use endobronchial inoculation with 10^3 CFU to induce infectious pigs. Unfortunately this inoculation method leads very often to diseased pigs, but the inoculated pigs are able to pass the infection on to the contact pigs, which seem to stay healthy. To be able to measure transmission between healthy pigs, the experiment is extended by replacing the group of inoculated pigs with a new group of contact pigs at the moment when the first group of contact pigs excretes enough *A. pleuropneumoniae* (Velthuis and De Jong 2001).

A further conclusion of this study was that the infection rate parameter is related to the fraction of infectious animals from which at least ten colonies from the nasal swab were isolated. When this fraction is zero, there is still a basic infectivity present in that group. On days where the fraction is above zero, the infectivity will increase with this fraction. It was quite surprising that the nasal swab is related to the infection rate parameter and the tonsillar swab not. Much more bacteria were isolated from the tonsillar swab and for a longer successive period than from the nasal swab.

In a lot of studies, nasal swabs were taken in order to detect *A. pleuropneumoniae* in an animal. Møller *et al.* (1993) compared the results of bacteriological examination of *A. pleuropneumoniae* from the tonsils with nasal swabs, measured in 303 slaughterhouse pigs. The tonsils were more often positive than the nasal swab (42% and 1.3% respectively). Wongnarkpet *et al.* (1999) found that about 14% of nasal swabs taken from 256 pigs of an endemic infected herd were found positive for *A. pleuropneumoniae*. The isolation rate peaked within the age class of 11 weeks. Willson *et al.* (1987) swabbed the nasal cavity of six groups of 20 pigs of an endemic infected herd that were 3, 6, 9, 12, 15 and 25 weeks of age. The isolation rate peaked at 12 weeks of age (30%), and by the age of 15 and 25 weeks *A. pleuropneumoniae* was no longer isolated from the nasal cavity. He suggested that shedding of the bacteria in nasal secretions take place only at the time of active infection. This agrees with the conclusion of this study but reflects probably not only the moment of active infection but also the status of individual infectivity of an animal. Kume *et al.* (1984) showed with taking nasal swabs of 619 healthy pigs that about 47.3% of those swabs were positive for *A. pleuropneumoniae*. This means within the framework of this study that this high proportion of pigs in the study of Kume *et al.* (1984) was highly infectious for *A. pleuropneumoniae*.

In this study it was shown that the tonsil is a good sampling site to diagnose infectious pigs for *A. pleuropneumoniae*. When from these infectious pigs more than ten colonies were isolated from the nasal swab these pigs are highly infectious to others. But pigs from which less than ten colonies were isolated from the nasal swab are still infectious, although on a low level.

In conclusion it can be stated that the variable transmission observed in the ten transmission trials could be explained by variability in infectivity present in the populations. It is possible to handle bacterial transmission in an experimental setting when taking this variation in infectivity into account, so that the effect of a control measure on the transmission can be tested.

5. ACKNOWLEDGEMENTS

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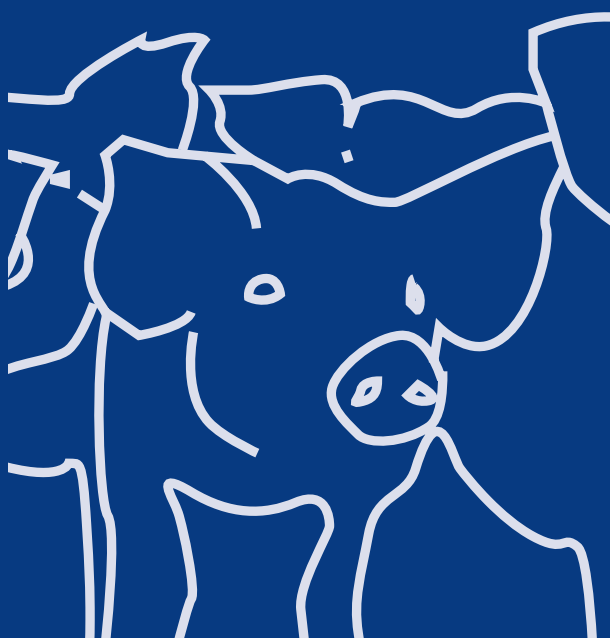
Quantification of transmission in one-to-one experiments

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SUMMARY

We study the statistical inference from data on transmission obtained from one-to-one experiments, and compare two algorithms by which the reproduction ratio can be quantified. The first algorithm, the transient state (TS) algorithm, takes the time course of the epidemic into account. The second algorithm, the final size (FS) algorithm, does not take time into account but is based on the assumptions that the epidemic process has ended before the experiment is stopped. The FS algorithm is a limiting case of the TS algorithm for the situation where time tends to infinity. So far quantification of transmission has relied almost exclusively on the FS algorithm, even if the TS algorithm would have been more appropriate. Its practical use, however, is limited to experiments with only a few animals. Here, we quantify the error made when the FS algorithm is applied to data of one-to-one experiments not having reached the final size. We conclude that given the chosen tests, the FS algorithm underestimates the reproduction ratio R_0, is liberal when testing $H_0 : R_0 \geq 1$ against $H_1 : R_0 < 1$, is conservative when testing $H_0 : R_0 \leq 1$ against $H_1 : R_0 > 1$ and calculates the same probability as the TS algorithm when testing $H_0 : R_{0-control} = R_{0-treatment}$ against $H_1 : R_{0-control} > R_{0-treatment}$. We show how the power of the test depends on the duration of the experiments and on the number of replicates. The methods are illustrated by an application to porcine reproductive and respiratory syndrome virus.

1. INTRODUCTION

Laboratory experiments are an important tool in the epidemiology of infectious diseases to estimate transmission parameters and to determine the effect of an intervention on transmission. A transmission experiment consists of a number of trials. In each transmission trial a number of infectious and susceptible animals are housed together and sampled regularly to monitor the epidemic process. An advantage of transmission experiments over field studies is that they offer a controlled environment in which the influence of a single factor on the transmission can be investigated, while minimising variation caused by other factors. This implies that more insight can be obtained into causative mechanisms underlying the transmission dynamics of the pathogen. Furthermore, transmission experiments are usually less expensive and less time-consuming than field studies, and make it possible to evaluate intervention measures that are not yet implemented or realisable in the field.

Data from transmission experiments may serve to construct and fit an epidemiological model. Once accepted as being appropriate, such a model can be used to estimate certain biologically interpretable parameters, and to test hypotheses. A model that is often used in the epidemiology is the SIR model, in which individuals are either susceptible, infectious, or recovered (Kermack and Mckendrick 1927; Bailey 1975). An interesting transmission parameter of the SIR model is the reproduction ratio (R_0) that is defined as the average number of secondary infections that would be caused by one infectious individual during its infectious period in a large population of susceptible individuals. If R_0 exceeds 1 the pathogen can spread and may cause a major outbreak, while if R_0 is smaller than 1 the pathogen cannot spread or it will at most produce a minor outbreak.

Transmission experiments have already proved to be useful in studies on viral pathogens such as pseudorabies virus (De Jong and Kimman 1994; Bouma *et al.* 1997a), classical swine fever virus (Bouma *et al.* 2000), porcine reproductive and respiratory syndrome virus (Nodelijk *et al.* 2001) and bovine herpes virus (Mars *et al.* 2000b). The main aim of these studies was to quantify the effect of interventions like vaccination on R_0 using the traditional final size (FS) algorithm. The observed data in these experiments were the 'final sizes' of the local epidemics, i.e., the total number of individuals ultimately infected in the experiment. Thus, it was assumed that either no infectious individuals or no susceptible individuals were left at the end of the transmission trial, so that the epidemic process had ended before the trial was stopped.

For some pathogens the final size approach may be feasible, but for others it may not. Consider, for instance, the bacterial pathogen *Actinobacillus pleuropneumoniae* in pigs. The length of the infectious period induced by this pathogen is unknown, and its excretion pattern varies widely between individual pigs (Velthuis *et al.* Accepted), making it difficult to

determine whether the epidemic process has ended when the transmission trial is stopped. For those pathogens it would be better to use an estimation method that does not rely on a final size situation.

An algorithm for the calculation of state probabilities that is not based on the final size assumption is available from the stochastic SIR model (Bartlett 1949). In this paper we will call it the 'transient state' (TS) algorithm. The TS algorithm takes the time course of the experimental epidemic into account with no need for a final size situation. Although an explicit solution for any population size is theoretically available from the TS algorithm, its practical use is restricted to experiments with few individuals. This is because its high degree of recursiveness may cause numerical problems, memory limitation or long computation time (Bailey 1975; Billard and Zhao 1993; Daley and Gani 1999). The high degree of recursiveness in the TS algorithm disappears if time tends to infinity, turning the TS algorithm into the readily applicable FS algorithm.

As long as the TS algorithm cannot be used for experiments with larger numbers of individuals the FS algorithm will have to be used, even if the final size has not been reached. In this paper we investigate the error made when the FS algorithm is applied to experiments where a final size situation has not been reached. We focus on what we call one-to-one transmission experiments. One such experiment will consist of replicated one-to-one trials in which a single infectious individual is housed with a single susceptible individual.

There are several reasons for preferring one-to-one experiments over experiments with more individuals. From a mathematical point of view, there is the advantage that a full analytic solution of the TS algorithm is within reach, and that the estimation methods can be based on binomial distributions, so that standard methods of estimation and testing are available. From a biological point of view, one-to-one trials have the advantage that there is no doubt as to which individual infected which other individual, and co-infection can be excluded. Furthermore, it is possible to estimate the probability of infection from one-to-one experiments without assuming an underlying model, so that the estimated parameter is robust. Therefore, one-to-one experiments are most appropriate compared to bigger experiments if the aim is to estimate R_0 or to test the effect of an intervention on R_0, knowing that R_0 in both treatment groups is higher than the threshold value 1 (Nodelijk *et al.* 2001).

The outline of the paper is as follows: (i) the stochastic SIR model is described briefly; (ii) an explicit solution for a single one-to-one trial is obtained; (iii) these solutions are converted to a binary outcome; (iv) the statistical inference with both algorithms is investigated; and (v) the error made when using the FS algorithm instead of the TS algorithm is investigated. This error is investigated on three topics: estimating R_0 and the corresponding confidence interval; testing the size of R_0 in relation to its threshold value 1; and testing the

reduction of R_0 due to an intervention. To illustrate the results we have added an example of a particular one-to-one experiment on porcine reproductive and respiratory syndrome virus (PRRSV) among pigs.

2. STOCHASTIC SIR MODEL

The stochastic SIR model, also called the General Stochastic Epidemic, was proposed by Bartlett (Bartlett 1949) and has been the subject of analysis by others (Bailey 1975; Billard and Zhao 1993; Daley and Gani 1999). In this model individuals are susceptible, infectious or recovered. Let $S(t)$ be the number of susceptible individuals at time t, and let $I(t)$ be the number of infectious individuals at time t. The total population size is constant, i.e., $N(t)=N$, so that the number of recovered individuals $R(t)$ at time t is given by $R(t)=N-S(t)-I(t)$. Hence, the population state at time t is denoted by the pair $(S(t),I(t))$, and a particular realisation by $(s(t),i(t))$ or simply (s,i).

Given that the population state is (s,i) at time t, it will be in state $(s-1,i+1)$ at some later moment if a susceptible individual becomes infectious upon an infection event. It will be in state $(s,i-1)$ if a infectious individual becomes immune upon a recovery event. The rate at which infection events occur is proportional to the number of susceptible individuals, the proportion of infectious individuals present, and the infection parameter β. This assumption is commonly referred to as the 'mass-action' assumption (De Jong et al. 1995). The rate at which recovery events occur is proportional to the number of infectious individuals and the recovery parameter α. Assuming that recovery events occur independently then the mean infectious period is given by $1/\alpha$.

Given the above assumptions, the dynamics of the model are governed by a Markov process. The one-step transition probabilities in a small time interval Δt are given by:

$$\Pr\{(S(t+\Delta t),I(t+\Delta t))=(s-1,i+1)\,|\,(S(t),I(t))=(s,i)\}=\beta\frac{s\,i}{N}\Delta t+o(\Delta t)$$

$$\Pr\{(S(t+\Delta t),I(t+\Delta t))=(s,i-1)\,|\,(S(t),I(t))=(s,i)\}=\alpha\,i\,\Delta t+o(\Delta t) \qquad (1)$$

$$\Pr\{(S(t+\Delta t),I(t+\Delta t))=(s,i)\,|\,(S(t),I(t))=(s,i)\}=1-(\beta\frac{si}{N}+\alpha\,i)\,\Delta t+o(\Delta t),$$

where $o(\Delta t)\to 0$ when $\Delta t\to 0$. Denoting the initial state of the process by (s_0,i_0), the state probabilities can be written as:

$$p_{s,i}(t)=\Pr\{(S(t),I(t))=(s,i)\,|\,(S(0),I(0))=(s_0,i_0)\} \qquad (2)$$

After rescaling time to units of the mean infectious period $1/\alpha$, the adjacent state probabilities satisfy the forward differential-difference equations:

$$\frac{d}{dt} p_{s,i}(t) = (i+1)p_{s,i+1}(t) + \left(R_0 \frac{(s+1)(i-1)}{N} \right) p_{s+1,i-1}(t) - \left(R_0 \frac{si}{N} + i \right) p_{s,i}(t) \, , \tag{3}$$

where

$$R_0 = \frac{\beta}{\alpha}$$

for $0 \le s+i \le s_0 + i_0$, $0 \le s \le s_0$, and hence $0 \le i \le s_0 + i_0$. Subject to the initial value $p_{s_0,i_0}(0) = 1$ this equation can be solved using standard methods. The solution that we call the transient state (TS) algorithm can be used to calculate a continuous-time state probability for each state in the epidemic process.

Despite the fact that the solution of equation (3) is formally available, an exact calculation of the continuous-time state probabilities for all states in the epidemic process is very laborious for all but the simplest cases. Attempts to find useful explicit solutions for the stochastic SIR model have been made (Gani 1965; Siskind 1965; Severo 1967; Severo 1969; Billard 1973), but calculation of the state probabilities is still recursive, and involves a considerable number of multiple summations and products.

The probability distribution used in the TS algorithm is given by the set of all five time-dependent state probabilities (Figure 3.1). The probability distribution of the FS algorithm is the limiting case of the TS algorithm where time tends to infinity. Infact, as $t \to \infty$ all state probabilities where $i \neq 0$ tend towards zero, so that the limiting probabilities of the states where $i=0$ approach the final size distribution of the experimental epidemic.

When using methods based on the SIR model to quantify R_0, one should remember that the results also depend on the assumptions underlying the model. Some of the assumptions are: all animals within the population have random contacts with each other; every class S, I and R consists of a homogeneous group of individuals; the infection rate is constant during the entire infectious period; the duration of the infectious period is exponentially distributed; and each recovered animal is fully immune towards infection. Thus, application of both the TS and the FS algorithm requires these assumptions to be checked carefully.
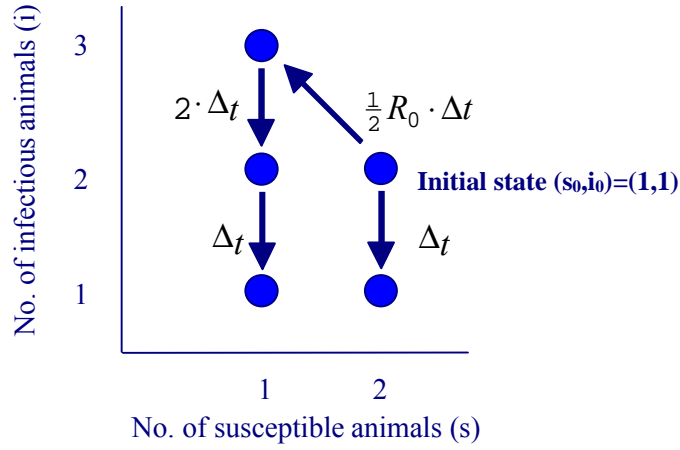
Figure 3.1. A schematic structure of the epidemic process in a one-to-one trial. Each state is given by the number of susceptible and infectious individuals. In the long run, the population ends up in one of the absorbing states (0,0) or (1,0). The transition rates are given next to the arrows, where time is scaled in units of infectious periods.

3. ONE-TO-ONE TRIAL

In a one-to-one trial a single infectious individual is housed together with a single susceptible individual. Hence, $s_0=1$, $i_0=1$ and $N=2$, at $t=0$. The following states are distinguished: *(1,1)*, *(1,0)*, *(0,2)*, *(0,1)* and *(0,0)*. In the long run the pair will always end up in one of the absorbing states *(1,0)* or *(0,0)* (Figure 3.1). The probability distribution belonging to the TS algorithm is:

$$p_{1,1}(t) = e^{-\frac{R_0+2}{2}t}$$

$$p_{1,0}(t) = \frac{2}{R_0+2}\left(1 - e^{-\frac{R_0+2}{2}t}\right)$$

$$p_{0,2}(t) = \frac{R_0}{R_0-2}\left(e^{-2t} - e^{-\frac{R_0+2}{2}t}\right) \tag{4}$$

$$p_{0,1}(t) = -2\frac{R_0}{R_0-2}e^{-2t} + \frac{4}{R_0-2}e^{-\frac{R_0+2}{2}t} + 2e^{-t}$$

$$p_{0,0}(t) = \frac{R_0}{R_0-2}e^{-2t} - \frac{8}{R_0^2-4}e^{-\frac{R_0+2}{2}t} - 2e^{-t} + \frac{R_0}{R_0+2}$$

if $R_0 \neq 2$. If $R_0=2$ the state probabilities for *(0,2)*, *(0,1)* and *(0,0)* are given by: $p_{0,2}(t) = te^{-2t}$, $p_{0,1}(t) = -2e^{-2t}\left(1 + t - e^t\right)$ and $p_{0,0}(t) = e^{-2t}\left(\frac{3}{2} + t\right) - 2e^{-t} + \frac{1}{2}$. Note that the probability distribution for the FS algorithm is a limiting case of the TS algorithm, and is given by the state probabilities of the two absorbing states:

$$p_{1,0} = \lim_{t \to \infty} p_{1,0}(t) = \frac{2}{R_0 + 2} \text{ and } p_{0,0} = \lim_{t \to \infty} p_{0,0}(t) = \frac{R_0}{R_0 + 2} , \tag{5}$$

while all state probabilities of the transient states are zero: $p_{1,1} = p_{0,2} = p_{0,1} = 0$.

Figure 3.2 shows an example of the dynamics of the model (4) where $R_0=3$. The probability of being in the initial state *(1,1)* equals one at $t=0$, and decreases in time. The state probabilities of the intermediate states *(0,2)* and *(0,1)* initially increase with time, and thereafter decrease asymptotically to zero. The state probabilities of the absorbing states *(0,0)* and *(1,0)* increase asymptotically to a non-zero value when t tends to infinity.
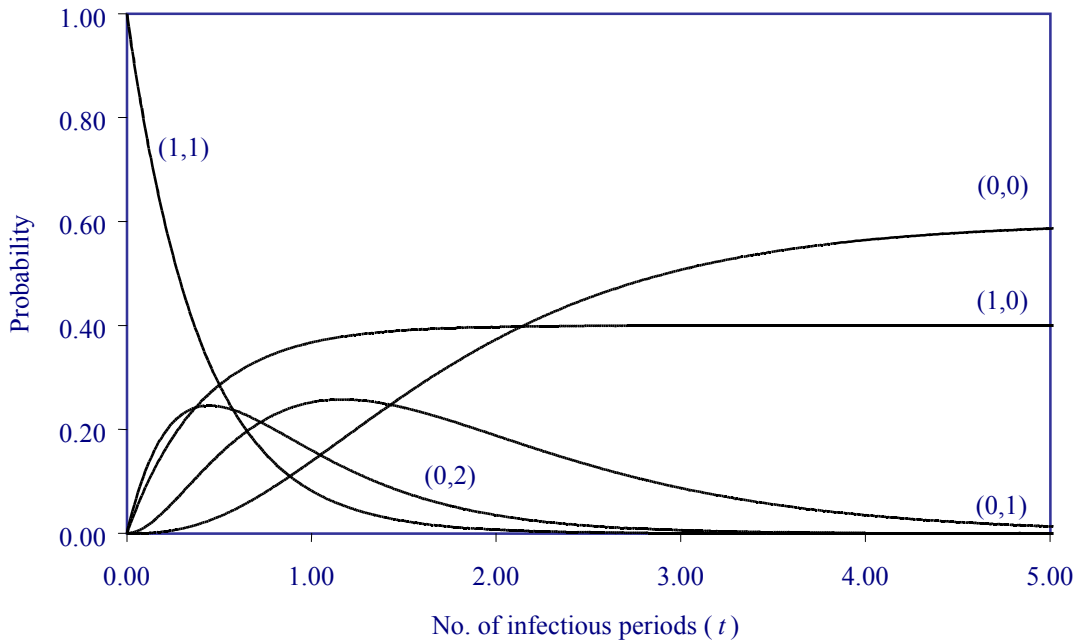

Figure 3.2.   The state probabilities of the five states in a one-to-one trial as a function of time. In this particular example $R_0=3$.

From the explicit solution, some interesting quantities can be determined, e.g., the mean time spent in state *(s,i)* and its variance. Assumptions (1) and the Markov property of the chain imply straightforwardly that the time spent in the transient states *(1,1)*, *(0,2)* and *(0,1)* are independently exponentially distributed with probability densities $\left(\frac{1}{2}R_0 + 1\right)e^{-\left(\frac{1}{2}R_0 + 1\right)t}$, $2e^{-2t}$ and e^{-t}, respectively (Cox and Miller 1965). Hence, the respective sojourn times have means $2/R_0 + 2$, $1/2$ and 1 with variances $(2/R_0 + 2)^2$, $1/4$ and 1, respectively.

The mean time before absorption takes place and its variance can be calculated as follows. There are two possible routes towards absorption, the first directly from *(1,1)* to *(1,0)*, and the second from *(1,1)* via *(0,2)* and *(0,1)* to *(0,0)*. The probability of the first route is equal to the probability that the waiting time for transition to *(1,0)* is less than the waiting time for transition to state *(0,2)*. Hence this probability is $2/R_0 + 2$, and the probability of the second route is $R_0/R_0 + 2$. Now let $T_{s,i}$ denote the time spent in state *(s,i)*, and $\delta = 0$ if the first route is followed, and $\delta = 1$ if the second route is followed. Then, for the time to absorption *Z* we have:

$$Z = T_{1,1} + \delta\left(T_{0,2} + T_{0,1}\right). \tag{6}$$

Calculation of the mean and variance of *Z* using the above formulations is straightforward:

$$E(Z) = \frac{4 + 3R_0}{2(R_0 + 2)}, \tag{7}$$

and

$$Var(Z) = \frac{R_0(4 + 5R_0)}{4(R_0 + 2)^2}. \tag{8}$$

The mean time to absorption of a highly infectious pathogen, $R_0 \rightarrow \infty$, is 1.5 infectious periods. Based on (7) and (8) 95% of the one-to-one trials will have reached an absorbing state within 3.69 infectious periods. In the case of a pathogen that is hardly infectious ($R_0 \rightarrow 0$), the mean time to absorption is 1 infectious period.

4. PRACTICAL CONSIDERATIONS

It is often difficult to distinguish between recovered and infectious individuals in transmission experiments. Consider, for instance, the bacterial pathogen *A. pleuropneumoniae* in pigs where under experimental conditions the excretion pattern varies widely between individuals (Velthuis *et al.* Accepted). It may occur that individuals cease to excrete the bacteria but that excretion is resumed after a few days. Therefore, it cannot be concluded that a pig has stopped being infectious when there has been no excretion of the bacteria for a few days. Susceptible individuals are easier to identify, because they are consistently negative in bacteriological culturing and serology during the experimental period.

To quantify *R_0* it is desirable to use a probability distribution over the number of susceptible individuals (*s*) instead of the number of infectious and susceptible animals (*s,i*). The probability of the number of susceptible individuals in a one-to-one trial is easily obtained by adding all state probabilities with equal numbers of susceptibles, i.e., *s=1* or *s=0*. Consequently, the number of infectious individuals becomes irrelevant in the quantification of *R_0*. The probability of having no susceptible individual at time *t* for the TS algorithm is the sum of the state probabilities $p_{0,2}(t)$, $p_{0,1}(t)$ and $p_{1,0}(t)$:

$$p_{s=0}(t) = \sum_i p_{s,i}(t) = \frac{R_0}{2+R_0} - \frac{R_0}{2+R_0} e^{-\frac{2+R_0}{2}t}. \tag{9}$$

The probability of having one susceptible individual left at time *t* is equal to the sum of state probabilities $p_{1,1}(t)$, and $p_{1,0}(t)$. The state probabilities of having one or no susceptible individual according to the FS algorithm equal the state probabilities of the two absorbing states (5), i.e., $p_{1,0}$ and $p_{0,0}$.

When planning a one-to-one experiment, it is possible to calculate the minimal experimental period depending on the expected *R_0*. Since the original number of state probabilities is reduced from five in (4) to the two state probabilities in (9) where only the number of susceptibles is considered, the minimal experimental period can be determined by use of the mean sojourn time and its variance in starting state *(1,1)*, i.e., $2/R_0 + 2$ and $(2/R_0 + 2)^2$, respectively. This because, the observable final size situation is reached immediately after state *(1,1)* has been left.

5. ONE-TO-ONE EXPERIMENTS

In a single one-to-one trial the outcome of the infection process is a binary variable, since an infection will occur or not. Hence, the total number of infection events k from n mutually independent replications of an one-to-one trial is binomially distributed with index n, and parameter $p_{s=0}(t)$ (9):

$$Q(K = k;t) = \binom{n}{k} \cdot p_{s=0}(t)^k \cdot (1 - p_{s=0}(t))^{n-k}. \qquad (10)$$

The binomial parameter for the FS algorithm is $p_{0,0} = \lim_{t \to \infty} p_{0,0}(t)$. Since the FS algorithm is a limiting case of the TS algorithm, it is interesting to investigate the effect of early stopping, i.e., using the FS algorithm instead of the TS algorithm. The error made when using the FS algorithm instead of the TS algorithm, where the latter should have been used, can be quantified by comparing infinite t (FS algorithm) to finite or even small t (TS algorithm). In the subsections to follow this will be done for different aspects.

5.1. Estimation of R_0

The maximum likelihood estimator (MLE) of parameter $p_{s=0}(t)$ is simply the observed proportion of successes, i.e., k/n. Hence, the MLE of R_0 is obtained as the solution of:

$$\frac{\hat{R}_0}{2 + \hat{R}_0} - \frac{\hat{R}_0}{2 + \hat{R}_0} e^{-\frac{2+\hat{R}_0}{2}t} = \frac{k}{n}. \qquad (11)$$

One-sided and two-sided statistical tests about R_0 can be performed on the basis of the probability distribution given in (10). A two-sided 95% confidence interval (CI) for R_0 can be constructed as usual. In case of extreme outcomes, i.e., $k=n$ or $k=0$, a one-sided interval would be more appropriate.

To quantify the behaviour of the two estimators of R_0 considered here, we simulated 1000 experiments of 20 one-to-one trials for a given pair of R_0 and t, by drawing 1000 random numbers from the binomial distribution (10). From each of the simulated experiment, R_0 was estimated with both the TS and the FS algorithm to give $R_{0\text{-TS}}$ and $R_{0\text{-FS}}$. This procedure resulted in two arrays of 1000 estimated R_0s. From these arrays we calculated the mean estimated $R_{0\text{-TS}}$ and $R_{0\text{-FS}}$, and the variance. This whole procedure was performed for several combinations of R_0 and t, and the results are given in Figure 3.3.

The top panel of Figure 3.3 shows that the $R_{0\text{-FS}}$ underestimates R_0 for small values of t, while it overestimates R_0 for high values of t. The $R_{0\text{-FS}}$ approaches $R_{0\text{-TS}}$ when t increases. So if the experimental period is relatively short and the FS algorithm is used instead of the TS algorithm, then it means that R_0 is underestimated. Note that $R_{0\text{-TS}}$ overestimates R_0 for all t and this overestimation increases with the real R_0.

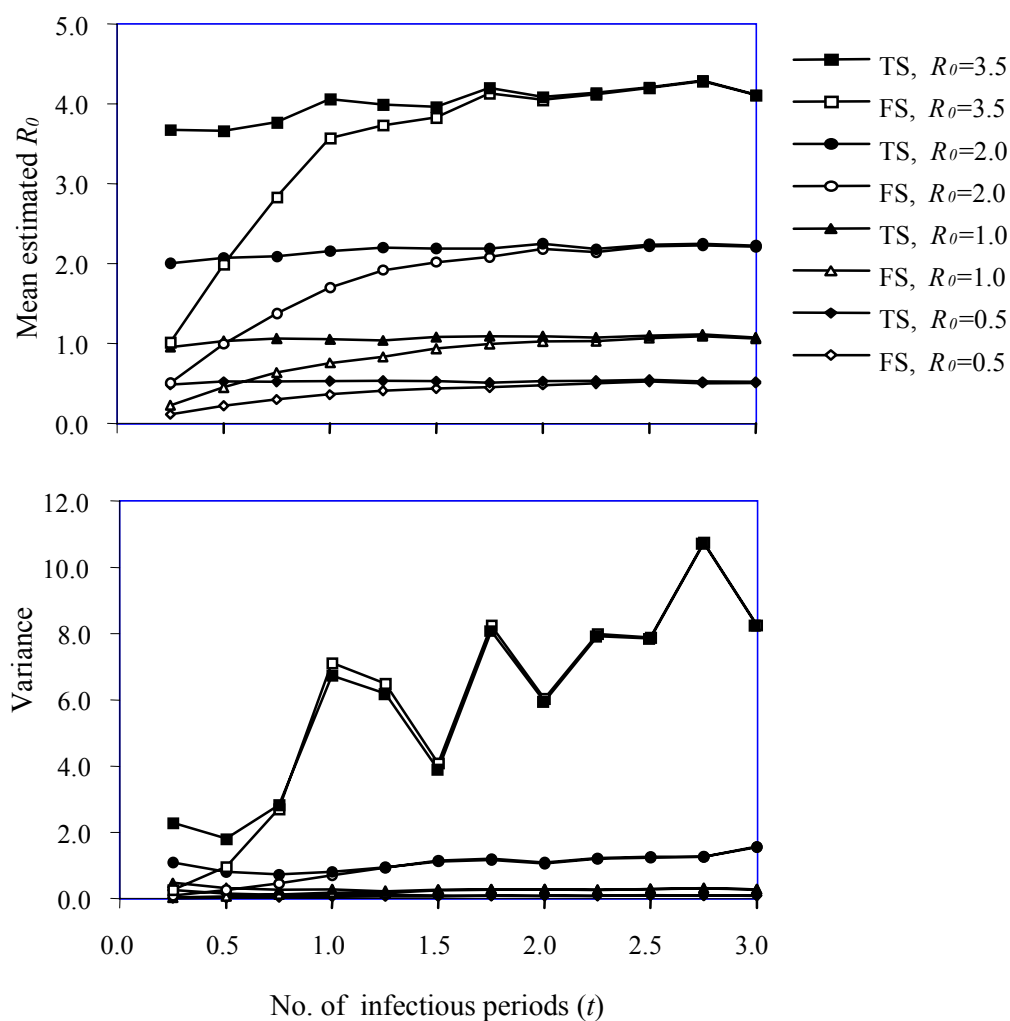Figure 3.3. The mean estimated R_0 (top panel) and the variance (bottom panel) as a function of the number of infectious periods (t) calculated from 1000 simulations for given combinations of R_0 and t.

5.2. *R₀* and the threshold value 1

An important purpose of transmission experiments is to assess whether a particular intervention can be used to eradicate an infectious agent. To achieve eradication R_0 should be

brought below 1 so that the infectious agent cannot persist, and only small outbreaks can occur. To test whether an intervention brings R_0 below 1 the hypothesis $H_0 : R_0 \geq 1$ against $H_1 : R_0 < 1$ should be considered. Application of the usual test for a binomial parameter, which in this case is $p_{s=0}(t)$ (9), and the observation that $p_{s=0}(t)$ is a monotone, increasing function of R_0, means that rejection of H_0 sustains H_1, i.e., R_0 is assumed to be smaller than 1. To this end, the probability that k or less infections have occurred is calculated under the null hypothesis, $R_0=1$, and should be lower than 0.05 to reject H_0:

$$\Pr(K \leq k; t | R_0 = 1) = \sum_{i=0}^{k} Q(K = i; t | R_0 = 1) \leq 0.05. \tag{12}$$

Probability $\Pr(K \leq k; t | R_0 = 1)$ is a decreasing function of t. Thus, if the FS algorithm $(t \to \infty)$ is used for testing the above-mentioned hypothesis it is possible to reject $H_0 : R_0 \geq 1$ with a higher probability than the indicated error rate. On the other hand, if the FS algorithm does not rejects H_0 the TS algorithm will not reject it either. In other words the FS algorithm is too liberal when testing $H_0 : R_0 \geq 1$ against $H_1 : R_0 < 1$.

Another hypothesis that may be of interest is $H_0 : R_0 \leq 1$ against $H_1 : R_0 > 1$. Rejecting H_0 suggests that R_0 is greater than 1. If this is so, it is unsure whether the infectious agent can be eradicated from the population and major outbreaks can occur. In this situation eradication will take place only by chance and minor outbreaks are possible depending on the size of R_0. Like (15), to reject H_0 the probability that k or more infections are observed should be lower than 0.05. This probability $\Pr(K \geq k; t | R_0 = 1)$ is an increasing function of t, so using the FS algorithm to test $H_0 : R_0 \leq 1$ may lead to the wrong acceptance of H_0. However, if H_0 is rejected with the FS algorithm, it will surely be rejected with the TS algorithm. So, the FS algorithm is conservative when testing $H_0 : R_0 \leq 1$.

5.3. The effect of an intervention on R_0

One application of transmission experiments is to assess the effect of an intervention on the transmission of an infectious agent. Here we compare the level of transmission in two populations, e.g., one vaccinated and the other unvaccinated. Although, in the simple case of a one-to-one experiment with equal stopping times in the control and treatment groups elementary tests like Fisher's one for testing equality of binomial proportions could be applied, we propose a more generally applicable method, which also can be applied to

experiments with larger numbers of animals per trial or with different stopping times. Methods to test the difference in transmission between two groups are available (Kroese and De Jong 2001). The hypothesis to be tested is that there is no difference in transmission between the treatment group and the control group, $H_0 : R_{0-control} = R_{0-treatment}$ against $H_1 : R_{0-control} > R_{0-treatment}$. Rejection of H_0 in favour of its alternative makes it plausible that the transmission in the treatment group differs from the control group. A natural test statistic is the difference in the number of contact infections between the two groups:

$$X = \left| K_{control} - K_{treatment} \right|.$$

To test $H_0 : R_{0-control} = R_{0-treatment}$, the probability that the observed difference in contact infections x or more has to be calculated under the assumption that the R_0 is equal in both groups. The probability to obtain a difference of x contact infections is twice the sum of all possible products of $Q(K = i;t)$ and $Q(K = i + x;t)$ for x≠0:

$$D(X = x;t) = 2 \cdot \sum_{i=0}^{n-x} Q(K = i;t) \cdot Q(K = i + x;t). \qquad (13)$$

H_0 is rejected if the probability of a difference of *x*, say $M(X = x;t)$, is smaller than 0.05, i.e., if

$$M(X = x;t) = \sum_{j=x}^{n} D(X = j;t) \leq 0.05. \qquad (14)$$

Note that we assume that the number of one-to-one trials is equal in both groups. It is also possible to calculate the above mentioned probabilities in situations where the numbers differ between treatment groups. However, for simplicity we only present results for the case where the numbers are equal.

Since the parameter $p_{s=0}(t)$ in $Q(K = k;t)$ depends on both parameters of interest R_0 and *t*, $M(X = x;t)$ depends also on R_0 and *t*. A conservative way to reject H_0 is to require that the maximum of $M(X = x;t)$ is smaller than 0.05 for any arbitrary R_0 and *t* for the TS algorithm, and for any arbitrary R_0 with *t*→∞ for the FS algorithm. In Figure 3.4 the surface of $M(X = x;t)$ is plotted against R_0 and *t* in the situation where the observed difference is 4 in 20 one-to-one trials per treatment group.

The whole surface of $M(X = x; t)$ is below 0.05 and its maximum is on the same height for any value of t. This is due to the fact that $M(X = x; t)$ depends on R_0 and t only through $p_{s=0}(t)$. Hence, for this test it is sufficient to use $p_{0,0}$ according to the FS algorithm.

In addition, one would also like to know how many trials one should conduct in order to find a significant difference between $R_{0\text{-}control}$ and $R_{0\text{-}treatment}$. Thus we have to calculate the power of the test, i.e., the probability to find a significant difference given that there is a difference. The power is determined by adding all probabilities $D(X = x; t)$ for all x for which the difference, given $R_{0\text{-}control}$, $R_{0\text{-}treatment}$ and n, is significant.
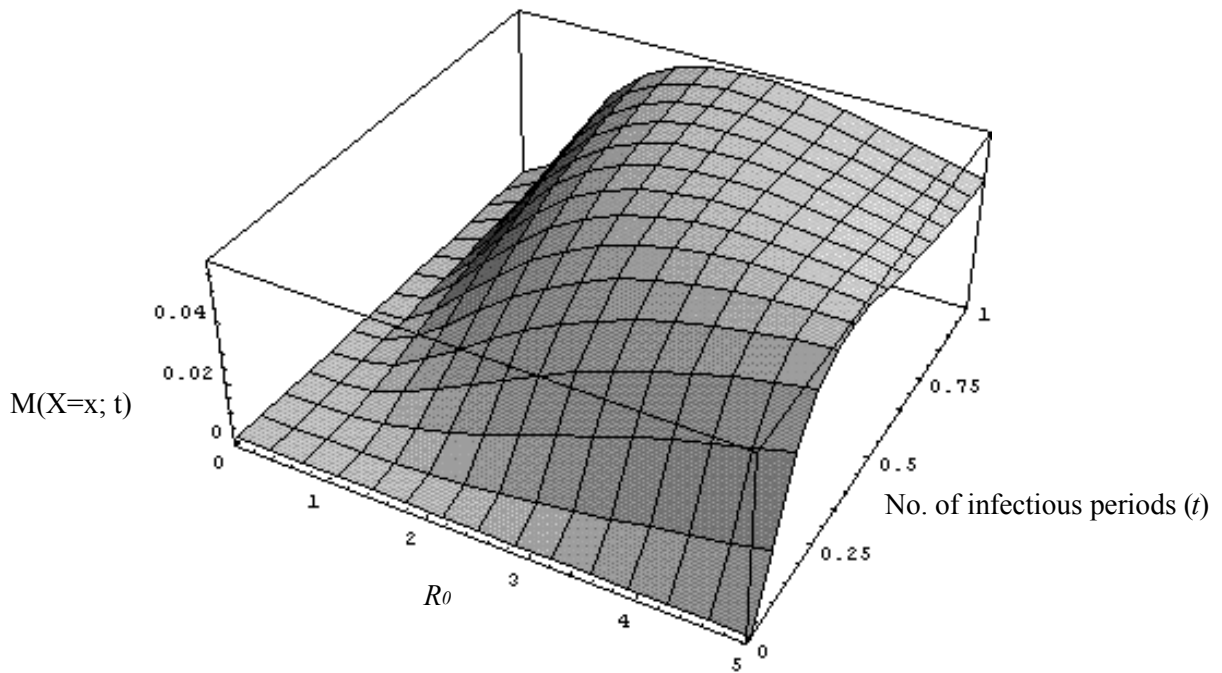
Figure 3.4. The cumulative probability $M(X = x; t)$ (14) to observe at least x contact infections, plotted as a function of R_0 and t.

Figure 3.5 shows an example of a power calculation. The top panel shows the results if the number of replicates is n=10, while n=20 in the bottom panel. Two scenarios are considered: $R_{0\text{-}control}$=3.5 versus $R_{0\text{-}treatment}$=0.5, and $R_{0\text{-}control}$=10.0 versus $R_{0\text{-}treatment}$=3.5. The error rate is set at 0.05.

It appears that high power can only be obtained if R_0 exceeds 1 in one treatment group and is less than 1 in the other. If R_0 exceeds 1 in both treatment groups, highest power is

obtained for small *t*. In this particular example reasonable power (say >0.80) is obtained if $R_{0\text{-}control}$>1 *and* $R_{0\text{-}treatment}$<1 and if the number of trials is large (here *n*=20).
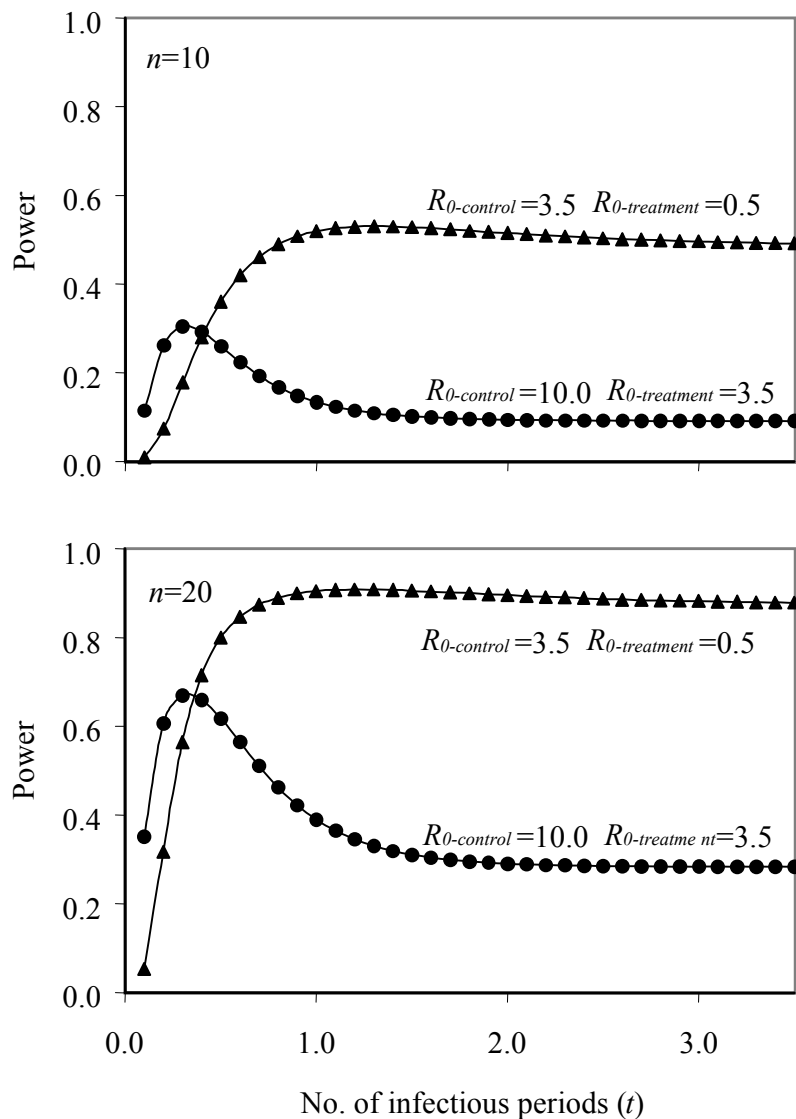
Figure 3.5. The power of the test $H_0 : R_{0-control} = R_{0-treatment}$ for various alternative hypotheses. In the top panel the number of replicates is *n*=10, while *n*=20 in the bottom panel. Two scenarios are considered: $R_{0\text{-}control}$=3.5 versus $R_{0\text{-}treatment}$=0.5, and $R_{0\text{-}control}$=10.0 versus $R_{0\text{-}treatment}$=3.5. The error rate is set at 0.05.

6. ILLUSTRATION

The results presented above are illustrated by application to the one-to-one experiment of Nodelijk *et al.* (2001), who investigated the effect of vaccination on the transmission of porcine reproductive and respiratory syndrome virus (PRRSV). Two sets of ten replicate one-

to-one trials were carried out. In one set of trials all pigs were vaccinated, while in the other they were left unvaccinated in the other experiment. At day 1, one pig from each couple was inoculated intranasally with PRRSV, while the other pig was placed in a separate pen. At day 2, the contact pigs were placed back to their original pens. To determine the onset and duration of viremia, sera were collected from all the pigs at day 1, and thereafter every third or fourth day. This continued until the end of the experiment, 56 days post inoculation. A PRRSV infection was confirmed by virus detection in the sera.

Table 3.1 shows the results of this experiment for the contact pigs. One inoculated pig from a vaccinated couple remained uninfected and was excluded from the analysis. All unvaccinated contact pigs were infected at day 14, while all vaccinated contact pigs were infected at day 17. Thus, in all one-to-one trials the final size of the outbreak had been reached in both treatment groups before the end of the experiment, making this experiment an ideal test case for an illustration.

Table 3.1.   Results of the one-to-one experiment carried out to test the effect of vaccination on the transmission of PRRSV.

Days post inoculation	Virus isolated from contact pig in the	
	Control group (n=10)	Vaccine group(n=9)
3	0	0
7	3	1
10	9	1
14	10	6
17	10	9
56	10	9

Now, let us assume that the experiment was not stopped at day 56, but at day 7, 10 or 14, thus before the final size was reached in all trials. The question is, would there be a difference in the conclusions drawn if the TS algorithm had been used instead of the FS algorithm?

Table 3.2 gives the estimated reproduction ratios in the unvaccinated (R_{0-c}) and the vaccinated group (R_{0-v}) together with the 95%-CIs and the p-values under the different H_0 hypotheses for the different scenarios. For the estimates with the TS algorithm we assumed that the duration of the infectious period was 56 days (Nodelijk *et al.* 2000).

Let us first assume that the experiment was stopped at day 7. On this day only 3 out of 10 unvaccinated, and 1 out of 9 vaccinated contact animals were infected. Table 3.2 shows to what extent the FS algorithm underestimates R_{0-c} and R_{0-v}. The FS algorithm does not reject

the hypothesis $H_0 : R_{0-c} \leq 1$ while the TS algorithm does. All other conclusions drawn with the FS algorithm are the same as with the TS algorithm.

Next, let us assume that the experiment was stopped at day 10. At this day, 9 out of 10 unvaccinated, and only 1 out of 9 vaccinated contact animals were infected. As before, the FS algorithm underestimates the R_{0-c} and R_{0-v}, but all other conclusions are the same for both algorithms.

Table 3.2.   The estimated R_{0-c} and R_{0-v}, 95%-CI, and the p-values under the different H_0 hypotheses for 4 different scenarios, assuming that the experiment was stopped at day 7, day 10, day 14 and day 17 post inoculation.

	Day 7		Day 10		Day 14		Day 17	
	FS	TS	FS	TS	FS	TS	FS	TS
R_{0-c}	0.86	6.12	18.0	43.41	∞	∞	∞	∞
95%-CI	0.14-3.75	1.18-18.5	2.49-∞	14.08-∞	4.48-∞	13.9-∞	4.48-∞	11.40-∞
H_0:R_{0-c}>1	0.56	1.00	1.00	1.00	1.00	1.00	1.00	1.00
H_0:R_{0-c}<1	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00
R_{0-v}	0.25	3.42	0.25	2.01	4.00	12.95	∞	∞
95%-CI	0.01-1.86	0.08-19.3	0.01-1.86	0.05-11.4	0.85-24.7	4.04-36.1	3.95-∞	10.49-∞
H_0:R_{0-v}>1	0.14	0.96	0.14	0.91	0.99	1.00	1.00	1.00
H_0:R_{0-v}<1	0.97	0.27	0.97	0.41	0.04	0.00	0.00	0.00
H_0:R_{0-c}=R_{0-v}	0.34	0.34	0.00	0.00	0.09	0.09	1.00	1.00
$R_0 \vert H_0$	5.58	39.58	2.29	13.25	2.75	10.06	∞	∞

Third, assuming that the experiment was stopped at day 14, by which time all unvaccinated couples, and 6 out of 9 vaccinated couples were infected, the final size was reached in all unvaccinated couples but not in all vaccinated couples. According to both algorithms R_{0-c} tends to infinity. The R_{0-v} remains underestimated with FS algorithm. All other conclusions are the same for both algorithms.

Overall, this example suggests that the FS algorithm is a good algorithm to test the different H_0 hypothesis, except for $H_0 : R_{0-c} \leq 1$ at day 7. However, the estimated reproduction ratios, R_{0-c} and R_{0-v}, will be underestimated when the FS algorithm is used instead of the TS algorithm.

7. DISCUSSION

In this paper we compared two algorithms to quantify the transmission of an infectious agent from one-to-one experiments. The first algorithm, the transient state (TS) algorithm, takes the time course of the experimental epidemic into account. The second algorithm, the

final size (FS) algorithm, does not take time into account, and assumes that the final size of the epidemic process has been reached before the experiment was stopped.

The stochastic SIR-model on which both algorithms are based was originally proposed by Bartlett (Bartlett 1949), and formal solutions are attributable to Billard (1973) and Kryscio (1975). Bailey (1975) derived a likelihood function for parameter estimation although it was not applied to real-world data. Inspired by observational data of human diseases, Becker (1989) described methods for the analysis of a single epidemic in a large community. Kroese and De Jong (2001) considered methods to analyse transmission experiments that have been applied to experimental data (De Jong and Kimman 1994; Bouma *et al.* 1997a; Bouma *et al.* 1997b; Bouma *et al.* 1998). Their methods, however, are restricted to the final size of the experimental epidemics.

Thus far, quantification of the reproduction number from transmission experiments has relied almost exclusively on the FS algorithm. This is not surprising since the FS algorithm is easy to understand and readily implemented on a personal computer, while the computational burden of the TS algorithm quickly becomes insurmountable as the size of the population increases. On the other hand, the applicability of the FS algorithm is not always warranted, as the epidemic process may not have ended in one or more of the trials when the experiment is stopped. The assumption that the epidemic processes have ended before the end of the experiment may be justified for viral infections with relatively fast transmission dynamics. However, the transmission dynamics of many bacterial infections are much slower, more variable, and less easy to keep track of.

Well-known examples of slow and highly variable infections include Salmonella enteritidis in chickens, Mycobacterium paratuberculosis in cattle, and Actinobacillus pleuropneumoniae in pigs. For instance, Velthuis *et al.*(Accepted) studied the transmission of Actinobacillus pleuropneumoniae among pigs by means of a transmission experiment. The excretion pattern of the bacterium in tonsil swabs and nasal swabs was highly variable. In all trials the bacterium could still be isolated from some pigs on the last day of the experimental period.

Hence, we are faced with the problem that while it is desirable to base the analysis of bacterial transmission experiments on the TS algorithm, it is not always feasible in practice. For one-to-one trials, however, this problem does not arise since a full analytical comparison of the TS algorithm is within reach. In this paper we have paper presented different aspects of the statistical inference based on the FS and TS algorithms on data from one-to-one trials:

First, in case of one-to-one trials it is still possible to estimate beforehand the time until absorption or an infection-event has occurred. In particular, the mean time to absorption and its variance are expressed in terms of R_0 by equations (7) and (8).

Second, the results show that the FS algorithm underestimates R_0 when the final size has not yet been reached. If the experimental period is short compared to the infectious period, the degree of underestimation is high. If, on the other hand, the experimental period is relatively long, R_0 will only slightly be underestimated. Furthermore, both algorithms lead to overestimated R_0. This bias is a consequence of the fact that R_0 is a convex function of the proportion of successful infection-events. Although this proportion is an unbiased estimate of the success probability, Jensen's inequality leads to a biased overestimation of R_0, which increases with time and R_0 (Rao 1973).

Third, we conclude that the FS algorithm is liberal (i.e., yielding incorrect significant results) if the null hypothesis $H_0 : R_0 \geq 1$ is tested against the alternative hypothesis $H_1 : R_0 < 1$. In other words, it is possible that the null hypothesis would be rejected with the FS algorithm, whereas it would not be rejected with the TS algorithm. The implication is that conclusions based on the FS algorithm may overestimate the possibility of eradication. On the other hand, the FS algorithm is conservative (ie, yielding incorrect non-significant results) if $H_0 : R_0 \leq 1$ is tested against its alternative $H_1 : R_0 > 1$. The implication is that the FS algorithm can safely be used for testing $H_0 : R_0 \leq 1$ even if the final size has not been reached in all trials.

Finally, there is no difference in p-value between both algorithms when testing: $H_0 : R_{0-control} = R_{0-treatment}$ against $H_1 : R_{0-control} > R_{0-treatment}$. This is due to the fact that M(X=x;t) depends on R_0 and t only through $p_{s=0}(t)$. Thus, in principle at least, the FS and TS algorithm are equally good when testing for the effect of an intervention. Note, however, that the power of the test does depend on both R_0 and t. In fact, the highest power is achieved if $R_{0-control}$ is higher than 1 while $R_{0-treatment}$ is smaller than 1. Moreover, the power of the test is highest at intermediate t. We conclude that both algorithms can safely be used to test for differences, but that the power of the test is affected by both R_0 and t.

To the best of our knowledge, there are two studies that use one-to-one experiments (Mars *et al.* 2000b). Nodelijk *et al.* (2001) carried out 20 one-to-one trials, ten with vaccinated pigs and ten with unvaccinated pigs. The aim of the study was to test whether vaccination reduces the transmission of porcine reproductive and respiratory syndrome virus (PRRSV) among pigs. All the suscecptible contact pigs were infected at the end of all the trials. In the control as well as in the vaccine groups both algorithms lead to the conclusion that R_0 exceeds 1. The authors concluded, with use of the FS algorithm, that there was no proof that vaccination reduced the transmission of PRRSV. However, it could still be that vaccination reduces the reproduction ratio, albeit not below 1. The analysis presented in this paper shows

that there is indeed evidence that vaccination has a significant effect on the transmission on day 10 (p=0.00) and a marginally significant effect at day 14 (p=0.09).

Mars *et al.*(2000b) carried out a one-to-one experiment with 32 trials to test if cows infected with a gE-negative bovine herpes virus 1 vaccine strain could re-excrete the strain and transmit it to contact-exposed cows. The number of trials was chosen such that the null hypothesis $H_0 : R_0 \geq 1$ should be rejected in favour of $H_1 : R_0 < 1$ when no contact infections would be observed. The experiment lasted 5 weeks, and no contact infections were observed. As a consequence Mars *et al.*(2000a) concluded that the R_0 the vaccine strain is below 1. In fact, using the FS algorithm the R_0 was estimated at 0.0 with a 95% confidence interval of (0.0; 0.91).

Finally, in this paper we have presented a first step towards the statistical inference of transmission experiments. Of course, much remains to be done. For instance, one could think of extension to experiments involving more animals per trial, to infectious processes with non-exponentially distributed infectious periods, or so that differentiation in individual levels of susceptibility and infectivity is allowed. To what extent the results the results of the present paper still hold in a more general setting, is at present an open question.

8. ACKNOWLEDGEMENTS

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Comparing methods to quantify experimental transmission of infectious agents

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SUMMARY

Transmission of an infectious agent can be quantified from experimental data using the transient state (TS) algorithm that was introduced in a previous paper. The TS algorithm is based on the stochastic SIR model and provides a time-dependent probability distribution over the number of infected individuals during an epidemic, with no need for a final size situation. However, because of numerical limitations the application of the TS algorithm is limited to populations with only a few individuals. So, we investigated the error of using the easily applicable, time-independent final size (FS) algorithm knowing that the final size situation was not reached in all trials. We conclude that the methods based on the FS algorithm: (i) underestimate the reproduction ratio R_0, (ii) is liberal when testing $H_0 : R_0 \geq 1$ against $H_1 : R_0 < 1$, (iii) is conservative when testing $H_0 : R_0 \leq 1$ against $H_1 : R_0 > 1$, and (iv) is conservative when testing $H_0 : R_{0-control} = R_{0-treatment}$ against $H_1 : R_{0-control} > R_{0-treatment}$. Furthermore, we present a new method to find a difference in transmission between two treatment groups (*MaxDiff* test). The *MaxDiff* test is compared to tests based on FS and TS algorithms and to a test based on a Generalised Linear Model (GLM). The GLM test was most powerful in finding a difference in transmission between two treatment groups. Next were the TS test and the *MaxDiff* test, which were approximately equally powerful, but more powerful than the FS test especially when both $R_{0-control}$ and $R_{0-treatment}$ are larger than 1.

1. INTRODUCTION

Quantification of experimental transmission has proved to be very useful to gain more insight into the transmission dynamics of an infectious agent and to find intervention strategies that can substantially reduce the transmission. Useful data for the quantification of transmission can be collected in transmission experiments in which the transmission of an infectious agent among individuals is observed.

A transmission experiment often consists of one or more independent transmission trials in each of which infectious and susceptible individuals are housed together in an isolated unit. The infection chain can be monitored by regular sampling. Accepted as plausible for the observed infection a mathematical transmission model will form the base of the quantification methods. The next step is to link the diagnostic results to the different infection classes assumed in the mathematical model so that the infection chain can be defined. If the classification and the mathematical model are plausible, transmission parameters can be estimated, and the effect of interventions on the transmission parameters can be tested.

An advantage of transmission experiments over field studies is that they offer a controlled environment in which the influence of a single factor on the transmission, like vaccination, can be investigated with a minimum of variation caused by other factors. Thus, more insight is obtained into causative mechanisms underlying the transmission dynamics of an infectious agent.

Of the many 'qualitative' transmission experiments with animals published (Jobert *et al.* 2000; Lechtenberg *et al.* 1994; Torremorell *et al.* 1997), and with humans (D'alessio *et al.* 1976; Dick *et al.* 1987) that have been published, all failed to address two crucial questions. First, to what extent does the infectious agent spread? Second, are the observed differences in transmission significant? To our knowledge, the first quantitative transmission experiments were published by Greenwood *et al.* (1936). They used experiments to quantify the transmission of several bacterial and viral infections among mice using endemic equilibria as a measure for transmission. Kermack and McKendrick (1936; 1939) estimated transmission from the data of the Greenwood experiments using a specific deterministic mathematical model. Later, Anderson and May (1979) fitted a simple deterministic mathematical model to the data in which they assumed that transmission (R_0) depends on population size. De Jong *et al.* (1995) showed at a later stage that the transmission observed in the Greenwood experiments could also be explained with a model that assumes that transmission (R_0) does not depend on population size. In transmission experiments using pseudorabies virus among pigs Bouma *et al.* (1995) demonstrated that given a constant density transmission did not depend on population size.

The first paper about a transmission experiment in which the effect of an intervention was tested and quantified, namely, the effect of vaccination on the transmission of pseudorabies virus among pigs, was published by De Jong and Kimman (1994). Subsequently, several other studies on the quantification of viral transmission have been published. An overview is given in Table 4.1.

Table 4.1.   An overview of published papers about transmission experiments in which transmission has been quantified with the help of mathematical models.

Year	Reference	Infectious agent	Host	D/St[a]
1936	Greenwood *et al.* 1936	*Bacterium aertrycke*	Mice	D
1936	Greenwood *et al.* 1936	Virus disease mouse ectromelia	Mice	D
1936	Kermack and Mckendrick 1936	Virus disease mouse ectromelia	Mice	D
1939	Kermack and Mckendrick 1939	*Bacterium aertrycke*	Mice	D
1994	De Jong and Kimman 1994	Pseudorabies	Pigs	St
1995	De Jong *et al.* 1995	*Bacterium aertrycke*	Mice	St
1995	Bouma *et al.* 1995	Pseudorabies virus	Pigs	St
1996	Bouma *et al.* 1996	Pseudorabies virus	Pigs	St
1997	Bouma *et al.* 1997a	Pseudorabies virus	Pigs	St
1997	Bouma *et al.* 1997b	Pseudorabies virus	Pigs	St
1998	Wit *et al.* 1998	Infectious bronchitis virus	Chickens	St
1998	Laevens *et al.* 1998	Classical swine fever virus	Pigs	St
2000	Bouma *et al.* 2000	Classical swine fever virus	Pigs	St
2000	Mars *et al.* 2000a	Bovine herpes virus	Cattle	St
2000	Mars *et al.* 2000b	Bovine herpes virus	Cattle	St
2000	Mars *et al.* 2000c	Bovine herpes virus	Cattle	St
2000	Nodelijk *et al.* 2001	Porcine reproductive and respiratory syndrome virus	Pigs	St
2001	Van Nes *et al.* 2001	Pseudorabies virus	Pigs	St
2001	Dewulf *et al.* 2001	Classical swine fever virus	Pigs	St
2002	Klinkenberg *et al.* 2002	Classical swine fever virus	Pigs	St
2002	Velthuis *et al.* Accepted	*A. pleuropneumoniae*	Pigs	St
2002	Velthuis *et al.* Accepted	*A. pleuropneumoniae*	Pigs	St
2002	Maurice *et al.* Accepted	Encephalomyocarditis virus	Pigs	St

[a] A deterministic (D) or stochastic (St) model was used for the quantification of transmissions

A mathematical transmission model that is often used for the analysis of transmission experiments is the stochastic SIR model, in which individuals are susceptible (S), infectious (I) or removed (R) (Bailey 1975; Bartlett 1949). An interesting feature of the stochastic SIR model is that it enables analysis on the basis of a single parameter, the reproduction ratio (R_0). R_0 is defined as the expected number of secondary cases produced by a typical infected individual during its entire period of infectiousness in a completely susceptible population

(Diekmann *et al.* 1990). Using the stochastic SIR model, a probability distribution can be derived as a function of R_0 over all possible states that can occur in the course of the experimental epidemic. By a state we mean a single combination of the number of susceptible individuals and the number of infectious individuals present in the population at a certain moment. Once the probability distribution has been obtained, transmission parameters can be estimated and hypotheses can be tested.

Several estimation methods based on the stochastic SIR model are available. The first papers on transmission experiments, for example Bouma *et al.* 1996; De Jong *et al.* 1994, made use of methods based on the probabilities of all possible final size (FS) situations of the outbreak, viz. the maximum likelihood estimator (FS-MLE) and the Martingale estimator (Kroese and De Jong 2001). A final size situation has been reached when no new infections can take place, because there are no susceptible or infectious individuals left. The probability distribution of the final size can be obtained with what we call the final size (FS) algorithm (Bailey 1975).

Methods based on a final size situation are very useful for infection chains that proceed rapidly like most viral infections, where a final size situation is reached within a few weeks. However, when doing transmission experiments with slow infection chains, like some bacterial infections where a final size situation has not been reached within a few weeks, a problem can arise when there are both susceptible and infectious individuals still present at the end of the experiment. Therefore, when using the FS-MLE or Martingale estimator, one has to be aware of the implicit final size assumption.

There is a maximum likelihood estimator available that is based on the number of contact infections at the end of the experiment and not on the final size assumption. We call this the transient state MLE (TS-MLE), which is a maximum likelihood estimator based on the probability distribution as a function of the two parameters R_0 *and time* over all possible states that can occur during the experimental epidemic. This probability distribution can be determined with the so-called transient state (TS) algorithm (Velthuis *et al.* 2002).

Use of the TS-MLE is not practical because the TS algorithm has certain limitations. First, the probability distribution can be obtained only if the length of the experimental period can be expressed in units of the average infectious period, which unfortunately is often unknown. Second, there is the computational burden that can grow as the number of individuals per trial increases. Consequently, one will be inclined to take refuge in the easily applicable FS algorithm even if the final size assumption is not justified. This brings about the following question: what error is made when the FS algorithm is used instead of the TS algorithm to quantify transmission from data of transmission experiments where the final size has not been reached in all trials? This question has already been addressed in the paper of

Velthuis *et al.* (2002) for replicated one-to-one trials, in which one susceptible individual is housed together with one infectious individual. In these experiments it was possible to make an analytic comparison of the two algorithms (the FS and the TS algorithm). Velthuis *et al.* (2002) concluded that the FS method: (i) underestimates the reproduction ratio R_0, (ii) is liberal when testing $H_0 : R_0 \geq 1$, (iii) is conservative when testing $H_0 : R_0 \leq 1$, and (iv) calculates exactly the same p-value as the TS algorithm when testing $H_0 : R_{0-control} = R_{0-treatment}$. Whether these conclusions also apply to more general transmission experiments remains unanswered.

A disadvantage of the FS or TS methods is that they make use of only a single observation, namely the number of contact infections eventually become infected by the end of the experiment (either in a final size situation or not). It means that information may be lost. For example, the difference between control and treatment groups in the number of contact infections during the experiment that had disappeared at the end of the experiment. This is the reason why we present an alternative method (the *MaxDiff* test) to detect a difference in transmission between two treatment groups. The *MaxDiff* test is based on the probability distribution of the maximum difference in the numbers of contact infections between the two treatments observed during the experimental period, and is able to detect a difference in transmission even if the R_0 in both treatment groups are both larger than 1. If this were the case, it implies that a treatment reduces the transmission – given that the transmission in the treatment group is slower than in the control group – although not enough to ensure that the infection will fade out. This result will be the subject of further research so that in a next experiment a combination of treatments, e.g. two vaccinations instead of one, can be used to see whether an additional intervention will result in a R_0 smaller than 1.

There is a statistical method available that takes the whole infection chain of the experimental epidemic into account: a method based on a generalised linear model (GLM) (Becker 1989; Mccullagh and Nelder 1989). This method, however, is less robust than the *MaxDiff* test since it relies on more assumptions. By comparing powers all methods – the FS test, the TS test, the *MaxDiff* test and the GLM test – were compared in their ability to test a difference in transmission between two treatments groups.

In summary, this paper is about investigation of the error made when using a method based on the final size (FS) algorithm instead of the transient state (TS) algorithm for the quantification of transmission from data from transmission experiments where the final size has not been reached in all trials. To find the difference in transmission between two treatment groups we present an alternative method (the *MaxDiff* test) that exploits at least some information on the infection chain at intermediate stages. Various tests to find a

difference in transmission between two treatments have been compared based on power calculations.

2. INVESTIGATION OF ERROR

2.1. Estimation and testing methods

2.1.1. Transient State algorithm

The time-dependent probability distribution over all possible outcomes (the number of contact infections) of an arbitrary transmission trial, as a function of R_0 *and time*, is based on the stochastic SIR model and obtained using the transient state (TS) algorithm. The stochastic SIR model is also called the General Stochastic Epidemic after Bailey (1975). Proposed by Bartlett (1949) it has been the subject of analysis by others (Bailey 1975; Billard and Zhao 1993; Daley and Gani 1999). The derivation of the TS algorithm is described in detail by Bailey (1975), and for the special case of transmission experiments by Velthuis *et al.* (2002).

In the SIR model individuals are susceptible (S), infectious (I) or recovered and immune (R). Infection or recovery events occur independently at corresponding rates. Let *S(t)* denote the number of susceptible individuals, *I(t)* the number of infectious individuals, *N* the total number of individuals, and (*s,i*) a particular realisation at time *t*. The rates at which infection events and recovery events occurs are $\beta s i / N$ and αi, respectively, where β is the infection parameter, α the recovery parameter, and $1/\alpha$ the average infectious period. If the initial state of the experimental epidemic is denoted by $(S(0), I(0)) = (s_0, i_0)$, the state probabilities are written as:

$$p_{s,i}(t) = \Pr\{(S(t), I(t)) = (s, i) \mid (S(0), I(0)) = (s_0, i_0)\} \tag{1}$$

Without loss of generality the unit of time can be $1/\alpha$, being the average infectious period. The adjacent state probabilities are described by the following forward differential-difference equations, which are derived from, the stochastic SIR model as described by Velthuis *et al.* (2002):

$$\frac{d}{dt} p_{s,i}(t) = (i+1)p_{s,i+1}(t) + \left(R_0 \frac{(s+1)(i-1)}{N}\right)p_{s+1,i-1}(t) - \left(R_0 \frac{si}{N} + i\right)p_{s,i}(t) \tag{2}$$

for $0 \leq s \leq s_0$, and $0 \leq i \leq s_0 + i_0 - s$, and where

$$R_0 = \frac{\beta}{\alpha} \ .$$ (3)

Subject to the initial condition $p_{s_0,i_0}(0) = 1$ expression 2 can be rewritten as:

$$p_{s,i}(t) = e^{-\left(R_0 \frac{si}{N} + i\right)t} \left(\int_0^t (i+1) e^{\left(R_0 \frac{si}{N} + i\right)\gamma} p_{s,i+1}(\gamma) \cdot d\gamma + \int_0^t R_0 \frac{(s+1)(i-1)}{N} e^{\left(R_0 \frac{si}{N} + i\right)\gamma} p_{s+1,i-1}(\gamma) \cdot d\gamma \right)$$ (4)

The evaluation of expression 4 (Velthuis *et al.* 2002) is called the TS algorithm and can be used to calculate a continuous-time state probability for each state (*s,i*) in the experimental epidemic. The limiting case of this probability distribution where time tends to infinity is the final size (FS) distribution. Note that state probabilities where $i \geq 0$ tend to zero as $t \to \infty$, reflecting the fact that in the SIR model eventually no infectious individuals are left.

For the estimation and testing on R_0 it is desirable to use the marginal probability distribution of *S(t)* instead of the joint distribution of *S(t)* and *I(t)*. This is because it is often difficult to distinguish between recovered and infectious individuals, while susceptible individuals are easier to define and detect. The marginal probability distribution of *S(t)* is readily obtained as:

$$pr(S(t) = s | R_0, t) = p\left(s | R_0, t\right) = \sum_I p_{s,i}(t)$$ (5)

An additional advantage of using the marginal probability distribution is that it makes it easier to investigate the error made when the TS algorithm solely considers the number of susceptible individuals and not the number of infectious individuals, which is also the case for the FS algorithm.

2.1.2. *Obtaining a point estimate for R_0*

Suppose *m* transmission trials have been performed, each with (*s_0,i_0*) as initial state and let *X* be the number of contact-infections in the *j*th trial at time τ, which may be the end of the experimental period. The probability distribution of outcomes $X_1 \ldots X_m$ in *m* trials at time τ is:

$$f(x_1,...,x_m | R_0, \tau) = \prod_{j=1}^{m} p(s_0 - x_j | R_0, \tau), \tag{6}$$

which changing our point of view we alternatively denote as the corresponding likelihood function $f(R_0 | \tau, x_1,...,x_m)$ and where $s_0 - x_j$ denotes the number of susceptible individuals in trial j escaping infection up to time τ. The value of R_0 that maximises the likelihood function is called the maximum likelihood estimate of R_0, $R_{0\text{-}MLE}$. Note, the assumption that all trials have identical initial states is not essential to use the method, but is made for notational convenience only.

2.1.3. Testing against threshold 1 and obtaining a confidence interval for R_0

One-sided and two-sided statistical tests on R_0 can be based on the likelihood function 6. R_0 has an important threshold property: if it exceeds 1, the infectious agent may cause a major outbreak, while if R_0 is smaller than 1 the infectious agent will only cause a minor outbreak. Thus, to achieve eradication of an infectious agent, R_0 should be brought below 1. To show that R_0 is smaller than 1 the composite null hypothesis $H_0 : R_0 \geq 1$ should be considered. The probability of k or less contact infections in m trials at time τ is calculated under the condition $R_0 = 1$:

$$g(k | R_0 = 1, \tau) = \sum_{y=0}^{k} \Pr(y | R_0 = 1, \tau), \tag{7}$$

which leads to the highest p-value (Kroese and De Jong 2001). Values of k for which $g(k | R_0 = 1, \tau) \leq 0.05$ form the critical region of the test, i.e. lead to rejection of H_0.

Another hypothesis that may be of interest is $H_0 : R_0 \leq 1$. Rejecting this hypothesis implicates that R_0 might well be larger than 1, making it unsure whether the infectious agent can be eradicated from the population and meaning that major outbreaks can occur. Like expression 7, to reject $H_0 : R_0 \leq 1$ the probability that k or more contact infections are observed in m trials under the null hypothesis should be smaller than 0.05.

A two-sided 95% confidence interval (95%-CI) for R_0 is constructed by finding all r such that $H_0 : R_0 = r$ is not rejected in a test with error level 0.05, as described by Kroese and De Jong (2001) for the FS case. A natural choice for a test statistic is K, the total number of contact infections in m trials at time τ:

$$K = \sum_{j=1}^{m} X_j , \qquad (8)$$

and k_{max} the maximum number of contact infections possible. Then, given $K = k$ the 95%-CI is constructed by collecting all values r such that the hypothesis $H_0 : R_0 = r$ would not be rejected. In case of extreme outcomes, where none or all susceptible individuals were contact-infected, a one-sided confidence interval would be more appropriate.

2.1.4.   Testing difference in R_0 between two treatment groups

An important motivation to conduct a transmission experiment is to assess the effect of an intervention on the transmission of an infectious agent. We can compare the levels of transmission under two different experimental conditions or treatments, e.g., vaccinated yes or no. A method to test the difference in transmission between two groups based on the FS algorithm is available (Kroese and De Jong 2001). The same methodology can be applied using the TS algorithm and will be briefly described below.

In the null hypothesis there is no difference in transmission between the two treatment groups, $H_0 : R_{0-control} = R_{0-treatment}$, whereas the alternative hypothesis is that the intervention has reduced the transmission, $H_1 : R_{0-control} > R_{0-treatment}$. Here $R_{0-control}$ denotes the R_0 in the control group, and $R_{0-treatment}$ the R_0 in the treatment group. Because we assume that the treatment will not result in an increased R_0 a one-sided test is discussed, but modification to a two-sided test is straightforward.

A natural test statistic for this test as described by Kroese and De Jong (2001) is the total number of contact infections observed in the control trials ($K_{control}$) minus the total number of contact infections in the treatment trials ($K_{treatment}$) at time τ:

$$Z = K_{control} - K_{treatment} . \qquad (9)$$

To test $H_0 : R_{0-control} = R_{0-treatment}$, the probability that the difference in contact infections would be larger than or equal to the observed difference in contact infections z, has to be calculated with an equal R_0 for both groups. The probability to observe difference z at (stopping) time τ is the sum of all possible products of the two probability functions 7 for each treatment group:

$$h(z|R_0,\tau) = \sum_{i=0}^{z_{max}-z} g_{control}(i|R_0,\tau) \cdot g_{treatment}(i+z|R_0,\tau),$$

(10)

where z_{max} is the maximum difference possible. H_0 is rejected if the probability of having difference z or more at (stopping) time τ is smaller than 0.05, i.e., if

$$m(z|R_0,\tau) = \sum_{j=z}^{z_{max}} h(z|R_0,\tau) \leq 0.05.$$

(11)

As $H_0 : R_{0-control} = R_{0-treatment}$ is a composite null hypothesis it is correct to require for its rejection that the maximum of $m(z|R_0,\tau)$ is smaller than 0.05 for any arbitrary R_0 and τ for the TS algorithm, and for any arbitrary R_0 with $\tau \to \infty$ for the FS algorithm. Note, we have assumed, for simplicity of notation, equal numbers and sizes of transmission trials, but generalisation to unequal numbers of trials or even unequal numbers of individuals per trial is straightforward.

To find a difference in transmission power calculations can be done to investigate which design is optimal. A design can be optimised by varying the number of trials per treatment group, and the number of individuals at the start of each trial (s_0, i_0). The power is calculated by adding all probabilities for all z for which the difference in the number of contact infections is significant according to the FS algorithm ($\tau = \infty$) given specific (expected) values of $R_{0-control}$ and $R_{0-treatment}$, i.e., where $m(z|R_{0-control}, R_{0-treatment}, \tau = \infty) \leq 0.05$:

$$power(z_1,...,z_m|R_{0-control}, R_{0-treatment}, \tau)$$
$$= \sum_{y=x}^{x_{max}} \sum_{i=0}^{x_{max}-y} g(K_{control} = i|R_{0-control}, \tau) \cdot g(K_{treatment} = i+x|R_{0-treatment}, \tau).$$

(12)

2.2. Application to a five-to-five experiment

2.2.1. *Five-to-five transmission experiment with two trials per treatment.*

To investigate the error made by falsely assuming FS conditions, we focus on a particular type of transmission experiment consisting of two five-to-five trials per treatment. In a five-to-five trial five infectious individuals and five susceptible contact individuals are housed together at the start. Five-to-five transmission experiments have been favoured in many

studies, e.g., De Jong and Kimman 1994; Bouma *et al.* 1996; Bouma *et al.* 1997a; Bouma *et al.* 1997b; Bouma *et al.* 1998; Bouma *et al.* 2000; Nodelijk *et al.* 2001; Velthuis *et al.* Accepted. This because, a five-to-five experiment has satisfactory power (according to the FS estimator) to find a difference in transmission if R_0 is smaller than 1 in one treatment group and far larger than 1 in the other group (Kroese and De Jong 2001).

2.2.2. Estimation of R_0 and testing against its threshold value 1

In a five-to-five trial the number of contact-infections can be 0, 1, 2… 5. The six continuous time state probabilities of these outcomes were derived from equation 2 with initial state $(s_0, i_0) = (5,5)$. In case of two replicated five-to-five trials 21 different outcomes (x_1, x_2) can occur (Table 4.2) and for each combination of these a likelihood function 6 can be derived to estimate R_0 or a probability function 7 to test R_0 against its threshold value 1. For all possible outcomes and for three different stopping times τ (0.5, 1.0 and ∞) $R_{0\text{-}MLE}$ and its approximated 95%-CI, and the p-values for testing $H_0 : R_0 \geq 1$ and $H_0 : R_0 \leq 1$ are given in Table 4.2.

Comparing the estimated $R_{0\text{-}MLE}$, there is a clear suggestion that $R_{0\text{-}MLE}$ decreases as τ, which is possible the stopping time, increases. The 95%-CI shifts downwards and becomes narrower with increasing stopping time. To get more insight into the time-dependent behaviour of $R_{0\text{-}MLE}$ we simulated 1000 transmission experiments consisting of two five-to-five trials, for given pairs of R_0 and τ. Trial outcomes were simulated by drawing from the marginal probability distribution of the number of contact-infections in a five-to-five trial. For each simulated outcome (x_1, x_2) the $R_{0\text{-}MLE}$ was estimated using both the FS and TS method, which resulted in two arrays of 1000 estimated R_0 from which the Mean Squared Error (MSE) was estimated. Experiments with outcome (5,5) implying that all individuals were contact infected, were excluded from the calculation of the MSE since the estimated $R_{0\text{-}MLE}$ will tend to infinity for both algorithms. This whole procedure was performed for several values of R_0 (0.5, 1.0, 2.0 and 3.5) and several values of τ (0.5, 1.0, 1.5… 3.5).

The calculated MSE are given in the top panel of Figure 4.1, and the frequencies of experiments with outcome (5,5) are given in the bottom panel. From Figure 4.1 it can be concluded that if the FS-method ($\tau = \infty$) is falsely used, thus if the final size has not been reached in all the trials, $R_{0\text{-}MLE}$ and the 95%-CI will be underestimated. This underestimation is large for small values of τ and decreases fast with increasing τ. So, if the FS estimation method is used instead of the TS method and the experimental period is relatively short $R_{0\text{-}MLE}$ will be underestimated.

Table 4.2.   All possible outcomes of a replicated five-to-five trial. The first column gives the numbers of infected individuals in each trial: x_1, x_2. The other columns give $R_{0\text{-MLE}}$, the corresponding 95% CI, and p-values for testing $H_0 = R_0 \geq 1$ and $H_0 = R_0 \leq 1$ for stopping times $\tau=0.5$, $\tau=1.0$ and $\tau=\infty$. The p-values < 0.05 are underlined.

| x_1,x_2 | $R_{0\text{-MLE 95\%-CI}}$ | | | p-value $H_0 = R_0 \geq 1$ | | | p-value $H_0 = R_0 \leq 1$ | | |
	$\tau=0.5$	$\tau=1.0$	$\tau=\infty$	$\tau=0.5$	$\tau=1.0$	$\tau=\infty$	$\tau=0.5$	$\tau=1.0$	$\tau=\infty$
0,0	$0.000_{\ 0.000\text{-}1.940}$	$0.000_{\ 0.000\text{-}1.248}$	$0.000_{\ 0.000\text{-}0.892}$	0.144	0.050	0.017	1.000	1.000	1.000
1,0	$0.510_{\ 0.013\text{-}2.958}$	$0.315_{\ 0.008\text{-}1.901}$	$0.195_{\ 0.005\text{-}1.354}$	0.421	0.189	0.068	0.856	0.950	0.983
1,1	$1.018_{\ 0.123\text{-}3.900}$	$0.623_{\ 0.076\text{-}2.501}$	$0.378_{\ 0.046\text{-}1.767}$	0.694	0.403	0.163	0.579	0.811	0.932
2,0	$1.045_{\ 0.123\text{-}3.900}$	$0.646_{\ 0.076\text{-}2.501}$	$0.394_{\ 0.046\text{-}1.767}$	0.694	0.403	0.163	0.579	0.811	0.932
2,1	$1.560_{\ 0.317\text{-}4.858}$	$0.951_{\ 0.194\text{-}3.114}$	$0.568_{\ 0.114\text{-}2.190}$	0.873	0.629	0.303	0.306	0.597	0.837
2,2	$2.120_{\ 0.568\text{-}5.901}$	$1.284_{\ 0.345\text{-}3.792}$	$0.755_{\ 0.198\text{-}2.685}$	0.959	0.810	0.476	0.127	0.371	0.697
3,0	$1.647_{\ 0.317\text{-}4.858}$	$1.024_{\ 0.194\text{-}5.114}$	$0.622_{\ 0.114\text{-}2.190}$	0.873	0.629	0.303	0.306	0.597	0.837
3,1	$2.177_{\ 0.568\text{-}5.901}$	$1.329_{\ 0.345\text{-}3.792}$	$0.790_{\ 0.198\text{-}2.685}$	0.959	0.810	0.476	0.127	0.371	0.697
3,2	$2.770_{\ 0.870\text{-}7.113}$	$1.676_{\ 0.527\text{-}4.614}$	$0.978_{\ 0.298\text{-}3.438}$	0.989	0.921	0.653	0.041	0.190	0.524
3,3	$3.478_{\ 1.228\text{-}8.564}$	$2.096_{\ 0.740\text{-}5.581}$	$1.210_{\ 0.414\text{-}4.276}$	0.998	0.974	0.801	0.010	0.079	0.347
4,0	$2.382_{\ 0.568\text{-}5.901}$	$1.503_{\ 0.345\text{-}3.792}$	$0.925_{\ 0.198\text{-}2.685}$	0.959	0.810	0.476	0.127	0.371	0.697
4,1	$2.942_{\ 0.870\text{-}7.113}$	$1.811_{\ 0.527\text{-}4.614}$	$1.092_{\ 0.298\text{-}3.438}$	0.990	0.921	0.653	0.041	0.190	0.524
4,2	$3.597_{\ 1.228\text{-}8.564}$	$2.186_{\ 0.740\text{-}5.581}$	$1.289_{\ 0.414\text{-}4.276}$	0.998	0.974	0.801	0.010	0.079	0.347
4,3	$4.418_{\ 1.654\text{-}10.430}$	$2.666_{\ 0.993\text{-}6.793}$	$1.548_{\ 0.550\text{-}5.219}$	1.000	0.994	0.905	0.002	0.026	0.199
4,4	$5.585_{\ 2.180\text{-}13.232}$	$3.364_{\ 1.306\text{-}8.587}$	$1.949_{\ 0.717\text{-}6.530}$	1.000	0.999	0.967	0.000	0.006	0.095
5,0	$3.432_{\ 0.870\text{-}7.113}$	$2.250_{\ 0.527\text{-}4.614}$	$1.480_{\ 0.298\text{-}3.438}$	0.990	0.921	0.653	0.010	0.190	0.524
5,1	$4.054_{\ 1.228\text{-}8.564}$	$2.560_{\ 0.740\text{-}5.581}$	$1.653_{\ 0.414\text{-}4.276}$	0.998	0.974	0.801	0.002	0.079	0.347
5,2	$4.849_{\ 1.654\text{-}10.430}$	$3.004_{\ 0.993\text{-}6.793}$	$1.889_{\ 0.550\text{-}5.219}$	1.000	0.994	0.905	0.000	0.026	0.199
5,3	$5.949_{\ 2.180\text{-}13.232}$	$3.646_{\ 1.306\text{-}8.587}$	$2.246_{\ 0.717\text{-}6.530}$	1.000	0.999	0.967	0.000	0.006	0.095
5,4	$7.830_{\ 2.879\text{-}19.481}$	$4.776_{\ 1.721\text{-}12.615}$	$2.923_{\ 0.939\text{-}9.493}$	1.000	1.000	0.993	0.000	0.001	0.033
5,5	$\infty_{\ 3.982\text{-}\infty}$	$\infty_{\ 2.378\text{-}\infty}$	$\infty_{\ 1.298\text{-}\infty}$	1.000	1.000	1.000	0.000	0.000	0.007
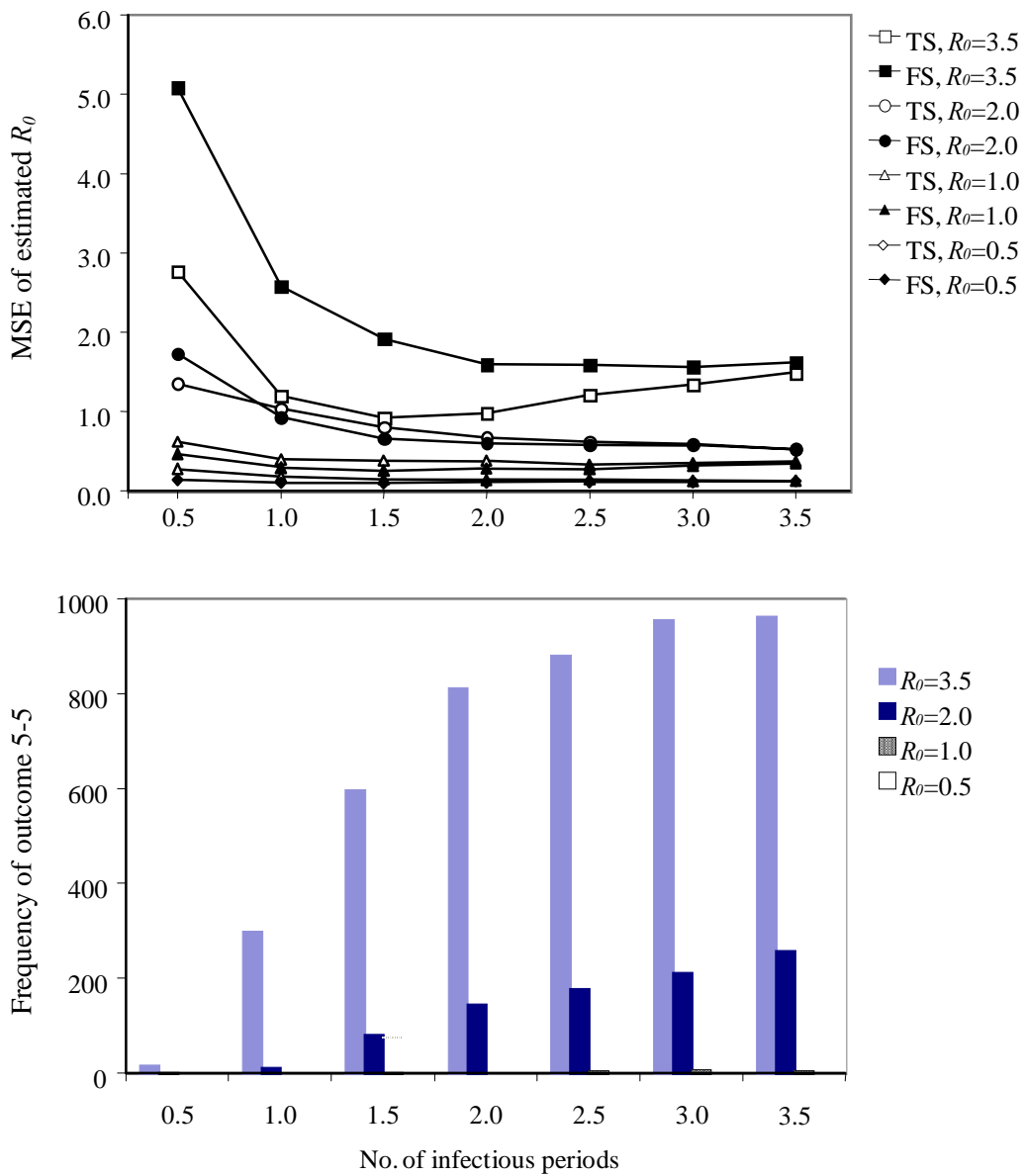
Figure 4.1. The Mean Squared Error (top panel) and the frequency of outcome 5-5 (bottom panel) as a function of the number of infectious periods τ calculated from 1000 simulated transmission experiments, consisting of two five-to-five trials, for given combinations of R_0 and τ.

The nominal p-value when testing $H_0 : R_0 \geq 1$ with the FS method is smaller than the actual p-value of the TS-method (Table 4.2), suggesting that if the FS method is used to test $H_0 : R_0 \geq 1$ (which corresponds to $\tau = \infty$) while actually the TS method should be used (e.g., $\tau = 0.5$) H_0 will more often be rejected. This would only happen for one specific outcome (0,0) in case of the five-to-five transmission experiment as presented in this paper. However, for

bigger experiments (with more individuals or more trials) this situation will become more common. So, the FS test is liberal in testing $H_0 : R_0 \geq 1$, with the result that it could wrongly be concluded that R_0 is smaller than threshold value 1.

The nominal p-value when testing $H_0 : R_0 \leq 1$ with the FS method is larger than the actual p-value of the TS-method (Table 4.2). This suggests that if the FS method is used to test $H_0 : R_0 \leq 1$ when the TS method should be used then H_0 will more often be sustained. Thus, the FS test is conservative in testing $H_0 : R_0 \leq 1$, so if with the FS test it is concluded that R_0 is larger than 1, it will also be the case if the TS algorithm is used (even when τ is small).

2.2.3.   Testing to find a difference in R_0 between two treatment groups

Using a five-to-five transmission experiment consisting of two replicated trials per treatment group to investigate whether the treatment reduces the transmission, the difference in the number of contact infections between the treatment groups (z) can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. The p-values for testing $H_0 : R_{0-control} = R_{0-treatment}$ for all possible outcomes are listed in Table 4.3 for three different stopping times τ: 0.5, 1.0 and ∞.

Table 4.3.   The p-values for testing $H_0 : R_{0-control} = R_{0-treatment}$ versus its alternative $H_1 : R_{0-control} > R_{0-treatment}$ for a five-to-five transmission experiment with two trials per treatment group. The test is based on the difference in the number of contact infections z observed at three different stopping times: τ=0.5, τ=1.0, and τ=∞. The p-values < 0.05 are underlined.

	p-value for testing $H_0 : R_{0-control} = R_{0-treatment}$		
Z	τ=0.5	τ=1.0	τ=∞
0	1.00	1.00	1.00
1	0.43	0.43	0.44
2	0.29	0.29	0.31
3	0.17	0.18	0.21
4	0.09	0.10	0.13
5	0.04	0.05	0.07
6	0.02	0.02	0.03
7	0.01	0.01	0.01
8	0.00	0.00	0.00
9	0.00	0.00	0.00
10	0.00	0.00	0.00

As can be seen in Table 4.3, the p-values between the different stopping times do not differ much, although there is a slight increase with increasing stopping time. This means that the FS test of $H_0 : R_{0-control} = R_{0-treatment}$ is just a little conservative. Only when according to the FS algorithm the p-value is slightly larger than the critical value (0.05), it is possible that when using the TS test ($\tau < \infty$) the p-value will be slightly smaller than the critical value (see $z=5$ in Table 4.3). In this case H_0 would not be rejected with the FS test, while it would be so with the TS test.

2.2.4.   *Power calculations for the TS algorithm*

Power calculations based on a one-sided test have been performed for a transmission experiment consisting of two replicated five-to-five trials per treatment group. The results for different combinations of $R_{0\text{-}control}$ and $R_{0\text{-}treatment}$ are presented in Figure 4.2.
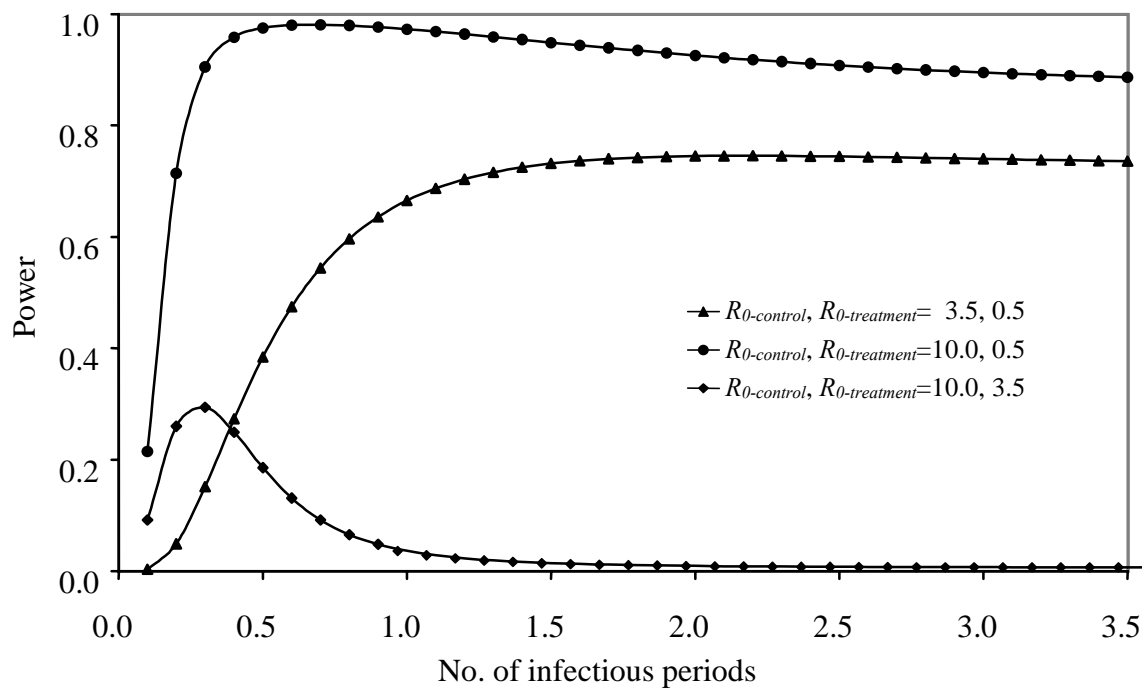

Figure 4.2.   The power of the one-sided test based on the TS algorithm to test a difference in transmission in a five-to-five transmission experiment consisting of two trials per treatment group plotted against the number of average infectious periods. Three different combinations of $R_{0\text{-}control}$, $R_{0\text{-}treatment}$ were considered.

It appears that in this particular example reasonable power (>0.80) can only be achieved if $R_{0\text{-}control}$ is much larger than 1 (e.g., 10.0), while $R_{0\text{-}treatment}$ is smaller than 1 (e.g., 0.5). The

maximal power for the combination $R_{0\text{-}control}$, $R_{0\text{-}treatment}$=10.0, 0.5 is achieved for a stopping time less than one, and for the combination $R_{0\text{-}control}$, $R_{0\text{-}treatment}$=10.0, 3.5 for a stopping time even smaller than half an average infectious period. For the combination $R_{0\text{-}control}$, $R_{0\text{-}treatment}$= 3.5, 0.5 the power seems to increase with stopping time, so there does not appear to be a maximum for intermediate stopping time. Eventually ($\tau \to \infty$) the power will asymptotically go to constant values for all scenarios.

Summarising, the maximum power is often achieved at an intermediate stopping time that is very varied. Intuitively, there are three possible scenarios if the experiment is terminated too early so that not all trials have reached a final size situation. First, the experiment is terminated before appearance of the maximum difference in the number of contact infections between the two treatment groups, consequently, the power is small. Second, the experiment is terminated after the maximum difference in the number of contact infections was reached with the result that the power is also small. Third, the experiment is terminated at the exact moment that the maximum difference in the number of contact infections is reached, so in this case the power is largest.

3. NEW TEST TO FIND A DIFFERENCE IN TRANSMISSION

3.1. Motivation for developing a new test

Although the TS method would theoretically be useful for the quantification of transmission, there is still a problem: the duration of the experiment expressed in units of the average infectious period must be known. The average infectious period $1/\alpha$ is unknown for most infections. Also unknown is whether the average infectious period of an infection is exponentially distributed, which is assumed in the stochastic SIR model. Another disadvantage of using a method based on the TS or FS algorithm for the statistical analysis of transmission experiments is that only a part of the information on the infection chain is used, namely, only the number of contact infections observed at the end of the experiment. This led to the development of a new test to find a difference in the number of contact infections in the course of the transmission experiment.

A difference in the number of contact infections between two treatment groups observed in the course of local epidemics or infection chains can have disappeared at the end of the experiment. In this situation both R_0 are probably larger than 1, but are different. It implies a difference in speed at which the final sizes of the local epidemics were reached. Results of this nature imply that a treatment tends to reduce the transmission – given that the moment of reaching the final size situation is in the treatment group than the control group – although not

enough to produce an R_0 smaller than 1. However, the observed reduction indicates that the transmission can be reduced, with the next step being to test whether a combination of treatments, e.g., two vaccinations instead of one, might result in a R_0 smaller than 1.

An intuitively more appropriate test statistic than the number of contact infections observed at the end of the experiment would be the maximum difference in the number of contact infections between the two treatment groups observed in the course of the experiment (*MaxDiff*). A test based on this statistic is described below together with its usefulness by comparison between power calculations and that of other tests.

3.2.   MCMC simulations of a five-to-five transmission experiment

The test statistic used in the *MaxDiff* test is the maximum difference in the number of contact infections that has occurred somewhere during the transmission experiment (we will call this the maximum difference): $MaxDiff = MAX\left(K_{control} - K_{treatment}\right)$. The probability distribution of *MaxDiff* can be determined by using the Markov Chain Monte Carlo (MCMC) technique (Gilks *et al.* 1996). With the MCMC technique a five-to-five transmission experiment consisting of two control and two treatment trials is simulated. A Markov Chain model describes the infection chain in each trial. The transmission dynamics follow the stochastic SIR model, which means that the infection rate equals $R_0 si / N$, and the recovery rate equals i. Interval Δt is set to 0.002 average infectious period so that only one infection or recovery event can occur in the same interval.

The four independent infection chains of the four five-to-five trials are generated by simulations, each time assuming a certain level of transmission in each treatment group ($R_{0\text{-}control}$, $R_{0\text{-}treatment}$), an interval between two subsequent observation moments or samplings and a length of the experimental period. From these four simulated infection chains the maximum difference was determined in the number of contact infections between two treatment groups, i.e., *MaxDiff*, the first moment at which *MaxDiff* was observed, and the longest sequence of successive intervals in which *MaxDiff* was observed.

3.3.   Probability distribution of *MaxDiff* and the critical region

The probability distribution of *MaxDiff* varying between -10 and 10 was obtained by doing 10,000 simulations of a transmission experiment under $H_0 : R_{0-control} = R_{0-treatment}$ for several values of R_0. The fraction of simulations showing a particular *MaxDiff* as being the maximum difference in the number of contact infections between the two treatment groups, represents the probability of observing that particular value of *MaxDiff* for given R_0. The interval between two subsequent observation moments or samplings was set at 0.1 average

infectious period, and the length of the experimental period was set at 8 infectious periods. This procedure was followed for many different values of R_0, from 0.5 to 13.0 with steps of 0.5. The contour plot of the probability distribution of *MaxDiff* is given in Figure 4.3. The probability distribution of *MaxDiff* is obviously bimodal, and the probability of observing a high or a very small difference under H_0 is very small (<0.05), whereas the probability of observing moderate values of *MaxDiff* is greater.
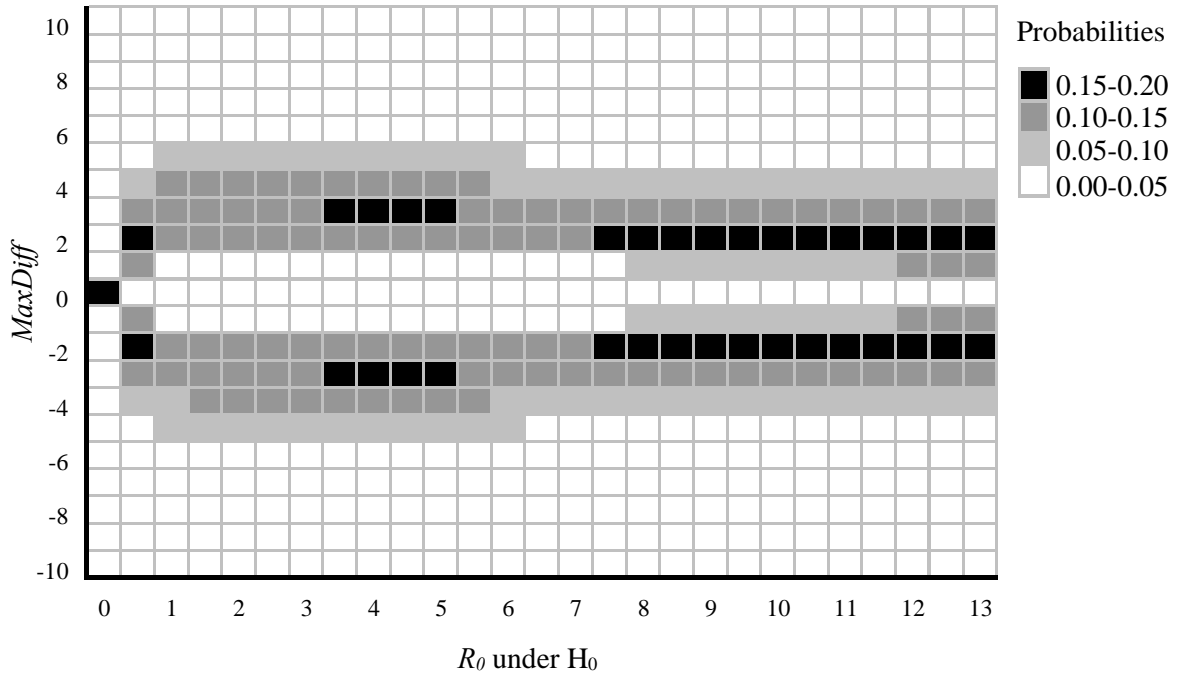

Figure 4.3. The probability distribution of *MaxDiff* for different values of R_0 under the null hypothesis $H_0 : R_{0-control} = R_{0-treatment}$

Based on the probability distribution of *MaxDiff*, one-sided and two-sided tests can be performed to find a difference in transmission between the control and the treatment group. A one-sided test of the null hypothesis, $H_0 : R_{0-control} = R_{0-treatment}$ against its alternative, $H_1 : R_{0-control} > R_{0-treatment}$ is considered, i.e. only to ascertain a possible decreasing effect of the treatment on the transmission. To test this hypothesis, the region in which observed values of *MaxDiff* are considered significant, i.e., the critical region of the test, was calculated for a 5% significant level and for the same set of R_0 as in Table 4.4. The critical regions of the test for this particular five-to-five transmission experiment presented in Figure 4.4 show that the critical region of the test is smallest for intermediate values of R_0 (1.5-2.5). The practical

problem is that a critical region has to be chosen for a common value of R_0 that is usually unknown. The most conservative critical region to choose is the minimal region over all common values of R_0. This test is conservative in the sense that incorrect significant results are found in less than five percent of the cases. However, the advantage is that if a significant result is found it can be trusted.
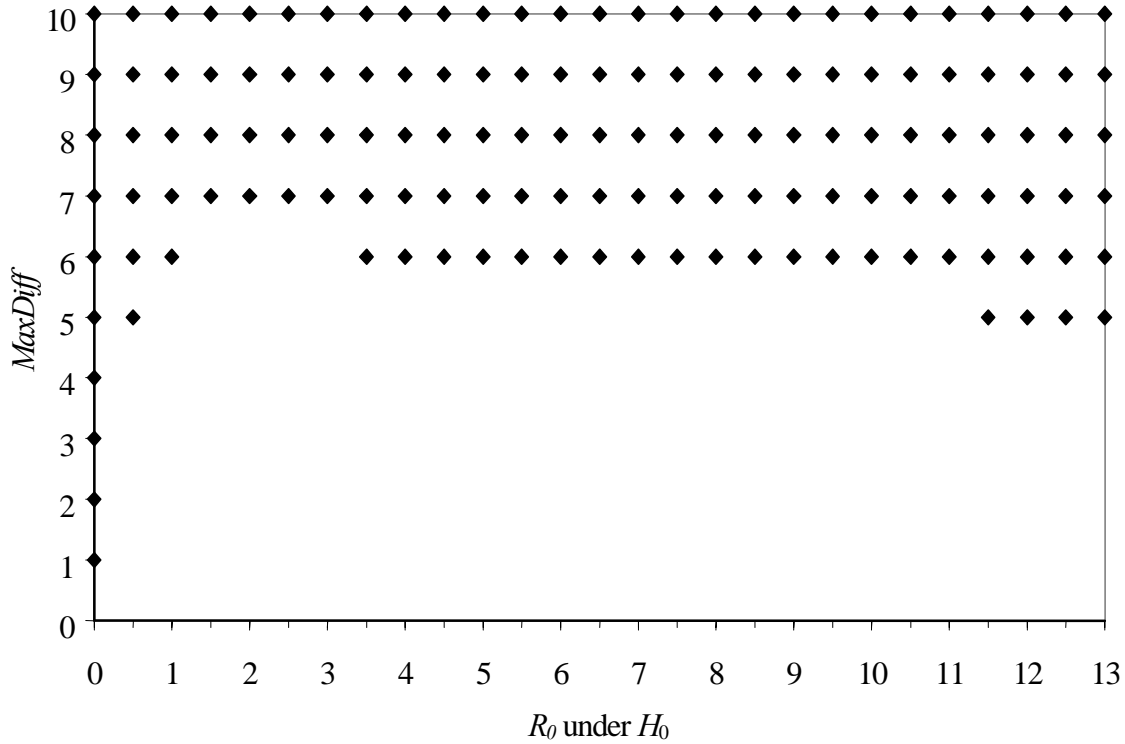

Figure 4.4. The critical region in which an observed *MaxDiff* is considered significant ($\blacklozenge$) at error level 0.05.

3.4. Power calculations

One of the main considerations in choosing a test is the power of the test. The power of the *MaxDiff* test is determined as the fraction of 10,000 simulated five-to-five transmission experiments with a significant result (*MaxDiff*>6) under the alternative hypothesis, i.e., for a given combination of $R_{0\text{-}control}$, $R_{0\text{-}treatment}$. The test is randomised by assigning each simulated outcome *MaxDiff*=6 as significant with a probability of 0.511 which is calculated as follows: $1 - (p(6) - 0.05)/(p(6) - p(7))$, where p(6) and p(7) are the p-values of *MaxDiff*=6 and *MaxDiff*=7, which are 0.0698 and 0.0293, respectively.

In Table 4.4 the calculated powers of the *MaxDiff* test are given for several combinations of $R_{0\text{-control}}$, $R_{0\text{-treatment}}$ and for several sampling intervals. Different sampling intervals are considered, since it is highly likely that a significant result is overlooked because of its brief presence. Therefore, (i) the length of the period in which *MaxDiff* could have been observed, and (ii) the first moment at which *MaxDiff* occurs, and (iii) the fraction of simulations in which the first period in which *MaxDiff* is observed equals the longest such period, are given in Table 4.4 too.

Table 4.4. Power calculations of the different tests to find a difference in transmission between two treatment groups applied on data of a five-to-five transmission experiment with two trials per treatment.

	Sampling interval[a]	$R_{0\text{-control}}$, $R_{0\text{-treatment}}$					
		1.5, 0.5	3.5, 0.5	3.5, 1.5	10.0, 0.5	10.0, 1.5	10.0, 3.5
Power FS		0.276	0.725	0.140	0.871	0.203	0.006
Power MaxDiff	0.50	0.263	0.775	0.265	0.982	0.695	0.140
	0.25	0.272	0.787	0.290	0.986	0.782	0.271
	0.10	0.271	0.797	0.317	0.990	0.827	0.351
	0.01	0.272	0.799	0.330	0.993	0.861	0.433
Power TS[b]		0.276	0.746	0.351	0.995	0.882	0.466
Power GLM	0.50	0.493	0.872	0.792	0.982	0.992	0.683
	0.25	0.498	0.863	0.809	0.929	0.993	0.858
	0.10	0.484	0.851	0.807	0.890	0.992	0.915
	0.01	0.486	0.859	0.805	0.894	0.991	0.928
Moment TS-max		∞	2.16	1.00	0.66	0.45	0.30
First moment *MaxDiff*		1.700	1.355	0.969	0.574	0.482	0.383
Length period *MaxDiff*		3.665	3.553	1.570	2.421	0.548	0.201
Variation length period		7.485	8.597	6.072	9.781	2.301	0.741
Fraction First=Longest[c]		0.765	0.777	0.559	0.791	0.581	0.399

[a] The interval between two subsequent observations or samplings used in the 10,000 simulations. Expressed in numbers of average infectious periods.
[b] The maximum calculated power according to the TS algorithm.
[c] The fraction of simulations in which the first period where *MaxDiff* is observed is also the longest period where *MaxDiff* is observed.

The usefulness of the new *MaxDiff* test can be ascertained by comparing its power to that of other (common) tests. Besides the two tests based on the FS and TS algorithms, there exists another slightly different type of test that is based on a Generalised Linear Model (GLM test). The GLM method is described in detail by Becker (1989) and an adapted version for use on transmission experiments is described by Velthuis *et al.* (2002). The GLM uses the whole course of the experimental epidemic or infection chain to estimate the transmission parameter

β (see Equation 3), and to test the effect of experimental treatments or variables on β. Data needed for the GLM method are the numbers of cases that have occurred in the period during two successive samplings, the number of susceptible individuals at the start of the period, and the average number or fraction of infectious individuals present in the previous period.

To calculate the power of the GLM test, each simulated infection chain was transformed to the appropriate data structure. From these data the infection parameter β and its 95%-CI were estimated for each treatment group ($\beta_{control}$ and $\beta_{treatment}$) in each simulated transmission experiment. If the 95%-CIs of $\beta_{control}$ and $\beta_{treatment}$ did not overlap, it was concluded that the difference in transmission between the two treatment groups was significant. Like with the power of the *MaxDiff* test, the power of the GLM equals the fraction of 10,000 simulated transmission experiments that gave a significant result.

Table 4.4 gives the calculated powers of the different tests for several combinations of $R_{0-control}$, $R_{0-treatment}$ and for several sampling intervals. The *MaxDiff* test is generally more powerful than the FS test, and should be preferred. The power of the *MaxDiff* test is generally less than the maximum power of the TS test, but will approach the maximum power if the sampling interval becomes extremely small. The power of the GLM test is large for almost all combinations of $R_{0-control}$, $R_{0-treatment}$.

The power of the *MaxDiff* test for the five-to-five transmission experiments, described here, is only sufficiently large (>0.80) if $R_{0-control}$, $R_{0-treatment}$ is 10.0, 0.5 or if $R_{0-control}$, $R_{0-treatment}$ is 10.0, 1.5 with a sampling interval of 0.01. Slightly smaller powers (>0.70) are obtained for the combinations 3.5, 0.5 and 10.0, 1.5. The power increases with the difference between $R_{0-control}$ and $R_{0-treatment}$, but is insufficient in all but one scenario where $R_{0-control}$ and $R_{0-treatment}$ are both greater than one. The sampling interval (in the range of 0.01 till 0.50 average infectious periods) has only a small influence on the power of the *MaxDiff* test when both $R_{0-control}$ and $R_{0-treatment}$ are small, whereas it has more influence when $R_{0-control}$ and $R_{0-treatment}$. The biggest influence is observed for the combinations 10.0, 1.5 and 10.0, 3.5, which can be explained by the on average small periods in which *MaxDiff* can be observed, 0.538 and 0.150 respectively.

4. DISCUSSION

In this paper we investigated the error made when quantifying transmission from data of transmission experiments on basis of FS assumptions, when the final size has not been reached in all trials. Furthermore, we present a new method to detect a difference in transmission between two treatment groups, and a comparison of this new method with existing quantification methods.

Thus far, quantification of the reproduction number from transmission experiments has relied almost exclusively on methods based on the FS assumption. This is not surprising since the FS assumption leads to methods that are easy to understand and readily implemented on a personal computer, while the computational burden involved in methods based on the TS algorithm quickly becomes insurmountable as the size of the population increases. On the other hand, the applicability of the FS methods is not always warranted, as the epidemic process may not have ended in one or more of the trials at the time that the experiment is terminated. The FS assumption may be justified for viral infections with relatively fast transmission dynamics, but the transmission dynamics of many bacterial infections are much slower and more variable so that it is less likely that the FS assumption is fulfilled at the end of the experiment.

The results show that R_0 is underestimated under violation of the FS assumptions. If the experimental period is short relative to the average infectious period, the extent of underestimation is substantial. If, on the other hand, the experimental period is relatively long, R_0 will only be slightly underestimated. This result was expected intuitively since the number of observed contact infections could still grow when a final size situation has not been reached.

Furthermore, it was shown that the FS test is liberal in testing $H_0 : R_0 \geq 1$ against its alternative $H_1 : R_0 < 1$. Thus, the actual size of the FS test may well be larger than nominal. The implication of this result is that the FS method overestimates the possibility of eradication. Also, it was shown that the FS test is conservative in testing $H_0 : R_0 \leq 1$ against its alternative $H_1 : R_0 > 1$. This means that the FS algorithm can safely be used for testing if R_0 is larger than its threshold value 1.

The FS test is slightly conservative in testing $H_0 : R_{0-control} = R_{0-treatment}$ against the alternative $H_1 : R_{0-control} > R_{0-treatment}$ if the final size has not actually been reached. For some differences in the number of contact infections the calculated p-value of the FS test came up slightly greater than the p-value calculated under the TS assumptions. The p-values for all differences are identical in both the FS and TS test in case of one-to-one experiments. The reason for this is that the likelihood function of this test in a one-to-one experiment depends on R_0 and time only through the binomial success probability (see Velthuis et $al.$ 2002). This does not apply to the more complicated likelihood functions of a five-to-five experiment. Since the FS test is somewhat conservative in finding a difference, it is still a very powerful test to use for the analysis of experiments where not all trials have reached a final size situation.

The second goal of this paper was to present a new test of equality of transmission for two treatment groups, i.e. to find a difference in transmission, and to compare this new test with methods commonly in use. The new test is based on the probability distribution of the maximum difference in number of contact infections between the two treatment groups observed in the course of the experiment, which we call *MaxDiff*. This probability distribution is generated using a MCMC technique and its accuracy depends, therefore, on the number of simulations on which the distribution is based, which in this case was 10,000.

One difference between the *MaxDiff* test and the tests based on the FS or TS assumptions is that the input is obviously different. The input needed for the *MaxDiff* test is the maximum difference in the number of contact infections observed somewhere during the experiment, while the input needed for FS and TS tests require only the numbers of contact infections at termination of the experiment. So both the FS and TS tests potentially ignore appreciable information, especially when $R_{0\text{-}control}$ and $R_{0\text{-}treatment}$ are larger than 1 and differ considerably.

The probability distribution of *MaxDiff* under the null hypothesis $H0 : R_{0-control} = R_{0-treatment}$ is bimodal. The explanation for this phenomenon is that if R_0 under H_0 is small, it is most improbable that a contact infection occurs in both groups during exactly the same sampling interval so that a positive difference in the number of contact infections is likely to occur. Similarly, if R_0 under H_0 is big, it is highly unlikely that exactly the same epidemics occur in both groups. So, in this situation a difference in the number of contact infections is likely to occur. Hence, there is little probability of observing no difference, whereas the probability observing a small difference is high. If *MaxDiff* is zero while a number of contact infections has been observed in both treatment groups, the assumed model, which in this case is the stochastic SIR model, should be reconsidered. This is because in the stochastic SIR model the outcome of *Maxdiff* equal to zero has always a very low probability.

The power of the *MaxDiff* test increases with the sampling intensity. In the range of the sampling intervals that we have investigated (which are 0.01, 0.1, 0.25 and 0.50 average infectious period) the influence is small. It is advisable to have an intensive sampling scheme provided it does not interfere with the transmission dynamics. The power of the *MaxDiff* test is greater than the power of the FS test, and less than the maximum power of the TS test and the GLM test. This is not surprising since the power of a test is related to the amount of information used as input. The GLM test uses the most information of the infection chain, the FS test the least information and the *MaxDiff* and TS tests somewhere in between. Note, the presented power of the TS test is the maximum power at a certain moment. This power will correspond to the power of the *MaxDiff* test in which an extremely intensive sampling scheme is assumed. So, the *MaxDiff* test is a better test to use than the FS or the TS test, especially if both $R_{0\text{-}control}$ and $R_{0\text{-}treatment}$ are larger than 1. In our example of a five-to-five experiment the

power of the *MaxDiff* test is unsatisfactory, however the power will increase rapidly with increasing numbers of trials per treatment group.

As mentioned above, each test needs a different amount of input. The information needed for the FS test is the number of infected individuals ultimately infected in the transmission experiment. The information needed for the TS test is again the number of infected individuals ultimately infected, but also the average length of the infectious period, which is often unknown. Information needed for using the *MaxDiff* test is the number of infected individuals in the course of each trial, so that it needs a more intensive sampling scheme than required for the tests based on the FS or TS algorithms. However, the average length of the infectious period does not need to be known for the *MaxDiff* test. For the GLM test the information needed is most specific, namely, the moment at which an individual becomes infectious must be known, which is sometimes hard to determine.

All tests are based on the stochastic SIR model, however, other transmission models can be considered if the transmission dynamics of the infection that needs to be quantified differs substantially from the SIR dynamics. The practical feasibility of the tests could be affected by using another transmission model.

The different tests are also based on different additional assumptions besides the assumptions of the stochastic SIR model. The FS algorithm is based on the final size assumption, while the TS test, the *MaxDiff* test and the GLM test are not. The GLM test, on the other hand, requires that the infection or recovery events in two different periods are stochastically independent, which might be disputable.

An extra advantage of using the *MaxDiff* test is that the transmission experiment can be terminated at the moment a significant difference in the number of contact infections has been reached, which is not possible with the other tests. This would be an advantage from an animal welfare point of view, especially if the individuals become very diseased, and it would be an advantage from a economical point of view since it would reduce the costs of the experiments. Note, when an experiment is stopped preliminary, information might be missed needed to estimate transmission parameters.

In this paper some exploration of the error made when using the FS test even though the final size situation has not yet been reached was based on a five-to-five experiment consisting of two identical trials per treatment group. Similar conclusions were drawn for one-to-one experiments in the paper of Velthuis *et al.* (2002). We think that these conclusions are also intuitively feasible for transmission experiments with different numbers of animals per trial and different numbers of trials. More research is needed to confirm this extrapolation. Furthermore, we did not investigate the robustness of the different tests in relation to more

heterogeneity in the study population than assumed, which might be subject for future research.

5. ACKNOWLEDGEMENTS

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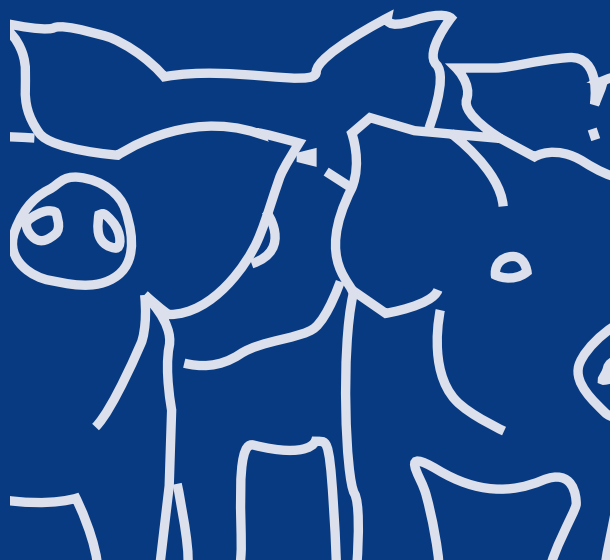
Design and analysis of an *Actinobacillus pleuropneumoniae* transmission experiment

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SUMMARY

To quantify experimentally the transmission of *Actinobacillus pleuropneumoniae* from subclinically infected carriers to susceptible contact pigs and to test the effect of possible interventions on the transmission, an experimental design with accompanying statistical method is needed. This paper includes the design of an *A. pleuropneumoniae* transmission experiment, and a method with which the transmission can be quantified and with which it is possible to test the effect of an intervention on the transmission. The experimental design consists of two parts. First, sub clinically infected carrier pigs are created by contact exposure of specific-pathogen-free pigs to endobronchially inoculated pigs. Second, transmission is observed from the group of carrier pigs to a second group of susceptible contact pigs after replacing the inoculated pigs by new contact pigs. The presented analysis method is a generalised linear model with which the effect of an intervention on the susceptibility and on the infectivity can be tested separately, if the transmission is observed in heterogeneous populations. The concept of the experimental design and the analysis method is illustrated by describing an *A. pleuropneumoniae* transmission experiment in which the effect of vaccination on the susceptibility is quantified. In this, it was shown that nasal excretion of *A. pleuropneumoniae* is related to infectivity, however the effect of vaccination on the susceptibility of pigs to become infected has not been demonstrated.

1. INTRODUCTION

Porcine pleuropneumonia due to *Actinobacillus pleuropneumoniae* causes great economic losses in the swine industry world wide (Hunneman 1986). Several studies have demonstrated an effect of vaccination on the interaction between bacteria and host (Oishi *et al.* 1995; Furesz *et al.* 1997; Magnusson *et al.* 1997; Wongnarkpet *et al.* 1999; Goethe *et al.* 2000; Savoye *et al.* 2000; Van Overbeke *et al.* 2001). Generally, the effect of vaccination on the development of clinical signs or on the immune response after an *A. pleuropneumoniae* infection was studied. However, these studies do not shed light on how vaccination affects the transmission of *A. pleuropneumoniae*, as they focus on characteristics of the infection that are important for the individual pig only. When vaccination has to be assessed as a tool for eradication it is important to know the effect of vaccination on the spread – or transmission – of the bacteria.

Direct transmission of *A. pleuropneumoniae* from pig to pig is believed to be the predominant transmission route, since the bacterium does not survive long in the environment (Nicolet 1992). Furthermore, it is believed that subclinically infected carriers are important in the transmission of *A. pleuropneumoniae* (Savoye *et al.* 2000). Therefore, prevention or reduction of direct transmission from subclinically infected carriers to susceptible pigs by an intervention – like vaccination – may lead to eradication of *A. pleuropneumoniae* from a population. However, it is unknown whether such feasible interventions can reduce the transmission of *A. pleuropneumoniae* among pigs.

The effect of interventions on the transmission can be experimentally investigated by doing transmission experiments. A transmission experiment consists often of one or more trials in which each of which the transmission is studied among a group of treated or among a group of untreated animals. At the start of each trial some infectious and some susceptible animals are housed together. The transmission of a pathogen can be estimated from the number of contact-infections that occurred during the trials, with a method that is based on a mathematical model (Kroese and De Jong 2001). The effect of an intervention can be quantified by comparing the number of contact infections in the trials with treated animals to the trials with untreated animals.

Transmission experiments have already proven to be useful in testing the effect of interventions on viral transmission, e.g. pseudorabies virus in pigs (De Jong and Kimman 1994; Bouma *et al.* 1996; Bouma *et al.* 1997a; Bouma *et al.* 1997b; Van Nes *et al.* 2001), Porcine reproductive and respiratory syndrome virus in pigs (Nodelijk *et al.* 2001) and bovine herpesvirus in cattle (Mars *et al.* 2000). However, as yet, no attempts have been made to evaluate the effect of interventions on the spread of bacterial infections like *A. pleuropneumoniae*. To test whether a possible intervention can reduce the transmission of *A. pleuropneumoniae* a transmission experiment is considered to be a useful tool.

A difficulty with bacterial infections like *A. pleuropneumoniae* is that it is not clear when animals are infectious, which is important to know for the quantification of transmission. Furthermore, the length of the infectious period is unclear for most bacterial infections. In consequence, it is unknown in bacterial transmission trials whether the infection chain has ended before the trials are stopped. Most methods to quantify transmission are based on the assumption that the infection chain has ended before the trials are stopped, which often applies for viral transmission trials (De Jong and Kimman 1994; Kroese and De Jong 2001). Since this assumption is not always applicable for bacterial transmission experiments other estimation methods than the traditional ones should be considered to analyse the data.

In this paper, a design for a transmission experiment to study the transmission of *A. pleuropneumoniae* is presented. This transmission experiment is a so-called extended transmission experiment in which the transmission is observed from a group of subclinically infected carrier pigs that were contact-infected (C_1-pigs) by endobronchially inoculated pigs (E-pigs) to a group of susceptible contact-exposed pigs (C_2-pigs), which resembles the transmission in the field. For the analysis of the experimental data we present a quantification method that is based on a Generalised Linear Model (GLM). With this method it is possible to quantify the effect on the susceptibility (i.e. the probability to become infected and infectious) and on the infectivity (i.e. the probability to infect another animal) separately.

To illustrate the practical implications of the presented design and analysis, data of a conducted transmission experiment with *A. pleuropneumoniae* serotype 9 consisting of four trials were used. In two trials the contact-exposed C_2-pigs were vaccinated, whereas the C_2-pigs in the other two trials were not vaccinated like the inoculated and carrier pigs in all four trials. As a result of the fact that only the C_2-pigs were vaccinated only the effect of vaccination on susceptibility could be tested.

2. EXPERIMENTAL DESIGN

2.1. Development history

In order to make the actual design more comprehensible we first sketch briefly the history that led to the design. From this history it will become clear which aspects of the design have been adjusted before getting at the experimental design that is suitable to study *A. pleuropneumoniae* transmission.

First, we want to say something about the requirements for a successful design. In the mathematical model on which the statistical analysis is based, the population in which the infection chain is observed is considered to be homogeneous (Bailey 1975). In other words,

all susceptible animals should be equally susceptible, and all infectious animals should be equally infectious, whether inoculated or contact-infected. Furthermore, in the case of *A. pleuropneumoniae* the transmission among clinically inapparent pigs is considered pivotal, because it cannot be observed in the field by clinical inspection only and is therefore difficult to control. For this reason we decided to study the transmission from subclinically infected carriers to susceptible pigs.

In the first four transmission trials, in which five pigs were intranasally inoculated and five pigs were contact-exposed, a broad range of effects was observed. Either a lot of transmission was observed or no transmission at all: some E-pigs got (per-) acute pleuropneumonia and others were not infected at all (Velthuis *et al.* Accepted). Moreover, all contact infected pigs stayed clinically inapparant after infection, whereas some inoculated pigs became diseased, which demonstrated that the 'naturally' infected pigs were different from the inoculated pigs, i.e., the infectious state was not homogeneous.

At this point we decided that artificial inoculation was not the appropriate way to induce a repeatable infectious but clinically inapparant state, and we switched to an extended transmission experiment (Bouma *et al.* 1997b). A schematic overview is given in Figure 5.1. In an extended transmission experiment infectious pigs are created by contact exposing susceptible C_1-pigs to artificially inoculated E-pigs. On the day when enough C_1-pigs are infected, the inoculated E-pigs are removed and replaced by a second group of susceptible C_2-pigs. This day is called the replacement day.

The first extended transmission experiment (consisting of two trials) started with intranasally inoculating five pigs per trial. All E-pigs in trial one became infected (which means that *A. pleuropneumoniae* was isolated from nasal or tonsillar swabs), and transmitted the infection to the five C_1-pigs within seven days. In trial two only one E-pig became infected whereas no C_1-pig. Since we had decided in advance to extend both trials simultaneously, the C_1-pigs in trial one had stopped being infectious before the replacement day – which was day 15 – so that no further contact infections were observed in the C_2-pigs.

In the next extended transmission experiment (consisting of two trials) we decided to extend the trials independently and not simultaneously. In trial one none of the intranasally inoculated E-pigs became infected, whereas in trial two four of the five E-pigs became infected and they transmitted the bacterium to all C_1- pigs before day 14. This trial was extended on day 15 and all C_2-pigs were infected before day 21. As there was still a group of susceptible contact pigs available – waiting for contact infections in trial one – we extended trial two for a second time on day 25. All pigs in the third group of contact pigs (C_3-pigs) were infected within 3 days, and no clinical disease was observed in the C_1, C_2 or C_3 pigs.

Because in some trials no infection chain or only a short infection chain – a so-called minor outbreak – was observed we concluded that intranasal inoculation was an inadequate method to start an *A. pleuropneumoniae* infection chain and we switched to endobronchial inoculation. At a later stage the probability to become infectious due to intranasal inoculation was quantified to be only 0.34 (Velthuis *et al.* 2000). In both trials of the next extended transmission experiment, the endobronchially inoculated E-pigs were able to transmit the bacterium to the C_1-pigs, although in trial one only three E-pigs became infectious after inoculation and only three C_1-pigs were contact-infected. Consequently, this trial was not successful after the replacement day (day 7), since none of the C_2-pigs became infected (probably due to the fact that the infectious C_1-pigs stopped being infectious before replacement). Trial two was very successful and all C_2-pigs were infected 7 days after the replacement day (day 4).

The moment of replacement had to be optimised, and therefore the design was adjusted again in the following way. The decision to replace the E-pigs by C_2-pigs was not based on '*A. pleuropneumoniae* isolated from most contact pigs' as it was until this moment, but based on 'at least 40 colonies *A. pleuropneumoniae* isolated from a swab of four contact pigs'. This criteria was based on the observation that when about more than 40 colonies were isolated from the tonsillar or nasal swab on one day, mostly a lot of colonies *A. pleuropneumoniae* will be isolated on the subsequent days (unpublished results). In the next extended transmission experiment, consisting of two trials and in the two control trials that are described in this paper, the induced infection chains developed very well and were reproducible. The replacement days were days 7, 5, 6 and 3, respectively, and all C_2-pigs were infected within, 9, 5, 12 and 11 days after replacement, respectively. In the next section the design of the *A. pleuropneumoniae* transmission experiment, which we found to be most appropriate to study the transmission of *A. pleuropneumoniae* is described.

2.2. Experimental design

The design of our *A. pleuropneumoniae* transmission experiment is a modification of the design of the so-called extended transmission experiment that was described by Bouma *et al.* (1997b). The experiment consists of a number of trials, in which the transmission from five infectious carrier pigs to five susceptible contact pigs is observed (a five-to-five trial). The number of ten pigs per trial is chosen because when conducting two of such trials per treatment group it has satisfactory power to find a meaningful difference in transmission, and secondly it resembles the situation in the field where pigs are often housed in groups of ten. For an extended five-to-five trial, three groups of five six-week-old, specific-pathogen-free pigs are needed. A schematic overview of a trial is given in Figure 5.1. The pigs are randomly

assigned to the different groups of each trial (E-pigs, C_1-pigs or C_2-pigs). One week before the start of each trial, the E-pigs and the C_1-pigs are housed together in an isolation unit, whereas the C_2-pigs are housed separately. On day zero, at the age of 6 to 7 weeks, the E-pigs and C_1-pigs are separated, by transferring the C_1-pigs to another unit. The remaining five E-pigs are endobronchially inoculated with 10-ml inoculum containing 10^3 colony forming units *A. pleuropneumoniae* serotype 9 (Van Leengoed and Kamp 1989). Briefly, the pigs are sedated and are restrained with their heads in a vertical position. A long sterile catheter is entered deep into the lungs, and the inoculum is injected. From an animal welfare point of view we decided that when an inoculated pig shows severe clinical symptoms of pleuropneumonia it would be treated with analgetic and antiphlogistic means, e.g. flunixine meglumine.

△ ▲ inoculated (E) pigs
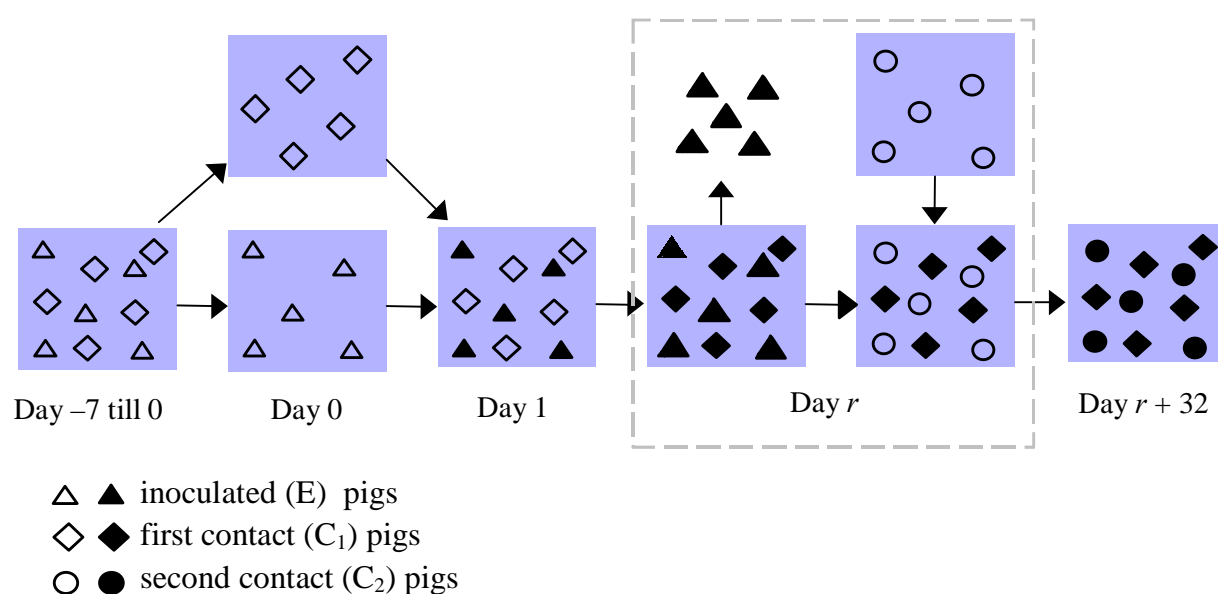◇ ◆ first contact (C_1) pigs
○ ● second contact (C_2) pigs

Figure 5.1. A schematic overview of the experimental design of an extended five-to-five trial. The open symbols represent non-infectious pigs whereas the black symbols represent the infectious pigs. At the replacement day *r* the E-pigs were replaced by C_2-pigs.

On day one, the transferred C_1-pigs are placed back so that they will be contact-exposed to the E-pigs. At the replacement day – when at least four C_1-pigs are cultured positive for *A. pleuropneumoniae*, which means that at least 40 colonies *A. pleuropneumoniae* are isolated from a tonsillar or nasal swab until that moment – the E-pigs will be removed from the trial and replaced by the C_2-pigs. The sampling procedure and the bacteriological examination of

the samples will be described in subsection bacteriology. From the replacement day on, the C_1-pigs and C_2-pigs remain together in the trial for about five weeks.

The infection chain before and after the replacement day should be monitored in detail. This can be done by (semi-) quantitative bacteriology on nasal and tonsillar swabs, which is described in the next section. All pigs were housed at about 25°C, with *ad libitum* feeding and drinking, and at a density of one pig per 0.85 m^2, which is similar to field conditions.

2.3. Bacteriology

Nasal and tonsillar swabs are taken and examined bacteriologically during the trials to determine whether E-pigs excrete *A. pleuropneumoniae* and whether C_1- and C_2-pigs have become infected. The swabs are taken daily until at least one week after the replacement day and subsequently at least three times a week for the rest of the experimental period. The swabs are streaked out directly on a specific plate that contains Heart Infusion Agar supplemented with 5% sheep blood, 0.2% Nicotinamide Adenine Dinucleotide, 0.75 µg/ml Clindamycin-HCl, 0.75 µg/ml Gentamycin, 4 µg/ml Vancomycin-HCl, 35 µg/ml Amphothericine B (Sigma, St. Louis, MO) (CGVA-plate). Subsequently, the swab is suspended in 3-ml saline solution and a 1:100 dilution of this suspension is made. The suspensions and its dilution are inoculated on two other plates. After 24 hours the number of suspected colonies is counted or estimated if there are too many. To confirm that the colonies are *A. pleuropneumoniae*, one typical colony per plate is tested for satellite growth and one for specific anti-serotype-9 serum agglutination. After *A. pleuropneumoniae* is confirmed, the number of suspected colonies counted on both the swabbed plate and the plate with the 3-ml suspension is used for the decision to extend the trial, and for further statistical analysis.

All pigs are examined at necropsy where the palatine tonsil and if present pneumonic parts of the lungs are collected for bacteriological examination. These specimens are analysed with the same bacteriological procedures as used for the swabs (Velthuis *et al.* Accepted). Briefly, they are added to 3-ml saline suspension and mingled, where after different dilutions are inoculated on CGVA-plates. The number of suspected colonies are counted and confirmed as described for the swabs.

2.4. Number of trials

The effect of an intervention on the transmission can be investigated in a transmission experiment consisting of several trials in each of which transmission is studied either among treated animals or among untreated animals. The number of trials needed per treatment group depends on the calculated power, which is defined as the probability to find a difference in

transmission between the treatment and control group given that there is a difference. The power is calculated by adding all probabilities (based on a final size situation) where the differences in the number of contact infections between the control and treatment group is significant (at 0.05 error rate) given a certain level of transmission in each treatment group. The level of transmission is in this case expressed as the reproduction ratio (R_0), which is defined as the average number of cases caused by one infectious individual during its infectious period in a large population of susceptible individuals. For power calculations it is often assumed that the reproduction ratio in the control group ($R_{0-control}$) is far above 1, whereas the reproduction ratio in the treatment group ($R_{0-treatment}$) is below 1. Based on the given reproduction ratios, $R_{0-control}=10$ and $R_{0-treatment}=0.5$, the minimal number of replicated trials per treatment group is calculated be two to have enough power (0.89) to find a difference. Thus, to test the effect of an intervention on the transmission at least four trials should be conducted, of which two trials serve as the treatment group and the other two as the control group.

2.5. Infection status of the pigs

For the analysis of data from transmission experiments it is essential to determine the infection status – susceptible or infectious – of the individual pigs during the trials. At the start of the trials all pigs are assumed to be susceptible, since the pigs are obtained from an *A. pleuropneumoniae* free SPF-herd. The onset of the infectious state is more difficult to determine. In a previous study (Velthuis *et al.* Accepted) it was shown that pigs with an *A. pleuropneumoniae* culture positive tonsil at necropsy had been infectious during the trials. We assume that the start of the infectious period of an infectious pig (with an *A. pleuropneumoniae* culture positive tonsil at necropsy) is the first day at which *A. pleuropneumoniae* is isolated from a nasal or tonsillar swab taken from it. By this assumption it is possible to determine for each day the numbers of infectious and susceptible animals, which is necessary for the statistical analysis.

3. STATISTICAL ANALYSIS

3.1. Generalised Linear Model

A stochastic susceptible-infectious model (SI-model) can be used to describe the transmission of *A. pleuropneumoniae* in the trials. The SI-model assumes that susceptible pigs can become infected and infectious, and that infectious pigs stay infectious during the rest of

the experimental period (Bailey 1975). According to the SI-model, infectious contacts can occur if infectious and susceptible individuals are present in a population, by which the susceptible individuals become infectious.

When a successful transmission – i.e. an infection – occurs, the number of susceptible pigs decreases by one whereas the number of infectious pigs increases by one. The rate at which a randomly chosen animal has infectious contacts is assumed to be proportional to the density of infectious individuals with proportionality constant β, which is also called the infection parameter. This implies that number of infectious contacts encountered by one individual in a period of length Δt follows a Poisson distribution with parameter: $\beta I/N \cdot \Delta t$, where I is the average number of infectious individuals present, and N the total number of individuals present.

Hence, the probability of a susceptible animal having no infectious contacts – i.e. escaping infection – during a period Δt is $e^{-\beta \cdot \Delta t \cdot I/N}$ and thus the probability to become infected is $1 - e^{-\beta \cdot \Delta t \cdot I/N}$. From this it can be seen that 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, which is the number of susceptible individuals at the start of the period. Consequently, the relation between the expected number of cases per unit of time $E(C)$, and I, N, β, and S is known:

$$E(C) = S\left(1 - e^{-\beta I/N}\right).$$

From data of transmission trials – i.e. the number of cases per day (C) within a period between two subsequent samplings – the infection parameter β can be estimated, since S, I and N are known. When using a GLM (Mccullagh and Nelder 1989) with a complementary-log-log link function and $\log I/N$ as offset variable, the infection parameter β can be estimated and variables can be tested on their effect on β. For the use of a GLM to analyse infectious disease data see Becker (1989).

The infection parameter β is a function of the infectivity of infectious individuals, and the susceptibility of the susceptible individuals. In a previous study (Velthuis *et al.* Accepted) it was shown that the infectivity of infectious individuals for *A. pleuropneumoniae* was not constant. It was concluded that β was tenfold higher on days where more than ten colonies were isolated from the nasal swabs of the infectious individuals than on other days. For that reason, it can also be assumed that β depends on this characteristic in future experiments, which leads to a model that differentiates between highly or little infectious individuals.

In extended transmission experiments for *A. pleuropneumoniae* as described in this paper it is possible to deal with even more heterogeneous populations (as in the vaccination

experiment described in section 4 of this paper). Besides the highly and little infectious individuals, also treated and untreated individuals may be present in the same experimental population. In this case, eight different infection parameters can be estimated from the data: β_{uu}^-, β_{uu}^+, β_{tu}^-, β_{tu}^+, β_{ut}^-, β_{ut}^+, β_{tt}^- and β_{tt}^+, in which the subscript indicates the treatment status of the susceptible and infectious individuals respectively, and the superscript the infectivity level of the infectious individuals. Thus, β_{tu}^- is the infection parameter between treated susceptible individuals and untreated infectious individuals that are little infectious, β_{tu}^+ is the infection parameter between treated susceptible individuals and untreated infectious individuals that are highly infectious, and so on.

As mentioned before, for estimation of the different β, the number of susceptible pigs at the start of each period between two sampling intervals (S_t and S_u), the average number of infectious pigs in each period (I_t and I_u), and the total number of pigs present should be known. By assuming that new cases in a period are induced by the infectious pigs of the previous period, the full model can be written as:

$$G(E(C)) = \log\left(-\log\left(1 - \frac{E(C)}{S}\right)\right)$$

$$= \log(\beta) + \log\left(\frac{I}{N}\right) = a + b \cdot H_u^+ + c \cdot G_t + d \cdot F_t + e \cdot H_t^+ + \log\left(\frac{I}{N}\right)$$

where $H_u^+ = I_t^+ / (I_t + I_u)$ is the fraction of untreated highly infectious pigs, $G_t = S_t / (S_t + S_u)$ the fraction of treated susceptible individuals, $F_t = I_t / (I_t + I_u)$ the fraction of treated infectious pigs, and $H_t^+ = I_u^+ / (I_t + I_u)$ the fraction of treated highly infectious pigs. The regression coefficients of the various variables are related to the various distinct β as can be seen in Table 5.1.

Every variable that leads to a significant reduction of the residual deviance after inclusion should be included in the model. The estimated coefficients of the included variables express the effect of these variables on the infection parameter β. It is possible to calculate the different β and the two sided limits of the 95% confidence interval using the estimated coefficients and their variances and covariances.

The effect of a treatment on the susceptibility is accepted if the effect of variable G_t differs significantly from zero, which will indicate that β_{uu}^+ differs from β_{tu}^+, β_{uu}^- from β_{tu}^-, β_{ut}^+ from β_{tt}^+, and β_{ut}^- from β_{tt}^-. The effect of a treatment on the infectivity is accepted if the effect of variable F_t differs significantly from zero, which will indicate that β_{tu}^+ differs from β_{tt}^+, β_{tu}^- from β_{tt}^-, β_{uu}^- from β_{ut}^-, and β_{uu}^+ from β_{ut}^+. Significance levels will be set at 0.05. All calculations can be done with the GLM facilities of Genstat 5, Release 4.1 (Trust 1999).

Table 5.1. The full model for a heterogeneous population, consisting of highly and little infectious individuals and treated and untreated individuals. The different infection parameters and their relation with the various variables of the model.

Model: $\log(\beta) = a + b \cdot H_u^+ + c \cdot G_t + d \cdot F_t + e \cdot H_t^+$

Symbol	Description	Expression
β_{uu}^-	Infection parameter between S_u and I_u^+ individuals	e^a
β_{uu}^+	Infection parameter between S_u and I_u^+ individuals	e^{a+b}
β_{tu}^-	Infection parameter between S_t and I_u^- individuals	e^{a+c}
β_{tu}^+	Infection parameter between S_t and I_u^+ individuals	e^{a+b+c}
β_{ut}^-	Infection parameter between S_u and I_t^- individuals	e^{a+d}
β_{ut}^+	Infection parameter between S_u and I_t^+ individuals	e^{a+d+e}
β_{tt}^-	Infection parameter between S_t and I_t^- individuals	e^{a+c+d}
β_{tt}^+	Infection parameter between S_t and I_t^+ individuals	$e^{a+c+d+e}$

4. THE EXAMPLE VACCINE EXPERIMENT

4.1. Materials and methods

4.1.1. *A. pleuropneumoniae experiment to test effect of vaccination*

To test whether vaccination reduces the transmission of *A. pleuropneumoniae* an extended transmission experiment as described in this paper was conducted. The transmission experiment consisted of four trials of which the first control and vaccine trial (trials I and II) were conducted simultaneously, and where after directly the second control and vaccine trial (trials III and IV) were conducted, also simultaneously.

The trials differed at two points from the above-described experimental design. First, because the vaccine could influence the number of excreted *A. pleuropneumoniae* bacteria – so that the moment of the replacement day becomes unsure – only the C_2-pigs in the vaccine trials (II and IV) could be vaccinated and not the C_1-pigs, which led to a heterogeneous population. Second, due to the fact that one of the original five vaccinated pigs of trial II died at day 0, the number of C_2-pigs in trials III and IV had to be increased to six and seven respectively to have enough power (0.89) to find a possible difference in transmission between the vaccinated and unvaccinated groups. Therefore, the number of C_2-pigs over the

four trials was four and seven for the vaccinated C_2-pigs in trials II and IV, and five and six for the unvaccinated C_2-pigs in trials I and III.

4.1.2.   Vaccination

The C_2-pigs in trials II and IV were vaccinated intramuscularly with 2-ml of a commercial *A. pleuropneumoniae* subunit vaccine (Porcilis App batch: 78106PK99/ 2378, Intervet International, Boxmeer, the Netherlands) at two and five weeks of age. The active components of the vaccine were – according to the manufacturer – the three Apx-toxins and outer membrane protein.

4.1.3.   Serology

To monitor humoral immune responses, blood samples were collected weekly starting on day -7 and additionally on the replacement day. Since *A. pleuropneumoniae* serotype 9 produces ApxI and ApxII, both with haemolytic and cytotoxic activity, we tested the sera in a haemolysin neutralisation test (HLN-test) and a cytotoxin neutralisation test (CTN-test) as described in detail elsewhere (Kamp *et al.* 1997). The ability of the sera to neutralise haemolysin of culture supernatant fluids of *A. pleuropneumoniae* serotype 9 and to neutralise cytotoxin of culture supernatant fluids of serotype 9 and serotype 8 was quantified. A raised titre in the serotype 8 CTN-test was expected on days before infection in the sera of vaccinated pigs, since *A. pleuropneumoniae* serotype 8 produces in addition to ApxII ApxIII, which is a strong cytotoxic protein. Serum antibodies against *A. pleuropneumoniae* serotype 9 were determined in a complement fixation test (CFT-test) (Nielsen 1975).

4.2. Results of the vaccine experiment

4.2.1.   Transmission experiment

The infection status of each pig – susceptible or infectious – on the different sampling days was determined as described in section 2.5. In Figure 5.2 the results of the four transmission trials are shown, and a summary of the serology is presented in Table 5.2.

All pigs, except three vaccinated C_2-pigs in trial II had an *A. pleuropneumoniae* culture positive tonsil at necropsy. Since we assumed that pigs with a positive tonsil at necropsy were infectious for *A. pleuropneumoniae* from the first day *A. pleuropneumoniae* was isolated from a swab, all C_1-pigs were infectious on the replacement day, which was day 6, 5, 3, and 3 for the four trials, respectively.

Table 5.2. Summary of the serological results of the C_2-pigs

Test	Day[a]	Unvaccinated				Vaccinated			
		Exp I		Exp III		Exp II		Exp IV	
		No. pigs[b]	Range[c]	No. pigs	Range	No. pigs	Range	No. pigs	Range
$HLN_{ser\,9}$	r	0/5	-	0/6	-	1/4	32-32	0/7	-
	r_{30}	3/5	16-128	2/6	16-16	$0/3^d$	-	6/7	16-64
$CFT_{ser\,9}$	r	0/5	-	0/6	-	1/4	40-40	1/7	20-20
	r_{30}	0/5	-	0/6	-	1/3	20-20	4/7	20-40
$CTN_{ser\,9}$	r	0/5	-	0/6	-	4/4	16-64	3/7	16-16
	r_{30}	4/5	16-128	3/6	16-16	$3/3^d$	16-32	7/7	16-64
$CTN_{ser\,8}$	r	0/5	-	0/6	-	0/4	-	0/7	-
	r_{30}	0/5	-	0/6	-	$0/3^d$	-	0/7	-

[a] *r* is the replacement day, and *r30* thirty days later.
[b] number of C2-pigs with a raised titre (with respect to 1/8) / total number of C2-pigs present.
[c] the minimum and maximum titres measured, and - if none of the pigs had a titre.
[d] one vaccinated C2-pig was culled on day 21.

All E-pigs showed clinical symptoms varying from mild to acute pleuropneumonia and had to be medicated. One E-pig in trial IV died due to sepsis (*A. pleuropneumoniae* was isolated from the liver and spleen) on day 2. Some of the C_1-pigs (five before and four after the replacement day) and a few C_2-pigs (three vaccinated and four unvaccinated) were a bit listless, had a slightly increased respiratory rate, and an increased rectal temperature (Figure 5.2). One vaccinated C_2-pig in trial II had to be culled on day 23 due to severe arthritis of the elbow joint from which *Actinomyces pyogenes* was isolated. From the tonsil of this pig *A. pleuropneumoniae* was isolated and the pig had in addition to the clinical signs due to arthritis a slightly increased respiratory rate.

Only 5 of the 11 unvaccinated C_2-pigs had a raised HLN titre and 7 pigs a raised $CTN_{serotype\,9}$ titre 30 days after the replacement day (r_{30}) (Table 5.2). This indicates an active immune response, probably against *A. pleuropneumoniae* serotype 9. However, despite the fact that all unvaccinated C_2-pigs became infected at least 18 days before the r_{30} titre was measured, not all pigs developed a measurable titre after infection.

None of the unvaccinated C_2-pigs and none of the C_1-pigs had a raised CFT titre. This was not surprising since no clinical disease was observed, and CFT antibodies might only develop as a consequence of clinical infection (Cruijsen *et al.* 1995). So, the absence of CFT antibodies in C_2-pigs and C_1-pigs indicates that carrier pigs were created, and that it was possible to observe transmission from carrier pigs to susceptible contact pigs.

Furthermore, *A. pleuropneumoniae* culture positive lung lesions were found in all E-pigs. In trials I and II no C_1-pig was found to have a lung lesion, but in both trials III and IV two of the five C_1-pigs had a lung lesion. In both trials I and III, three unvaccinated C_2-pigs had a lung lesion, whereas in trials II and IV none of the vaccinated C_2-pigs had (Figure 5.2).
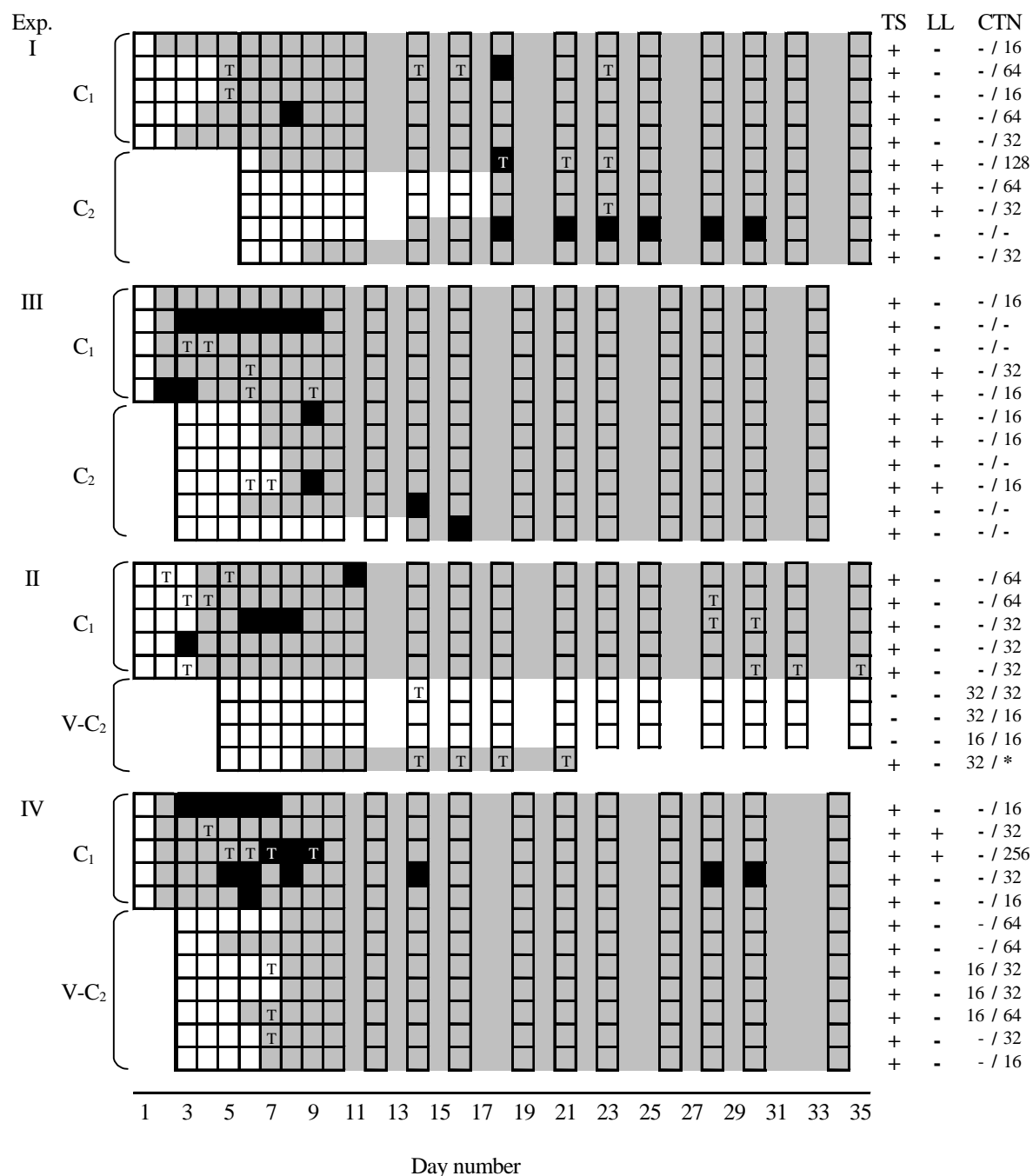
Figure 5.2. An overview of the four conducted transmission trials with *A. pleuropneumoniae*, C_1 = pig infected by endobronchially inoculated pigs, C_2 = unvaccinated contact pig, $V-C_2$ = vaccinated contact pig, the thick black line indicates the replacement day, blocks = days at which swabs were taken, grey shaded blocks = the period at which the pig was assumed to be infectious, black blocks = days at which the pig was extra infectious, T = days at which the pig had a body temperature >40.5°C, TS = pig with an *A.pleuropneumoniae* culture positive tonsil at necropsy, LL = pig with an *A. pleuropneumoniae* culture positive lung lesion, CTN = $CTN_{serotype\ 9}$ titre measured on the replacement day / titre measured 30 days after the replacement day, + = yes, - = no, and *= missing value.

4.2.2. *Statistical analysis: quantification of the transmission*

Since we consider only transmission among naturally infected pigs in our trials as an acceptable reflection of transmission in the field, we used only information of C_1- and C_2-pigs, i.e. information after the replacement day.

In principle, the search for an acceptable model was performed by judging the deviance reduction from adding each candidate variable in turn after eliminating the other ones. A variable was included in the model if the deviance reduction so obtained was significant in a chi-squared test with one degree of freedom.

Unfortunately, variables F_t and H_t^+ could not be included in the model, because of limited information in the data, i.e. there were only a few periods where F_t and H_t^+ were unequal to zero. Variable F_t could indicate whether vaccination has an effect on the infectivity of pigs, and variable H_t^+ could have given insight into whether the nasal excretion of *A. pleuropneumoniae* by vaccinated pigs was related to infectivity. However, these effects and consequently the difference between several infection parameters could not be tested on significance.

The full model and the eventually fitted model are presented in Table 5.3 together with the estimated coefficients of the variables H_u^+ and G_t, b and c, respectively. The residual deviance of the fitted model was 36.77 with df=34 (p-value = 0.34), indicating an acceptable fit. The dispersion parameter was set to one. From the coefficients of the full model, the different infection parameters and their 95%-CIs were estimated (Table 5.4). Details of the search and the interpretation of the model as to the various explanatory variables will be discussed in the subsections to follow.

Table 5.3. The full model and the fitted model used in the analysis of the vaccine experiment

| Variable | $\log \beta = a + b \cdot N_u^+ + c \cdot G_t$ | | | | $\log \beta = a + b \cdot N_u^+$ | | | |
	Change of deviance	d.f.[a]	Estimated coefficient	s.e.[b]	Change of deviance	d.f.[a]	Estimated coefficient	s.e.[b]
Constant	-	-	a= −2.28	0.58	-	-	a= −2.49	0.49
N_u^+	8.68[c]	1	b= 5.93	2.11	9.61	1	b= 6.13	2.01
G_t	0.51[c]	1	c= −0.36	0.52				

[a] degrees of freedom
[b] standard error of the estimated coefficient
[c] eliminating the other variable

Table 5.4.   Estimates of the infection parameters.

Infection parameter	Estimate	95% CI	Differs significantly from (0.05)
β_{uu}^{-}	0.10	0.033 ; 0.32	β_{uu}^{+}
β_{uu}^{+}	39	1.4 ; ∞	β_{uu}^{-}
β_{vu}^{-}	0.072	0.024 ; 0.22	β_{vu}^{+}
β_{vu}^{+}	28	0.91 ; ∞	β_{vu}^{-}

4.2.3.   Effect of excretion on infectivity of unvaccinated pigs

In a previous study (Velthuis *et al.* Accepted) an effect of excretion via the nose on the infectivity of unvaccinated pigs has been demonstrated. Therefore, variable H_u^{+} was added to the model first – so ignoring the other variables – resulting in a change of deviance of *9.61* with *df=1*, which confirms the effect of excretion on the infectivity. The reduction of deviance was slightly smaller (8.69) when adding H_u^{+} after eliminating G_t. So the inclusion of H_u^{+} in the model is well founded, and again an effect of excretion via the nose on the infectivity of unvaccinated pigs has been demonstrated in this study. The estimated effect of H_u^{+} in this study was *b=5.93* with *s.e.=2.11*, and the effect of H_u^{+} in the previous study was *b=2.39* with *s.e.=0.63*. It can be concluded that infection parameters β_{uu}^{-} and β_{uu}^{+} differ, as do the infection parameters β_{tu}^{-} and β_{tu}^{+} (Table 5.4, last column). The relative difference in infectivity due to extra excretion of *A. pleuropneumoniae* via the nose, i.e. the ratio between β_{uu}^{-} and β_{uu}^{+}, was $e^{5.93} = 376$. This means that an unvaccinated pig is about 376 times as infectious on days where more than 10 colonies *A. pleuropneumoniae* were isolated from the nasal swab than on other days.

4.2.4.   Effect of vaccination on susceptibility

The change in deviance when adding G_t to a model that already contains variable H_u^{+} was *0.51* with *df=1*. This indicates that vaccination has no effect on the susceptibility to become infected by an *A. pleuropneumoniae* infection. The estimated effect of G_t differed not significantly from zero (*c=–0.36* with *s.e.=0.52*). It was concluded that vaccination did not have an effect on the susceptibility. In other words, vaccinated pigs are as likely to become infected as unvaccinated pigs.

From a serological point of view the effect of vaccination was insufficient too (see Table 5.2). Although, the HLN titre of one vaccinated C$_2$-pig, CTN$_{serotype\ 9}$ titres of seven vaccinated C$_2$-pigs, and CFT titres of two vaccinated C$_2$-pigs were raised at the replacement day (9 to 12 days after second vaccination), the titres were low. Moreover, not all vaccinated C$_2$-pigs had a raised titre. This indicates that vaccination not always induces a measurable immune

response, and if it does, it is very low. All vaccinated C_2-pigs had a no measurable CTN$_{serotype}$ $_8$ titre. This is another indication that vaccination induced an insufficient serological effect, since it shows that there was no measurable immune response against Apx III, which was present in the vaccine.

Six of the ten vaccinated C_2-pigs had a raised HLN titre 30 days after replacement. This indicated an immune response against an *A. pleuropneumoniae* infection, since the three uninfected vaccinated C_2-pigs did not have a raised HLN titre. All vaccinated C_2-pigs had a raised CTN$_{seroptype\ 9}$ titre, including the three uninfected vaccinated C_2-pigs. This could be a response to an infection but also to vaccination.

5. DISCUSSION

The aim of this paper was to present the design of an *A. pleuropneumoniae* transmission experiment, including a method to analyse the data. Subsequently, the concept has been illustrated by presenting a conducted transmission experiment in which the effect of vaccination on the susceptibility for *A. pleuropneumoniae* was investigated.

An advantage of transmission experiments over field studies is that causal relations can be demonstrated, since the treatment is the only difference between control and treatment group, and variation due to other factors is kept to a minimum. Thus, with the presented design it becomes possible to test all kinds of interventions and combinations of interventions on their effect on the transmission – divided in susceptibility and infectivity – of *A. pleuropneumoniae*. Consequently, the feasibility of an eradication program for *A. pleuropneumoniae* can be investigated.

It is believed that subclinically infected carriers play an important role in the transmission of *A. pleuropneumoniae* in the field (Kume *et al.* 1984; Nielsen and Mandrup 1977; Savoye *et al.* 2000). That's why we believe that it is important to study transmission of *A. pleuropneumoniae* from carrier pigs to susceptible contact pigs. So, a suitable method to create carriers is needed. In a previous study it was shown that the probability to induce an infectious state by intranasal inoculation was very low (*p=0.34*) (Velthuis *et al.* Accepted). Because of this result, we decided to inoculate the E-pigs endobronchially in the experimental design of the present study. Since endobronchial inoculation induces clinical symptoms as well as an infectious state, the E-pigs could not be used directly. We had to extend the trials in the transmission experiment by replacing the E-pigs by new susceptible contact pigs. Thus, carriers were created in our study by contact exposing pigs to endobronchially inoculated E-pigs.

A disadvantage of extending the trials is that C_1-pigs cannot always be treated with the intervention that is tested, e.g. vaccination. This is because the moment of replacing depends on the excretion of *A. pleuropneumoniae* by the C_1-pigs and the treatment might influence this excretion. From a mathematical point of view it is more correct to study the transmission in a homogeneous population than in a heterogeneous population. So that standard quantification methods in which a homogeneous population is assumed, i.e. Final Size estimator and Martingale estimator, can be used to analyse the data (Kroese and De Jong 2001). However, the GLM method as described in this paper can be used to analyse data of heterogeneous populations, and is therefore the most appropriate method to analyse data of *A. pleuropneumoniae* transmission experiments.

Defining the infection status – susceptible or infectious – of an individual animal during the trial is very important for the outcome of the study. In a previous study (Velthuis *et al.* Accepted) it was demonstrated that if a pig carries *A. pleuropneumoniae* in its tonsils at necropsy it has been infectious for other pigs during the experimental period. Additionally, it was assumed that the onset of infectivity started at the first day at which *A. pleuropneumoniae* was isolated from a swab taken from the infectious pig. In other studies of bacterial infections an animal is often called infected if the bacteria was isolated from more than two successive samples, e.g. *Staphylococcus aureus* intramammary infections (Lam *et al.* 1996). If this definition had been applied to the tonsillar swabs taken in the transmission trials as described in this study, a lot more pigs would have been called infectious than there actually were. Or, if this definition was applied to the nasal swabs taken in the transmission trials, a lot less pigs would have been called infectious than there actually were. So, a good foundation for the classification of individuals is important. Furthermore, in case of an *A. pleuropneumoniae* infection it is also important to determine the periods in which pigs are highly or little infectious. This, to allow for variable infectivity in the model. Therefore, a regularly and intensive sampling scheme is needed when conducting *A. pleuropneumoniae* transmission trials.

So far in literature three methods to estimate infection parameters from transmission experiments are described: the Final Size estimation (Kroese and De Jong 2001), the Martingale estimation (De Jong and Kimman 1994), and an estimation method based on a Generalised Linear Model (Becker 1989, De Jong, 1996). The Final size and Martingale estimators require that the final size situation of the local epidemics in the trials have been reached before the end of the experiment. For bacterial infections like *A. pleuropneumoniae* this requirement is not always met. It is possible that there are still infectious pigs present together with susceptible pigs at the end of a trial, like in trial II of the vaccine experiment.

The GLM method does not rely on the final size assumption and is therefore more appropriate for analysis of data from transmission experiments with *A. pleuropneumoniae*.

We could quantify the effect of vaccination on susceptibility and on infectivity separately with the present analysis. This was possible since we were dealing with a heterogeneous population. In an earlier paper dealing with heterogeneity by De Jong *et al.* (1996) were different β estimated for the transmission of bovine respiratory syncytial virus between sero-positive and sero-negative animals. They fitted two models, one for sero-negative susceptible individuals and one for sero-positive susceptible individuals, whereas in the present study one model was fitted for both vaccinated and unvaccinated susceptible individuals. Whether fitting one or two separate models in the GLM depends on the data structure. For example, if the treatment group consists of only treated infectious and susceptible individuals, and the control group only of untreated infectious and susceptible individuals, it would be better to fit two models, one for each treatment group. However, when dealing with a heterogeneous population, consisting of treated and untreated infectious and susceptible individuals, it is more appropriate to fit one model.

To our knowledge the presented vaccine experiment was the first study in which the effect of vaccination on the transmission of *A. pleuropneumoniae* was quantified. So far, the available vaccine studies concerned clinical trials and/or field studies to investigate reduction of clinical symptoms of pleuropneumonia and mortality or induction of antibody responses (Furesz *et al.* 1997; Oishi *et al.* 1995; Wongnarkpet *et al.* 1999; Goethe *et al.* 2000). In the present study the effect of a commercial sub-unit vaccine on the transmission was assessed in a transmission experiment consisting of four conducted trials. With the accompanying analysis it was possible to demonstrate the effect of nasal excretion on the infectivity, however it was not possible to demonstrate an effect of vaccination on the susceptibility or infectivity.

In this study it could not be demonstrated that vaccination had an effect on the susceptibility (to become infected and infectious) for *A. pleuropneumoniae*. The serological results did support this conclusion, although the only three vaccinated pigs with no HLN titres and low CTN titres in trial II did not become contact-infected at all. A plausible explanation for the generally low titres in the vaccinated pigs may be that vaccination was not carried out at six and ten weeks of age as advised by the manufacturer, but at two and five weeks of age.

The effect of nasal excretion on the infectivity was already demonstrated in a previous study (Velthuis *et al.* Accepted). In this study the estimated effect of H_u^+ was 2.39 (with s.e. 0.63) whereas we found 6.13 (with s.e 2.01) in this study. In the previous study more data from more (ten) transmission trials that were very similar to the ones in the present study were available for the estimation of the effect of H_u^+. So, the estimated coefficient in the study of

Velthuis *et al.* (Accepted) is more reliable than the one in this study, which is also indicated by the smaller standard error. Thus, an unvaccinated pig is about ten times ($e^{2.39} = 10.6$) more infectious towards other pigs on days where more than ten colonies *A. pleuropneumoniae* were isolated from the nasal swab than on other days. Anyhow, all data suggest that excretion via the nose is very important for the transmission of *A. pleuropneumoniae*. This could indicate that the nose is the main exit-route or that nose-nose contact is the most important transmission-route.

Finally the question whether our findings could possibly have practical relevance as to an eradication program for *A. pleuropneumoniae* is briefly addressed. In general, an infectious disease can be eradicated when the reproduction ratio is smaller than its threshold value one. This reproduction ratio equals the product of infection parameter *β* and length of the infectious period. Unfortunately, the length of the infectious period is unknown for *A. pleuropneumoniae*. So at present conclusions about the feasibility of an eradication program can not be drawn.

6. CONCLUSION

− Carrier pigs of *A. pleuropneumoniae* serotype 9 can be created by exposing contact pigs to endobronchially inoculated pigs.
− The transmission of *A. pleuropneumoniae* from carriers to susceptible contact pigs can be studied in a transmission experiment.
− By using a GLM it is possible to test susceptibility and infectivity separately.
− The effect of an intervention on the transmission of *A. pleuropneumoniae* can be quantified with the presented design and analysis method.
− The nasal excretion of *A. pleuropneumoniae* is positively correlated to the infectivity of pigs.
− Vaccination has no demonstrable effect on the susceptibility with respect to *A. pleuropneumoniae* infection.

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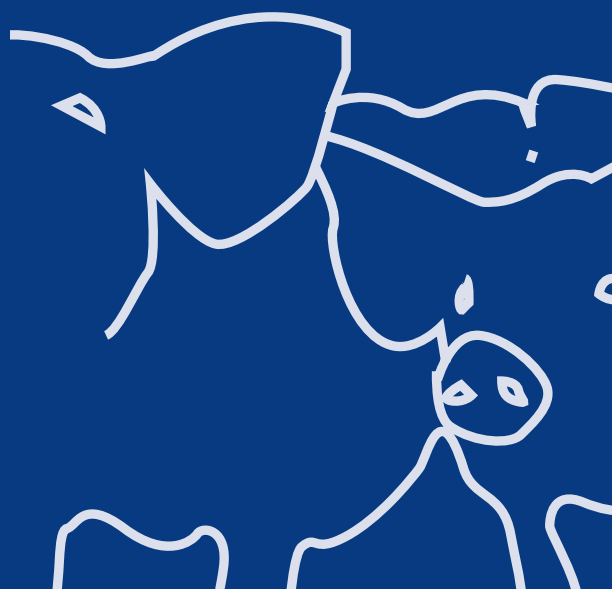
6

General discussion

A. G. J. Velthuis

1. INTRODUCTION

Infectious bacteria can be transmitted from infected individuals to others, but how? We set out to gain more insight into the transmission dynamics of bacteria between individuals with help of transmission experiments. By studying the effect of interventions on the transmission, better-directed intervention strategies can be devised. Therefore, the main goal of the research described in this thesis was:

'Development of methods to quantify bacterial transmission in an experimental setting'

To achieve this goal we developed an experimental transmission model able to quantify the transmission of *A. pleuropneumoniae* from subclinically infected carrier pigs to susceptible pigs. We decided to start with the simple experimental design that was successfully used to study the transmission of pseudorabies virus (Bouma 1997). In each of the first eight transmission trials five pigs were intranasally inoculated with 10^4 colony forming units (CFU) at day zero. At day one, five susceptible contact pigs were placed with the inoculated ones so that the infection chain could start. We were not sure whether this approach would also work for *A. pleuropneumoniae*, so we collected as much information as possible to be able to find and solve any bottlenecks. It appeared that this simple approach was not appropriate for studying the transmission dynamics of *A. pleuropneumoniae* and needed some changes in order to end up with a workable design.

In this general discussion we present how we gained more insight into the transmission mechanism of *A. pleuropneumoniae*. We will discuss the approach of trial and error to arrive at the optimal design to study and quantify the transmission of *A. pleuropneumoniae*. After discussing the mathematical and statistical methods used, we will discuss: (1) whether this experimental and statistical approach is also applicable for other bacterial infections; (2) whether the results obtained for *A. pleuropneumoniae* infections can be extrapolated to the field; and (3) whether the new statistical methodology can also be applied to viral infections.

2. EXPERIMENTAL DESIGN

2.1. Starting the infection chain

To start an infection chain deliberately an appropriate challenge model to create infectious pigs is needed. To study the transmission of *A. pleuropneumoniae* from subclinically infected pigs to susceptible pigs, we needed a challenge model that would

induce infectious, but subclinically infected pigs, i.e. carriers. Furthermore, from a mathematical point of view we needed homogeneous populations of infectious individuals and susceptible individuals (Hethcote and Van Ark 1987). This means that infectious individuals at the start of the transmission experiment should be equally infectious as the contact infected pigs.

The most obvious method to create infectious pigs is by using artificial inoculation. A successful inoculation method should consist of a carefully considered infection site or route and an optimised dose. If the dose is too high, animals can become severely diseased and the immune system can be unnaturally stimulated, resulting in inoculated animals that differ from contact infected individuals. If the dose is too low, animals might not become infectious at all. The latter situation is hard to interpret when analysing the infection chain: animals have been in contact with the infectious agent by inoculation, but have not become infected or infectious: should we classify them as susceptible or as recovered?

In the first eight transmission trials, we used intranasal inoculation with 10^4 CFU *A. pleuropneumoniae* serotype 9. However, it appeared that intranasal inoculation led to very variable results (Chapter 2). Some inoculated pigs became seriously diseased, some even died as result of per-acute pleuropneumonia and others did not show any sign of disease. The contact infected pigs, on the other hand, had only mild symptoms of pleuropneumonia or none at all. This was quite a contrast to the homogeneity that we needed to have when analysing the data.

By looking at the characteristics of the intranasally inoculated pigs more closely and by comparing these characteristics to the amount of transmission that was observed in the trials, we concluded that the probability to create an infectious pig by intranasal inoculation was 0.32 (Chapter 2). This was much less than expected. Summing up, intranasal inoculation does not guarantee infectious pigs that are more or less identical to the contact infected susceptible pigs in the same transmission trials.

Since this artificial inoculation did not directly led to the desired infectious state we decided to use an extended transmission experiment. In extended transmission experiments, infectious animals are created by contact infection, thus by housing them with artificially inoculated pigs. We decided on endobronchial inoculation with 10^3 CFU *A. pleuropneumoniae* to infect pigs (Van Leengoed and Kamp 1989) instead of intranasal inoculation with 10^4 CFU, since the latter could not guarantee an infectious state. The majority of the endobronchially-inoculated pigs became infected and regrettably showed severe signs of pleuropneumonia. They were, however, well able to transmit the infection to the contact pigs, which in turn had no or at most only mild symptoms of pleuropneumonia.

2.2.   Timing of mixing the susceptible animals with the challenged ones

Correct timing of mixing the susceptible animals with the challenged ones is very important. If the mixing takes place too late, an amount of 'infectivity' of the infectious individuals will have already been wasted in an environment without susceptible individuals. Consequently, the transmission might be underestimated. If the mixing takes place too early it is possible that the challenged individuals have not all reached their infectious states yet. It will make it difficult to determine the infection state of the (non-infectious) challenged individuals. For example, suppose that a challenged individual is not infected at the moment of mixing and does not become infected during the rest of the experimental period. Should we classify it as susceptible or as recovered? Or suppose that a challenged individual was not infected at the moment of mixing, but has been infected at the end of the experimental period. Was this animal infected by the challenge or was it infected by contact infection? To quantify transmission in a small group of animals, small differences in the number of animals per infection state can lead to very different results.

2.3.   Interpretation of the observations

Before starting a transmission experiment it is important to consider the observations that can be made and how these observations relate to the infection states in the assumed epidemic model. Transmission parameters can be estimated by linking measurements at individual level and measurements at population level. Whether or not an animal has been infected (and became infectious) is important to determine. This is the minimum amount of information needed to quantify transmission. For more distinctive quantification methods, we need to know the moment at which animals become infected (Chapters 3 and 4). Note, from an epidemiological point of view, an infected state means that the individual will also be or become infectious to others. And an infected state does not automatically mean that the animal is or will become diseased.

Several diagnostic tools are available to detect the presence of an infectious agent or a former contact with the agent. Serology based on detection of antibodies directed against the infectious agent in sera is a commonly used method in veterinary practice. Serology can give useful information about the infection chain if one is only interested in the number of contact infections at the end of the experimental period. Three conditions need to be fulfilled when using serology as measurement for the infected state. First, all infected individuals should develop antibodies against the infectious agent. Second, one must be able to classify all individuals that develop antibodies against the infectious agent as infected, which means that they have also been infectious. Third, the experimental period needs to be long enough to give

infected individuals enough time to develop a measurable immune response. Serology has proved to work well for viral infections, like pseudorabies virus (De Jong and Kimman 1994; Bouma *et al.* 1996; Bouma *et al.* 1997), classical swine fever virus (Laevens *et al.* 1998; Bouma *et al.* 2000), or porcine respiratory and reproductive syndrome virus (Nodelijk *et al.* 2001). However, serology is of no use for the determination of the infected state for *A. pleuropneumoniae*. This is because not all infected individuals develop a measurable immune response and the time needed to develop a measurable titre is relatively long, i.e. 10 to 14 days (Chapters 2 and 5).

Another way of determining an infected state is to isolate the infectious agent from the animal. Isolation is, of course, the best proof that the agent is present in the sampling site. But, what does it say about the (real) infection status of the individual? If, for example, *A. pleuropneumoniae* is isolated from the pleura at necropsy does it also mean that the animal has been infectious? The pleura is not an exit route and is therefore of no direct relevance for the transmission of *A. pleuropneumoniae*. Furthermore, it is highly probable that the pathogen is missed due to a minimal detection level, or due to the fact that the pathogen is present elsewhere in the body or exit route.

To determine an infected state with regard to transmission often more is needed than the minimal detection level or more subsequent positive samplings. Hence, the minimal detection level does not restrict the usefulness of an isolation method to detect an infected state.

A very labour-intensive diagnostic technique is PCR with which the smallest amount of DNA or RNA originating from the infectious agent can be detected. A PCR is assumed to be very sensitive, but this does not automatically mean that it is a useful method to study and quantify transmission. For example, a positive PCR does not prove colonisation of the bacterium or virus in the animal. More importantly, it does not prove that the animal has been or is infectious for others. An illustrative example is the study of Dewulf *et al.* (2001) in which the transmission of classical swine fever among gilts was quantified (although there were some criticism of the quantification method used in this study). PCR-based calculations resulted in a R_0 of 14.8 whereas it was 3.3 when based on virus isolation. This (large) difference illustrates that the PCR technique might be too sensitive to classify animals in the infected state relevant for transmission, with the result that R_0 might be overestimated.

Savoye *et al.* (2000) used PCR results to study qualitative transmission of *A. pleuropneumoniae* among pigs by either direct contact or contact by air (housed in two isolated units that were connected with a tube). The authors concluded that PCR produced positive results far more often than traditional bacteriology, implying that transmission might be underestimated if the analysis was based on bacteriology. This conclusion might be

questionable, since the sensitivity is not directly related to the transmission of *A. pleuropneumoniae* as discussed below.

We determined an appropriate measurement for the infected state of *A. pleuropneumoniae* (Chapter 2). After comparing several measurements we concluded that confirmation of whether a pig had been infected and became infectious in the transmission trial could be obtained if *A. pleuropneumoniae* was isolated from its tonsils at necropsy. This definition better fitted the observed data than definitions based on '*A. pleuropneumoniae* isolated from swabs taken' (nasal or tonsillar, in subsequent samplings or not, with a certain minimal number of colonies) and 'positive serological results' (from several tests).

2.4.   Variability in transmission

When the simple stochastic SIR model is used to analyse the experimental transmission a certain amount of variation in transmission is implicitly assumed. One way to check whether the assumed model fits the observed transmission is to compare the amount of variation assumed in the model with the amount of variation that is observed. Significantly more variation in transmission was observed in the *A. pleuropneumoniae* transmission trials than was assumed in the stochastic SIR model (Chapter 2). So, the assumed transmission mechanism did not represent the observed transmission of *A. pleuropneumoniae*. This discrepancy was probably because the number of infectious pigs at the start of the trials was less than expected as a result of the very variable results of the intranasal inoculation. An additional explanation was that there was a large variation in infectivity within infected pigs. An infected pig was ten times more infectious on days when more than ten colonies of *A. pleuropneumoniae* were isolated from its nasal swab than on other days (Chapters 2 and 5).

A quantification method to estimate the transmission of *A. pleuropneumoniae* could account for this variable infectivity. Another implication of this result is that an intensive sampling scheme should be applied in *A. pleuropneumoniae* transmission experiments, so that data on nasal excretion of *A. pleuropneumoniae* are available to correct for the extra infectivity.

2.5.   Duration of the experiment

For the analysis to be sound, the duration of an experiment should be long enough and the sampling scheme intensive enough to collect enough information about the infection chains. The amount of information needed depends on the quantification method that will be used to analyse the data. For example, for a quantification method based on the final size (FS) algorithm, the local epidemics in the different trials should have reached a final size situation before the experiment ends. So, ideally the experiment must last long enough to reach this

final size. This is not feasible for bacterial infections, like *A. pleuropneumoniae*. Even for other (more rapidly proliferous) infectious agents this is also not always feasible, since it is possible that an intervention slows down the transmission dynamics so much that reaching a final size situation will take a considerable length of time. Therefore, we investigated the risk of making wrong conclusions if an FS based method is used when the final size has not been reached in all trials (Chapters 3 and 4).

When the final size is not reached at the moment at which the transmission experiment is terminated important information might be missed. In Figure 6.1 two possible scenarios are presented. In the left panel of the figure the number of infections per treatment group in a
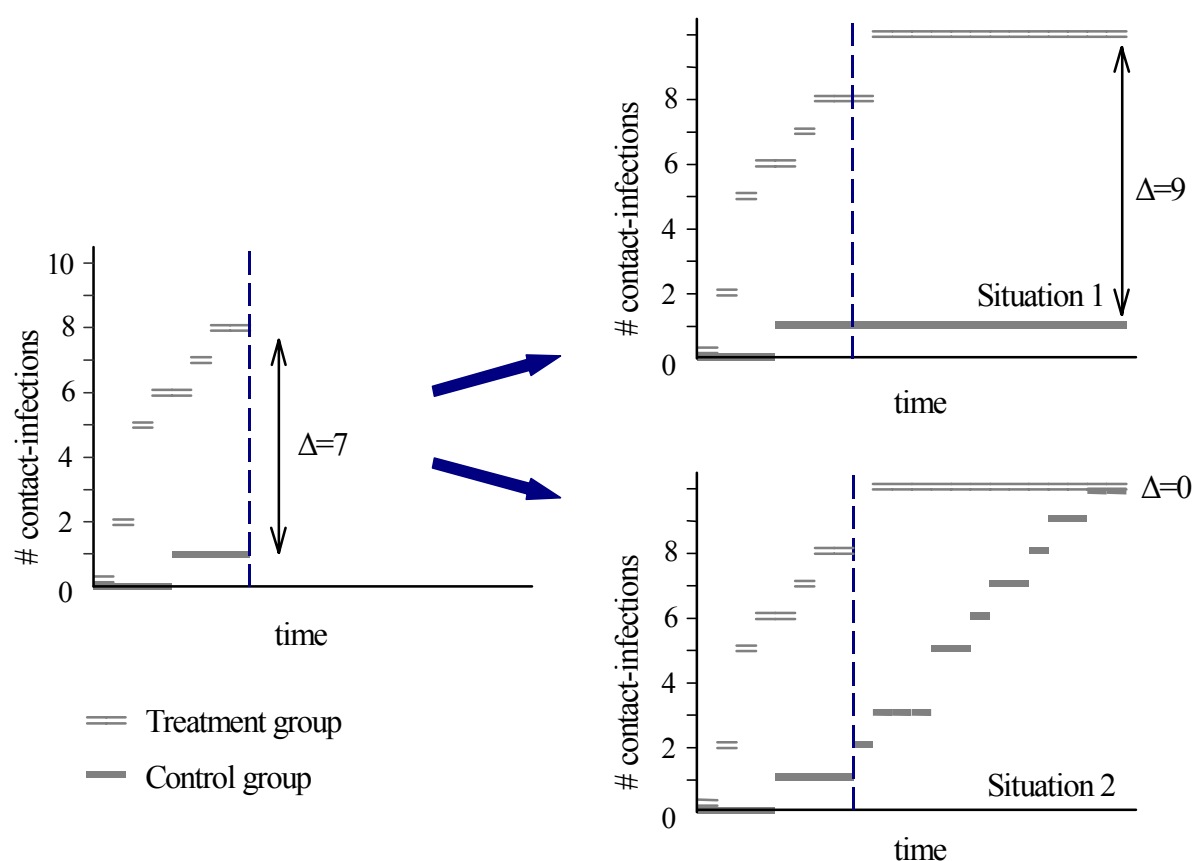
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Figure 6.1. Theoretical results of a transmission experiment that is stopped (dashed vertical line) before a final size situation has been reached in all trials (left panel) and two possible end-situations that could have been observed when the final size situations have been reached (right panels).

transmission experiment is presented against time. The experiment was terminated at the moment where the final size situation had not been reached in all trials (dashed vertical line).

If this experiment had lasted longer the results could have been different. For example, the difference in the number of contact infections could have increased (see the right top panel of Figure 6.1) or this difference could have decreased (see the right bottom panel of Figure 6.1). The different end-situations would lead to different conclusions as to the significance of the difference in transmission between the two treatments.

For analyses of a transmission experiment with a method based on the transient state (TS) algorithm (Chapters 3 and 4), the final size situation does not need to be reached in all trials. However, one of the inputs needed for this method is the duration of the experiment expressed as the number of average infectious periods, which is often unknown. A solution for this problem is to assume a worst-case scenario, namely, to assume that the average infectious period is equal to the expected live span of the animals, which is very well defined for most farm animal species. With this assumption the duration of the transmission experiment can be expressed in terms of the number of average infectious periods, and the methods based on the TS algorithm can be used to analyse the experiment.

For the use of the *MaxDiff* method the average infectious period needs not to be known. When using the *MaxDiff* method to test for a difference in transmission between two treatment groups (Chapter 4) the experiment should last long enough to observe a significant difference in the number of contact infections (see Figure 6.1). In this case, it should be possible to stop the experiment immediately when a significant difference in the number of contact infections has been reached. This would be good for animal welfare and, of course, cost-reduction. Note, when stopping an experiment preliminary extra information will be missed and estimation of transmission parameters will become unsure. In a latter section we will discuss this point further.

3. ANALYSIS OF TRANSMISSION EXPERIMENTS

Various mathematical models and subsequently various statistical methods can be used for estimating transmission parameters or for testing hypotheses about the size of these parameters (Chapters 3 and 4). Generally, the statistical methods can be divided into two groups. The first group uses the total number of infected individuals that are infected at the end of the experiment as input, whereas the second group uses the number of infected individuals during the whole experiment as input. The two groups will be discussed in the next sections, and we will discus the issue of heterogeneity in the last section. There are many other aspects in which quantification methods differ and which are not discussed in this chapter. Table 6.1 gives an overview of the characteristics of the different quantification methods.

Table 6.1. An overview of the characteristics of the different methods available to quantify transmission from transmission data.

Characteristics	Martingale estimator	FS-algorithm	TS-algorithm	*MaxDiff* method	GLM method
Epidemiologic model					
SI	–	+	+	–	+
SIR	+	+	+	+	+
SEIR[1]	+	+	–	–	+
Assumptions					
Final size reached	+	+	–	–	–
Random mixing	+	+	+	+	+
Defined I-state	+	+	+	+	+
Defined moment infection	–	–	–	+	+
Defined moment recovery	–	–	–	+	+
Exponentially distributed infectious period	–	+	+	+	+
Independent periods between two samplings	–	–	–	+	+
Data needed					
# infections at end of trials	+	+	+	+	+
Moment of infections	–	–	–	+	+
Moment of recovery	–	–	–	–	+
Length average infectious period	–	–	+	–	–
Statistical inference					
Estimation of R_0	+	+	+	–	–
Estimation of β	–	–	–	–	+
Testing size of R_0	–	+	+	–	–
Testing size of β	–	–	–	–	+
Testing difference in transmission	–	+	+	+	+
Power to find difference	n.a.[2]	–	+/–	+	++
Robust results	++	++	+	+	+/–
Easy to compute?	++	++	–	+/–	++
Standard software available?	–	–	–	–	+

[1] 'E' indicates the latent state
[2] not applicable

3.1.   Methods based on the number of individuals *eventually* infected

Quantification methods based on the final size, e.g. FS maximum likelihood and the martingale estimators (Kroese and De Jong 2001) are not appropriate to quantify the transmission of *A. pleuropneumoniae* for two reasons. First, the final size assumption required by those methods is not always achieved in experiments with *A. pleuropneumoniae*. Second, those methods do not easily account for the variable infectivity, which is an important characteristic of the transmission of *A. pleuropneumoniae*. Therefore, we developed other quantification methods, which are presented in Chapters 3 and 4 of this thesis.

In principle, it is possible to write down a mathematical model in which the time-dependent transmission dynamics of *A. pleuropneumoniae* is described, while accounting for variable infectivity. This would fulfil the extra conditions needed to quantify the transmission from *A. pleuropneumoniae* transmission experiments. However, even a simple version of this model – based on the transient state (TS) algorithm not yet allowing for variable infectivity – was analytically unsolvable and numerically could only be solved for very low numbers of animals per trial (Chapters 3 and 4). Therefore, we ignored the variable infectivity for the development of quantification methods and focused on methods that did not rely on the final size assumption.

As previously said, quantification methods based on the transient state (TS) algorithm are *not* based on the final size assumption (Chapters 3 and 4), but are based on the number of infected individuals at an earlier stopping time. The TS algorithm generates the time-dependent probability distribution on which several estimation and testing methods are based. The TS algorithm takes the time course of the experimental epidemic into account with no need for a final size situation. Although an explicit solution for any population size is theoretically available from the TS algorithm, its practical use is restricted to experiments with only few individuals. This is because its high degree of recursiveness may cause numerical problems and memory limitations (Bailey 1975; Billard and Zhao 1993; Daley and Gani 1999). The recursiveness in the TS algorithm disappears if time tends to infinity, turning the TS algorithm into the readily applicable FS algorithm.

When using the TS algorithm, the duration of the experiment in terms of the average infectious period needs to be known, which is often not the case. Furthermore, as already stated the application of TS methods is not yet feasible due to computational limitations. Therefore one will probably resort to the easily applicable FS methods. We investigated the error made when FS methods are used instead of TS methods even though the final size situation will not always be reached. It was concluded that the error made in such situations is acceptable concerning one-to-one and five-to-five transmission experiments (Chapters 3 and 4).

3.2. Methods based on number of infected animals *during* the experiment

Although the methods based on the number of infected individuals at the end of the experiment – FS and TS methods – are flexible in that no strong assumptions have to be made, they do not make use of all available information. Consequently, important information may be lost, like for example, an observed difference in the number of contact infections between control and treatment groups during the experiment that has disappeared at the end of the experiment, as is illustrated in the right bottom panel of Figure 2. A statistical method that

takes the whole infection chain of the experimental epidemic into account does exist and is based on a generalised linear model (GLM) (Chapters 4 and 5). Also, there is a statistical test available that uses the maximal difference in the number of contact infections between two treatment groups as input to test whether there is a difference in transmission: the *MaxDiff* test (Chapter 4).

The GLM method is based on the stochastic SIR model, and with which the infection parameter β can be estimated and hypothesis about its size can be tested. Becker (1989) described the application of a GLM to epidemiological models and used a specific version of the SIR model. In Chapters 2 and 5 a GLM based on an improved model choice is described. A disadvantage of the GLM method is that it assumes periods between two subsequent samplings are independent of each other. This means that each susceptible animal at the start of a period has the same probability of becoming infected in the subsequent period. This is questionable, since the resistance of a susceptible animal to become infected may be different at the start or at the end of the experiment.

For the *MaxDiff* test it is also assumes that all periods on which the simulations are based – which is in this case 0.002 average infectious period – are independent. The test statistic used in the *MaxDiff* test is the maximum difference in the number of contact infections that has occurred somewhere during the course transmission experiment (we will call this the maximum difference). The probability distribution over all possible maximal differences under the null hypothesis that there is no difference in transmission is generated by markov-chain-monte-carlo simulations, which are based on the transmission processes assumed in the stochastic SIR model (Chapter 4). With the probability distribution it is easy to test whether the observed maximal difference lies within the critical region or not. The power of the *MaxDiff* test is sufficient to detect a difference in transmission between two treatments even when the reproduction ratios of both groups are above 1. In case that the transmission in the treatment group seems slower, these results would imply that a treatment tends to reduce the transmission, although not enough to have an R_0 smaller than 1. However, the observed reduction indicates that the transmission can be reduced. The next step might be to test whether a combination of treatments, for instance, two vaccinations instead of one, might result in a R_0 smaller than 1.

3.3.   Accounting for heterogeneous populations

One of the assumptions of the stochastic SIR model is that the classified groups in the different infection states (S, I or R) are homogeneous. Thus, all S animals are assumed to be equally susceptible, all I animals equally infectious and all R animals equally immune. In contrast to this assumption is the finding that the infectivity of infectious pigs for

A. pleuropneumoniae appears to be more variable than is assumed in the stochastic SIR model (Chapters 2 and 5). Only with the GLM method it is practical feasible to allow for heterogeneity like the extra variable infectivity, therefore, it is the most appropriate method for the quantification of *A. pleuropneumoniae* transmission (Chapters 4 and 5). Heterogeneity is included in the GLM model by adding variables that describe which fraction of an infection group meets a specific characteristic, like the fraction of I animals from which more than ten colonies *A. pleuropneumoniae* were isolated from the nasal swab.

The other quantification methods described in this thesis cannot account for this variable infectivity. They can probably be adjusted in that direction, for example, by assuming a stochastic SI_1I_2R model (susceptible, high infectious, low infectious, recovered). However, it might not be very feasible to use a more complex stochastic model for estimation or testing methods.

Apart from variable infectivity, we had to accept an extra form of heterogeneity due to the experimental nature of the design. This heterogeneity resulted from mixing untreated infectious pigs with treated susceptible pigs in the treatment trials at the day of replacement (Chapter 5). When a treated susceptible pig gets infected and becomes infectious, there are two categories of I animals present: treated and untreated. This situation could not be avoided, because the exact moment to replace the inoculated pigs by the second-generation susceptible contact pigs (the C_2-pigs in Chapter 5) depends on the isolation of *A. pleuropneumoniae* from swabs taken from first generation infected contact pigs (the C_1-pigs in Chapter 5). This excretion might be influenced by a treatment like vaccination. Consequently, only the effect of the treatment on susceptibility could be tested by GLM analysis, which is only part of the transmission. As already discussed above, here too the GLM analysis is the most appropriate method to use for the quantification.

4. EXTRAPOLATION

4.1. Methodology applicable for other bacterial infections and animals?

More insight into the transmission dynamics of bacterial infections other than *A. pleuropneumoniae* can be gained with help of transmission experiments. At this moment research is being carried out on the transmission dynamics of *Salmonella enteritidis* and *Campylobacter jejunum* between chickens (as yet unpublished) and of *E. coli* O157:H7 and *Mycobacterium paratuberculosis* between calves (also as yet unpublished). All these studies struggle with the same problems that arose during the development of the *A. pleuropneumoniae* experiment. These include finding the optimal challenge model, finding

the optimal design, deciding on which diagnostic tests should be used, classifying animals into different infection states based on diagnostics, etc. Preliminary results of the experiments show that it is possible to quantify transmission of other bacteria, also in other animal species. Using the current experience with regard to bacterial transmission experiments, better and quicker decisions can be made on the design of an experiment, so that the development of transmission experiments for other bacterial infections might also become easier.

The quantification methods as presented in this thesis might be applicable for a whole range of infections, namely, for the transmission of all infectious agents following the transmission dynamics as assumed in the stochastic SIR model (Table 1). If the transmission dynamics are different, for example, if there are only two states (SI) or if there is an extra latent state (SEIR), some quantification methods are still applicable.

4.2.   Extrapolation of *A. pleuropneumoniae* results to the field

The results of the transmission of *A. pleuropneumoniae* described in this thesis are based on experiments in which many factors have been controlled, so these results may differ from the field situation and extrapolation is questionable. We chose to do transmission experiments so that we could study the transmission dynamics as purely as possible and to find causal relations between interventions and transmission parameters. This is hard to achieve in the field where many external factors cannot be controlled.

Confirmation that a pig has been infectious for *A. pleuropneumoniae* in the transmission trial is obtained when the bacterium is isolated from its tonsils at necropsy. We think that this result is also valid in the field, as 'A. pleuropneumoniae positive tonsils' is a host-specific characteristic, which we believe is hardly affected by external factors. However, if a pig has *A. pleuropneumoniae* negative tonsils in the field it is hard to determine it has not been infectious, because it is unknown whether an infectious pig carries *A. pleuropneumoniae* in its tonsils for the rest of its life. The age of a pig at necropsy in the transmission experiments is about 60 days, while the slaughter age in the field is 180 days. Thus, it is quite probable that tonsils of infectious pigs will be free of the bacterium after some time. Another reason why the tonsils of an infectious pig in the field might be negative in bacteriological examination is that other bacteria might oust *A. pleuropneumoniae* from this body site or might overgrow and thus mask *A. pleuropneumoniae* during the bacteriological examination.

The infectivity of an infectious pig was ten times higher on days when more than ten colonies *A. pleuropneumoniae* were isolated from the nasal swab than on other days. We think that this result also applies – although not so specific – to the field situation, since it is also a host-specific characteristic. It is possible that the cut-off of ten colonies might be different in the field, since other bacteria present in a field sample will influence the isolation

of *A. pleuropneumoniae*. From the experiments it also appeared that the tonsillar swab was by far more often positive for *A. pleuropneumoniae* than the nasal swab, which was positive only once or twice and not even in all of the infectious pigs. Nasal swabs were taken in a lot of field studies and appeared to be positive for *A. pleuropneumoniae* quite often (Kume *et al.* 1984; Møller *et al.* 1993; Wongnarkpet 1999). This would imply that the farms from which those positive pigs originated were probably heavily infected. Another implication of this result is that nasal swabs are not appropriate for a test and removal eradication strategy, since many infectious pigs will be missed.

The infection parameter *β* for *A. pleuropneumoniae* was estimated at 0.15 when less than 10 colonies were isolated from the nasal swab and 1.54 otherwise (Chapter 2). Whether the same level of transmission is the same in the field situation is not very obvious, since external factors might have come into play. Other hygiene levels, different climate control, other infections, maternal immunity, stress, other type and age of hosts, etc., might all have an effect on the excretion of bacteria, on the mobility of the bacteria, on the success rate to find another host and on the inhalation of the bacteria.

The effect of vaccination on the susceptibility to become infected (and infectious) of pigs has not yet been demonstrated. If it would have been demonstrated in our experiments, we believe that this result can be extrapolated to the field since it is a host-specific characteristic. However, extrapolation in this case is not very useful since in the field all pigs on a farm will be vaccinated or not and the effect of vaccination on the infectivity of pigs is as yet unknown.

4.3. Viral versus bacterial transmission experiments

The aim of this thesis was to develop methods to quantify bacterial transmission in an experimental setting. To achieve this aim for *A. pleuropneumoniae*, some biological and mathematical problems had to be overcome. There has been experience with addressing such biological and mathematical issues regarding viral transmission (Bouma 1997). From the experience that we gained during this project and from studies as yet unpublished studies on other bacterial infections (see Section 4.1.) we can now identify points in which the quantification of bacterial transmission differs from viral transmission. One point is that the excretion patterns of bacterial infections are intermittent and not very predictable, while viral excretion follows a more or less definite pattern. This makes the interpretation of the results of bacterial transmission experiments more difficult.

Another point is that the I state of a bacterial infection is harder to define (due to the intermittent excretion and due to incomplete serology) than that of viral infections. In most viral transmission studies virus excretion or a measurable antibody response against the virus is considered to be sufficient to indicate a specific individual as infected and infectious. After

a closer study for *A. pleuropneumoniae* it appeared that the presence of the bacterium inside the tonsils was more closely related to the I state than the amount of excretion via nasal or tonsillar swabs. Furthermore, the R state of a bacterial infection is harder to define (also due to intermittent excretion and incomplete serology) than of viral infections. Most viral studies consider that the animal has recovered when the virus excretion ceased. From the *A. pleuropneumoniae* trials it appeared that some pigs kept on excreting the bacterium – although intermittently – until the end of the experiment, while others ceased excreting quite a while before the experiment was stopped.

The last important point that makes quantification of bacterial transmission more difficult than viral transmission is that bacterial transmission chains proceed more slowly than viral transmission chains. Consequently, a final size situation will not have been reached in a lot of bacterial transmission experiments. It means that other quantification methods are needed for bacterial transmission than the methods used for viral transmission.

5. AREAS FOR FUTURE RESEARCH

The problems with *A. pleuropneumoniae* in the field are closely related to that of pleuropneumonia. There are two strategies to prevent problems: preventing transmission or preventing disease. We chose to study the transmission in this thesis, however studying the mechanism of becoming diseased is important and requires future research. More insight into the disease mechanism can be obtained with the help of the challenge model in which sub-clinical infected carrier pigs are created (as presented in Chapter 5).

Below, are some issues related to this thesis – subdivided into transmission of *A. pleuropneumoniae* and quantification of transmission – requiring further research presented.

5.1. Transmission of *A. pleuropneumoniae*

■ Will the transmission of *A. pleuropneumoniae* serotype 9 among SPF pigs be similar to the transmission among conventional pigs? In other words, and as discussed above, can the results be extrapolated to the field?

■ Is the transmission mechanism of other *A. pleuropneumoniae* serotypes similar to that of serotype 9? And, will interventions that reduce the transmission of serotype 9 also reduce the transmission of other serotypes?

■ Which interventions (or combination of interventions) can reduce the transmission of *A. pleuropneumoniae* serotype 9 and other serotypes? Are there possibilities for an eradication programme?

- What is the role of immunity (including maternal) in the transmission dynamics of A. pleuropneumoniae?

5.2.   Quantification of transmission

- Can the TS algorithm be approximated numerically? Is it possible to program the FS algorithm more efficiently so that larger transmission experiments can be analysed as well?
- Can the conclusions about the error made when using the FS algorithm instead of the TS algorithm, and which are drawn from one-to-one experiments and from five-to-five experiments being generalised to all sizes of experiments?

6. ACKNOWLEDGEMENTS

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We set out to gain more insight into the transmission dynamics of bacteria between individual animals with help of transmission experiments. In a well-designed transmission experiment various aspects of transmission can be studied. By studying the infection chain closely better insight into the transmission dynamics will be gained and transmission parameters can be quantified. Moreover, by studying the effect of interventions on transmission, better-directed intervention strategies can be devised. The main goal of the research described in this thesis was the development of methods to quantify bacterial transmission in an experimental setting.

In order to define a more workable aim we restricted the research to the study of the transmission of one specific bacterium, i.e. *Actinobacillus pleuropneumoniae* serotype 9 in pigs. We designed an experimental method to study the transmission of *A. pleuropneumoniae* among pigs in a controlled environment in order to:

- gain a better understanding of the transmission dynamics of A. pleuropneumoniae;
- test the effect of interventions on the transmission of A. pleuropneumoniae;
- develop new statistical or mathematical methods to quantify (bacterial) transmission.

Before all this could be achieved we encountered several difficulties that had to be overcome. For instance, the observed transmission in the first ten transmission trials with *Actinobacillus pleuropneumoniae* was highly variable, which was surprising since the design of the trials was very similar (Chapter 2). This needed further investigation before we could continue. Therefore, it was investigated whether the variation in observed transmission could be explained by variation in infectivity of *A. pleuropneumoniae* infected pigs. After exploring the data of the trials, it was concluded that the variation in observed transmission was caused by two factors. First, the intranasal inoculation method did not lead to carriers in a repeatable and consistent way (Chapter 2). Second, the infectivity of *A. pleuropneumoniae* appeared to be very variable and was closely related to nasal excretion (Chapters 2 and 5). More precisely, the characteristic that was highly correlated to the level of infectivity was the number of *A. pleuropneumoniae* colonies isolated from the nasal swab. We concluded that the probability for an infectious pig to infect a susceptible pig was tenfold higher on days where at least ten colonies were isolated compared to other days.

Another problem was that artificial inoculation was not the appropriate way to induce an infectious but clinically inapparant state with respect to *A. pleuropneumoniae* in a repeatable way, so we switched to an extended transmission experiment. In an extended transmission experiment infectious pigs are created by contact exposing susceptible pigs to artificially inoculated pigs. When enough susceptible pigs are subclinically infected, the inoculated pigs are removed and replaced by a second batch of susceptible pigs. The first extended transmission experiment (consisting of two trials) started with five intranasally inoculated

pigs per trial. These trials turned out to be not satisfactory so it was decided to use endobronchial inoculation in all future experiments (Chapters 2 and 5). Thus, in the final design subclinically infected carrier pigs are created by exposing susceptible pigs to endobronchially inoculated pigs, where after the experiment was extended to start the infection chain between carrier pigs and a second batch of susceptible pigs (Chapter 5).

Another issue to solve was how to analyse data of the *A. pleuropneumoniae* transmission experiments. First, it had to be determined when pigs could be considered as infectious, and for this a good definition of the infectious state for *A. pleuropneumoniae* was needed. We looked for measurable characteristics, which could be indicative for infectious pigs or for the level of infectivity. With help of a Principal Component Analysis a definition of the infectious state was determined, concluding that pigs from which *A. pleuropneumoniae* was isolated from the tonsil (at necropsy) had been infectious during the experiment (Chapter 2).

Another problem that cropped up in the analysis phase was the appropriateness of the traditional Final Size (FS) methods. These methods were developed for viral infections and are based on the assumption that the infection chain has reached a final size situation before the experiment was terminated. In other words, at the end of the experiment there should be no possibilities for new infections to occur as there would be no infectious individuals or no susceptible individuals left. From the conducted *A. pleuropneumoniae* experiments it appeared that reaching a final size situation with those experiments was not always feasible. So, other statistical methods not dependent on the final size assumption were developed (Chapters 3 and 4). Some of these methods are based on the Transient State (TS) algorithm. The TS algorithm is based on the stochastic susceptible-infectious-removed (SIR) model and provides a time-dependent probability distribution over the number of infected individuals during an epidemic. So, a probability distribution for all situations with arbitrary stopping time could be calculated. The FS algorithm is a limiting case of the TS algorithm for the situation where time tends to infinity. With both methods it is possible to estimate or test hypothesis about the level of transmission.

Because of numerical limitations the application of the TS algorithm is limited to experiments with only a few individuals. Another problem that occurs when using the TS algorithm is that the duration of the experiment expressed in units of the average infectious period must be known, which is mostly not the case. Therefore, one will probably resort to the easily applicable FS methods. So, we investigated the error made when FS methods are used instead of TS methods even though the final size situation will not always be reached (Chapters 3 and 4). At first, we investigated the error analytically based on one-to-one experiments (Chapter 3). It was concluded that the methods based on the FS algorithm:

■ underestimate the reproduction ratio R_0;

- are liberal when testing the null hypothesis 'R_0 is equal or larger than 1' against its alternative 'R_0 is smaller than 1';
- are conservative when testing the null hypothesis 'R_0 is equal or smaller than 1' against its alternative 'R_0 is larger than 1';
- give the same probability as the method based on the TS algorithm when testing the null hypothesis 'there is no difference in transmission between control and treatment group' against its alternative 'the R_0 in the treatment group is smaller then the R_0 in the control group'.

Furthermore, we showed how the power of the test depends on the duration of the experiments and on the number of replicates. Finally, we applied the statistical methods to porcine reproductive and respiratory syndrome virus infections for illustrative purpose.

Next we investigated the error made numerically for five-to-five experiments which have been frequently used to investigate viral transmission (Chapter 4). It was concluded in this chapter that the methods based on the FS algorithm in case of a five-to-five experiment:

- underestimate the reproduction ratio R_0;
- are liberal when testing the null hypothesis 'R_0 is equal or larger than 1' against its alternative 'R_0 is smaller than 1';
- are conservative when testing the null hypothesis 'R_0 is equal or smaller than 1' against its alternative 'R_0 is larger than 1';
- are slightly liberal when testing when testing the null hypothesis 'there is no difference in transmission between control and treatment group' against its alternative 'the R_0 in the treatment group is smaller then the R_0 in the control group'.

The last problem we tackled was to test for a difference in the number of contact infections between two treatment groups that was observed during the course of the experiment, which faded away at the end of the experiment. Here, both the FS method and the TS method would not reject the null hypothesis 'there is no difference in transmission between the two treatment groups'. This phenomenon is likely to occur when the R_0 is different in both treatment groups, and greater than 1 in both cases. Therefore, a third method, the *MaxDiff* method, was developed. The *MaxDiff* method can detect a difference in the number of contact infections in the course of the experiment (Chapter 4). The *MaxDiff* method is based on a probability distribution that can be derived numerically using the Markov Chain Monte Carlo technique. We compared the effectiveness of the *MaxDiff* method to other testing methods based on the FS and TS algorithms, and to the existing method that is based on a Generalised Linear Model (Chapter 4). It was concluded that the GLM test was most powerful in finding a difference in transmission between two treatment groups. The TS

test and the *MaxDiff* test, which were approximately equally powerful, were more powerful than the FS test especially when both $R_{0\text{-control}}$ and $R_{0\text{-treatment}}$ are larger than 1.

In Chapter 5 we describe the ultimate experimental design with the most appropriate statistical method to quantify experimentally the transmission of *A. pleuropneumoniae* from subclinically infected carriers to susceptible contact pigs and to test the effect of possible interventions on the transmission. As already stated, the experimental design consists of two parts. First, subclinically infected carrier pigs are created by contact exposure of specific-pathogen-free pigs to endobronchially inoculated pigs. Second, transmission is observed from the group of carrier pigs to a second group of susceptible contact pigs after replacing the inoculated pigs by new contact pigs. The most appropriate quantification method is a generalised linear model (GLM) with which the effect of an intervention on the susceptibility and on the infectivity can be tested separately, if the transmission is observed in heterogeneous populations. The GLM is most appropriate because of two reasons: first this method is able to account for the variable infectivity of infectious pigs and second this method is able to deal with a heterogeneous population consisting of treated and untreated individuals. The experimental design and the quantification method are illustrated by describing an *A. pleuropneumoniae* transmission experiment in which the effect of vaccination on the susceptibility is quantified. In this, it was shown that nasal excretion of *A. pleuropneumoniae* is related to infectivity, however the effect of vaccination on the susceptibility of pigs to become infected has not been demonstrated.

Chapter 6 discusses several aspects of the methodological approaches that have been presented in this thesis. The main aspects of this discussion concern:

- the approach of trial and error to arrive at the optimal design to study and quantify the transmission of *A. pleuropneumoniae*;
- the mathematical and statistical methods used to quantify transmission. We discussed whether the experimental and statistical approach would also be applicable for other bacterial infections;
- whether the results obtained for *A. pleuropneumoniae* infections can be extrapolated to the field;
- whether the new statistical methodology can also be applied to viral infections.

Finally, some issues requiring further research subdivided into transmission of *A. pleuropneumoniae* and the quantification of transmission are presented.

Samenvatting

AANLEIDING

Het bestrijden en uitroeien van infectieuze aandoeningen op een bedrijf, of in een regio of in een land is in de moderne veehouderij erg belangrijk. Voor de individuele veehouder is het vrij zijn van infectieuze aandoeningen ook belangrijk omdat veestapels met een hoge gezondheidsstatus in het algemeen hogere productiecijfers realiseren, hogere prijzen opbrengen en lagere kosten aan medicatie met zich meebrengen. Ook kunnen infectieuze aandoeningen invloed hebben op de internationale handel tussen landen. In landen, die vrij zijn van een bepaalde infectieuze aandoening, kunnen overheden dieren en dierlijke producten weigeren van landen die niet vrij zijn van de aandoening. Zij kunnen op deze wijze de voedselveiligheid en diergezondheid in eigen land beschermen.

Voor het uitroeien van een infectieus agens, zoals een bacterie of virus, moet de overdracht van het agens tussen individuen (ofwel de transmissie) gereduceerd worden. Deze reductie moet zodanig groot zijn, dat het voor het agens niet meer mogelijk is zich te handhaven en daardoor op termijn zal verdwijnen. Er bestaan twee typen transmissie: verticale en horizontale transmissie. Verticale transmissie is de overdracht van een infectie van ouder naar nakomeling tijdens de dracht of geboorte. Horizontale transmissie is elke andere vorm van overdracht van een agens tussen individuen.

De mate van transmissie (verticaal of horizontaal) wordt vaak uitgedrukt door de reproductie ratio: R_0. R_0 is gedefinieerd als het gemiddeld aantal nieuw geïnfecteerde individuen dat in een volledig vatbare populatie door één typisch infectieus individu wordt geïnfecteerd. Met andere woorden, R_0 is een getal dat de mate waarin een infectie zich verspreidt aan het begin van een uitbraak aangeeft. Voor een epidemie – de situatie waarin het aantal geïnfecteerde dieren toeneemt – moet een infectieus individu gemiddeld meer dan één vatbaar individu infecteren. Dus wanneer R_0 hoger is dan drempelwaarde 1, kan de infectie zich verspreiden en grote uitbraken veroorzaken. Wanneer de R_0 van een infectie kleiner is dan drempelwaarde 1, kan de infectie nog wel aanleiding geven tot nieuwe gevallen, maar gemiddeld zal de omvang alleen maar afnemen. Daarom zal de infectie nooit tot een grote uitbraak leiden, maar zal onder deze omstandigheden altijd uitsterven. Voor een succesvolle uitroeiingstrategie – ofwel eradicatie – moet de R_0 gereduceerd worden tot een waarde lager dan 1. Om te onderzoeken welke maatregelen de R_0 kunnen reduceren is kwantitatieve kennis nodig over het transmissiemechanisme van het infectieuze agens.

Kennis omtrent het transmissiemechanisme kan worden verkregen met behulp van epidemiologische studies. De epidemiologie is de wetenschap die de mate waarin een ziekte of agens voorkomt en zich ontwikkeld bestudeerd, die zoekt naar factoren die van invloed zijn op de mate waarmee een ziekte voorkomt of van invloed zijn op de verspreiding van het agens en die onderzoekt hoe een ziekte of agens het beste bestreden kan worden. Binnen één

richting in de epidemiologie worden vaak studies gedaan met modellen waarin de relaties tussen risicofactoren en de mate van voorkomen van een ziekte of agens geschat worden. In deze modellen worden de transmissiedynamica van het infectieuze agens en de interacties tussen individuen in een populatie buiten beschouwing gelaten. Beiden hebben echter invloed op de transmissiesnelheid van een agens. Een andere richting van de epidemiologie houdt zich bezig met methoden waarmee de transmissiedynamica van een infectieus agens bestudeerd kan worden en waarmee transmissieparameters geschat kunnen worden. Hierbij worden wiskundige transmissiemodellen ontwikkeld die de transmissiedynamica van het agens in een populatie theoretisch beschrijven. Gebaseerd op deze wiskundige modellen kunnen met behulp van statistische methoden transmissieparameters geschat worden uit transmissiedata. Deze transmissiedata kunnen worden verzameld in transmissie-experimenten of in het veld (bijvoorbeeld tijdens een uitbraak).

Met behulp van dierexperimenten is het mogelijk de transmissie van een agens in een gecontroleerde omgeving te bestuderen en te kwantificeren. Een transmissie-experiment bestaat vaak uit meerdere deelexperimenten waarin de infectieketen – het aantal nieuwe infecties in de loop van de tijd – van een infectieus agens wordt gevolgd. In elk deelexperiment wordt een aantal infectieuze dieren en een aantal vatbare dieren gezamenlijk gehuisvest (bijvoorbeeld in een isolatieruimte). De infectieketen kan nauwkeurig worden gevolgd door regelmatig monsters van de dieren te nemen en deze te testen (zoals speeksel, bloed of mest) en soms door het scoren van klinische verschijnselen.

Voor de analyse van de data is het classificeren van de dieren in de mogelijke infectieklassen, zoals 'vatbaar', 'infectieus' of 'hersteld', cruciaal. Hierbij is het belangrijk te weten dat de geïnfecteerde en infectieuze dieren geïdentificeerd moeten worden en niet de zieke dieren. Lichamelijke processen die ziekteverschijnselen veroorzaken zijn namelijk niet altijd relevant voor de verspreiding van de infectie. Bovendien zijn geïnfecteerde dieren die infectieus zijn voor anderen niet altijd ziek. De term 'vatbaarheid' wordt ook vaak verschillend geïnterpreteerd: in transmissiestudies wordt vaak de vatbaarheid om geïnfecteerd (en later infectieus) te worden bedoeld en niet de vatbaarheid om ziek te worden. Deze verschillen zijn belangrijk om over na te denken wanneer waarnemingen vertaald worden in infectiegebeurtenissen.

Transmissie-experimenten kunnen geanalyseerd worden door het vergelijken (ofwel fitten) van de experimentele data met een wiskundig model. Een simpel epidemiologisch model dat vaak gebruikt wordt, is het vatbaar-infectieus-immuun (SIR-) model. In het SIR-model worden twee gebeurtenissen beschreven: infectie- en herstelgebeurtenissen (Tabel 8.1). Als het model past bij de transmissiedynamica van de infectie kunnen biologisch interpreteerbare parameters, zoals R_0, geschat worden om de mate van transmissie te bepalen.

Naast het schatten van transmissieparameters kunnen ook verschillende hypotheses getoetst worden. Een relevante nulhypothese is bijvoorbeeld 'er is geen verschil in de mate van transmissie tussen de behandelingsgroep en de controle groep' tegen het alternatief 'er is wel verschil'. Als de bovenstaande nulhypothese verworpen wordt, kan geconcludeerd worden dat de behandeling een effect heeft op de transmissie van het agens. Een andere nulhypothese die getoetst zou kunnen worden is 'R_0 is groter of gelijk aan 1' tegen het alternatief 'R_0 is kleiner dan 1'. Als deze nulhypothese wordt verworpen kan geconcludeerd worden dat R_0 significant kleiner is dan 1. Dit betekent dat de infectie zeker zal uitdoven onder de desbetreffende omstandigheden. Een tegenovergestelde nulhypothese is 'R_0 is kleiner of gelijk aan 1' tegenover het alternatief 'R_0 is groter dan 1'. Als deze nulhypothese wordt verworpen kan geconcludeerd worden dat R_0 significant groter is dan 1. In dit geval kunnen zich twee mogelijke scenario's voordoen. Het eerste scenario is dat de infectie zich verspreidt en dat een groot deel van de populatie geïnfecteerd zal worden. Het tweede scenario is dat slechts enkele individuen worden geïnfecteerd en dat de infectieketen al zeer snel na introductie zal uitdoven.

Tabel 8.1.   Beschrijving van het stochastisch SIR model.

Gebeurtenis	Symbolische weergave [a]	Snelheid waarmee de gebeurtenis plaatsvindt
Infectie	$(S,I) \rightarrow (S-1,I+1)$	$\beta SI/N$
Herstel	$(S,I) \rightarrow (S,I-1)$	αI

[a] S = het aantal vatbare individuen; I = het aantal infectieuze individuen; N = het totaal aantal individuen; β = de infectie snelheid parameters; α = de herstel snelheid parameter.

Transmissie-experimenten hebben een belangrijk voordeel ten opzichte van veldstudies. In de gecontroleerde omgeving van een transmissie-experiment kan namelijk het effect van één factor (bijvoorbeeld van vaccinatie) op de transmissie bestudeerd worden, terwijl variatie veroorzaakt door mogelijke andere factoren geminimaliseerd wordt. Dit impliceert de relatie tussen de interventie en het transmissiemechanismen mogelijk causaal is. Verder zijn experimenten over het algemeen goedkoper en minder tijdrovend dan veldstudies. Hierdoor kunnen meer interventies getest worden en ook interventies met betrekking tot infecties die in het veld niet getest kunnen of mogen worden. Voorbeelden zijn studies naar ziekten die in het land niet voorkomen (zoals mond- en klauwzeer) of vaccins die wettelijk nog niet zijn toegestaan.

Een nadeel van transmissie-experimenten is dat extrapolatie naar de veldsituatie niet altijd makkelijk is (wat ook geldt voor andere dierexperimenten). Maar er is aangetoond dat de resultaten van transmissie-experimenten, waarin de transmissie van pseudorabies virus tussen

conventionele varkens is bestudeerd, goed overeenkomen met wat in het veld wordt waargenomen. Een goede volgorde van onderzoek zou zijn: eerst het effect van interventies op de transmissie testen in een experiment en daarna die interventies die zich al in een experimentele setting bewezen hebben testen in het veld.

De eerste transmissie-experimenten waarin het de effecten van interventies op de transmissie zijn gekwantificeerd, zijn uitgevoerd met virale infecties zoals pseudorabies virus, bovine herpes virus, PRRSV virus en later het varkenspestvirus. Tot nu toe was de ontwikkeling van transmissie-experimenten met bijbehorende analysemethoden alleen gebaseerd op virale infecties en was deze methodologie zo ver bekend nog nooit toegepast op bacteriële infecties. Dit leidde tot het algemene doel van het onderzoek beschreven in dit proefschrift:

'Het ontwikkelen van methodieken om bacteriële transmissie in een experimentele setting te kwantificeren'.

Om een meer werkbaar doel te creëren hebben we het onderzoek gericht op de transmissie van één specifieke bacterie, namelijk *A. pleuropneumoniae* serotype 9 in varkens.

2. *ACTINOBACILLUS PLEUROPNEUMONIAE*

De bacterie *A. pleuropneumoniae* veroorzaakt longontsteking bij varkens, een ziekte die wereldwijd veel schade met zich meebrengt in de varkenssector. Acute (klinische) uitbraken van de ziekte worden gekarakteriseerd door een hoog percentage zieke dieren en veel sterfte. Vooral vleesvarkens van circa drie maanden oud zijn gevoelig voor deze ziekte. Op endemisch geïnfecteerde vermeerderingsbedrijven – waar de infectie constant circuleert – krijgen de meeste biggen via de biest maternale antilichamen tegen de infectie. Hierdoor zijn ze beschermd tegen de ziekte. Op endemisch geïnfecteerde vleesvarkensbedrijven is de aanwezigheid van de bacterie vaak gerelateerd aan een verminderde groei en een lagere voederconversie. Via studies die op slachthuizen zijn uitgevoerd is bekend dat veel varkens oude longlaesies hebben en dat veel varkens *A. pleuropneumoniae* in de keeltonsillen meedragen tot op de slachtleeftijd.

Er zijn 15 verschillende serotypen van de bacterie beschreven welke verdeeld kunnen worden over twee subgroepen: biotype 1 en 2. De verschillende serotypen variëren in virulentie – het gemak waarmee ze ziekte veroorzaken –, maar alle serotypen zijn in staat ernstige ziekteverschijnselen te veroorzaken en kunnen zelfs tot de dood leiden. Het ontstaan van longontsteking veroorzaakt door *A. pleuropneumoniae* is nog niet volledig begrepen. Er

zijn al wel veel virulentiefactoren (zoals de drie Apx-toxinen) van *A. pleuropneumoniae* beschreven, die het de bacterie mogelijk maken in het dier te overleven en zich te vermenigvuldigen.

Een behandeling met anti-bacteriële middelen is effectief bij klinische *A. pleuropneumoniae*-uitbraken. Klinische problemen kunnen voorkomen worden door middel van continue of herhaaldelijke toepassing van anti-microbiële behandelingen. Aanpassingen in het management en in huisvestingssystemen kunnen de kans op een klinische uitbraak ook reduceren.

Varkens die geïnfecteerd zijn geweest met *A. pleuropneumoniae*, krijgen geen klinische symptomen meer wanneer ze nogmaals door hetzelfde serotype worden geïnfecteerd. Verder zijn ze gedeeltelijk beschermd tegen andere *A. pleuropneumoniae* serotypen. Vaccins die dode *A. pleuropneumoniae* bacteriën van een bepaald serotype bevatten, beschermen alleen tegen klinische symptomen van hetzelfde serotype en niet tegen andere serotypen. Vaccins die de toxinen en eventueel andere bestanddelen van *A. pleuropneumoniae* bevatten, beschermen wel tegen de klinische symptomen veroorzaakt door alle serotypen. Helaas voorkomen deze vaccins niet dat dieren sub-klinisch drager worden van de bacterie.

De directe transmissie van *A. pleuropneumoniae* van dier naar dier is waarschijnlijk de belangrijkste transmissieroute, omdat de bacterie niet lang in de omgeving kan overleven. Men denkt dat sub-klinisch geïnfecteerde dragers de meest belangrijke bron vormen voor het introduceren en verspreiden van de bacterie op een bedrijf. Wanneer dit waar zou zijn, zou het voorkomen van het ontstaan van dragers of een voldoende reductie van de transmissie van dragers naar vatbare dieren kunnen leiden tot de eradicatie van *A. pleuropneumoniae* van een populatie. Kennis omtrent de transmissiedynamica van *A. pleuropneumoniae* van dragers naar vatbare dieren is dan ook nodig voor een goed onderbouwd eradicatie- of controleprogramma.

Er zijn veel experimentele infectiemodellen voor *A. pleuropneumoniae* ontwikkeld om de pathogenese van longontsteking te onderzoeken en om het effect van interventies op de klinische verschijnselen te testen. Tot nu toe was er geen experimenteel transmissiemodel voor *A. pleuropneumoniae* beschikbaar om de transmissiedynamica van deze bacterie te bestuderen en om het effect van interventies op de transmissie te testen. Dit transmissiemodel is in dit proefschrift beschreven.

3. DOEL EN BESCHRIJVING VAN HET PROEFSCHRIFT

Het doel van het onderzoek beschreven in dit proefschrift was de ontwikkeling van een experimenteel transmissiemodel om de transmissie van *A. pleuropneumoniae* te bestuderen in

een gecontroleerde omgeving. Met dit experimentele transmissiemodel – ofwel transmissie-experiment – wordt het mogelijk om:

- Meer inzicht in de transmissiedynamica van *A. pleuropneumoniae* te verkrijgen;
- Nieuwe statistische en wiskundige methoden te ontwikkelen om bacteriële transmissie te kwantificeren;
- Het effect van interventies op de transmissie van *A. pleuropneumoniae* te testen.

Voor een optimaal experimenteel ontwerp is het kiezen van experimentele procedures van cruciaal belang. Deze procedures moeten passen bij de biologische interacties tussen gastheer en agens. Voorbeelden van experimentele procedures zijn het bepalen van de optimale infectieroute en de dosis om infectieuze individuen te creëren en het bepalen van de definitie van geïnfecteerde én infectieuze dieren. Verder moeten geschikte wiskundige en statistische methoden gevonden worden die passen bij de transmissiedynamica van de specifieke bacteriële infectie.

Ook tijdens de ontwikkeling van het *A. pleuropneumoniae* transmissie-experiment moesten verschillende experimentele procedures geoptimaliseerd worden en wiskundige en statistische methoden ontwikkelt worden. Hierbij moesten meerdere vraagstukken opgelost worden. Bijvoorbeeld, de transmissie in de eerste tien transmissie-experimenten varieerde veel meer dan verwacht, terwijl de opzet en de uitvoering van deze experimenten erg vergelijkbaar waren. Daarom werd nader onderzocht of de variatie in transmissie verklaard kon worden door variatie in de infectiviteit – de snelheid waarmee een dier andere dieren kan infecteren – van geïnfecteerde dieren.

Na het bestuderen van de experimentele data kon geconcludeerd worden dat er twee oorzaken voor de variabele transmissie bestonden. Als eerste bleek dat de intranasale inoculatie (het druppelen van het inoculum in de neus) niet altijd leidt tot een infectieus dier. Ten tweede bleek dat de individuele infectiviteit van dieren voor *A. pleuropneumoniae* zeer variabel was en
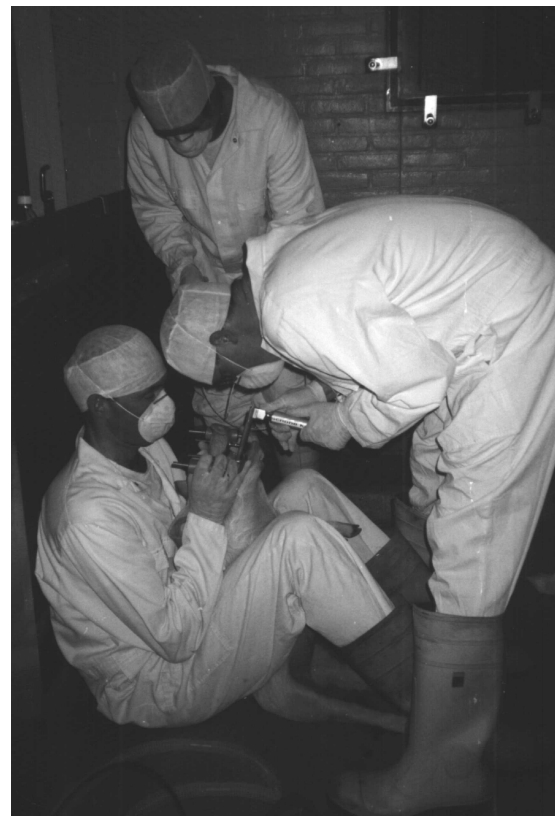
Endobronchiale inoculatie: via een slangetje wordt het inoculum diep in de longen van een verdoofd varken gebracht

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deze gerelateerd is aan de nasale *A. pleuropneumoniae* uitscheiding. Dieren waren tien keer zo infectieus op dagen waar meer *A. pleuropneumoniae* koloniën uit de neusswab worden geïsoleerd dan op andere dagen.

Een andere inoculatiemethode leidde wel tot het gewenste resultaat. Met deze methode worden vatbare dieren geïnfecteerd door ze bloot te stellen aan dieren die zelf met behulp van endobronchiale inoculatie (het via een slangetje inbrengen van het inoculum diep in de longen) geïnfecteerd zijn. Wanneer er genoeg contact-geïnfecteerde dieren zijn, worden de endobronchiaal geinoculeerde dieren vervangen door een tweede groep vatbare dieren. Deze tweede groep wordt dan blootgesteld aan de infectie via de sub-klinisch geïnfecteerde dieren die als eerste contact-geïnfecteerd zijn. Dit wordt ook wel een doorschuifexperiment genoemd. De variatie in infectiviteit kon niet worden ondervangen door aanpassingen in het experimentele ontwerp, maar bij de analyse van de transmissiedata kon hier wel rekening mee worden gehouden.

Andere vraagstukken waren gerelateerd aan de analyse van de data van de *A. pleuropneumoniae* experimenten. Aan het begin van de analyse van een transmissie-experiment moet worden bepaald wanneer een dier als infectieus moet worden geclassificeerd. Hiervoor is een definitie voor de infectieuze status nodig die is uitgedrukt in termen van de verzamelde data, zoals bijvoorbeeld drie dagen achtereenvolgens keelswab positief. Met behulp van een 'principale componenten analyse' hebben we de infectieuze status gedefinieerd. Aangenomen is dat wanneer *A. pleuropneumoniae* uit de tonsillen van een dier (bij sectie) kan worden geïsoleerd, het dier infectieus is geweest tijdens het experiment.

Selectieve plaat met mix van bacteriën die uit een keelswab zijn geïsoleerd

Ook bleek dat de statistische methoden die altijd gebruikt werden bij de virale transmissie-experimenten niet geschikt zijn voor de kwantificering van *A. pleuropneumoniae* transmissie. Deze methoden zijn namelijk gebaseerd zijn op de 'final-size' (FS) aanname die veronderstelt dat de infectieketen is afgelopen voordat het experiment wordt beëindigd. Met andere woorden, aan het einde van een experiment zouden er geen vatbare én infectieuze dieren tegelijkertijd meer aanwezig mogen zijn. Dit is vaak niet het geval bij *A. pleuropneumoniae* experimenten, omdat dieren heel lang drager van de bacterie kunnen blijven.
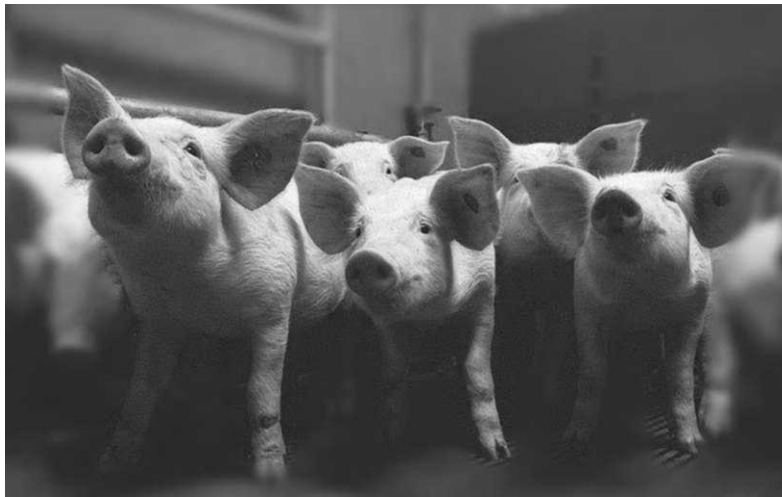
Andere statistische methoden – die geen 'final-size' aanname veronderstellen – zijn ontwikkeld en zijn in dit proefschrift gepresenteerd. Sommige methoden zijn afgeleid van het zogenaamde 'Transient-State' (TS) algoritme. Deze TS-methoden bleken in de praktijk niet goed toepasbaar: de numerieke oplossing voor specifieke experimenten groeit exponentieel zodat de computercapaciteit al snel overschreden wordt. Mede hierdoor is te verwachten dat voor de analyse van transmissie-experimenten waarin geen final-size situatie is bereikt toch de FS-methoden worden gebruikt. Daarom is onderzocht hoe groot de fout is als een FS-methode gebruikt wordt in plaats van een TS-methode. In eerste instantie is dit analytisch onderzocht op basis van 'paartjes-proeven', waarin één infectieus en één vatbaar dier samen worden gehuisvest. Later is de fout door het toepassen van FS-methoden ook numeriek onderzocht voor experimenten waarin vijf infectieuze en vijf vatbare dieren samen worden gehuisvest. Voor beide type experimenten is geconcludeerd dat de fout acceptabel was. Dus de kans is klein dat foute conclusies worden getrokken wanneer FS-methoden worden gebruikt in plaats van een TS-methoden.

Als laatste is een statistische methode ontwikkeld die ook een verschil in transmissie kan aantonen als er tijdens een experiment – bestaande uit een behandelingsgroep en een controlegroep – het verschil in het aantal contact infecties tussen de twee groepen groeit en vervolgens weer kleiner wordt. Deze situatie kan zich voordoen als de R_0 tussen de groepen verschilt, maar in beide gevallen groter is dan 1. De FS- en TS-methoden zullen dit verschil niet blootleggen terwijl er wel een verschil is. Misschien lijkt het verschil in transmissie niet relevant – R_0 is immers in beide groepen groter dan 1 –, maar wanneer één interventie al een significante reductie in transmissie tot gevolg heeft, kan een combinatie van twee of meer interventies wel de R_0 tot onder de 1 reduceren.

Voor het detecteren van deze verschillen in transmissie is een nieuwe methode ontwikkeld, de *MaxDiff* methode. Met deze methode is het mogelijk om een statistisch significant verschil in het aantal contact infecties tussen twee behandelingsgroepen te detecteren dat tijdens de experimentele periode aanwezig is, maar aan het eind (bijna) verdwenen is. De effectiviteit van deze methode, uitgedrukt in het onderscheidingsvermogen van de toets, hebben we vergeleken met die van de FS en TS methoden en met een geheel andere methode, die gebaseerd is op een generalised linear model (GLM). Hieruit bleek dat de *Maxdiff* methode krachtiger is dan de FS en TS methoden om een verschil tijdens een experiment te detecteren. De TS-methode is alleen krachtiger wanneer het experiment exact op het moment waarop het verschil maximaal is gestopt wordt. De GLM methode is krachtiger dan de andere methoden, maar voor deze methode moet per geïnfecteerd dier het moment van infectie bepaald worden wat soms erg moeilijk is.

Het uiteindelijke ontwerp voor een *A. pleuropneumoniae* transmissie-experiment is een doorschuifexperiment waarin de eerste groep van vijf vatbare (C_1-) dieren contact-geïnfecteerd wordt door vijf endobronchiaal geïnoculeerde (E-) dieren. Wanneer er genoeg C_1-dieren contact-geïnfecteerd zijn, worden de E-dieren vervangen door een tweede groep van vijf vatbare (C_2-) dieren. Vanaf dit 'doorschuifmoment' kan de te observeren infectieketen van start gaan. Een gevolg van deze experimentele opzet is dat alleen de C_2-dieren behandeld kunnen worden als het om een individuele behandeling, zoals vaccinatie, gaat. Dit komt omdat een behandeling effect kan hebben op de bacteriële uitscheiding van dieren. Als dus ook de C_1-dieren behandeld zouden worden, wordt het bepalen van het doorschuifmoment moeilijk en onzeker.

Voor de analyse van *A. pleuropneumoniae* transmissie-experimenten wordt een SI model verondersteld. De herstelfase (R) wordt genegeerd omdat bleek dat de geïnfecteerde dieren tijdens het tijdsbestek van het experiment drager bleven. Voor het schatten en toetsen van transmissie-parameters van *A. pleuro-pneumoniae* experimenten zijn de GLM methoden de meest geschikte methoden, omdat *A. pleuropneumoniae* transmissie gekarakteriseerd wordt door variabele infectiviteit en deze variatie kan alleen in GLM model-

De C_1- en C_2-dieren in een *A. pleuropneumoniae* transmissie-experiment

len goed worden meegenomen. Verder kunnen GLM methoden beter dan andere methoden omgaan met verschillende typen dieren in één infectie klasse, zoals gevaccineerde en niet-gevaccineerde vatbare en infectieuze dieren in 1 experiment. Deze situatie ontstaat wanneer alleen de C_2-dieren behandeld worden.

Met het beschreven experiment is het effect van een commercieel *A. pleuropneumoniae* vaccin getest op de transmissie. Omdat alleen C_2-dieren gevaccineerd konden worden, kon alleen het effect van vaccinatie op de vatbaarheid (om geïnfecteerd te worden) statistisch getoetst worden. Het effect kon niet aangetoond worden, maar de relatie tussen de neusuitscheiding van *A. pleuropneumoniae* en de infectiviteit werd nogmaals aangetoond.

Transmissie-experimenten worden steeds belangrijker, omdat het effectief beperken van transmissie tijdens uitbraken, maar ook het efficiënt uitroeien van infectieuze aandoeningen heel belangrijker is in de moderne veehouderij waar voedselveiligheid en de internationale handel belangrijke topics zijn. Dit onderzoek heeft een bruikbare methodiek opgeleverd om interventies met betrekking tot de transmissie van bacteriële infecties, zoals *A. pleuropneumoniae* te testen en in een experimentele setting te kwantificeren. De opgedane kennis vormt een basis voor studies naar de transmissie van andere bacteriële infecties bij varkens maar ook bij andere diersoorten. Op dit moment lopen er onderzoeken waarin de transmissie van *Salmonella enteritidis* en *Campylobacter jejunum* tussen kippen, *Mycobacterium paratuberculosis* en *E.coli* O157:H7 tussen kalveren en *E.coli* O149K91F4ac tussen biggen met behulp van transmissie-experimenten onderzocht wordt.

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Met zeer veel trots kan ik nu zeggen: hier is mijn 'boekje'. Ik wil toch even terug kijken naar hoe ik dit vakgebied ben ingerold en hoe ik het heb ervaren. Bij ons op de boerderij was ik vaak tussen de dieren te vinden. Ze hebben me altijd geboeid. Katten, honden, koeien, kalfjes, kippen, konijnen, parkieten, dwergpapegaaien, zebravinkjes, varkens en zelfs goudvissen: ik heb ze om me heen gehad en ik heb er veel plezier aan beleefd. Ik voelde mij ongelukkig wanneer deze dieren zwak, ziek en misselijk waren. Hoe kun je een dier dan helpen? Maar vooral ook: hoe had dit voorkomen kunnen worden? Juist op die momenten heb ik mijzelf voorgenomen om later 'iets' te gaan doen om te voorkomen dat dieren ziek worden. De keuze tussen diergeneeskunde en zoötechniek (ofwel veeteelt) was voor mij lastig omdat beide richtingen mij konden helpen mijn doel te bereiken. Ik heb de keuze dan ook maar van de loting af laten hangen. Dat ik als afstudeerrichting epidemiologie heb gekozen was voor mij een logische keuze. Tijdens mijn afstudeervak epidemiologie zag ik het licht: onderzoek doen is leuk! Gelukkig kreeg ik mijn eerste baan aangeboden bij het voormalig praktijkonderzoek rundvee, schapen en paarden. (Ik was bijna als baconverkoper bij een grote slachterij begonnen.) Bij het PR heb ik onderzoek verricht naar de risicofactoren van stofwisselingsaandoeningen bij rundvee. Aan het einde van dit onderzoekstraject trok een vacature mijn aandacht: er werd een Aio gezocht om de transmissie van een bacterie met een hele lange en ingewikkelde naam te kwantificeren. Ondanks dat ik mij nog zó had voorgenomen om géén onderbetaalde Aio te worden, heb ik toch een brief geschreven. En hier is dan mijn boekje... een ervaring rijker en wat spaargeld armer. Ik heb mijn Aio-traject met veel plezier doorlopen, mede door de gezellige en ongedwongen sfeer bij QVE en op het lab van bacteriologie. Slechts enkele schaarse momenten heb ik spijt gehad van mijn beslissing om Aio te worden. Eén van die momenten was toen ik en Hans gingen informeren naar een hypotheek. Nadat ik vol trots vertelde dat ik Aio was en dat mijn salaris de komende 4 jaren alleen maar zou stijgen en dat het na mijn promotie helemaal goed zat, deelde de hypotheekadviseur mij vrolijk mee. "Ach meisje… , op basis van dat Aio-salaris kan je geen hypotheek krijgen, want er is absoluut geen zekerheid. De meeste Aio's maken dat boekje toch niet af". Ik denk dat ik de dag na mijn promotie naar die hypotheekadviseur ga om hem met mijn boekje om de oren te slaan!

Annet V

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meedenken
medewerking
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Ik, Annette Gezina Johanna Velthuis, werd op 27 april 1972 geboren in Oldenzaal. In 1989 behaalde ik mijn HAVO diploma en vervolgens in 1991 mijn VWO diploma aan het Twents Carmel Lyceum te Oldenzaal.

In hetzelfde jaar begon ik met de studie Zoötechniek aan de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit) te Wageningen. In 1996 ben ik afgestudeerd, met als oriëntatie Veehouderij en afstudeerrichting Kwantitatieve Epidemiologie en Kwantitatieve Fokkerij.

In het verlengde van dit laatste afstudeervak ben ik voor twee maanden werkzaam geweest als toegevoegd onderzoeker bij het ID-DLO (nu ID-Lelystad) bij de voormalige afdeling Fokkerij en Genetica te Lelystad.

Hierna ben ik één jaar werkzaam geweest als onderzoeker bij het Praktijkonderzoek Rundvee, Schapen en Paarden (nu Praktijkonderzoek Veehouderij) bij de afdeling Dier en Product.

In augustus 1997 werd ik aangesteld als Assistent In Opleiding (AIO) bij de leerstoelgroep Dierhouderij van Wageningen Universiteit en was gedetacheerd bij de groep Kwantitatieve Veterinaire Epidemiologie (QVE) van ID-Lelystad. Ik verrichtte daar het onderzoek dat in dit proefschrift is beschreven.

Sinds 1 januari 2002 ben ik als universitair docent werkzaam bij de leerstoelgroep Agrarische Bedrijfseconomie van Wageningen Universiteit met de economie van voedselveiligheid als invalshoek.

Aantekeningen