Transmission of *Actinobacillus pleuropneumoniae* in pigs is characterized 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 an *A. pleuropneumoniae* positive tonsil 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.

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 trans-mission [1]. 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 [1–5], classical swine fever virus [6] and bovine herpes virus [7, 8]. The vaccination scheme

used in the Dutch eradication programme for pseudo-rabies virus was developed with knowledge about the transmission obtained from transmission experiments combined with mathematical modelling [9].

To get more knowledge about the transmission of bacterial infections, ten transmission trials were carried out with the bacterium *A. pleuropneumoniae*. *A. pleuropneumoniae* is regarded as a primary pathogen that causes pleuropneumonia in pigs and great economic losses worldwide [10]. 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 [11]. 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 [1].

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 [1]. 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.

MATERIALS AND METHODS

Pigs

One hundred and twenty-two, 5-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.

Experimental design

Ten transmission trails within 6 experiments were carried out. To create infectious animals, pigs were intranasally inoculated in 8 trials and endobronchially inoculated in 2 trials. One trial included only 3 (intranasally) inoculated pigs and 4 contact pigs, whereas the others included 5 inoculated pigs and 5 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 temporarily housed in another isolation unit. One day later, the contact pigs were housed with the inoculated pigs again to minimize 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 a 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 (Fig. 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 became infected. The experimental periods of the ten trials and the replacement days of the extended trials are shown in Figure 1.

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 m², 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 tonsil, nasal lymph node and, if present, pneumonic parts of the lungs (lesions) were collected for bacteriological examination.
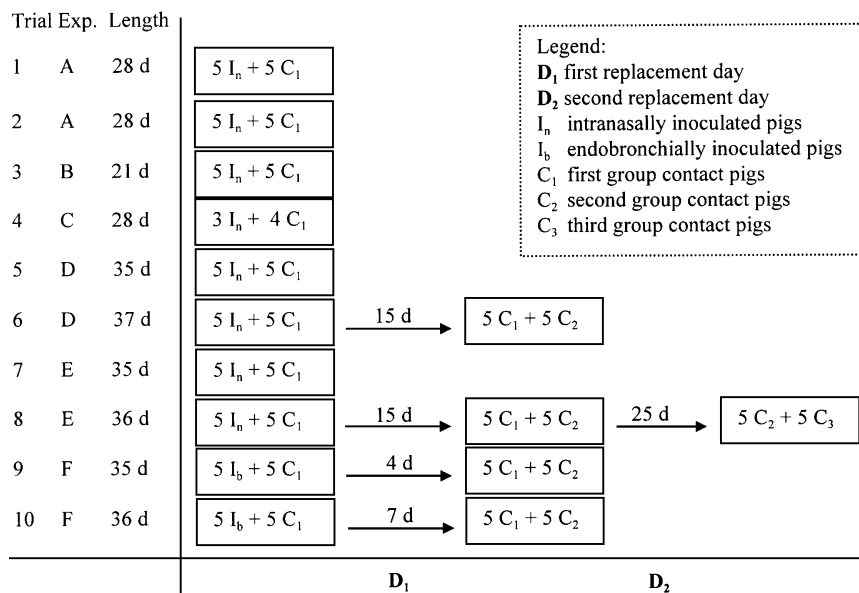
Fig. 1. Design of the ten transmission trials carried out with *A. pleuropneumoniae*. Four of the ten trials were extended, and one of those four was double extended. See text for details.

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 h 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 were counted the next morning. For both inoculation methods the pigs were anaesthetized shortly with halothane gas and were held in upright position. The pigs were intra-nasally 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 [12].

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 gentamicin, 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 a 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 3 sec 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.

Haemolysin neutralization assay

The ability of the sera to neutralize haemolytic culture supernatant fluids of *A. pleuropneumoniae* serotype 9 was determined, using sheep erythrocytes [13]. Two-fold dilutions of 50-μl volumes of the sera in GVS (gelatine veronal saline buffer) were incubated for 30 min 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 h 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.

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 [14] 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 performed according to the true mass-action theory [15]. The rate at which an infection event occurs depends on the density of S-pigs, the number of I-pigs present and an infection
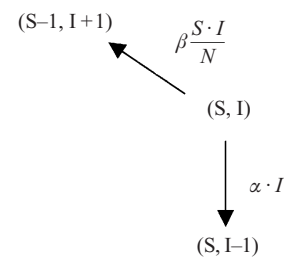

Fig. 2. The transition probabilities from state (S, I) to the two following states due to an infection $(S-1, I+1)$ or recovery event $(S, I-1)$.

rate parameter β (Fig. 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 a given time only one of both events can occur, transmission parameters can be estimated according to the generalized linear model method of Becker [14].

De Jong and Kimman [1] described an algorithm for calculating the probability distribution of the final size of the outbreak given transmission parameters and start conditions. 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 characterize the transmission. This R_0 is also called the basic reproduction ratio and is biologically defined as the average number of secondary cases caused by one typical infectious pig [16].

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 by the transmission mechanism described in the SIR-model:

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

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 $\text{var}(X_i)$ 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 the probability distribution

of the final size of the outbreak [1]. So, we tested whether $H_0 : R_{0,\exp1} = R_{0,\exp2} = \cdots = R_{0,\exp n} = R_0$ 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 has a low value (< 0.05).

Principal component analysis

To examine which of the possible indicators for individual infectivity has a high variation, we performed 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 summarizes the variability in the data [17]. In a PCA, principal components are created that 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.

Estimating variation in individual infectivity

Variability in individual infectivity is characterized 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 [1], 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.

$$\text{Prob}[C \mid S_1, q] = \text{Bin}(S_1, q), \quad q = 1 - \exp\left(-\beta \frac{I_1}{N}\right).$$

The above-mentioned statistics are sufficient to estimate the infection rate parameter β using a generalized linear model (GLM) with a complementary-log–log link, a binomial error term, and $\ln(I_1/N)$ as the offset [18].

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 N_{10} is the fraction of I_1-pigs from which on average of two subsequent samples more than ten colonies *A. pleuropneumoniae* was 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:

$$\beta = \exp\left(b_0 + \sum_1^i b_i \cdot x_i\right).$$

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

Table 1. *Overview of the results of the ten transmission trials*

Trial	I/C*	Number of pigs		A. pleuropneumoniae isolated from						HN-positive	
		Total	Found dead	Tonsil	Lesion†	T_1‡	T_{10}§	N_1‖	N_{10}¶	Start**	End††
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	$5_{(35)}$

* Intranasally or endobronchially inoculated (I_n or I_b) or first, second or third contact pig (C_1, C_2 or C_3).
† Lung lesion.
‡ $\geqslant 1$ colony isolated from a tonsillar swab.
§ $\geqslant 10$ colonies isolated from a tonsillar swab.
‖ $\geqslant 1$ colony isolated from a nasal swab.
¶ $\geqslant 10$ colonies isolated from a nasal swab.
** Number of pigs with raised titre (with respect to 1/8) at the start of the trial (day 0).
†† End of the trial: the day number is given in parentheses.

RESULTS

In Table 1 an overview of the results of the ten transmission trials is shown. It is clear that the observed transmissions are highly variable when looking at the results of the bacteriological examination of the specimens of the C_1-pigs.

Variation in transmission between trials

We assumed that the transmission mechanism of *A. pleuropneumoniae* is described by the stochastic general SIR-model of Becker [14]. 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

Table 2. *The variables, the descriptions and their loadings in the first principal component*

Variable	Description	Loading*
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

* 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.

be highly variable. There were two trials with no contact infection at all, six trials where all C_1-pigs became infected, and two trials where some but not all C_1-pigs seemed infected (Table 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*.

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.

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).

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 tonsil at necropsy.

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 tonsil 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 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 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.

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
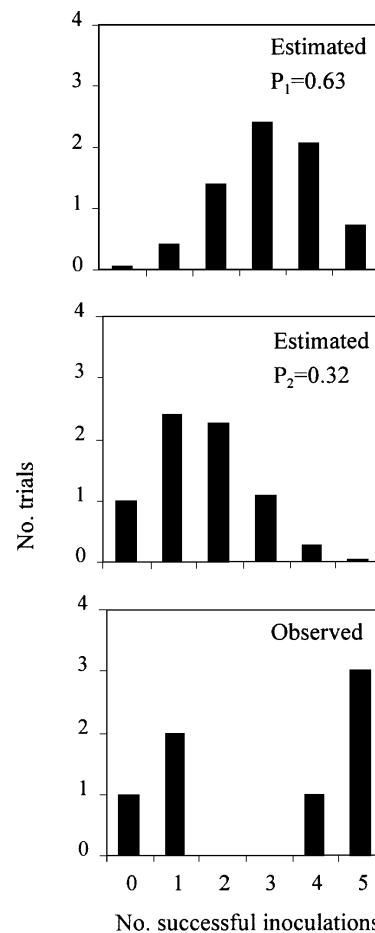

Fig. 3. The estimated and observed frequencies of seven trials over the number of successful intranasal inoculations x when executing five intranasal inoculations per trial. The top panel shows the estimated frequency that was based on the success probability p_1, the middle panel gives the frequency that was based on p_2, and the bottom panel represents the observed frequency of trials over the number of successful intranasal inoculations.

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 an *A. pleuropneumoniae* positive tonsil 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 N_{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 N_{10}. The estimated constant b_0 was -1.95 with standard error 0·24 and the estimate for N_{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:

$$\beta = \exp(-1.95 + 2.36 \cdot N_{10}).$$

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 where more than ten colonies were isolated from the nasal swab than on the other days.

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 in-

fectivity. 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 tonsil at necropsy it was infectious for *A. pleuropneumoniae* during the trial. It was quite surprising that an eventual carrier status in the tonsil 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 [19]. 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 nasal swabs taken in the trials a lot less pigs were called infectious than there actually were. Thus, having an *A. pleuropneumoniae* positive tonsil is a better indicator for infectious pigs than successive swabs positive for *A. pleuropneumoniae*. Unfortunately the status of the tonsil can only be measured at necropsy or maybe earlier when taking tonsil biopsies, which was not tested in this study.

The importance of the colonization of *A. pleuropneumoniae* in the tonsil is also mentioned in studies from Chiers et al. [20], Møller et al. [21] and Nicolet [11]. Møller et al. [21] 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. [22] 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 itself 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 colonized

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 colonization of *A. pleuropneumoniae* in the tonsil and also excretion of *A. pleuropneumoniae* from the tonsil 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 tonsil. Little is known about this interaction. Chiers et al. [20] looked at early interactions of *A. pleuropneumoniae* with the tonsil of 1-week-old gnotobiotic pigs that were inoculated onto their tonsils. He showed that in a few hours the bacteria attached to 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 tonsil could be a good diagnostic tool to detect carrier pigs within a population. Savoye et al. [23] 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 optimize the sampling strategy of the tonsil 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 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 colonization 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* [24].

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. [21] 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 [25] 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. [26] swabbed the nasal cavity of 6 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. [27] 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. [27] 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.

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