DETECTION AND TRANSMISSION OF EXTRACELLULAR FACTOR PRODUCING *STREPTOCOCCUS SUIS* SEROTYPE 2 STRAINS IN PIGS

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2009

Swildens, B. 2009. Detection and transmission of extracellular factor producing *Streptococcus suis* serotype 2 strains in pigs

Printing of this thesis was financially supported by the Department of Farm Animal Health of the Faculty of Veterinary Medicine, Utrecht University

ISBN: 978-90-393-5202-1

On the cover: fibrinopurulent meningitis, published in "Color Atlas of Veterinary Pathology", 2nd edition, edited by Jaap E. van Dijk, Erik Gruys, and Johan M.V.M. Mouwen, printed by Sanders Ltd (2007), ISBN 9 7807 0202 7581

Printed by: Atalanta Drukwerkbemiddeling, Houten

DETECTION AND TRANSMISSION OF EXTRACELLULAR FACTOR PRODUCING *STREPTOCOCCUS SUIS* SEROTYPE 2 STRAINS IN PIGS

De detectie en overdracht van extracellulaire factor producerende *Streptococcus suis* serotype 2 stammen in varkens

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op
dinsdag 17 november 2009 des middags te 12.45 uur

door

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geboren op 10 februari 1960 te Utrecht

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CONTENTS

Chapter 1

General Introduction

INTRODUCTION

Streptococcus suis (*S. suis*) has been associated in the etiology of a wide range of clinical diseases in swine. The symptoms caused by *S. suis* include meningitis, arthritis, pneumonia, septicemia, endocarditis, polyserositis and sudden death. Outbreaks have been recognized worldwide in countries where pig production is well developed (Staats et al., 1997). Farmers and veterinarians classified the impact of a *S. suis* serotype 2 meningitis on the well-being of affected pigs as very severe in comparison with swine influenza or enteric diseases (Bennett and IJpelaar, 2003). The control of diseases caused by *S. suis* demands great efforts at swine farms. The costs of drugs, extra labor, and high mortality rates have a significant impact on their economics. Concomitant with the high use of antibiotics to control streptococcal disease at swine farms, the hazard of antibiotic resistance increases, which is a threat for animal as well as public health (Hendriksen et al., 2008).

S. suis is also a zoonotic agent which affects predominantly people who handle pigs routinely like farmers and butchers (Arends and Zanen, 1988). Sometimes the bacterium provokes larger outbreaks in man like in the Sichuan province in China (WHO, 2005; Yu et al., 2006; Yu et al., 2005). Experts in communicable diseases classify *S. suis* meningitis in man as an extremely violent disease (Bennett and IJpelaar, 2003).

Although much research has been done on the pathogenesis and transmission of *S. suis*, standardized protocols for the control of *S. suis* related clinical disease are not available. Diagnostic tools to detect the carriers of *S. suis* play a pivotal role in the understanding of *S. suis* transmission, but are not well defined so far. This thesis adds new insight in the detection and transmission of *S. suis* and the prevention of disease caused by *S. suis*.

STREPTOCOCCUS SUIS

Since De Moor (1963) defined *S. suis* as Lancefield group R, S and T several reallocations of *S. suis* within the Lancefield classification system have been suggested (Elliott, 1966; Facklam, 2002; Windsor and Elliott, 1975). The gram-positive, facultative anaerobic, alpha-haemolytic coccus is now categorized into at least 35 different serotypes based on specific capsular antigens (Gottschalk et al., 1989; Higgins et al., 1995; Perch et al., 1983). Serotype 2 was the most isolated serotype in Europe while in the Netherlands, Germany and Belgium serotype 9 was predominant (Wisselink et al., 2000).

Several virulence factors such as the capsular polysaccharide, muramidase released protein (MRP), extracellular factor (EF), hemolysin, adhesions factors and environmentally regulated genes are associated with disease (Segura and Gottschalk, 2004; Smith et al., 2001). The EF producing *S. suis* serotype 2 strain (*S. suis* 2 EF^{+}) is positively associated with the occurrence of diseases in pigs (Vecht et al., 1991).

In serotypes 7 and 9, several other putative virulence factors have been described whereas in these serotypes EF is not associated with disease (Wisselink et al., 2000).

In most European countries as well as in the United States and Australia, EF positive strains of serotype 2 are the most frequent isolates from diseased pigs (Berthelot Herault et al., 2000; Galina et al., 1996; Mwaniki et al., 1994; Salasia and Lammler, 1995; Wisselink et al., 2000). That is why the well-documented EF producing *S. suis* 2 strain was used in our epidemiological studies.

THE PREVENTION AND CONTROL OF STREPTOCOCCAL DISEASE AND TRANSMISSION

The prevention of streptococcal diseases can be based on two basic strategies. In the first strategy the endemic occurrence of *S. suis* on the farm is permanent and accepted, while in the second strategy the objective is to accomplish elimination of *S. suis* strains and subsequently maintain freedom of these strains.

In the endemic situation, disease is controlled by management measurements that are focused on pig health in general as well as by preventing the transmission of *S. suis* between animal groups using separation and/or medication. Vaccination with farm-specific whole bacterin vaccines and medications are applied in order to decrease the incidence of diseases. Strategies focused on the elimination of virulent *S. suis* strains from herds could provide a more sustainable method to prevent *S. suis* diseases. Expensive depopulation-repopulation systems with hysterectomy derived piglets have been performed to achieve this, but breakdowns of the *S. suis*-free status have been reported (Clark et al., 1994; Fangman and Tubbs, 1997).

1. The route of infection

The upper respiratory tract is mostly mentioned as porte d'entrée for *S. suis* (Gottschalk and Segura, 2000; Madsen et al., 2002). Other routes of infection like skin lesions and translocation from the intestinal tract may be possible too. Although translocation of different bacteria through the intestinal mucosa is a well-known phenomenon, the translocation of *S. suis* has not been examined as a potential route of infection so far.

Frequently small numbers of bacteria, mostly normal indigenous species, translocate from the intestine across the intact mucosal epithelium, whereupon they are effectively killed by the host's immune defenses. However, if the integrity of the mucosal barrier is damaged bacteria readily translocate further on to extra-intestinal sites, such as mesenteric lymph nodes, spleen or bloodstream (Berg, 1992). This especially occurs when 'stress'-mediating conditions diminish the blood supply in the superior mesenteric artery. The accompanying reduction in oxygenation of the intestines eventually gives rise to intestinal ischemia-reperfusion injury (Meddah et al., 2001; Pargger et al., 1997; Parks et al., 1982; Spreeuwenberg et al., 2001; Steffen et al., 1988).

Many risk factors for the occurrence of *S. suis* meningitis in weaned pigs are described in literature. They often include stressful events like weaning, mixing of litters, change of climate and transition to solid food provision (Dee et al., 1993; Dee and Corey, 1993; Villani, 2003). In line with the translocation of indigenous bacteria it could be possible that under such stressful circumstances, *S. suis* translocates the intestinal mucosa. In that case, it would be justified to focus weaning strategies on the prevention of intestinal ischemia-reperfusion injury, especially at farms with streptococcal disease problems. To examine the possibility of *S. suis* translocation, we inoculated the pathogenic EF-producing *S. suis* serotype 2 strain in pigs that were thereafter transported (Chapter 5).

2. Transmission of *Streptococcus suis*

Carriers of *S. suis* are found in all age categories at farrowing farms: sows, piglets and weaned pigs. Most clinical cases are seen in piglets between one and three weeks after weaning. Transmission of *S. suis* was described from the sow to her litter as well as between pigs after weaning. Other, however less frequent demonstrated routes of transmission are for instance intravaginal and by aerosols over short distance (Berthelot-Herault et al., 2001; Cloutier et al., 2003; Robertson et al., 1991).

At farms where *S. suis* is endemic, several interventions with the aim to eliminate *S. suis* or to raise piglets free of *S. suis* were performed with different success rates (Fangman and Tubbs, 1997; Higgins and Gottschalk, 2006). The authors could not bring about durable *S. suis* -free herds after early weaning programs neither without nor with medication of sows and piglets. The elimination of *S. suis* from the tonsils of fattening pigs by medication alone was not achieved as well (Clifton Hadley et al., 1984). However, Torremorell (1998) and Amass (2000) did observe lower prevalences in piglets after vaccination of the sows. Unfortunately, a standard vaccination strategy that covers all local *S. suis* strains is hampered by the absence of industrially produced protective vaccines (De Greeff et al., 2003; Li et al., 2006; Staats et al., 1997; Wisselink et al., 2002). Instead, farm specific autovaccines are frequently used. This requires specialized pathological examination to isolate the strain and laboratory facilities for bacterial growth and determination as well as the vaccine production.

Breeding piglets free from specific virulent *S. suis* strains would have many advantages in health-management after weaning. To accomplish this at farms where *S. suis* is endemic under sows, a strategy to prevent infection of the offspring is required. To study the feasibility of such a strategy, a transmission experiment is performed. Sows, carriers of *S. suis* 2 EF^+, were vaccinated and medicated to halt *S. suis* 2 EF^+ transmission to their litter. As described in a study in chapter 4, we combined both these treatments to obtain the best chance to prevent *S. suis* 2 EF^+ transmission. The effect of this combination was not examined before.

Summarizing, there is no general strategy to prevent streptococcal disease in an endemic situation. The same goes for *S. suis* eradication procedures without an expensive depopulation. Prevention and control of streptococcal disease is still ham-

pered by the lack of knowledge about the pathogenesis and *S. suis* transmission within farms. In order to enable *S. suis* transmission studies in the field, possibly followed by monitoring programs at farm level we designed a convenient method for pig sampling and laboratory analysis.

DETECTION OF *STREPTOCOCCUS SUIS*

S. suis can be found in many organs of diseased animals, such as the nose, tonsils, lungs and the intestinal and genital tracts (Baele et al., 2001; Clifton Hadley and Alexander, 1980; Devriese et al., 1994; Robertson et al., 1991; Williams et al., 1973). The presence of *S. suis* in organs of clinically healthy pigs is mostly transient. However the tonsils can harbor *S. suis* colonies deep in the crypts for prolonged periods, without any clinical signs of disease (Davies and Ossowicz, 1991). Therefore the tracing of carriers of *S. suis* is preferably performed by sampling of the tonsils.

A polymerase chain reaction (PCR) developed by Wisselink and others (1999) for the detection of EF-positive *S. suis* serotype 2 strains was in use to analyze clinical specimens such as tonsil homogenates. This PCR demonstrated high sensitivity on purified chromosomal DNA; the equivalent of 14 *S. suis* cells was easily detected. No amplification products were obtained with any other examined strain. Till this point, the test qualities were not known on specimen taken from live pigs.

Characterization of a diagnostic test typically focuses on how correct the test classifies subjects in two subpopulations (classes) with a different status i.e. animals that are healthy or diseased, or likewise non-carriers and carriers. With regard to the true status of an animal, the quality of a diagnostic test is expressed as sensitivity and specificity. Sensitivity is defined as the probability that a test classifies a subject that is truly diseased or carrier as positive and specificity is defined as the probability that a test classifies a subject that is truly healthy or non-carrier as negative.

To determine both parameters the true disease status of the animals must be indisputably defined by a gold standard. For the characterization of the *S. suis* 2 EF^+ PCR in living sows, there was no gold standard. By adding at least one extra test and sampling from more populations with distinct prevalences, the need for a gold standard can be evaded. Assuming that the tests are valid, conditionally independent and behave the same across the populations, the unknown prevalences, sensitivities and specificities can be estimated from the test outcomes (Hui and Walter, 1980).

To perform this test-characterization, Bayesian statistical analysis has been increasingly applied in the past few years (Enoe et al., 2001; Frossling et al., 2003; Joseph et al., 1995; Orr et al., 2003). The basic principle of the Bayesian approach is that every valid test (a test that performs better than chance) correctly classifies a proportion of the true positive and true negative test-samples. Because these proportions usually do not completely overlap for different tests, an iterative procedure can generate the best fitting sensitivity and specificity from the test results (Casella and George, 1992). To obtain narrower credibility intervals, the Bayesian model can be

extended with more test or populations. Furthermore, information from previous studies carried out on the applied or comparable tests and additional parameters that account for possible dependence between the diagnostic tests can be added (Enoe et al., 2000; Gelman et al., 1995; Lee, 1997).

We performed such a Bayesian analysis on the results of two *S. suis* tests on tonsil-swabs taken from live sows and on the removed whole tonsils after slaughter. To fulfill the criteria for Bayesian analyses, the laboratory tests differed substantially from each other in methodology (PCR and Bacterial Examination) what potentially minimizes the dependency between the tests. To have sufficient true carriers and non-carriers, the sows were obtained from four herds with different prevalences. The results are discussed in chapter 2. In order to acquire smaller credibility intervals for sensitivities and specificities of all tests, two additional continuous antibody tests were added and different ways to incorporate prior available information are compared in chapter 3. The statistical and in particular the biological implications of these Bayesian analyses are discussed in chapter 6.

SCOPE AND OUTLINE OF THE THESIS

We focused our research primarily on the endemic *S. suis* status at farms. To be able to perform transmission studies, we estimated the sensitivity and specificity of a polymerase chain reaction assay (PCR) on swabs taken from live sows. This test was developed by Wisselink et al. (1999) for the detection of EF-positive *S. suis* serotype 2 strains (chapter 2). The outcomes of the PCR together with two other *S. suis* laboratory tests, but without a gold standard, were analyzed in a Bayesian analysis using WinBUGS (Spiegelhalter et al., 2003). To optimize the model, different parameterizations of expert information about the tests and parameters for dependency between tests were compared (chapter 3). Using the PCR on tonsil-swabs, we selected *S. suis* 2 EF^+ carrier sows and studied the transmission between these dams and their own offspring in isolated farrowing units. This is considered to be the first step in the complex and largely unresolved chain of *S. suis* transmission at farms (chapter 4). Finally, to offer possible tools in the control of streptococcal diseases in weaned pigs, which is the most often affected group at commercial farms, we examined the hardly ever studied route of *S. suis* infection through intestinal translocation (chapter 5). In chapter 6 some important points and implications of the thesis are discussed in more depth, supported by additional, so far unpublished results.

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Chapter 2

Detection of Extracellular Factor-positive *Streptococcus suis* serotype 2 strains in tonsillar swabs of live sows by PCR

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Veterinary Microbiology 109 (2005) 223–228

ABSTRACT

In this study the sensitivity (Se) and specificity (Sp) of a PCR for the detection of EF-positive *Streptococcus suis* serotype 2 strains in tonsillar swabs of live sows were assessed. We sampled 471 sows originating from four farrow-to-finish farms, by tonsillar swabbing and collected their tonsils after slaughter. On these specimens a PCR, a bacterial examination (BE) or both were performed for the detection of EF-positive *S. suis* serotype 2 strains. Swab-PCR, Tonsil-PCR and Tonsil-BE were regarded as three integral tests. A Bayesian approach was used to calculate the Se and Sp of the tests. Se and Sp for Swab-PCR were 0.63 (95% Credibility Interval <0.52, 0.74>) and 0.96 (<0.92, 0.99>), respectively. Values for Se and Sp of Tonsil-PCR amounted to 0.88 (<0.75, 0.96>) and 0.94 (<0.87, 0.99>), respectively. For Tonsil-BE Se was 0.65 (<0.51, 0.76>) and Sp was 0.97 (<0.91, 0.99>). Repetition of the swabbing procedure after 10 minutes resulted in a higher Se (0.85 (<0.67, 0.96>) than the Se of the first Swab-PCR. Repetition of the PCR on the same sample did not result in any significant changes in the outcome of the analysis.

Keywords
Streptococcus suis, sensitivity and specificity, Bayesian, carrier, tonsillar swabs

INTRODUCTION

Streptococcus suis is a gram-positive coccus that has been implicated in the etiology of a wide range of clinical diseases in swine. The symptoms caused by *S. suis* in swine include meningitis, arthritis, pneumonia, septicaemia, endocarditis, polyserositis and sudden death. Outbreaks have been recognized worldwide in countries where pig production is well developed (Staats et al., 1997). In most European countries as well as in the United States and Australia, muramidase-released-protein (MRP) and extracellular factor (EF) positive strains of serotype 2 are most frequently isolated from diseased pigs (Galina et al., 1996; Mwaniki et al., 1994; Salasia and Lammler, 1995; Wisselink et al., 2000).

Sows carrying *S. suis* strains in their tonsils are considered the most important source of infection for susceptible young pigs (Clifton Hadley et al., 1984; Robertson and Blackmore, 1989). Detection of these carriers can contribute to the development of control measures. In diagnostic assays, the whole tonsil will probably be the best specimen to detect animals carrying *S. suis*, however this can only be achieved after slaughter. Tonsillar swabbing seems to be the most practical method for sampling live pigs, although it is possibly not the most sensitive method, because only the surface of the tonsil is sampled and *S. suis* tends to form confined groups of bacteria deep in the tonsillar tissue (Davies and Ossowicz, 1991).

For the detection of *S. suis* serotype 2 strains from clinical specimens, various laboratory techniques have been developed. Most test methods are time consuming and lack sensitivity or specificity or both for virulence markers. Recently, a polymerase chain reaction for the detection of EF-positive *S. suis* strains (PCR) was developed. This PCR assay demonstrated good sensitivity (Se) and specificity (Sp) on tonsillar specimens of slaughter pigs when compared with bacteriological examination of the same specimens (Wisselink et al., 1999). However, Se and Sp of this PCR on tonsillar swabs of live pigs were not determined.

Generally, sensitivity (Se) and specificity (Sp) of a test are determined by comparing its results with the results obtained in a reference test (gold standard). However, as virtually no reference test has perfect Se and Sp, classification errors may lead to serious bias in the assessment of test characteristics of a new test. To solve this problem, Bayesian statistical analysis has been increasingly applied in test-characterization in the past few years (Enoe et al., 2001; Frossling et al., 2003; Orr et al., 2003). The basic principle of the Bayesian approach is that every valid test (a test that performs better than chance) correctly classifies a proportion of the true positive and true negative test-samples. Because these proportions usually do not completely overlap for different tests, an iterative procedure can generate Se and Sp from a priori defined probability distributions and the new test results (Casella and George, 1992). These probability distributions are based on previous studies carried out on the applied test or on comparable tests (Enoe et al., 2000; Gelman et al., 1995; Lee, 1997). In the current study we assessed the quality of a PCR for the detection of EF-positive *S. suis* serotype 2 strains on tonsillar swabs of live sows. A Bayesian approach enabled us to calculate Se and Sp without the use of a gold standard.

MATERIALS AND METHODS

1. Sample collection

From four farrow-to-finish farms, all sows selected for slaughter during eight months were included into the study. At these farms pigs suffered from clinical problems related to EF-positive *S. suis* serotype 2 strains. Culling was not in any-way related to carriership of these virulent strains. Sows were not medicated before and during the sampling period. In the waiting area of the slaughterhouse, the sows were caught and fixated using a nose sling. An iron wedge was pushed between the teeth. The swab (a sterilised 40 cm plastic stalk with a cotton wool tip of 5 cm, Heinz- Herens) was inserted through the 5 cm (2 inches) round hole in the middle of the wedge, lightened with a torch. Both tonsils were swabbed during ten seconds, while turning the swab between forefinger and thumb. The whole procedure took less than 1 minute on average. For calculation of the repeatability of the tonsil-swab-procedure, randomly selected sows were swabbed a second time 10 minutes after the first time (second-Swab-PCR). Within one hour after tonsil swabbing, the sows were slaughtered. After scrubbing and superficial burning of the carcass both whole tonsils were collected. The experimental protocol was approved by the Animal Ethical Committee of the Utrecht University in accordance with Dutch law on animal experiments.

2. Sample handling

Directly after sampling tonsillar swabs and tonsils were transported to the laboratory. Tonsillar swabs were transported in 15 ml Todd-Hewitt broth (Oxoid, CM 189,) with 0.25% Streptococcus Selective Supplement (Oxoid, SR 126^{E}) and 0,2 µg/ml cristalviolet in individual 50 ml Falcon tubes on ice. Whole tonsils were transported in individual plastic bags on ice.

At the laboratory, swab samples were grown in the transport medium for 18 hours at 37°C. Whole tonsils were processed into homogenates as described by Wisselink et al. (1999) and 15% glycerol was added. All samples were stored in 2 ml Greiner cryo tubes at -70°C.

3. The PCR assay and bacteriological examination

The PCR assay and the preceding DNA preparations were performed as described by Wisselink et al. (1999). *S. suis* strains were isolated from the tonsillar specimens (bacteriological examination, BE) as described by Wisselink et al. (1999). Three hundred bacterial examinations were performed on the tonsil homogenates (Tonsil-BE); 152 were randomly selected from Tonsil-PCR positive samples, whereas 148 were randomly selected from Tonsil-PCR negative samples. For calculation of the repeatability of the PCR-assay, PCR's (including the preceding DNA preparations

from the stored samples) were repeated on 299 randomly selected samples, equally distributed over positive- and negative Swab-PCR and Tonsil-PCR samples.

4. Statistical analysis

The combinations of a sampling method and an isolation technique were each regarded as one complete test, thus resulting in three tests: the PCR on tonsillar swabs (Swab-PCR), the PCR on the whole tonsil (Tonsil-PCR) and the bacterial examination of the whole tonsil (Tonsil-BE). The values of Se and Sp of the three tests were determined by performing a Bayesian analysis, as implemented in the winBUGS program (Spiegelhalter et al., 2003). The required prior probability distributions about the Se of Tonsil-PCR and Tonsil-BE were based on the work of Wisselink et al. (1999). Further information about the Se of the Tonsil-BE based on studies of (Davies and Ossowicz, 1991; Moreau et al., 1989) was inconclusive. Therefore normal probability distributions that allow for values on a wide interval (0.01 – 0.99) were assumed for all Se parameters. For the Sp's of Swab-PCR and Tonsil-PCR more constraining probability distributions were considered valid, because of the good Sp of the PCR in a validation study by Wisselink et al. (1999). These distributions emphasized on values larger than 0.5. The Sp of Tonsil-BE was assumed to concentrate on values higher than 0.73, because the combination of slide agglutination, hybridisation and PCR in this BE likely result in a high Sp (Vecht et al., 1985; Wisselink et al., 1999). Herd-prevalence was calculated in a pilot study in the involved herds to be at least 0.15 and at the most 0.80. Consequently, a distribution with equal probabilities between 0.01 and 0.99 for all herd-prevalences was a cautious assumption.

Se and Sp calculations by means of comparing the tests with a gold standard test were performed as described by Martin et al. (1987). With BE as the gold standard, correction for sampling bias was performed (Greiner and Gardner, 2000) because the 300 tonsil samples in BE were not equally selected from the 468 Tonsil-PCR positive and negative samples.

As measure of the repeatability of the tonsillar swabbing procedure, the kappa (proportion agreement beyond chance) of the first and second-Swab-PCR was calculated (Fleiss, 1981). Moreover, for calculation of Se and Sp of the second-Swab-PCR, this test was added as a fourth test in the Bayesian analysis in addition to Tonsil-PCR, Swab-PCR (first time) and Tonsil-BE. The repeatability of the PCR-assay on the same sample was evaluated in a similar fashion.

RESULTS

1. Detection of EF-positive *S. suis* serotype 2 strains

We sampled 471 sows alive by tonsil swabbing just before slaughter and collected the tonsils of 468 of these sows after slaughter. From 463 animals both tonsillar swabs and tonsil-homogenate were available for analysis in PCR. A summary of the isolation results of EF-positive *S. suis* serotype 2 strains in the three applied test-combinations is shown in Fig. 1. A cross tabulation of Swab-PCR and Tonsil-PCR (Fig. 1A) resulted in 270 matching results in both tests, 73 samples positive in Tonsil-PCR but negative in Swab-PCR and 20 samples dissimilar the other way around. This led to an agreement of 80% between both tests. The apparent prevalence indicated by Tonsil-PCR was 11% higher than by Swab-PCR in the sampled group of sows.

Out of the 300 samples selected for BE, 54 (18%) were positive in Tonsil-PCR and negative in Tonsil-BE while only 4 (1,3%) samples were discordant the other way around (Fig. 1C). The proportion of equal results of both tests was 80,6%. The proportion of equal results of Swab-PCR and Tonsil-BE was slightly lower (77,3%), while the discordant results were more evenly divided (36 and 32) (Fig. 1B).

A		Tonsil-PCR	
		+	-
Swab-PCR	+	105	20
	-	73	265

B		Swab-PCR	
		+	-
Tonsil-BE	+	66	36
	-	32	166

C		Tonsil-PCR	
		+	-
Tonsil-BE	+	98	4
	-	54	144

Fig. 1. Cross tabulations of the results of the detection of EF-positive *S. suis* serotype 2 strains: (A) Swab-PCR versus Tonsil-PCR, (B) Swab-PCR) versus Tonsil-BE and (C) Tonsil-PCR versus Tonsil-BE.

2. Sensitivity and specificity using the Bayesian approach

Using Bayesian analysis, the calculated values for Se, Sp and 95% credibility intervals (confidence intervals) of tests are summarized in Table 1. Swab-PCR and Tonsil-BE had a Se of 0.63 and 0.65, respectively; Tonsil-PCR had a Se of 0.88. Sp's of all tests were around 0.96.

Table 1
Sensitivities, specificities and 95% credibility intervals of Swab-PCR, Tonsil-PCR and Tonsil-BE, in Bayesian analysis.

Test	Sensitivity (credibility interval)		Specificity (credibility interval)	
Swab-PCR	0.63	(<0.52, 0.74>)	0.96	(<0.92, 0.99>)
Tonsil-PCR	0.88	(<0.75, 0.96>)	0.94	(<0.87, 0.99>)
Tonsil-BE	0.65	(<0.51, 0.76>)	0.97	(<0.91, 0.99>)

3. Repeatability of the swabbing procedure

The repeatability of the tonsil swab procedure was intermediate with a kappa of 0.63 (Fig. 2). A number of 19 samples were negative in the first Swab-PCR and positive in the second-Swab-PCR while only 3 samples had results the other way around. In the Bayesian analysis with the second-Swab-PCR added as a fourth additional test, the Se of the second-Swab-PCR was higher than, and the Sp was similar to those of the first Swab-PCR (Table 1):

Se second-Swab-PCR: 0.85 (95% credibility interval <0.67, 0.96>)
Sp second-Swab-PCR: 0.93 (95% credibility interval <0.84, 0.98>)

4. Repeatability of the PCR-assay

The results of the repeated PCR test on the same sample were very similar to the first PCR as indicated by a kappa of 0.81 for tonsillar swabs and of 0.82 for whole tonsil specimens (Fig. 2). This agreement was also found when the repeated PCR test on the same sample was added as a fourth additional test in the Bayesian analysis (results not shown).

A		Swab-PCR +	Swab-PCR -
Second-Swab-PCR	+	29	19
	-	3	104

B		First PCR +	First PCR -
On swabs			
Repeated-PCR	+	65	5
	-	9	70

C		First PCR +	First PCR -
On tonsils			
Repeated-PCR	+	71	8
	-	4	67

Fig. 2. Repeatability of Swabbing-procedure and PCR-assay. (A) Cross tabulation of the results of Swab-PCR versus the second-Swab-PCR (Kappa of 0.63); (B) Cross tabulation of the results the first PCR-assay versus the repeated PCR-assay on the same tonsillar swab specimens (Kappa 0.81); (C) Cross tabulation of the results the first PCR-assay versus the repeated PCR-assay on the same tonsil specimens (Kappa 0.82).

DISCUSSION AND IMPLICATIONS

We calculated Se and Sp of a method to detect live sows carrying EF-positive *S. suis* serotype 2 strains by tonsillar swabbing and PCR. Up till now, no reliable calculations for Se and Sp of any combination of a live-sow-sampling method and an isolation technique for *S. suis* serotype 2 were available. In this study, a very reliable calculation of Se and Sp of Swab-PCR was done by performing a Bayesian analysis on the results of two sampling methods, two well-defined tests and repetition of all procedures. Se of Swab-PCR was 0.63 (<0.52, 0.74>) and Sp was 0.96 (<0.92, 0.99>) (Table 1).

The Bayesian approach has the advantage over traditional statistical methods, which use gold standard comparisons that by using prior scientific information about the parameters combined with all test-results more precise calculations can be made. We calculated the Se of Tonsil-BE at 0.65 (<0.51; 0.76>) and Sp at 0.97 (<0.91, 0.99>). This indicates that BE cannot be used as gold standard without taking its less than perfect test characteristics into account.

However, bacteriological examination is frequently used as the gold standard in test evaluations. When the characterization of Swab-PCR in this study was done with Tonsil-BE as gold standard, a different conclusion as in the presented Bayesian analysis was drawn: the Se of Swab-PCR was 0.58 (<0.47, 0.69>) and the Sp 0.87 (<0.81, 0.93>). The Se was within the credibility intervals of the Bayesian analysis in this study, but the Sp was underestimated (negative bias), mainly due to false negative samples in the Tonsil-BE as a result of a less than perfect Se of the latter test.

The Tonsil-PCR showed a higher Se compared to the Swab-PCR: 0.88 (<0.75; 0.96>) and 0.63 (<0.52, 0.74>), respectively. The Se of the second-Swab-PCR (0.85 (<0.67, 0.96>)) also tended to be higher than that of the first Swab-PCR. These higher Se's might be due to the harvesting of more bacteria out of deep crypts and adjacent lymphoid tissue than from the surface of the tonsils of carrier sows (Arends et al., 1984; Williams et al., 1973), or of some other unknown time dependent process. Further research to unravel the underlying mechanisms of this phenomenon might demonstrate ways to improve Se of the Swab-PCR.
Sp's of all three tests for the EF-positive strains were calculated around 0.96 (Table 1). The sampling method did not seem to have effect on the Sp of the PCR. The Sp of Tonsil-PCR is in line with the Sp of the Tonsil-PCR as determined by Wisselink et al. (1999), who calculated 0.94 (<0.88, 1.0>).

The agreement between results of the PCR-assay and its repeat on the same sample was high as indicated by a kappa of 0.81 (Fig. 2), which was also concluded from the Bayesian analysis. This suggests that differences in Swab-PCR results mainly reflect differences in the amount of bacteria at the tonsils, the sampling procedure and the sample processing before the PCR-assay.

The results from this study open possibilities to develop a test-based eradication program for EF-positive *S. suis* serotype 2 strains. Sampling protocols to certificate herds that are free of EF-positive *S. suis* serotype 2 strains can now be de-

signed. Moreover, to develop eradication strategies like test and removal, studies on the dynamics of the infection between animals within herds can be implemented.

CONCLUSIONS

The Se and Sp of the Swab-PCR for the detection of EF-positive *S. suis* serotype 2 strains in live sows were calculated at 0.63 (<0.52, 0.74>) and 0.96 (<0.92, 0.99>), respectively.

ACKNOWLEDGEMENTS

We thank K. van der Stelt and the personnel of the slaughterhouse Ballering at Son, the Netherlands, for their permission and helpfulness during the sampling of the sows and the collection of the tonsils. Helmi Fijten, Dick van de Wiel and Willem Buist of the Animal Science Group at Lelystad are thankfully acknowledged for the elaborate work on bacterial examinations and assistance in performing the statistical calculations. Personnel of the Veterinary Faculty's Department of Herd Health at Utrecht, are thanked for assistance at the time of sampling and various other colleagues and students for their help to process the samples.

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Chapter 3

Estimation of Sensitivity and Specificity of Three Conditionally Dependent Diagnostic Tests in the Absence of a Gold Standard

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Journal of Agricultural, Biological, and Environmental Statistics
Volume 11, Number 4, Pages 360–380

ABSTRACT

A model is presented to evaluate the accuracy of diagnostic tests. Data from three tests for the detection of EF-positive *Streptococcus suis* serotype 2 strains in sows were analysed. The data were collected in a field study in the absence of a gold standard, i.e. the true disease status (non-infected or infected) of the tested animals was unknown. Two tests were based on a polymerase chain reaction (PCR); one test was applied to a tonsil swab (taken from the live animal) and the other test was applied to the whole tonsil (collected at slaughter). The third test was based on a bacterial examination (BE) of the whole tonsil. To reduce experimental cost BE was only performed for a subset of the animals in the sample. The model allows for dependence between tests, conditional upon the unknown true disease status of the animals. Accuracy was expressed in terms of sensitivity and specificity of the tests. A Bayesian analysis was performed that incorporated prior information about the accuracy of the tests. The model parameters have a simple interpretation and specification of priors is straightforward. Posterior inference was carried out with Markov chain Monte Carlo (MCMC) methods, employing the Gibbs sampler, as implemented in the WinBUGS program. Different parameterisations to allow for selection and missing values, use of different priors, practical problems in the analysis and some interesting issues in a joint analysis of the binary (positive or negative) results of PCR and BE and two additional continuous enzyme-linked immunosorbent assays (ELISA) are discussed.

Keywords: Bayesian analysis; Evaluation of diagnostic test; Gibbs sampling; Latent class model.

INTRODUCTION

When a reference or gold standard test (De Bock and Van Houwelingen, 1998) is available to establish whether an animal is diseased or not, the accuracy of other (imperfect) tests can be readily determined. Common measures for accuracy of a binary test (healthy or diseased, non-infected or infected, negative or positive, - or +) are the sensitivity (probability of a positive test result for a truly positive animal) and specificity (probability of a negative test result for a truly negative animal). Often however, no reference test is available, either because no test with acceptable accuracy exists, which is the case for EF-positive *Streptococcus suis* serotype 2 strains (*S. suis* from now on), or because a possible reference test is too expensive for practical use in a large-scale experiment. To obtain a sample of truly positive animals, healthy animals can be infected. But accuracy, as determined under such controlled conditions, may be seriously biased. For a realistic impression of test accuracy, data have to be collected in the field. However, in the absence of a reference test, for such field data, the true disease status of the animals involved is unknown.

When a new test is applied to a single herd, at least 3 parameters (a prevalence, sensitivity and specificity) have to be estimated, while there is only a single sufficient statistic (the number of animals in the sample that test positive). By adding a second test and sampling from two herds, all 6 parameters (two prevalences, two sensitivities and two specificities) can be estimated, as shown by Hui and Walter (1980). Hui and Walter assume a mixture model, the herd prevalences representing the mixture probabilities for two latent classes (truly negative or truly positive animals). The multinomial distributions that are involved in the mixture are simplified by assuming equal specificity and sensitivity for the two herds and independence between the tests conditional upon the true disease status of the animals. The assumption of equal specificity and sensitivity over herds is common to most studies presented in the literature and will be made in the present paper as well.

Studies by Vacek (1985) and Torrance-Rynard and Walter (1997) show that an analysis assuming independence, while (some) tests are actually dependent, may yield biased results. Moreover, conditional dependence between tests may affect the efficiency of a testing program based on multiple tests (Gardner et al., 2000). Therefore, the model discussed in the present paper comprises parameters for conditional dependence.

Often there will be information available about the accuracy of the additional tests from previous studies or biological background. For instance, a bacterial examination of cultures grown from faeces or saliva will typically have a high specificity. When the information is not specific enough to assume that some parameters are actually known, a Bayesian analysis employing prior distributions for the parameters is appealing. In a Bayesian analysis, in principle there is no need for parameters to be estimable on the basis of the likelihood alone, see for instance, Joseph et al. (1995), Dendukuri and Joseph (2001) and Georgiades et al. (2003). When the model lacks identifiability, this can be mitigated by prior information.

In the present paper a Bayesian analysis will be performed. We will show that all model parameters have a clear interpretation in terms of accuracy or conditional dependence. Consequently, the formulation of prior distributions for *S. suis* was straightforward. Similarly constructed priors will be useful for analysis of other data as well. Posterior inference was carried out with the Gibbs sampler (Casella and George, 1992; Gelman et al., 1995), as implemented in the WinBUGS program (Spiegelhalter et al., 2003).

The paper is organised as follows. First, the data are presented with a brief discussion of *S. suis* and the diagnostic tests. In the third section, two equivalent parameterisations of the model are presented. The first parameterisation is expressed in terms of multinomial distributions. In WinBUGS the multinomial distribution can be specified when there are no missing data. The second parameterisation is specified in terms of product (conditional) binomial distributions. This specification can be handled with WinBUGS in conjunction with missing data (for BE). It corresponds naturally to the sampling method and can be extended to incorporate the continuous ELISA tests. In the fourth section the choice of prior distributions is considered. Thereafter the results for the *S. suis* data are presented. Finally, aspects of the model and posterior inference, such as choice of priors and parameter identifiability, are discussed.

THE DATA

S. suis has been implicated as the cause of a wide range of clinical diseases in swine. Symptoms caused by *S. suis* in swine include meningitis, arthritis, pneumonia, septicaemia, endocarditis, polyserositis and sudden death. Outbreaks have been recognised worldwide in countries where pig production is well-developed (Staats et al., 1997). Sows carrying *S. suis* strains in their tonsils are considered the most important source of infection for susceptible young pigs. Detection of these carrier sows can contribute to the development of effective control measures. The present study was performed to determine the accuracy of a PCR test directed towards detection of the epf-gene encoding the EF protein, a virulence marker of S. *suis* serotype 2 strains. The PCR was applied to tonsil swabs of the live sows (swab-PCR) and to the whole tonsils (tonsil-PCR) that were collected at slaughter. BE is based on bacterial growth followed by serotyping by a slide agglutination test and further phenotyping by PCR. The BE was applied to the whole tonsils. For details we refer to Swildens et al. (2005).

From 4 farms with clinical problems related to *S. suis*, all 476 sows that were selected for slaughter between September 2002 and April 2003 were included in the present study and tested by tonsil- and swab-PCR (positive / + or negative / - test result). For 13 animals one of the PCR measurements was missing. For BE (also + or -) only a sub sample of 300 animals was tested to reduce the (considerable) expense. All 152 animals that tested positive on tonsil-PCR were submitted to BE, but

only a random selection of 148 tonsil-PCR negative animals were submitted to BE. The data are presented in Table 1.

The experiment also involved two ELISA tests; swab-ELISA and tonsil-ELISA that were measured for all animals in the sample. ELISA isolates the EF-protein produced, after growth in a culture medium. These measurements were not binary, but continuous. Suitable cut-off points had to be determined. The ELISA tests performed quite poorly and were eventually discarded from the analysis. However, these tests are of interest from a methodological point of view. We will focus on the two PCR tests and BE, but some aspects of the analysis of all 5 tests will be discussed as well.

Table 1. The *S. suis* data for the PCR tests and BE
The data as counts per combination of test results for tonsil-PCR, swab-PCR and BE (* = missing observation).

test results/herd	herd 1	herd 2	herd 3	herd 4
- - -	88	23	16	8
- - +	0	2	0	0
- + -	4	2	3	0
- + +	1	1	0	0
+ - -	16	4	2	9
+ - +	18	6	4	6
+ + -	8	3	10	2
+ + +	37	6	12	9
- - *	89	21	10	8
- + *	3	0	3	3
- * *	2	1	1	0
+ - *	1	5	0	2
+ + *	5	1	4	8
+ * *	0	0	1	0
* - *	6	1	0	0
* + *	0	0	0	1

THE MODEL

True disease status, denoted by $D = 1$ for a positive (+, infected, diseased) animal and $D = 0$ for a negative (-, not infected, healthy) animal, was assumed to be independently distributed with prevalence $\pi_m = P(D = 1 \mid herd\ m)$ for animals sampled from herd m, $m = 1...4$. In paragraph 1 the model is expressed in terms of joint probabilities of multinomial distributions for test results y_1, y_2 and y_3 of tests t_1, t_2 and t_3; tonsil-PCR, swab-PCR and BE respectively. In this formulation of the model all tests are involved in a similar fashion; i.e. expressions for joint probabilities of test results are symmetric for the tests. However, this representation of the model cannot be used for the *S. suis* data in conjunction with WinBUGS because of the missing

values for BE. An equivalent representation, based on product conditional distributions, which can be used with WinBUGS, is introduced in paragraph 2. Because a specific order of the tests is assumed, this parameterisation is asymmetric for the tests. The analysis of the *S. suis* data was performed under the latter model representation. Our main interest was in the sensitivity $\alpha_i = P(y_i = 1 | D = 1)$ and specificity $\beta_i = P(y_i = 0 | D = 0)$ of test t_i, $i = 1, 2, 3$.

1. The Multinomial Parameterisation

1.1 Joint probabilities

For truly positive and negative animals, the joint conditional probabilities for the test results y_1, y_2 and y_3 are expressed in terms of basic parameters θ_+ and θ_-:

$$P_{+jkl} = P(y_1 = j, y_2 = k, y_3 = l | D = 1) = \exp(\theta_{+jkl}) / \Sigma_{j,k,l} \exp(\theta_{+jkl}),$$
$$P_{-jkl} = P(y_1 = j, y_2 = k, y_3 = l | D = 0) = \exp(\theta_{-jkl}) / \Sigma_{j,k,l} \exp(\theta_{-jkl}).$$

This ensures that the probabilities are positive and sum to 1. The conditional probabilities are assumed to be the same for all herds. Throughout the text, indices + and - will refer to truly positive and negative animals respectively. Parameters θ_+ and θ_- are expressed in terms of 'main effects' δ_+ and δ_- and 'two-factor interactions' ε_+ and ε_-, e.g.

$$\theta_{-jkl} = \delta_{-1j} + \delta_{-2k} + \delta_{-3l} + \varepsilon_{-12jk} + \varepsilon_{-13jl} + \varepsilon_{-23kl}.$$

For a parsimonious model, attention is restricted to two factor interactions only. Appropriate constraints result in expressions for parameters θ (Table 2) that offer a convenient interpretation for the δ and ε parameters as shown in the next paragraph. Per herd the test results are multinomially distributed with probabilities:

$$P(y_1 = j, y_2 = k, y_3 = l \mid herd\ m) = \pi_m P_{+jkl} + (1 - \pi_m) P_{-jkl}, \quad j,k,l = 0,1, \quad m = 1...4.$$

1.2 Interpretation of main effects δ and interactions ε

The log odds for, say, test t_1 and truly negatives or positives, given fixed values for tests t_2 and t_3, are:

$$\text{logit}(P(y_1 = 1 \mid D = 0, y_2 = j, y_3 = k)) = -\delta_{-1} + j\varepsilon_{-12} + k\varepsilon_{-13},$$
$$\text{logit}(P(y_1 = 1 \mid D = 1, y_2 = j, y_3 = k)) = \delta_{+1} + (j-1)\varepsilon_{+12} + (k-1)\varepsilon_{+13}.$$

Table 2. Parameterisation of the multinomial representation

Truly negative animals ($D = 0$)

$l = 0$ (test t_3 negative)	$k = 0$ (test t_2 negative)	$k = 1$ (test t_2 positive)
$j = 0$ (test t_1 negative)	$\theta_{-000} = 0$	$\theta_{-010} = -\delta_{-2}$
$j = 1$ (test t_1 positive)	$\theta_{-100} = -\delta_{-1}$	$\theta_{-110} = -\delta_{-1}-\delta_{-2}+\varepsilon_{-12}$

$l = 1$ (test t_3 positive)	$k = 0$ (test t_2 negative)	$k = 1$ (test t_2 positive)
$j = 0$ (test t_1 negative)	$\theta_{-001} = -\delta_{-3}$	$\theta_{-011} = -\delta_{-2}-\delta_{-3}+\varepsilon_{-23}$
$j = 1$ (test t_1 positive)	$\theta_{-101} = -\delta_{-1}-\delta_{-3}+\varepsilon_{-13}$	$\theta_{-111} = -\delta_{-1}-\delta_{-2}-\delta_{-3}+$ $\varepsilon_{-12}+\varepsilon_{-13}+\varepsilon_{-23}$

Truly positive animals ($D = 1$)

$l = 0$ (test t_3 negative)	$k = 0$ (test t_2 negative)	$k = 1$ (test t_2 positive)
$j = 0$ (test t_1 negative)	$\theta_{+000} = -\delta_{+1}-\delta_{+2}-\delta_{+3}$ $\varepsilon_{+12}+\varepsilon_{+13}+\varepsilon_{+23}$	$\theta_{+010} = -\delta_{+1}-\delta_{+3}+\varepsilon_{+13}$
$j = 1$ (test t_1 positive)	$\theta_{+100} = -\delta_{+2}-\delta_{+3}+\varepsilon_{+23}$	$\theta_{+110} = -\delta_{+3}$

$l = 1$ (test t_3 positive)	$k = 0$ (test t_2 negative)	$k = 1$ (test t_2 positive)
$j = 0$ (test t_1 negative)	$\theta_{+001} = -\delta_{+1}-\delta_{+2}+\varepsilon_{+12}$	$\theta_{+011} = -\delta_{+1}$
$j = 1$ (test t_1 positive)	$\theta_{+101} = -\delta_{+2}$	$\theta_{+111} = 0$

So, main effects δ are logit transformed conditional specificities or sensitivities. For example, δ_{-1} is the logit of the specificity of t_1 conditional upon the correct classification results $y_2 = y_3 = 0$ of t_2 and t_3. Under positive dependence, the conditional specificity is maximal when $y_2 = y_3 = 0$, minimal when $y_2 = y_3 = 1$, and in between otherwise. The (marginal) sensitivities and specificities are functions of the main effects and interactions, e.g. for the specificity of t_1:

$$logit(\beta_1) = \delta_{-1} + log\left(\frac{\exp(\delta_{-2}) + \exp(\delta_{-3}) + \exp(\delta_{-2} + \delta_{-3} + \varepsilon_{-23})}{\exp(\delta_{-2}+\varepsilon_{-13})+\exp(\delta_{-3}+\varepsilon_{-12})+\exp(\delta_{-2}+\delta_{-3}+\varepsilon_{-12}+\varepsilon_{-13}+\varepsilon_{-23})} \right)$$

When test t_1 is conditionally independent of tests t_2 and t_3 for truly negative animals: $\varepsilon_{-12} = \varepsilon_{-13} = 0$ and $logit(\beta_1) = \delta_{-1}$.

The interaction terms ε are log odds ratios. For example:

$$\varepsilon_{+13} = \log\left(\frac{P(y_1 = 1 \mid D = 1, y_2 = k, y_3 = 1)}{P(y_1 = 0 \mid D = 1, y_2 = k, y_3 = 1)} \Big/ \frac{P(y_1 = 1 \mid D = 1, y_2 = k, y_3 = 0)}{P(y_1 = 0 \mid D = 1, y_2 = k, y_3 = 0)} \right)$$

Conditional upon the result $y_2 = k$ of test t_2, this is the logarithm of the cross product ratio for tests t_1 and t_3, a familiar measure for association in contingency tables (Bishop et al., 1975). The parameterisation (Table 2) is such that positive conditional dependence corresponds to positive interaction terms. Similar to Vacek (1985), Torrance-Rynard & Walter (1995) and Yang & Becker (1997), there are two separate parameters for dependence in the model, one for truly positives and one for truly negatives, for each pair of tests. Since a substantial number of combinations of test results may hardly or not at all occur in the data, from a practical point of view such a parsimonious model for possible dependence between tests is often desirable.

2. The Logistic Regression Parameterisation

2.1 Conditional probabilities

The tests were placed in the order t_1, t_2, t_3 (tonsil-PCR, swab-PCR, BE). The marginal distribution for y_1 was specified, then the conditional distribution for y_2 given y_1 and for y_3 given y_1 and y_2. This involved binomial distributions only. For our sampling design the ordering was convenient since our first interest was in the PCR tests t_1 and t_2 and any relationship between them. BE was observed depending on the result for tonsil-PCR, and was therefore a natural third in the order. All effects were introduced on the logit scale:

$$\text{logit}(P(y_1 = 1 \mid D = 0)) = -\gamma_{-1},$$
$$\text{logit}(P(y_1 = 1 \mid D = 1)) = \gamma_{+1}.$$
$$\text{logit}(P(y_2 = 1 \mid D = 0,\, y_1 = j)) = -\gamma_{-2} + \lambda_{-12} j \ ,$$
$$\text{logit}(P(y_2 = 1 \mid D = 1,\, y_1 = j)) = \gamma_{+2} + \lambda_{+12}(j-1),$$
$$\text{logit}(P(y_3 = 1 \mid D = 0, y_1 = j,\, y_2 = k)) = -\gamma_{-3} + \lambda_{-13} j + \lambda_{-23} k,$$
$$\text{logit}(P(y_3 = 1 \mid D = 1, y_1 = j,\, y_2 = k)) = \gamma_{+3} + \lambda_{+13}(j-1) + \lambda_{+23}(k-1), \quad j,k = 0,1.$$

2.2 Additional continuous test results

The logistic regression representation can be extended with linear regression for, say, tonsil-ELISA (test t_4 with response y_4) on PCR and BE and swab-ELISA (t_5 with response y_5) on PCR, BE and tonsil-ELISA (see also Congdon, 2001, Section 6.6.4), e.g. for truly negatives for test t_4:

$$(y_4 \mid D = 0, y_1 = j,\, y_2 = k, y_3 = l) \sim \text{N}(\gamma_{-4} + \lambda_{-14} j + \lambda_{-24} k + \lambda_{-34} l,\, \tau_4),$$

where the residual precision τ_4 is the inverse of the residual variance σ_4^2.

2.3 Interpretation of constants γ and coefficients λ

Parameters γ_{+1} and γ_{-1} are the logit transforms of the sensitivity α_1 and specificity β_1 of test t_1, i.e.

$$\alpha_1 = \{1 + \exp(-\gamma_{+1})\}^{-1} \text{ and } \beta_1 = \{1 + \exp(-\gamma_{-1})\}^{-1}.$$

For test t_2, conditional upon a correct result y_1 of test t_1, γ_{+2} and γ_{-2} are the logit transforms of a conditional sensitivity and specificity, respectively. For instance:

$$\gamma_{-2} = \text{logit}(P(y_2 = 0 \mid D = 0,\, y_1 = 0)).$$

Parameters λ_{+12} and λ_{-12} are log odds ratios, e.g. for truly positives

$$\lambda_{+12} = \log\left(\frac{P(y_2 = 1 \mid D = 1,\, y_1 = 1)}{P(y_2 = 0 \mid D = 1,\, y_1 = 1)} \Big/ \frac{P(y_2 = 1 \mid D = 1,\, y_1 = 0)}{P(y_2 = 0 \mid D = 1,\, y_1 = 0)} \right).$$

This is the logarithm of the cross product ratio for tests t_1 and t_2 for truly positives. So, λ_{+12} and λ_{-12} represent conditional dependence between tests t_1 and t_2. These parameters are 0 under conditional independence and positive under positive conditional dependence, e.g. $\lambda_{+12} > 0$ when there is a tendency for concordant observa-

tions between tests t_1 and t_2 for truly positives. The sensitivity and specificity of t_2 are:

$$\alpha_2 = \frac{\alpha_1}{1+\exp(-\gamma_{+2})} + \frac{1-\alpha_1}{1+\exp(-\gamma_{+2}+\lambda_{+12})},$$

$$\beta_2 = \frac{\beta_1}{1+\exp(-\gamma_{-2})} + \frac{1-\beta_1}{1+\exp(-\gamma_{-2}+\lambda_{-12})}.$$

Under conditional independence between t_1 and t_2, say for truly negatives; $\lambda_{-12} = 0$ and $\text{logit}(\beta_2) = \gamma_{-2}$. A similar interpretation holds for the parameters in the logistic regression for test t_3. For instance, constant γ_{+3} is the logit transformed sensitivity of test t_3, conditional upon results $y_1 = y_2 = 1$ for tests t_1 and t_2. Coefficient λ_{+13} is the logarithm of the cross product ratio of tests t_1 and t_3, conditional upon $y_2 = k$, $k = 0, 1$ for test t_2 (and is equivalent with interaction ε_{+13}). Derivation of (more lengthy) expressions for the sensitivity α_3 and specificity β_3 is straightforward. Again, similar to Vacek (1985), Torrance-Rynard & Walter (1995) and Yang & Becker (1997), there are two separate parameters for dependence in the model, one for truly positives and one for truly negatives, for each pair of tests.

Some algebra shows that the multinomial and logistic representations (when only two-factor interactions are considered) are equivalent and parameters of one parameterisation can be expressed in terms of the other, e.g.

$$\gamma_{+2} = \delta_{+2} + \log\{(1+\exp(-\delta_{+3}))/(1+\exp(-\delta_{+3}+\varepsilon_{+23}))\}.$$

When $\varepsilon_{+23} = 0$, it follows that $\gamma_{+2} = \delta_{+2}$. It can also be shown that $\varepsilon_{+13} = 0$ implies that $\lambda_{+12} = \varepsilon_{+12}$. Furthermore, $\gamma_{-3} = \delta_{-3}$, $\lambda_{-13} = \varepsilon_{-13}$ and $\lambda_{-23} = \varepsilon_{-23}$, with similar expressions for truly positives. The condition $\varepsilon > 0$ imposes a stronger constraint on the dependence structure than $\lambda > 0$; $\varepsilon > 0$ implies that $\lambda > 0$, but $\lambda > 0$ does not necessarily imply that $\varepsilon_{+12} > 0$ or $\varepsilon_{-12} > 0$. Dependence parameters may be constrained to be positive through the choice of prior distributions, as discussed in the next section.

2.4 Measuring dependence

An appealing measure for conditional dependence between two tests is the percentage increase for true positives (or negatives) in the sensitivity (or specificity) of one test given that the other test is positive (or negative), e.g. for test t_1 and t_2

$$R_{se,12} = \frac{P(y_2 = 1 \mid D = 1, y_1 = 1) - \alpha_2}{\alpha_2} * 100\% = \frac{\{1+\exp(-\gamma_{+2})\}^{-1} - \alpha_2}{\alpha_2} * 100\%.$$

Since

$$R_{se,12} = Corr(y_1, y_2 \mid D = 1)\sqrt{(1-\alpha_1)(1-\alpha_2)/(\alpha_1\alpha_2)} * 100\%,$$

$R_{se,12}$ (and likewise $R_{sp,12}$) is symmetric in t_1 and t_2. Like the correlation between y_1 and y_2 (Gardner et al., 2000), the R parameters have lower and upper bounds, e.g.:

$$-\text{minimum}\left(\frac{1-\alpha_1}{\alpha_1}\frac{1-\alpha_2}{\alpha_2}, 1\right) * 100\% \;\le\; R_{se,12} \;\le\; \text{minimum}\left(\frac{1-\alpha_1}{\alpha_1}, \frac{1-\alpha_2}{\alpha_2}\right) * 100\%,$$

with a lower bound of 0 for positive dependence. When $P(y_2 = 1 \mid D = 1, y_1 = 1) = 1$, which is mentioned as an example of complete dependence in Gardner et al. (2000), it follows that $R_{se,1,2} = (1-\alpha_2)/\alpha_2 * 100\%$. In that case $\alpha_2 \ge \alpha_1$, with equality when t_1 and t_2 are equivalent for truly positives, i.e. $P(y_1 = y_2 \mid D = 1) = 1$. Expressions for other R parameters can be readily derived, for instance:

$$R_{se,13} = \frac{\{1+\exp(-\gamma_{+3})\}^{-1}\{1+\exp(-\gamma_{+2})\}^{-1} + \{1+\exp(-\gamma_{+3}+\lambda_{+23})\}^{-1}\{1+\exp(\gamma_{+2})\}^{-1} - \alpha_3}{\alpha_3} * 100\%$$

CHOICE OF PRIORS AND POSTERIOR INFERENCE

1. Choice of priors

For the γ (and δ) parameters, that are logit transforms of (conditional) specificities and sensitivities, 3 different priors were employed. Values for lower and upper percentage points and for the median were transformed to logit scale and a mean and precision were chosen for the normal distribution that yielded an acceptable compromise between these values (Table 3). A low informative prior for (conditional) sensitivities and specificities, with 2.5 and 97.5 percentile points equal to 0.06 and 0.94 and mean and median 0.5, is presented in Figure 1a. A prior for the (conditional) specificity of swab- and tonsil-PCR with emphasis on values larger than 0.5, with percentile points 0.15 and 0.98, mean 0.70 and median 0.77, is presented in Figure 1b. A quite informative prior for the conditional specificity of BE, with percentile points 0.73 and 0.99, mean 0.92 and median 0.94, is presented in Figure 1c. The last prior is a fairly cautious reflection of the fact that the specificity of culture growth should be quite high. Final results were obtained with the low informative prior (Figure 1a) for the sensitivities, the prior with emphasis on values larger than 0.5 (Figure 1b) for specificities of tonsil- and swab-PCR and the informative prior (Figure 1c) for the specificity of BE. For considerations that led to this choice of priors we refer to Swildens et al. (2005) and the discussion.

Attention was restricted to positive conditional dependence between the tests since negative dependence was biologically quite unlikely. Because little was known about conditional dependence, a wide gamma prior was employed for the dependence parameters λ (and ε) (Figure 1d). This prior was derived analogous to Smith et al. (1995), employing the log odds interpretation. Values for an odds ratio around 1.6, e.g. $(0.65/0.35)/(0.55/0.45)$, $(0.75/0.25)/(0.65/0.35)$ or $(0.90/0.10)/(0.85/0.15)$, were plausible under dependence, while an odds ratio of 6, e.g. $(0.9/0.1)/(0.6/0.4)$, was considered to be fairly extreme. The mean and median of the gamma prior in Table 3 are 0.48 = log(1.61) and 0.21 = log(1.23). The probability to exceed 1.79 = log(6) is 0.07. Additional analyses were also performed with a normal prior for the λ parameters, with mean 0 and precision 0.8429 (5 and 95 percentile points –log(6) and log(6) respectively), to gather evidence for conditional positive dependence by evaluation of the posterior probability for $\lambda > 0$. For the prevalence π_m per herd a uniform prior (Table 3) was employed. Extreme values for π_m were avoided for reasons to be explained in the next section.

When the priors from Table 3 are applied to both representations, posterior inference will not be the same, because (some) parameters in the two representations do not have the same interpretation. In principle, we can make sure that fully equivalent priors are used in WinBUGS by (1) formulation of priors for parameters δ, ε, (2) introduction of γ, λ parameters as functions of the δ, ε parameters and (3) logistic representation of the model in terms of γ, λ parameters. This implicitly induces dependent priors for the γ, λ parameters (including additional constraints for positive ε dependence). Plots and percentile points of the induced priors for the γ, λ parameters can be easily derived by WinBUGS (without use of data). However, there are many possible choices for prior distributions and the priors in Table 3 offer reasonable choices for both parameterisations. Here, we use the priors from Table 3 directly for the γ, λ parameters of the logistic representation without additional constraints. Plots and percentile points for induced priors of marginal sensitivities, specificities and odds ratios can be derived by WinBUGS (again without the use of data). A marked difference in posterior inference between the two representations would imply that the choice of priors is more critical than expected, probably because the data convey relatively little information. In that case the order of the tests in the logistic representation would probably also markedly affect posterior inference and further careful attention should be given to the choice of prior distributions. We will return to this issue in the next section.

2. Posterior inference

No provision for selection of samples for BE was needed in the posterior analysis of *all* data collected, since neither the kernel of the likelihood nor the priors are affected. Posterior inference was carried out with the Gibbs sampler (Casella and George, 1992; Gelman et al., 1995), as implemented in the WinBUGS program (Spiegelhalter et al., 2003). The logistic regression representation was used, because

of the missing values for BE. The (posterior) means from the values for the parameters generated by the Gibbs sampler were used as point estimates and the 2.5 and 97.5 percentile points as end points of 95 % credible intervals. We focussed on PCR and BE, but some results of joint analyses of PCR, BE and ELISA will be briefly discussed as well. We will present a direct comparison between the multinomial and logistic representation for the data restricted to the animals with observed BE. Of course restriction to animals with observed BE will bias the parameter estimates. The sole purpose was to compare posterior inference with WinBUGS for the two representations and maximum likelihood inference with the popular TAGS program (Pouillot et al., 2002).

Table 3. Prior distributions

Prior distributions for PCR tests and BE	
Parameters γ (and δ), i.e. logit of (conditional) sensitivity and specificity:	
• uninformative prior	N(0, 0.5)[1]
• more informative prior (specificities PCR)	N(1.22, 0.45)[1]
• informative prior (specificity of BE)	N(2.85, 1.13)[1]
parameters λ (and ε), i.e. log odds ratios that model conditional dependence:	
• positive valued wide gamma(0.48,1)[2] prior	
• normal N(0, 0.8429) [1] prior (to find evidence for positive dependence in the data)	
Prevalence π per herd:	
• a uniform distribution on the interval (0.01, 0.99)	
Additional priors for continuous ELISA measurements	
Coefficients λ in the additional linear regressions:	
• a lognormal(0, 0.2)[3] prior	
Residual standard deviations σ in the additional linear regressions:	
• a uniform distribution on the interval (0, 10)	

[1] normal distribution N(m, τ) with mean m and precision τ, i.e. variance $1 / \tau$,
[2] gamma distribution gamma(v, w) with mean v / w and variance v / w^2,
[3] mean 12.2, median 1, 2.5% point 0.01 and 97.5% point 80.1.

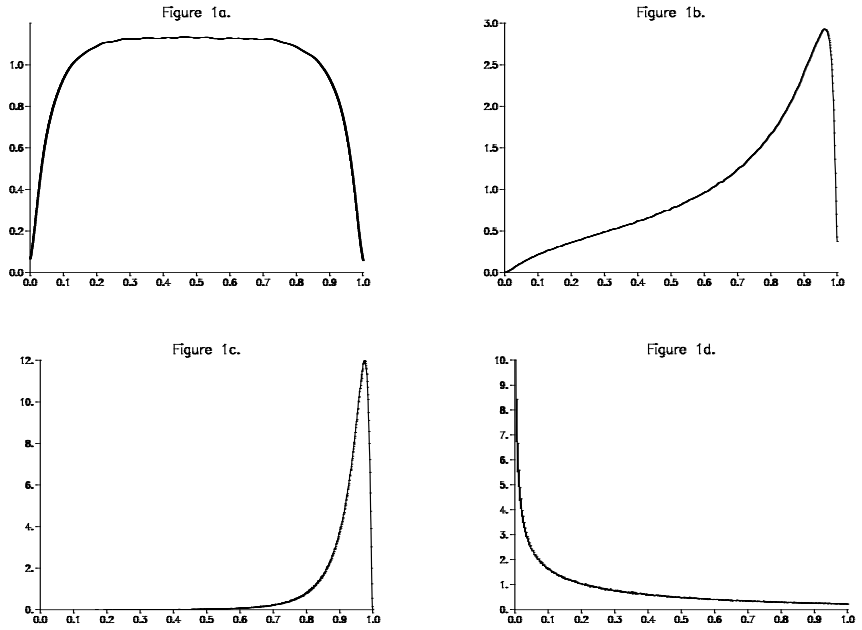

Figure 1
The low informative prior (a) for (conditional) sensitivity and specificity, the prior (b) for (conditional) specificity that focuses on values larger than 0.5, the informative prior (c) for conditional specificity of BE and the low informative prior (d) for coefficients λ (and interactions ε) (parameters for conditional dependence) (details in Table 3).

RESULTS

1. Preliminary analysis of simulated data

For a first impression of the performance of the posterior analysis for the different prior distributions, data was simulated with GenStat (2000) and analysed with WinBUGS. There are some tricky aspects in fitting latent class models that may complicate the analysis, depending on the size and structure of the data. The impact of these aspects is much easier to recognise on simulated data, where the true parameter values are known.

There is always the tendency to switch labels (truly negative or positive). For any set of sensitivities α, specificities β and prevalences π, there exists a mirror image with sensitivities $(1-\beta)$, specificities $(1-\alpha)$ and prevalences $(1-\pi)$ with the same likelihood. Even with a judicious choice of starting values and informative priors on (some of) the parameters, label switching may occur (sometimes after more than a 100.000 runs). The chain may get trapped for quite a large number of runs around this mirror image, with dire consequences for means and percentile

points. Label switching becomes apparent from inspection of the chain, and from unrealistic values for prevalences, specificities and sensitivities. Possible remedies are priors with zero probability mass for the offending mirror image or, equivalently, constraints like $(\gamma_{+3} + \gamma_{-3}) > 0$ implying that a positive result has a higher probability for truly positives than for truly negatives, conditional upon positive PCR results. For the present data, that proved to be quite informative, no additional requirements except careful inspection of the Markov chains was needed. For many chains label switching did not occur in the first 50.000 - 750.000 runs. Results of these chains agreed well with the true parameter values in the simulation.

When a specificity or sensitivity gets close to 1, successive values in the Markov chain may become highly correlated and the chain may have difficulty in reaching a less extreme value. This was observed when the precision of the normal distribution in the low informative prior for γ was chosen quite small, e.g. 0.001, as often used in WinBUGS examples. In that case the prior is U-shaped (as if Figure 1a was turned upside down). With a precision of 0.5 as specified in Table 3, the prior avoids extreme values for sensitivities and specificities (and is perhaps slightly conservative for the specificity of BE). Occasionally, problems were also observed with extreme herd prevalences, therefore a uniform prior on (0.01, 0.99), excluding values close to 0 or 1, was chosen. We will now proceed with results for the real data of Table 1.

2. Sensitivities and specificities

Markov Chains of length 50.000 - 750.000 were employed. Repeated chains that did not trap into single states within herds and did not switch labels produced similar results. The results in the second column of Table 4 were derived with the fairly uninformative priors (Figure 1a). The results in the 3rd column were derived with more prior emphasis on values larger than 0.5 for the (conditional) specificities of tonsil- and swab-PCR (Figure 1b) and the prior for the specificity of BE that largely concentrates on the interval 0.73 - 0.99 (Figure 1c). For the more informative priors, estimated specificities were slightly higher with narrower credible intervals. It was reassuring that the specificities and sensitivities under the different prior distributions were similar, indicating that the data were reasonably informative.

Table 4. Prevalences, sensitivities and specificities for tonsil-PCR , swab-PCR and BE for different priors for all the data

Parameters in logistic regressions: γ = constant term (for (conditional) se and sp) and λ = coefficient (dependence parameter).

Prior distributions $\pi \sim$ uniform(0.01,0.99) , $\lambda \sim$ gamma(0.48,1) except in column 4 where $\lambda = 0$, column 5 where $\lambda \sim N(0, 0.8429)$ and columns 6 and 7 where $\lambda \sim$lognormal(0, 0.2) for the coefficients of the ELISA measurements (see Table 3).

Priors for γ 's as mentioned in the table.

parameter	Fairly uninformative priors	More informative priors for specificities	Analysis under independence, all coefficients $\lambda = 0$	Analysis including negative dependence as well	Tests 4 and 5 (tonsil- and swab-ELISA) included as continuous measurements	Tests 4 and 5 included as binary measurements, boundary values -2.1 and -1.15 respectively.
	$\gamma \sim N(0,0.5)$	$\gamma \sim N(0,0.5)$, except	$\gamma \sim N(0,0.5)$, except	$\gamma \sim N(0,0.5)$, except	$\gamma \sim N(0,0.5)$, except	$\gamma \sim N(0,0.5)$, except
		γ-1~N(1.22,0.45)	γ-1~N(1.22,0.45)	γ-1~N(1.22,0.45)	γ-1~N(1.22,0.45)	γ-1~N(1.22,0.45)
		γ-2~N(1.22,0.45)	γ-2~N(1.22,0.45)	γ-2~N(1.22,0.45)	γ-2~N(1.22,0.45)	γ-2~N(1.22,0.45)
	□	γ-3~N(2.85,1.13)	γ-3~N(2.85,1.13)	γ-3~N(2.85,1.13)	γ-3~N(2.85,1.13)	γ-3~N(2.85,1.13)
Prevalence farm 1	0.28 (0.08, 0.37)	0.31 (0.23, 0.39)	0.29 (0.23, 0.35)	0.31 (0.23, 0.40)	0.10 (0.06, 0.16)	0.31 (0.21, 0.45)
Prevalence farm 2	0.31 (0.12, 0.46)	0.34 (0.20, 0.48)	0.32 (0.20, 0.44)	0.34 (0.21, 0.49)	0.10 (0.03, 0.21)	0.35 (0.19, 0.73)
Prevalence farm 3	0.55 (0.39, 0.72)	0.57 (0.42, 0.74)	0.54 (0.40, 0.67)	0.57 (0.42, 0.74)	0.07 (0.01, 0.30)	0.58 (0.41, 0.86)
Prevalence farm 4	0.67 (0.48, 0.84)	0.70 (0.53, 0.86)	0.66 (0.50, 0.80)	0.69 (0.52, 0.87)	0.25 (0.12, 0.42)	0.70 (0.52,0.92)
Sensitivity Ton-PCR	0.89 (0.75, 0.97)	0.88 (0.75, 0.96)	0.92 (0.85, 0.97)	0.89 (0.75, 0.97)	0.87 (0.73, 0.97)	0.87 (0.57, 0.97)
Sensitivity Swab-PCR	0.65 (0.53, 0.80)	0.63 (0.52, 0.74)	0.65 (0.56, 0.73)	0.63 (0.52, 0.75)	0.69 (0.44, 0.94)	0.62 (0.41, 0.76)
Sensitivity BE	0.65 (0.47, 0.78)	0.65 (0.51, 0.76)	0.71 (0.62, 0.80)	0.65 (0.52, 0.77)	0.80 (0.43, 0.95)	0.65 (0.42, 0.78)
Specificity Ton-PCR	0.92 (0.74, 0.98)	0.94 (0.87, 0.99)	0.94 (0.89, 0.98)	0.95 (0.88, 0.99)	0.68 (0.63, 0.74)	0.94 (0.84, 0.99)
Specificity Swab-PCR	0.95 (0.83, 0.99)	0.96 (0.92, 0.99)	0.96 (0.92, 0.98)	0.97 (0.93, 0.99)	0.78 (0.73, 0.86)	0.96 (0.91, 0.99)
Specificivity BE	0.95 (0.79,0.99)	0.97 (0.91, 0.99)	0.98 (0.95, 1.00)	0.98 (0.94, 0.99)	0.79 (0.74, 0.84)	0.97 (0.98, 0.99)

Figure 2

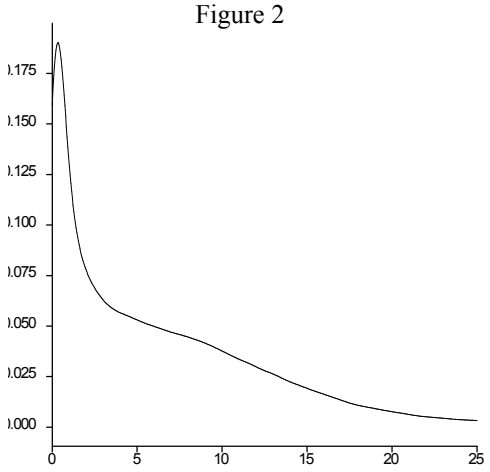

Figure 3

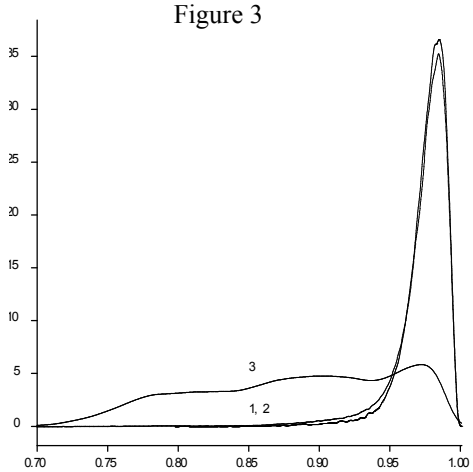

Figure 2
The posterior distribution of R_{se13} (corresponding to 3rd column of Table 4).

Figure 3
Posterior distributions of specificity β_3 of BE under positive conditional dependence (1) (3rd column of Table 4, gamma priors for λ from Table 3), positive or negative dependence (2) (5th column of Table 4, normal priors for λ from Table 3 with precision 0.8429) and positive or negative dependence (3) (wide normal priors for λ with precision 0.075).

3. Conditional dependence

The opportunity to detect conditional dependence for truly negatives was slim, because of the fairly high specificity of the tests. For the PCR tests, the percentages increase in sensitivity and specificity were $R_{se,12}$ = 2.0 (0.00, 13.4) % and $R_{sp,12}$ = 0.2 (0.00, 1.3) %. To gain more insight into the possible presence of positive conditional dependence, the data was re-analysed with a normal prior for λ (Table 3) that allowed for negative dependence as well. The credible intervals for coefficients λ_{+12} and λ_{-12} were wide, covering both relatively large negative and large positive values, with posterior probabilities $P(\lambda_{+12} > 0 \mid \boldsymbol{y})$ = 0.42 and $P(\lambda_{-12} > 0 \mid \boldsymbol{y})$ = 0.48 for positive dependence. Incidentally, when a λ parameter is poorly determined, extreme values may easily occur, since the likelihood does not change perceptibly from extreme to very extreme values for a λ parameter. The precision of the normal prior had a moderating influence on the interval length. Although there is no evidence for any marked dependence, we cannot draw any firm conclusions about independence between tonsil- and swab-PCR.

For tonsil- and swab-PCR and BE, for true negatives, the estimated R-parameters were small; $R_{sp,13}$ = 0.6 (0.00, 6.2) % and $R_{sp,23}$ = 0.1 (0.00, 0.9) %. When negative dependence was allowed, credible intervals for λ_{-13} and λ_{-23} for true

Table 5. A comparison between Bayesian posterior inference with the multinomial and logistic representation and maximum likelihood estimation (under independence) for the 300 animals without missing values

Priors for (conditional) sensitivities and specificities: $\gamma, \delta \sim N(0,0.5)$, *except* $\gamma_1, \delta_1 \sim N(1.22, 0.45)$, $\gamma_2, \delta_2 \sim N(1.22, 0.45)$ *and* $\gamma_3, \delta_3 \sim N(2.85, 1.13)$.
Priors for coefficients or interactions (dependence parameters) $\lambda, \varepsilon \sim$ *gamma(0.48, 1) (unless stated otherwise).*

Priors for prevalences $\pi \sim$ *uniform(0.01, 0.99).*

parameter	multinomial model	logistic model	multinomial model under conditional independence, i.e. $\varepsilon = 0$	Logistic model under conditional independence, i.e. $\lambda = 0$	maximum likelihood (with TAGS) under conditional independence
Prevalence farm 1	0.43 (0.32, 0.53)	0.44 (0.33, 0.54)	0.42 (0.33, 0.50)	0.42 (0.33, 0.50)	0.41 (0.33, 0.50)
Prevalence farm 2	0.40 (0.23, 0.57)	0.41 (0.24, 0.58)	0.40 (0.25, 0.55)	0.40 (0.25, 0.55)	0.39 (0.24, 0.56)
Prevalence farm 3	0.59 (0.43, 0.75)	0.61 (0.44, 0.77)	0.58 (0.43, 0.73)	0.58 (0.43, 0.73)	0.58 (0.42, 0.72)
Prevalence farm 4	0.70 (0.48, 0.87)	0.71 (0.49, 0.89)	0.67 (0.47, 0.84)	0.67 (0.47, 0.84)	0.69 (0.44, 0.86)
Sensitivity Ton-PCR	0.95 (0.87, 0.99)	0.94 (0.84, 0.99)	0.96 (0.91, 0.99)	0.96 (0.91, 0.99)	0.97 (0.88, 1.00)
Sensitivity Swab-PCR	0.61 (0.51, 0.73)	0.61 (0.50, 0.72)	0.63 (0.53, 0.73)	0.63 (0.54, 0.73)	0.64 (0.53, 0.73)
Sensitivity BE	0.68 (0.57, 0.79)	0.67 (0.55, 0.78)	0.71 (0.61, 0.81)	0.71 (0.61, 0.80)	0.72 (0.60, 0.81)
Specificity Ton-PCR	0.91 (0.78, 0.99)	0.92 (0.80, 0.99)	0.90 (0.82, 0.98)	0.90 (0.82, 0.98)	0.90 (0.75, 0.96)
Specificity Swab-PCR	0.94 (0.87, 0.98)	0.94 (0.88, 0.99)	0.94 (0.89, 0.98)	0.94 (0.89, 0.98)	0.94 (0.88, 0.97)
Specificivity BE	0.97 (0.90,0.99)	0.97 (0.91, 0.99)	0.98 (0.95, 0.99)	0.98 (0.95, 0.99)	0.99 (0.88, 1.00)

negatives were wide with posterior probabilities $P(\lambda_{-13} > 0 \mid \boldsymbol{y}) = 0.55$ and $P(\lambda_{-23} > 0 \mid \boldsymbol{y}) = 0.51$. For tonsil- and swab-PCR and BE, for true positives, the estimated R-parameters were larger; $R_{se,13} = 6.8$ (0.02, 25.5) % (posterior shown in Figure 2) and $R_{se,23} = 6.1$ (0.1, 22.0) %. When negative dependence was allowed, credible intervals for λ_{+13} and λ_{+23} for true positives tended to concentrate on positive values, with posterior probabilities $P(\lambda_{+13} > 0 \mid \boldsymbol{y}) = 0.89$ and $P(\lambda_{+23} > 0 \mid \boldsymbol{y}) = 0.81$. Although evidence is not conclusive, conditional dependence cannot be ruled out, particularly between tonsil-PCR and BE for true positives. When the precision of the normal priors for the λ parameters was reduced, the posterior probabilities for positive dependence increased. However, posteriors obtained with a very low prior precision did not look very acceptable (Figure 3). Further discussion will be postponed to the last section.

When possible dependence was ignored ($\lambda = 0$), estimated sensitivities for tonsil-PCR and BE increased and credible intervals became narrower (4th column of Table 4). Since conditional dependence could not be excluded, the results under positive dependence in the third column of Table 4, albeit perhaps somewhat conservative, were considered the most appropriate to present as final results in Swildens et al. (2005).

4. Additional continuous tests

First, a separate analysis was performed for the two ELISA tests only. ELISA measurements were log-transformed prior to analysis. Normal distributions were assumed with common residual variances for truly positives and negatives. Herd prevalences, ranging from 0.05 (0.01, 0.12) to 0.16 (0.07, 0.29) (extra priors are presented in Table 3), were quite low. In histograms of ELISA measurements (not shown) no mixture was apparent, except that some 6% of the animals showed markedly large values for tonsil-ELISA. These animals tended to be collected into one component of the mixture (the supposed positives).

Second, the logistic model for the PCR tests and BE was extended with linear regression for tonsil- and swab-ELISA. Again the ELISA measurements were log transformed prior to analysis and normality was assumed with common residual variances in the regressions of tonsil-ELISA (on tonsil-PCR, swab-PCR and BE) and swab-ELISA (on tonsil-PCR, swab-PCR, BE and tonsil-ELISA). Although a joint analysis with the PCR tests and BE improved upon the results (Table 4, column 6), prevalences remained low and specificities for PCR and BE showed a severe downward bias compared with previous results from analysis of PCR tests and BE only. For a grid of candidate cut-off points, the ELISA tests were transformed into binary variables and a joint analysis of all 5 tests was performed. Now, results for PCR and BE were similar to the results obtained before without the ELISA tests (Table 4, column 7). ROC curves (Greiner et al., 2000) (not shown), as obtained from the posterior means for specificities and sensitivities corresponding to the grid of cut-off points, showed that the ELISA tests performed poorly. We will return to these results in the discussion.

5. Comparison of posterior inference of the multinomial and logistic representations

In Table 5 (columns 2 and 3) results are presented for the multinomial and logistic representation for the data set restricted to the 300 animals where BE was observed. As explained before it is not recommended to discard animals with structurally missing values, for instance in order to analyse the data with the TAGS program (Pouillot et al., 2002) (that does not allow for missing values). The difference in prior information for the two parameterisations yielded only minor differences between posterior results. Under assumption of independence the models and priors are completely equivalent. Indeed the results under conditional independence (Table 5, columns 4 and 5) were virtually the same. The TAGS program (Pouilot et al., 2002) performs a maximum likelihood analysis under conditional independence for complete data only. Maximum likelihood estimates from TAGS (Table 5, column 6) were similar to posterior means with the multinomial or logistic representation under independence (Table 5, columns 4 and 5). Some of the 95% confidence intervals from TAGS were wider than the credible intervals from the Bayesian analysis, e.g. for specificity β_3 of BE. With the fairly uninformative priors (Figure 1a) credible intervals (not shown) under the multinomial and logistic representations were similar to the confidence intervals from TAGS.

DISCUSSION

Two equivalent representations (multinomial or product binomial) were presented of a model for analysis of data from 3 (possibly) conditionally dependent diagnostic tests in the absence of a gold standard. A popular and common mechanism to introduce dependence is the use of random effects and associated components of variance, see for instance Qu et al. (1996), Qu and Hadgu (1998), Hadgu and Qu (1998), Goetghebeur et al. (2000) and Dendukuri and Joseph (2001). These models are subject specific rather than population averaged in the terminology of Zeger et al. (1988). Parameters of interest, such as sensitivity or specificity, are only available after integration over the random effects and will involve both the fixed (systematic) effects and the components of variance of the random effects. Consequently, the formulation of appropriate priors in random effect models can be quite laborious. Random effects models can be useful for biologically similar tests where random effects may represent an individual's attributes that are not related to the true disease status, but affect all tests in a similar fashion, see e.g. Hadgu and Qu (1998) and Albert et al. (2001). In general however, with three or more tests, we prefer population-averaged models with basic parameters that have a more straightforward interpretation, thus avoiding undue effort in the construction of appropriate prior distributions.

Hanson et al. (2003) present a population-averaged model for two tests and several, typically many, herds. Their model can be extended to three or more tests.

For the joint distribution of the tests for truly positive and negative animals Dirichlet distributions were used. Although the Dirichlet is a natural choice for a prior on joint probabilities, it is something of a disadvantage, especially for 3 or more tests, that the joint probabilities are not of primary interest. Finding Dirichlet priors that adequately reflect prior information about specificities and sensitivities is not straightforward.

The parameterisation in the population-averaged model discussed in the present paper is basically the same as for log-linear models for contingency tables (Bishop et al., 1975). The log-linear parameterisation was recently employed by Hanson et al. (2000) to study dependence structures when true disease status was known and by Cook et al. (2000) for two tests in combination with hidden Markov chains for longitudinal data. The parameters of both the logistic regression and multinomial representations of our model have a simple interpretation and specification of priors will be straightforward for most problems. Parameters in the model of Yang & Becker (1997) have a more direct interpretation as marginal logit-transformed sensitivities and specificities and log odds ratios for pairs of tests, compared with their conditional counterparts in our model. However, the Yang & Becker marginal model involves imposition of a set of constraints such that joint probabilities are only available implicitly. For a Bayesian analysis (with WinBUGS) the absence of explicit forms for joint probabilities is a disadvantage.

Often, like for the *S. suis* data, there will be little information about conditional dependence. In that case in our model priors for conditional sensitivity and specificity may be based on information about marginal sensitivity and specificity, assuming modest dependence. If there is positive conditional dependence, which is biologically more likely (Gardner et al., 2000), these priors will be somewhat cautiously formulated, leading to slightly conservative inference. So the need for marginal basic parameters is small and in our opinion overruled by the advantages of the Bayesian analysis employing WinBUGS. Note that in the logistic representation, for test t_1 the basic parameters are the logit transformed marginal sensitivity and specificity and for t_1 and t_2 a marginal log odds ratio. In the rare case that reliable and substantial information about conditional dependence between several tests is available, initially priors can be formulated ignoring the conditional character of the parameters. Then priors for the basic conditional parameters can be systematically varied around the initial priors such that desired priors for sensitivity, specificity and odds ratios are obtained. This can be easily achieved with WinBUGS (without use of data). In that case the imposition of constraints in the Yang & Becker marginal model is replaced by the choice by trial and error of priors in our model. The priors used for the *S. suis* data range from fairly uninformative to quite informative and similarly constructed priors will be useful for other problems as well. The logistic representation can be used with missing data in WinBUGS. Extension with tests with continuous outcomes is straightforward. In that case the data will be introduced per individual, rather than grouped over individuals.

For the *S. suis* data test specificity and sensitivity were of primary interest. The prevalences per herd were nuisance parameters. The present model can be modified to include more intricate structures for prevalence. For instance, in Bouma et al.

(2001) the number of herds was large with quite small samples per herd. For each herd the prevalence was sampled from a beta distribution. The present model can be extended with a beta, logit-normal or probit-normal distribution for herd prevalence, with appropriate priors for the parameters of this distribution. When the posteriors suggest a U-shaped distribution, a more extensive family that includes bimodal distributions or an additional positive probability for zero prevalence may be considered. Quite intricate priors for the prevalence structure based on a Dirichlet Process are presented in Hanson et al. (2003).

In Guggenmoos-Holzmann and Van Houwelingen (2000) it is stated, perhaps somewhat indiscriminately, but not without some truth, that latent class models are invented "for detecting things you cannot see", like "introversion" or "sensation seeking" in psychology. Although we tend to think that "disease status (infected / non-infected)" has a more solid basis, the latent class model offers an elegant mathematical framework to give substance to a disease status that we cannot directly measure. Diagnostic tests are directed to certain aspects of the disease. A panel of tests directed at similar aspects of the disease, like traces of a gene possibly present in dead sample material, may correspond to a different mixture of distributions, than a panel of tests based on culture growth for live sample material. The latent classes for tonsil-ELISA are associated with a small fraction of clearly outlying observations and quite different from those underlying PCR and BE. PCR is directed towards detection of the epf- gene encoding for the EF protein, while ELISA isolates the protein itself after growth in a culture medium. Possibly the product is not always produced although the gene is present. So, a panel of tests based on varying aspects of the disease offers a safer background for the latent class model. When many different tests are available, an obvious alternative approach is to define a quasi-gold standard on the basis of a "majority vote" of the tests. An important difference with the latent class approach is that the quasi reference offers a "true" status for each animal, while the latent class model offers an underlying structure of true status in a probabilistic sense. The latent class model is the safer choice. But the fact remains that, like Baron Munchhausen, the model more or less pulls itself out of the swamp by its own hair.

Without a proper gold standard, lack of sufficient structure in the latent class model may lead to non-identifiability of parameters. Such is for instance the case with two dependent tests (Georgiades et al., 2003). Identifiability of parameters in the multinomial and logistic regression model representations was not formally (see e.g. Catchpole and Morgan, 2001) established. As a referee pointed out, even with higher order interactions set to 0, the two representations may at least sometimes lack identifiability. Therefore it is important to use all reliable prior information that is available. In this study, use of different priors suggested that the data were reasonably informative with respect to sensitivity and specificity of the tests. This was not the case with respect to conditional dependence. Although quite unlikely for the *S.suis* data from a biological point of view, negative dependence was considered to find evidence in the data for positive dependence by inspection of posterior probabilities $P(\lambda > 0 \mid \boldsymbol{y})$. Although there was an indication for moderate conditional dependence, evidence was not conclusive. In many WinBUGS applications the stan-

dard uninformative prior for parameters on the logit scale is a normal distribution with very low precision. Very low precision for the priors of the λ parameters resulted in posteriors with heavy left hand tails and a wobbly appearance with multiple modes (Figure 3). This may be explained by a conflict between priors and data, similar to Liu and Hodges (2003) (in the different context of a variance components model), or by lack of identifiability of the dependence parameters. This argues in favour of fairly uninformative priors obtained from more realistic considerations, such as the priors for log odds ratios λ that are presented in this paper (Table 3).

It remains imperative to inspect the Markov chains carefully and study the impact of different prior distributions. We find analysis of simulated data, with the same structure as the actual problem, indispensable in this respect.

ACKNOWLEDGEMENTS

We thank Cajo ter Braak, Joop de Bree, Paul Goedhart, Herman van Roermund and the referees for their constructive comments on the manuscript.

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Chapter 4

Elimination of strains of *Streptococcus suis* serotype 2 from the tonsils of carrier sows by combined medication and vaccination

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Veterinary Record (2007) 160, 619-621

ABSTRACT

The effect of vaccination with a killed whole-cell vaccine of extracellular factor-positive *Streptococcus suis* serotype 2 (*S. suis* 2 EF$^+$) combined with medication with amoxicillin on the presence of virulent *S. suis* 2 EF$^+$ strains on the tonsils of sows and their offspring was evaluated. In two herds, 14 pregnant sows that carried these virulent strains, as detected by PCR in three consecutive tonsillar brush samples, were selected and randomly assigned to treated or left untreated as controls. The treated sows were vaccinated at six and three weeks before the expected farrowing date and medication from one week before expected farrowing until the end of the experiment. Two weeks before parturition, the sows were housed in individual isolation farrowing rooms, and the sow and its litter were sampled by using tonsil-brushes and tonsil-swabs, respectively. Approximately 27 days postpartum, the sows and their piglets were euthanased and their tonsils were collected and analysed by PCR. No *S. suis* 2 EF$^+$ could be detected from the tonsils of the seven treated sows, but the tonsils of seven untreated sows remained positive. Only one litter of the untreated sows became infected, five days after birth, and none of the litters of the treated sows became infected.

Keywords
Streptococcus suis, vaccine, amoxicillin, carrier, tonsil

INTRODUCTION

Streptococcus suis has been implicated in the aetiology of meningitis, arthritis, pneumonia, septicaemia, endocarditis, polyserositis and sudden death in pigs. *S. suis* infection is a zoonosis that has repeatedly been reported in pig-rearing and pork-consuming countries (Arends and Zanen, 1988; WHO, 2005). The virulent extracellular factor-positive strains of *S. suis* serotype 2 (*S. suis* 2 EF^+) have frequently been isolated from diseased pigs in many European countries as well as in the USA and Australia (Galina et al., 1996; Mwaniki et al., 1994; Salasia and Lammler, 1995; Wisselink et al., 2000). Sows carrying *S. suis* strains in their tonsils are considered the most important source of infection for their susceptible offspring (Clifton Hadley, 1984; Robertson and Blackmore, 1989), which can become infected very early in their lives with different *S. suis* serotypes including virulent strains (Amass et al., 1996a; Cloutier et al., 2003; Robertson et al., 1991; Torremorell et al., 1998). Carrier sows can be detected using a PCR for the detection of *S. suis* 2 EF^+ strains in tonsillar swabs (Swildens et al., 2005).

Several intervention studies have been carried out to try to reduce the clinical signs associated with *S. suis* strains and the costs of control. For example, the vaccination of pregnant sows with a *S. suis* serotype 14 bacterin resulted in central nervous signs in significantly fewer of their 13-day-old piglets after an experimental intravenous challenge with a homologous strain than in the control group (Amass et al., 2000), and the vaccination of pregnant sows with a commercial serotype 2 vaccine significantly reduced the percentage of carriers of *S suis* serotype 2 in their one- to 15-day-old offspring (Torremorell et al., 1998). However, the medication of early-weaned piglets was not successful in raising piglets free of *S. suis* (Amass et al., 1996b), and Clifton-Hadley and others (1984) reported failures to eliminate *S. suis* from herds of slaughter pigs by different antibiotic regimens. However, to the best of the authors' knowledge, the effect of a combination of medication and vaccination of sows in order to protect their offspring from carrying *S. suis* has not been investigated.

The aim of this experiment was to examine the effect of a combination of vaccination and medication of carrier sows on the *S. suis* 2 EF^+ carrier status of the sows and their offspring.

MATERIALS AND METHODS

The experiment was carried out according to a protocol approved by the Animal Ethical Committee of Utrecht University, as required by Dutch law.

At two farrow-to-finish farms, a total of 16 *S. suis* 2 EF^+ carrier sows (eight per farm) were selected for inclusion in the experiment. At both farms the weaned pigs suffered from clinical problems related to *S. suis* 2 EF^+ strains. In a previous survey, the proportion of carrier sows had been estimated to be at least 0·55 (Swildens et al., 2005). Four batches of four sows were studied. For each experimental

batch, over 25 sows were sampled at the farms 10, nine and eight weeks before the expected date of farrowing, and sows that tested positive in three consecutive weekly samplings were considered to be carriers. On each farm, the selected carrier sows were randomly allocated to the treatment or control group. Two to three weeks before the expected farrowing date, one sow of each group was randomly selected at each farm and transported to the experimental unit. These four sows were randomly assigned to individual isolation rooms, where they farrowed. In the treatment group a mean (sd) of 9,86 (0.38) piglets per sow were born, and in the control group 8,57 (2,30) piglets per sow were born. Samples were collected regularly from the sows and individual piglets. Twenty-two to 32 days after the piglets' birth; the sows and piglets were euthanased and their tonsils were removed using sterile scalpels and scissors, without making contact with other parts of the carcass.

1. Sampling of sows

The sows were sampled by tonsillar brushing five times during the selection procedure, and in the week before they farrowed, within 24 hours after farrowing and weekly thereafter (table 1). The samples were taken as described by (Swildens et al., 2005) with a slight modification; briefly, after the sow had been immobilised, a wedge was pushed between its teeth, and each tonsil was brushed for at least eight seconds using a sterile toothbrush attached to a 40 cm long metal rod.

Vaginal swabs were taken with a sterilised 40 cm plastic rod with a 5 cm cotton wool tip (Heinz-Herens) within 24 hours after parturition.

Table 1: Sampling and treatment schedule for two groups of *Streptococcus suis* serotype 2-positive sows.

Action	Material/method	Group	Timing
Tonsils sampling	Brush	T/C	10, 9, eight, six, three and one week before the expected farrowing date, within 24h after farrowing and every week thereafter
Vaginal sampling	Cotton wool swab	T/C	Within 24h after farrowing
Vaccination	*Streptococcus suis* serotype 2 strain 4005	T	Six and three weeks before the expected farrowing date
Medication	Amoxicillin	T	Twice a day from one week before the expected farrowing date until euthanasia

T = treatment group, C = control group

2. Sampling of piglets

The piglets were sampled for the first time within 24 hours after birth, daily during the first week, and then three times a week until they were euthanased (Table 2). To sample the piglets, a small wedge was gently inserted between their teeth, and a sterilised 15 cm plastic rod with a 1·5 cm cotton wool tip (Applimed 0538) was used to swab both tonsils for 10 seconds, while turning the swab between forefinger and thumb; on average, the whole procedure took less than 45 seconds.

Table 2: Sampling schedule for the piglets from the seven treated sows and seven control sows.

Action	Material/method	Timing
Tonsils sampling	Cotton wool swab	Day of birth and daily for the first seven or eight days; thereafter, three times a week

3. Housing and hygiene protocol

The sows were housed in individual isolation rooms which, to prevent cross-contamination, were ventilated under negative pressure through high-efficiency particle arresting (HEPA) filters. A farrowing crate was placed in each isolation room, and food, water and medication were provided through closed systems, to minimise the need for technicians to enter the isolation rooms. Only technicians who had no other contacts with pigs sampled the animals, and before entering an isolation room they changed their clothes and put on disposable protective respiratory masks, gloves and hair caps.

4. Treatment

4.1 Vaccine

Six and three weeks before expected farrowing, the sows in the treatment group were vaccinated (Table 1). A formalin-killed, whole-cell vaccine of the EF-positive *S. suis* serotype 2 strain 4005 was prepared as described by (Wisselink et al., 2002). The vaccine contained approximately 109 colony forming units/dose; a water-in-oil emulsion (Stimune; Cedi-Diagnostics) was used as adjuvant. The sows received doses of 2 ml intramuscularly.

4.2 Medication

The sows in the treatment group were medicated twice a day from one week before the expected farrowingdate until they were euthanased. Each dose consisted of 40 mg/kg bodyweight of amoxicillin (Octacilline; Eurovet Animal Health, containing 80 per cent amoxicillin) in 2 litres of water.

5. Sample transport and storage

Directly after the samples were taken, the tonsillar and vaginal swabs and the tonsillar brushes were transported to the laboratory in a transport medium consisting of Todd-Hewitt broth (CM189; Oxoid) containing 0·25 per cent *Streptococcus* selective supplement (SR126; Oxoid) and 0·2 µg/ml crystal violet. At the laboratory the specimens were incubated in this medium for 18 hours at 37°C. Whole tonsils were transported in individual plastic bags on ice and processed within two hours of collection into homogenates, as described by Wisselink and others (1999); Glycerol was added to a final concentration of 15 percent. Individual samples and pooled samples from all the piglets in a litter were stored in 2 ml Greiner cryotubes at -70°C.

6. PCR test

The PCR assay for the detection of *S. suis* 2 EF$^+$ strains and the preceding DNA preparations were performed as described by Wisselink and others (1999). The homogenates of the post-mortem samples from the sows and piglets and the vaginal swabs from the sows were analysed by PCR. In addition, eight tonsillar brush samples taken during lactation from six treated sows and four taken from two untreated sows were analysed. All the pooled litter samples, and the individual samples taken within 24 hours after birth from the only litter of piglets that had positive individual homogenates, were also analysed by PCR.

7. Statistical analysis

To estimate the differences between the two treatment groups in the tonsillar carriage of *S. suis* 2 EF$^+$, a Fisher's exact test was performed in SPSS 9.0. Values of $P<0·05$ were considered significant.

RESULTS

A pair of sows from the same farm in the first batch were excluded from the experiment because the control sow was treated erroneously, leaving 14 sows in the experiment. No *S. suis* 2 EF$^+$ strains could be detected postmortem in the tonsils of the seven treated sows, whereas they were detected by PCR in all the tonsils of the seven untreated sows ($P<0·001$). The same contrast was observed in the tonsil brush samples taken during the lactation period; the eight samples from the treated sows tested negative but the four samples from the untreated sows tested positive by PCR ($P=0·036$). No *S. suis* 2 EF$^+$ could be detected in the vaginal swabs from the 14 sows.

None of the litters of the treated sows became infected with *S. suis* 2 EF$^+$ strains, but one of the seven litters of the control sows became infected. In this litter, *S. suis* 2 EF$^+$ strains were first detected in the pooled sample taken on day 5. The

pooled sample taken on day 6 and five pooled samples taken between day 16 and day 25 were also positive, but the four pooled samples taken between day 7 and day 14 were negative.

DISCUSSION

No *S. suis* 2 EF^+ strains could be detected by PCR in the whole tonsils of the treated sows postmortem. Earlier attempts by Amass and others (1996b) and Clifton-Hadley and others (1984) to eliminate *S. suis* from the tonsils of weaned pigs and slaughter-pigs by medication with therapeutic doses of antibiotics during five days and several months respectively, failed. In contrast with those trials, the present experiment used sows instead of pigs and used a combination of antibacterial medication and vaccination rather than antibacterial treatment alone. It is not possible to attribute the successful results to either the medication or the vaccination of the sows, although the results of vaccination trials by Amass and others (2000) and Torremorell and others (1998) suggest that the vaccination may have made a significant contribution.

Owing to the imperfect test sensitivity of 0.88 (Swildens et al., 2005), it is not possible to be certain that the treated sows were not still positive carriers at the end of the experiment, although the PCR on the whole tonsil was negative. However, in support of the negative postmortem findings, all the tonsillar brush samples taken from the treated sows during lactation also tested negative. The specificity of the test was 0·96 (Swildens et al., 2005), and it is therefore very unlikely that the sows were free of *S. suis* 2 EF^+ strains at the start of the experiment and were included in the trial erroneously as carriers.

Only one of the seven litters of the untreated sows was infected with *S. suis* 2 EF^+. This litter was probably not infected at birth, because *S. suis* 2 EF^+ was first detected in the pooled-litter-sample of day 5. Moreover, the vaginal swabs taken directly after parturition were all negative, which is consistent with the findings of Clifton-Hadley (1984), who did not detect *S. suis* on the vaginal samples of 81 sows and gilts in two herds with a high carrier rate. The number of litters infected by the untreated sows was low compared with the results of Robertson and others (1991), who cultured *S. suis* serotype 2 from all five litters in a trial. Torremorell and others (1998) cultured *S. suis* serotype 2 from tonsillar swabs from 13 of 24 piglets from an unknown number of litters, five days after birth. However, both these studies were carried out in commercial farrowing units where infectious pigs could have infected susceptible pigs in neighbouring pens. In the present experiment a strict biosecurity protocol was used so that the role of the sow as the initiator of infections within its own litter could be evaluated.

By the use of individual isolation rooms for the sows and their litters and the use of a well characterised test, the results of this experiment show that it is possible to wean piglets free of *S. suis* 2 EF^+ from positive sows, and that the incidence of vertical transmission in farrowing pens is low. By using the *S. suis* 2 EF^+-free weaners as replacement gilts, *S. suis* 2 EF^+ could be eliminated from a farm provided that

the gilts were not infected later on and that the incidence of transmission between gilts and sows and between sows was low. As a result, the costs of labour and the use of antibiotics to control disease associated with *S suis* could be reduced substantially. To determine the feasibility of this strategy, studies on the dynamics of animal contacts and the transmission of *S. suis* 2 EF$^+$ within a farm are needed.

ACKNOWLEDGEMENTS

The authors thank the personnel of the Department of Farm Animal Health from the Faculty of Veterinary Medicine of Utrecht, especially Jacomijn Stouten, for assistance at the time of sampling and all other colleagues and students for their help in processing the samples.

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Chapter 5

Intestinal translocation of *Streptococcus suis* type 2 EF^{+} in pigs

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Veterinary Microbiology 103 (2004): 29-33

ABSTRACT

Sepsis with subsequent multisystem organ failure after translocation of bacteria from the gut is a serious risk associated with stress situations. We showed that intestinal bacterial translocation can be one of the pathways for pathogenic *Streptococcus suis* infections in the pig. In 24 piglets weighing 10-14 kg, free of the extracellular factor (EF^+) producing phenotype of *S. suis* serotype 2, a silicon canula was placed in the proximal jejunum to enable intestinal inoculation and bypassing the upper alimentary tract. The pigs were individually housed. After stress induction in eighteen pigs by means of a truck drive in individual cages for one hour, pigs were inoculated through the intestinal canula either with *S. suis* type 2 EF^+ or with growth medium only, and put back in their original housing. The six not transported pigs were also inoculated with the same strain. To prevent oral self-infection, faeces were collected in a bag that was glued around the anus. Clinical and behavioural symptoms were recorded for 72 hours post inoculation, and then the animals were sacrificed for pathological and bacteriological examination. In three animals the inoculation strain was re-isolated from mesenterial lymph nodes and typically affected organs. No *S. suis* type 2 EF^+ was detected by specific Polymerase Chain Reaction (PCR) in any of the tonsil-swabs and -homogenates. We concluded that infection of the organs had taken place after bacterial translocation out of the gut and that the intestinal tract can be a porte d'entrée for *Streptococcus suis* type 2 EF^+.

Key words
Intestinal bacterial translocation / *Streptococcus suis* / stress / canula / pig

INTRODUCTION

The mucosal layer of the gut is an important mechanical barrier that prevents bacteria, endotoxin and other luminal contents to invade the extraintestinal tissues. However, endogenous bacteria can escape the intestinal tract and infect otherwise sterile tissues. This process of intestinal bacterial translocation into organs is a well-known phenomenon (Berg, 1992; Lemaire et al., 1997; Ljungdahl et al., 2000; Marston et al., 1989; Naaber et al., 2000; Steffen et al., 1988).

Streptococcus suis serotype 2 strains are often present in swine herds and are causing substantial losses (Staats et al., 1997; Vecht et al., 1985). In this experiment we concentrated on intestinal translocation of the EF-positive *S. suis* serotype 2 strain because this is the most frequently isolated phenotype of *S. suis* serotype 2 from diseased pigs in most European countries as well as in the United States and Australia (Galina et al., 1996; Salasia and Lammler, 1995; Vecht et al., 1991; Wisselink et al., 2000). Moreover, it can be isolated with a very sensitive and specific PCR (Wisselink et al., 1999).

Most *S. suis* clinical signs occur around the weaning period. In this period the gut morphology undergoes substantial changes. Under stressful circumstances such as weaning, the defence mechanisms of the gastrointestinal tract fail, thus facilitating bacteria to pass the stomach and translocate from the gut to (Nieuwenhuijs et al., 1998). Transport stress reduces the blood flow in the superior mesenteric artery which lowers the intramucosal pH (Nabuurs et al., 2001; Pargger et al., 1997; Van der Meulen et al., 2000). This mucosal acidosis increases the intestinal macromolecular permeability and the bacterial translocation from the gastrointestinal tract (Niewold et al., 2000). However, the generally accepted infection route of *S. suis* is through replication in the tonsils (Gottschalk and Segura, 2000). We investigated whether *S. suis* type 2 EF^+ can translocate from the intestinal tract to the internal organs of pigs. To exclude the tonsil as infection route we bypassed the upper gastrointestinal tract through a duodenal canula.

METHODS

Twenty-four male pigs, weighing 10-14 kg, were obtained from the high health status farm of the Lelystad Centre for Pig Husbandry. *S. suis* type 2 EF^+ was neither found by PCR in repeated examination of tonsil-swabs of 207 weaned piglets and sows during the 20 month of existence of the farm, nor in the entry-tonsil-sampling of the 24 experimental pigs. Clinical signs of streptococcal arthritis or meningitis were never observed in this herd. The 24 pigs were randomly allocated over individual cages to prevent cross infection. The absence of *S. suis* type 2 EF^+ at the tonsils was confirmed during the trial by *S. suis* type 2 EF^+ PCR analysis of tonsil-swabs twice before experimental inoculation and twice after inoculation and of the tonsil-homogenates after necropsy (Wisselink et al., 1999).

In the week after arrival, in all animals, a 2,5 mm diameter silicon canula with was surgically placed in the proximal jejunum about 10 cm distally from the flexura duodenojejunalis. The canula was guided sub-dermal to the top of the shoulder blade. Pigs were allowed to recover from surgery for 10 to 14 days.
To induce transport stress, 18 animals were transported in individual cages during one hour in a small truck over a meandrous road. Thereafter 12 of these pigs and the six pigs that were not transported, were inoculated through the canula with 5 ml Todd Hewitt broth with 2,5x 10^8 CFU of *S. suis* type 2 EF^+ strain 4005 (Vecht et al., 1992). The other six transported pigs were inoculated with Todd Hewitt broth (medium) only.

Table 1: Overview of treatment groups.

Group	Number of pigs	Transport	S. suis inoculation	Number of successful procedures
1	12	Yes	Yes	11
2	6	Yes	Growth medium only	4
3	6	No	Yes	4

Stoma bags were glued around the anus to prevent oral re-infection and possible contamination of the tonsils. The pigs were put back in their original cages. No changes in the food were made. The days after, clinical symptoms and rectal temperature were checked twice daily. The areas under the curves (AUC) of the five rectal temperature measurements were calculated relative to the normal temperature of the pig (39,5°C). Tonsil swabs were taken every morning. Sixty-nine to 74 hours after inoculation, all animals were killed for pathological examination. Swabs taken from joint cavities, kidney, brain, liquor, pericardium, peritoneum, pleura and lung-heart valve were plated directly at Columbia plates with 6% horse blood. Ten grams of tissue from tonsils, liver, mesenteric lymph nodes, spleen and lungs were each thoroughly crushed and mixed 1:1 with phosphate buffered saline (pH 7,2). 100 µl was plated at Columbia plates with 6% horse blood. Scrapings of proximal, mid- and distal jejunum mucosa were plated on the same plates and on Columbia plates with 6% horse blood, 0,4% Streptococcus Selective Supplement (Oxoid SR 126^E) and 0.01% Cristalviolet (2 mg/ml) to minimize Gram-negative bacterial and staphylococcal overgrowth. The experimental protocol was approved by the ethical committee of the ID-Lelystad in accordance with Dutch law on animal experiments.

RESULTS

In three animals (animal nr 1, 2 and 3 in table 2) out of 11 successfully inoculated and transported animals, *S. suis* type 2 EF^+ was re-isolated from organs, indicating intestinal bacterial translocation. In two of these pigs (nr 1 and 2) typical *S. suis* infected organs, such as the mesenteric lymph nodes, liver, spleen, joints and peritoneum, were positive. In addition these 2 pigs showed gross lesions typical of *S. suis*,

namely a fibrinosuppurative peritonitis, pleuritis, perisplenitis and arthritis. In the third animal (nr 3), *S. suis* type 2 EF^+ was re-isolated from the liver, spleen and peritoneum, but not from the mesenteric lymph nodes and joints. In this animal, pathology was less pronounced.

This was in contrast to four non-transported and four growth-medium inoculated animals where *S. suis* type 2 EF^+ was not cultured and only in one animal non-typical pathological lesions were seen. In the other five animals, in which the canula was erroneously placed in the ileum, there were also no positive cultures or pathological lesions.

Table 2: Summary of major results.

Identification numbers of affected animals

	Clinical Examination			Pathological Examination	Bacteriological Examination	
Group	Pigs with lameness	pigs with nervous symptoms	Pigs with clinical depression	Pigs with typical S. suis lesions [1)]	Pigs with S. suis in mesenterial lymph nodes and joints	Pigs with S. suis in liver, spleen and peritoneum
1 (n=11)	1, 2, 3	1, 2	1, 2, 4	1, 2	1, 2	1, 2, 3
2 (n=4)	-	-	5	-	-	-
3 (n=4)	-	-	-	-	-	-

[1)] Fibrinosuppurative peritonitis, pleuritis, perisplenitis and arthritis.

All three animals with translocation (nr 1, 2 and 3) of *S. suis* type 2 EF^+ showed lameness at least at one control moment and two of them (nr 1 and 2) showed central nervous symptoms, while none of the other animals in the experiment showed these symptoms. Two of the animals with translocation (nr 1 and 2) had a lower food intake and were clinically depressed and one of the other animals (without translocation) (nr 4) had a lower food intake (see table 2). The three animals with intestinal bacterial translocation (nr 1, 2 and 3) were by far highest in rank with regard to area under the temperature curves (Fig.1). There was a difference in AUC between the groups 1 and 3 ($p=0.019$), but this was after Bonferroni correction not significant in the Mann-Whitney Test (level of significance with three groups is 0,0167).

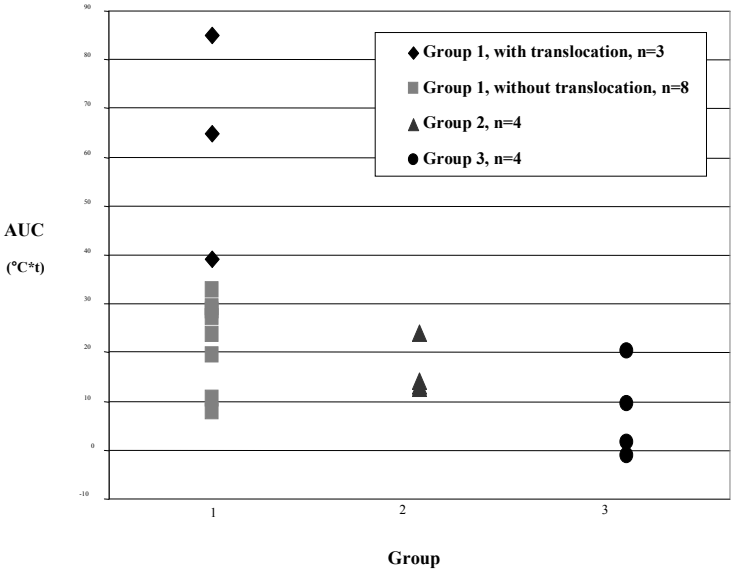

Figure 1. Area under the curve of rectal temperatures.
* AUC= area under the curve of five consecutive rectal temperature measurements with 39,5°C as reference temperature (°C*t).

The PCR analysis on *S. suis* type 2 EF^+ of the tonsil-swabs and the tonsil-homogenates did not reveal any positive samples. All small intestinal mucosa scrapings were negative for *S. suis*. The duodenal canula's were grown well together with the intestinal wall without signs of inflammation in all animals.

DISCUSSION AND CONCLUSION

In this experiment we have shown that *S. suis* type 2 EF^+ can translocate from the gut to different organs. In three originally *S. suis* type 2 EF^+ negative pigs we isolated the inoculation strain from multiple organs after intestinal inoculation.
In this experiment the *S. suis* infection pathway through replication in the tonsils and the negative effect of acid in the stomach, was bypassed by means of a canula to the duodenum. All tonsils stayed negative for *S. suis* type 2 EF^+ as demonstrated by PCR of daily tonsil-swabs after inoculation and PCR of the tonsil-homogenate after necropsy.

S. suis has been found in the intestine of pigs although most of the cultured strains probably belong to serotypes 9-22 (Devriese et al., 1994). In germfree pigs *S. suis* type 2 EF^+ can pass the stomach and survive in the small intestines (Vecht et al., 1992). We did not culture *S. suis* from the intestinal mucosal scrapings after inoculation. The bacteria were probably already excreted by intestinal transit during the experimental period.

(Nabuurs et al., 2001) and (Seidler et al., 2001) showed that transport stress leads through intestinal acidosis to an increase in bacterial translocation. However, it was not possible to show a significant effect on the translocation of the bacteria by transportation in this study. Taking into account the rather high bacterial count of the inoculum and the small number of animals showing sepsis, translocation from the intestine does not seem to be a very efficient route of infection. Nevertheless, considering the important changes in defensive mechanisms of the gastrointestinal tract during weaning, a possible role of translocation from the gut in the pathway of *S. suis* type 2 EF^+ infections can not be excluded. This is the first time that, by exclusion of the tonsil route, translocation from the gut is demonstrated to be one of the pathways for *S. suis* type 2 EF^+ infections.

ACKNOWLEDGEMENTS

The authors specially appreciated the technical assistance of the personal of the experimental facilities of the Institute for Animal Science and Health in Lelystad.

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Chapter 6

General Discussion

INTRODUCTION

Streptococcus suis (*S. suis*) has been associated with meningitis, arthritis, pneumonia, septicemia, endocarditis, polyserositis and sudden death in pig-husbandry all over the world. Streptococcal disease not only causes economic losses due to treatment and control costs, extra labor and mortality, but it also affects the well-being of pigs. Although much research has been done on the pathogenesis and epidemiology of *S. suis*, it remains difficult to design robust preventive strategies.

S. suis can often be isolated from clinically healthy pigs. The prevalence of *S. suis* carrier pigs in a herd does not correlate with the incidence of related disease outbreaks (Berthelot Herault et al., 2000; Clifton Hadley et al., 1984a; Clifton Hadley et al., 1984b; van Leengoed et al., 1987). Still, carriers of *S. suis* comprise the most important risk of infection for susceptible animals, whereby the moment of transmission is largely unknown. Infections during and shortly after birth are described in literature. A second important risk moment of *S. suis* transmission is when piglets are weaned and litters are mixed to form large homogeneous groups (Amass et al., 1996a; Cloutier et al., 2003; Robertson et al., 1991; Torremorell et al., 1998). Pigs can be carriers of different streptococcal strains at the same time (Monter Flores et al., 1993). Probably many of these *S. suis* strains do belong to the indigenous *S. suis* population that plays a role in the colonization resistance of skin, mucous membranes and intestines, thus preventing overgrowth of pathogenic bacterial strains (Guarner and Malagelada, 2003; Vollaard and Clasener, 1994; Wilson, 2005). However, especially the pathogenic strains are of primary interest in respect of prevention and control strategies.

The aim of the studies as described in chapter 2-5 was to provide better insight in the transmission of virulent *S. suis*. This is essential to improve strategies in the prevention of streptococcal disease. The extracellular protein producing serotype 2 strain (*S. suis* 2 EF^+) is often isolated from diseased pigs in most European countries as well as in the United States and Australia pigs (Berthelot Herault et al., 2000; Galina et al., 1996; Mwaniki et al., 1994; Salasia and Lammler, 1995; Wisselink et al., 2000). Therefore this strain was used in our experiments as a representative of virulent strains. In the first place a reliable diagnostic tool was needed. Therefore the quality of a *S. suis* 2 EF^+ PCR on swabs taken from live sows was determined. This was established using a Bayesian analysis of a latent class model that included the results of three *S. suis* 2 EF^+ test methods from four farms. This PCR was subsequently used to analyze the carrier status of lactating *S. suis* 2 EF^+ carrier-sows and their offspring before and after an intervention consisting of vaccination and medication of the sows. The vaccine contained an EF-positive serotype 2 strain and the medication consisted of long-term oral amoxicillin administration. After this intervention *S. suis* 2 EF^+ was not detectable anymore at the tonsils of the sows. Moreover, six out of seven non-treated carrier-sows did not transmit *S. suis* 2 EF^+ to their offspring during lactation. Finally, intestinal translocation of *S. suis* 2 EF^+ was demonstrated to be a possible pathway for infection in newly weaned pigs.

This chapter discusses some aspects of the presented studies in the light of additional experimental results and the literature. In the first section the validation of the *S. suis* 2 EF$^+$ PCR on swabs taken from live sows is explained in more detail. Firstly the choice of tonsillar swabs to sample live sows will be illustrated and thereafter some methodological difficulties in the characterization of the *S. suis* serotype 2 EF$^+$ PCR in a Bayesian analysis will be discussed. The second section is dedicated to the prevention and control of streptococcal transmission and disease. The possible importance of intestinal translocation as an infection route of *S. suis* and appropriate preventive measures will be commented. Thereafter, some unpublished data about the effect of medication on the transmission of *S. suis* serotype 9 are shown and compared with the data about *S. suis* serotype 2 EF$^+$ transmission. The chapter concludes with summarizing the conclusions and recommendations for future research.

DETECTION OF *S. SUIS* 2 EF$^+$ CARRIERS

A well-defined diagnostic method was needed for research on the prevalence and transmission of *S. suis* 2 EF$^+$. The polymerase chain reaction (PCR) developed by (Wisselink et al., 1999) for the detection of EF-positive *S. suis* serotype 2 strains performed very well under laboratory circumstances. To design a field-test for the detection of *S. suis* 2 EF$^+$ carrier sows, the most sensitive way of sampling was needed. *S. suis* can be detected in many organs of diseased pigs. The tonsils can harbor *S. suis* for prolonged periods without clinical signs of disease while in most other organs the presence of *S. suis* is transient (Davies and Ossowicz, 1991; Gottschalk and Segura, 2000). Therefore the tracing of carriers of *S. suis* is preferably performed by sampling the tonsils. In the first paragraph the results of a small study on the most sensitive sampling methods that are easy to combine with the PCR is shown.

To characterize the performance of the *S. suis* 2 EF$^+$ PCR on tonsil-swab samples of live sows, this test method was compared with two other available tests methods on the same sows, namely the PCR and a bacterial examination (BE) applied on the homogenates of whole tonsils removed after slaughter. The quality of the PCR and BE was established using a Bayesian analysis of a latent class model as described in chapter 2. The credibility intervals of the sensitivities of all three tests were larger than 0.2. To obtain smaller intervals, more valid and conditionally independent tests can be incorporated into the latent class model. Accordingly we tested all whole tonsil samples again in an Enzyme-Linked Immuno Sorbent Assay (ELISA) as described in chapter 3. This test was based on the detection of the EF-protein in the supernatants of *S. suis* 2 EF$^+$ cultures and was therefore expected to be conditionally independent from the other tests that were based on the presence of DNA of the bacterium (PCR) or growth of the bacterium (BE). The results were added to the model for Bayesian analysis. The outcomes however were not much better than the estimates and credibility intervals of sensitivities and specificities derived from the

original model with only three tests. Moreover, the sensitivity and specificity of the ELISA itself were disappointing. Therefore this EF-test was not further examined at that moment.

When a Surface Plasmon Resonance technique (SPR) was developed as an alternative EF detection method (Bergwerff and van Knapen, 2006), the model from chapter 2 was used again to characterize this test. In paragraph 2, we describe the way the cut-offs for both continuous antibody-based tests (ELISA and SPR) were chosen. The outcomes from the Bayesian analysis of the models with these continues tests included are discussed and compared with former results from chapter 2 and 3.

1. Sampling method

A small study was performed to compare two potential sample techniques on tonsils of live sows (tonsil swabbing and biopsy) to a sample of the homogenate of the whole tonsil obtained after slaughter. The analysis of these specimens was done by *S. suis* 2 EF^+ PCR (Wisselink, 1999).

Table 1. Results of the PCR on tonsil-swabs and tonsil-biopsies compared to the PCR on a sample of the homogenate of the whole tonsil of the same sows.

		Tonsil homogenate PCR* (post mortem)	
		+	-
Tonsil swab PCR (live sow)	+	12	3
	-	6	28

		Tonsil homogenate PCR* (post mortem)	
		+	-
Tonsil biopsy PCR (live sow)	+	7	1
	-	11	30

* The PCR on the whole tonsil homogenate sample is defined as gold standard. The resulting sensitivity and specificity of the PCR on tonsil swabs is 0.67 (CI95%: <0.45-0.88>) and 0.90 (<0.80-1.0>) respectively and the sensitivity and specificity of PCR on tonsil biopsies is 0.39 (<0.16-0.61>) and 0.97 (<0.91-1.0>) respectively.

Carriers of *S. suis* can be found slightly easier by PCR on swabs than on biopsies when compared to the PCR on the whole tonsil homogenate defined as the gold standard (Table 1). Similar results are described by Marois et al. (2007). The higher sensitivity of tonsil-swabs compared to tonsil-biopsy is plausible in view of the results of Davies and Ossowiscz (1991) who found colonies of *S. suis* confined to some locations at the tonsil by IFT. A colony can be missed using biopsy samples because of the limited sampling area involved. In other words, the biopsy is too local, and consequently, less suited for the detection of *S. suis* carrier sows. Deeper colonies, on the other hand, can be missed when a superficial swab is taken. However, we found that testing a second swab of the tonsils of a sow has a better sensitivity

than the first swab (Chapter 2). This suggests that a slightly more invasive technique like tooth-brush samples can overcome the drawbacks of superficial swabbing. Furthermore, swabbing the tonsils of a sow with a toothbrush instead of taking a biopsy is easier to perform. This sampling technique was utilized for the sampling of the sows in the transmission experiment as described in chapter 4. The Dutch Animal Health Service advises this sampling method now as a routine technique in farm screening programmes.

2. Characterization of the *S. suis* 2 EF$^+$ PCR without a gold standard

2.1 Cut-off threshold levels for two EF-tests with continuous outcomes

2.1.1 ELISA

The supernatants of the swabs and whole tonsil homogenates used in the experiment described in chapter 2 were tested with an ELISA as described by U. Vecht et al. (1993). This ELISA is aimed on the extracellular factor, a 110 kDa protein in the supernatant of *S. suis* 2 EF$^+$ broth. An optimal cut-off for the ELISA was determined after constructing a receiver operating characteristic (ROC) curve using the outcomes of Bayesian analyses of several models that included the results of the PCR on swabs, the PCR and BE on tonsils, each time extended with the binary results of different cut-offs for the ELISA. The constructed ROC-curve shows the pairs of sensitivity and specificity that correspond to these cut-off points of the continuous test. For the analysis described in chapter 3, the used cut-off was -2.1, being the titer at the maximum sum of sensitivity and specificity (Greiner et al., 2000). With this threshold 117 from 281 samples were classified positive for EF in the ELISA. In a comparison with Tonsil-PCR, many results were discordant (36%) (Table 2).

Table 2. The results for the *S. suis* 2 EF$^+$ PCR and ELISA on tonsils.

	PCR		ELISA -2.1	ELISA 1.0
pos	143	pos	80	23
		neg	63	120
neg	138	pos	37	1
		neg	101	137

An alternative way to determine the threshold level for a continuous test without the easy use of a 'gold standard' is based on modeling the available results of the test in question. In most situations the binominal frequency distribution of test results is composed of two underlying normal distributions (representing the positive and negative populations). A cut-off that minimizes the proportion of false negatives and false positives can be estimated using a simplified mixture population modeling

spreadsheet (Cameron and Eamens, 2000). From this MS Excel spreadsheet an obvious cut-off for the ELISA would be around 1. In that case 24/281 (8.5%) of the samples would be classified positive (Figure 1 and table 2).

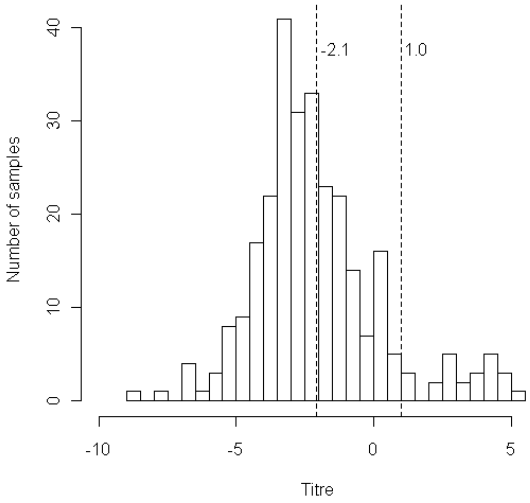

Figure 1: frequency histogram of ELISA results. Titres of 281 samples that are analysed in both ELISA and SPR. Cut-offs at -2.1 and 1.0 are indicated.

2.1.2 SPR

A new Surface Plasmon Resonance technique (SPR) was developed as an alternative EF detection method (Bergwerff and van Knapen, 2006). This test method is a very promising technique for the specific detection of proteins in e.g. Salmonella diagnostics (Jongerius-Gortemaker et al., 2002). In short, a quantified amount of antibodies against EF was added to the filtered supernatant of the *S. suis* 2 EF^+ broth. Purified EF was coated at the test-chip surface. The antibodies which did not bind to the EF in the sample attached to the EF at the chip and were measured at that place. In this sandwich method a low SPR response (RU) corresponded with high EF levels in the sample.

To dichotomize the test signals in a high and low response group, in each batch a cut-off value at different percentiles of the dilution series of the positive control (purified EF dilutions) was calculated (Ritz and Streibig, 2005). The optimal collective cut-off value for the SPR was determined using the mixture population modeling spreadsheet, similar to the establishment of the ELISA cut-off (Cameron and Eamens, 2000). The 30^{th} percentile (SPR30) was chosen as cut-off between positive and negative response samples in each batch (the histogram of all SPR results (Fig. 2) and the histogram for one of the batches with percentile lines (Fig. 3) are depicted below).

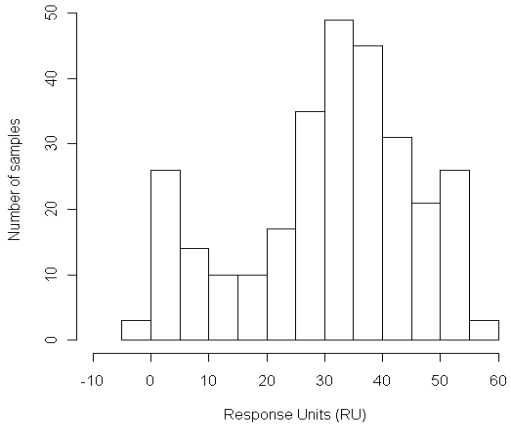

Figure 2: frequency histogram of SPR results. RU of 281 samples that are analysed in both ELISA and SPR.

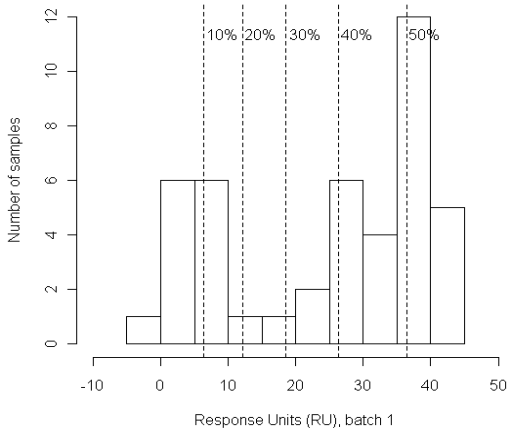

Figure 3: frequency histogram of SPR results. RU of 44 samples from SPR batch 1 that are also analyzed in the ELISA. Cut-offs at 10^{th} to 50^{th} percentile of the positive control dilution curve are indicated.

Using this 30% threshold level, 59 out of 281 samples (20%) were classified positive for EF in the SPR30. In a comparison with Tonsil-PCR, 33% of the test results were discordant (Table 3). This is similar to the ELISA 1.0. In both tests the misclassifications were mainly concentrated in the PCR positive samples. Nevertheless, 38% (<30,5%-46,4%>) of the SPR30 samples agreed with the positive PCR samples against only 16% (<10,0%-22,1%>) of the ELISA 1.0 samples. These values would represent the sensitivities of the tests when the PCR is defined as gold standard.

Finally, to compare the effect of the SPR threshold levels in the Bayesian models we also chose an arbitrary non-differential cut-off at the 50th percentile of each batch of the SPR (SPR50)(Table 3). Similar to the ELISA –2.1, 42% of the results were discordant with the Tonsil-PCR results.

Table 3. The results for the *S. suis* 2 EF^{+} PCR and SPR on tonsils.

	PCR		SPR30	SPR50
pos	143	pos	55	98
		neg	88	45
neg	138	pos	4	74
		neg	134	64

2.1.3 Summarizing the data

The PCR and BE test results of 281 whole tonsil samples that are also analyzed in both ELISA and SPR are shown in table 4 below. If the PCR is negative, the other test-results are predominantly negative, suggesting that at least the specificities of ELISA 1.0 and SPR30 are relatively good. PCR positive samples vary much more in the other three tests.

Table 4. The results for the *S. suis* 2 EF^{+} PCR, BE and SPR or ELISA on tonsils

PCR		BE			SPR30	ELISA 1.0	SPR50	ELISA -2.1*
pos	143	pos	94	pos	49	22	75	59
				neg	45	72	19	35
		neg	49	pos	6	1	23	21
				neg	43	48	26	28
neg	138	pos	4	pos	0	0	2	3
				neg	4	4	2	1
		neg	134	pos	4	1	72	34
				neg	130	133	62	100

* ELISA -2.1 results are analyzed in chapter 3. The numbers are slightly different because samples with missing SPR results are not included in this table.

To better visualize the agreement between the results of the SPR30, ELISA and PCR on whole tonsils homogenates, the test results are put in one scatterplot below (figure 4). The results of the SPR30 (Y-axis) are expressed as the difference between the RU of the sample and the 30% cut-off RU, relative to the range of the dilution series of the positive sample in the batch: the sample to cut-off 30 ratio = $(RU_{sample} - RU_{cutoff})/-(RU_{max} - RU_{min})$. This way, variation between batches is taken

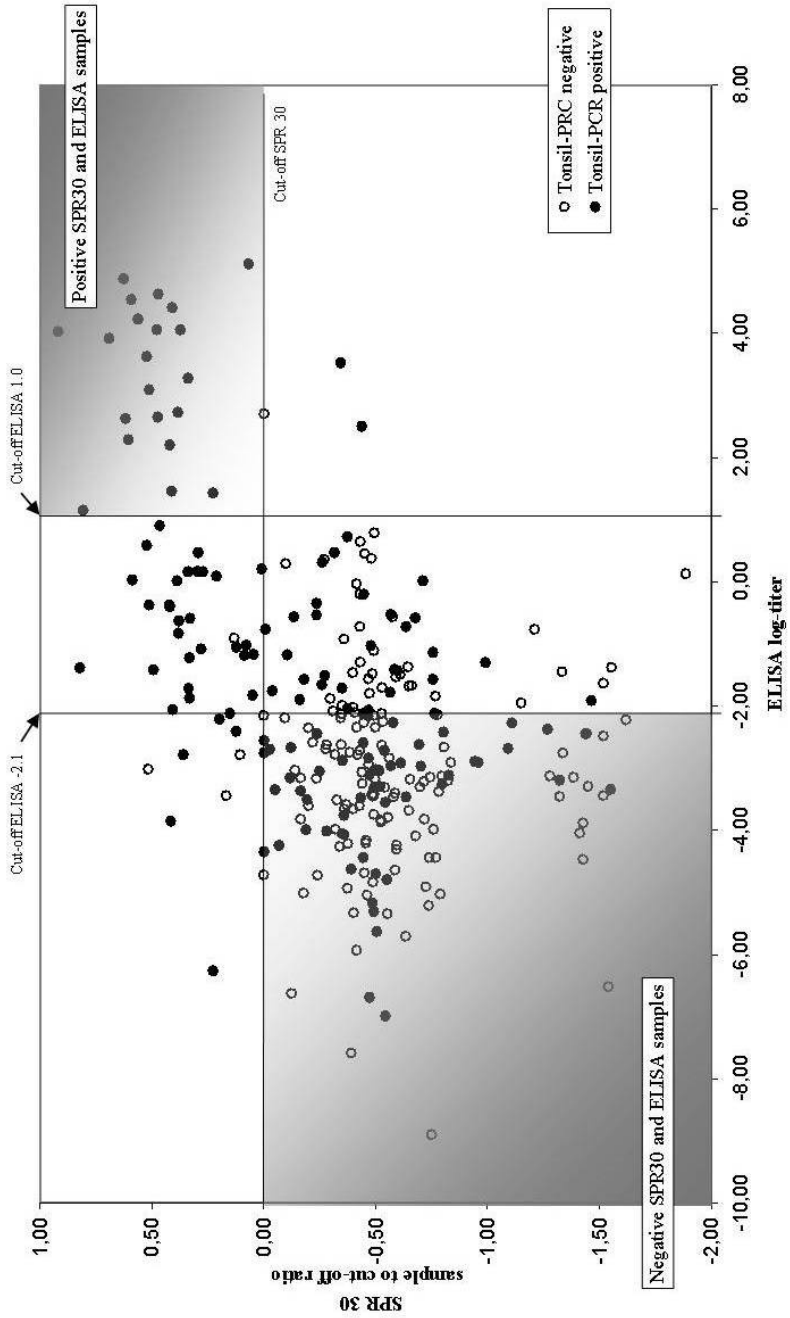

Figure 4: SPR30, ELISA and PCR results. The SPR30 is the response relative to the response range within each batch. The ELISA cut-offs at -2.1 and 1.0 are indicated. The areas with samples that have equal results in the ELISA -2.1 and the SPR30 are shaded.

into account. Thus, a sample with a ratio above 0 is classified as an EF-positive sample.

Samples that are tested EF-negative in the SPR30 as well as in the ELISA 1.0 or -2.1 (corner left-below) often have unequal PCR results, whereas EF-positive SPR30 and ELISA results (corner right-top) correspondent well with a positive PCR result. Presuming the PCR as gold standard, a relatively good specificity of the two EF-tests can only be expected at quite extreme cut-offs. To increase the sensitivity of the EF-tests, such a cut-off is not obvious. Altogether, the main conclusion from these descriptives is that although SPR30 and ELISA are both aimed at the presence of EF, their results are very discordant. The consequences on the estimates and credibility intervals for sensitivity and specificity as derived by a Bayesian analysis are discussed in the next paragraphs.

2.2 Bayesian analysis of test results

2.2.1 Results

The results of the Ton-ELISA 2.1 were added to the latent class model (Ton-PCR, Ton-BE and Swab-PCR) that was used for the characterization of the *S. suis* 2 EF^+ PCR on tonsil-swabs of live sows as described in chapter 2. In the Bayesian analysis in chapter 3, the corresponding estimates of sensitivity (0.58, Credibility Interval (CI) 95%: <0.49; 0.68>) and specificity (0.70, CI95%: <0.64; 0.78>) for the ELISA -2.1 were both relatively low. The estimates and credibility intervals for herd prevalences as well as the characteristics of the other three tests did not differ much from the outcomes of the original analysis as described in chapter 2 (2^{nd} and 3^{rd} column table 5). We concluded that the Tonsil-ELISA with a non-differential cut-off of -2.1, performed without problems in the Bayesian model, but did not add any new information either. However, a diagnostic test is most useful if at least one predictive value, either sensitivity or specificity, is close to 1 while the other is still reasonable. Therefore it seemed logical to add the ELISA with a cut-off of 1.0 to the original model, because it was expected to discriminate much better as described in paragraph 2.1.1. Thereafter the results of the newly developed SPR with a cut-off at the 30^{th} and the 50^{th} percentile were successively added to the same latent class model.

The ELISA with a cut-off of 1.0 did not perform well in the Bayesian analysis. The Bayesian algorithm was not capable to identify the most likely EF-positive or EF-negative samples. The very unstable Markov chains constantly produced double-topped posteriors (figure 5). As a consequence, the estimated parameters for prevalence, sensitivity and specificity of all four tests as shown in the 4^{th} column of table 5 cannot be interpreted. These estimates are the medians of very irregular densities. Probably, the very low number of positive samples in the ELISA 1.0 gave rise to the unsatisfactory outcomes of the Bayesian analysis.

Table 5. Prevalences, sensitivities and specificities for Tonsil-PCR, Swab-PCR and Tonsil-BE (original) as well as Tonsil-ELISA or Tonsil-SPR with different cut-offs expressed as median estimates and their 95% Credibility Intervals.

Priors: as described in table 3 of chapter 3, such as fairly uninformative priors for Se's (~N(0, 0.5)) and priors with emphasis on values larger than 0.5 for Sp's (~N(1.22, 0.45)).

Model*	Original	ELISA -2.1	ELISA 1.0 **	SPR30	SPR50 **
Prevalence farm 1	0.31 <0.23-0.39>	0.29 <0.21-0.40 >	*0.91 <0.65-0.99>*	0.94 <0.84-0.99>	*0.48 <0.02-0.99>*
Prevalence farm 2	0.34 <0.20-0.48>	0.33 <0.19-0.53>	*0.84 <0.59-0.97>*	0.96 <0.65-0.98>	*0.51 <0.06-0.96>*
Prevalence farm 3	0.57 <0.42-0.74>	0.56 <0.41-0.77>	*0.48 <0.22-0.69>*	0.39 <0.07-0.60>	*0.49 <0.16-0.73>*
Prevalence farm 4	0.70 <0.53-0.86>	0.69 <0.51-0.88>	*0.46 <0.16-0.77>*	0.45 <0.15-0.72>	*0.61 <0.13-0.89>*
Sensitivity TPCR	0.88 <0.75-0.96>	0.89 <0.68-0.97>	*0.28 <0.05-0.35>*	0.29 <0.22-0.36>	*0.43 <0.07-0.93>*
Sensitivity SPCR	0.63 <0.52-0.74>	0.63 <0.49-0.76>	*0.16 <0.02-0.23>*	0.18 <0.13-0.23>	*0.30 <0.02-0.86>*
Sensitivity TBE	0.65 <0.51-0.76>	0.66 <0.48-0.78>	*0.21 <0.18-0.28>*	0.23 <0.17-0.28>	*0.28 <0.03-0.73>*
Sensitivity TELISA	-	0.58 <0.49-0.68>	*0.06 <0.00-0.10>*	-	-
Sensitivity TSPR	-	-	-	0.18 <0.13-0.23>	*0.62 <0.09-0.72>*
Specificity TPCR	0.94 <0.87-0.99>	0.94 <0.85-0.99>	*0.23 <0.08-0.42>*	0.29 <0.18-0.48>	*0.61 <0.15-0.98>*
Specificity SPCR	0.96 <0.92-0.99>	0.96 <0.91-0.99>	*0.33 <0.08-0.53>*	0.39 <0.18-0.58>	*0.72 <0.15-0.99>*
Specificity TBE	0.97 <0.91-0.99>	0.98 <0.90-0.99>	*0.55 <0.32-0.77>*	0.62 <0.46-0.76>	*0.75 <0.40-0.99>*
Specificity TELISA	-	0.70 <0.64-0.78>	*0.90 <0.74-0.98>*	-	-
Specificity TSPR	-	-	-	0.93 <0.80-0.99>	*0.47 <0.30-0.91>*

* Included in the original model are the results of Tonsil-PCR, Swab-PCR and Tonsil-BE (column 2). This model is extended with Tonsil-ELISA or Tonsil-SPR in column 3-6.

** The medians are estimated from densities with more than one top and consequently cannot be interpreted.

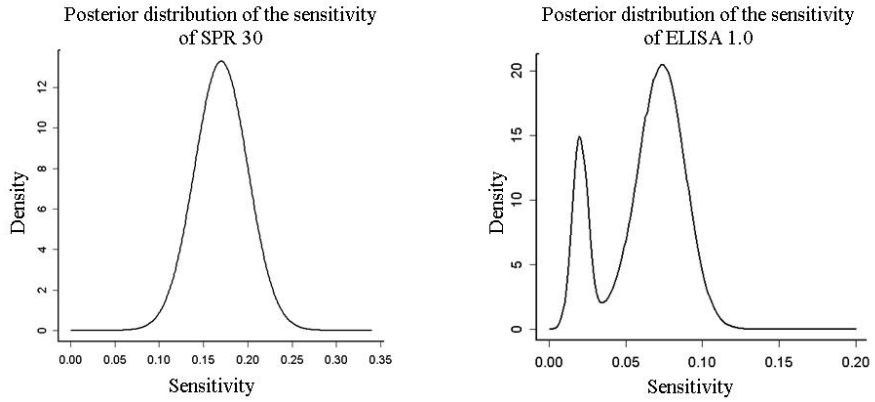

Figure 5. Posterior distributions of the sensitivities of SPR and ELISA in the models with SPR30 and ELISA 1.0 respectively. Examples of stable and unstable densities (in table 5 column 5; row 10 and column 4, row 9 respectively).

The SPR30 results were also added as a fourth test to the former latent class model similar to the ELISA's (5th column in table 5). As expected from the 2x2 comparison, the Bayesian analysis produced a low sensitivity (0.18, CI95%: <0.13-0.23>, fig.2.) and a high specificity (0.93, CI95%: <0.80-0.99>) for the SPR30. The Tonsil-PCR, Swab-PCR and Tonsil-BE had remarkable low estimates for sensitivities, relatively much lower than in the original analysis without the SPR30. This downward bias was probably data driven to compensate for both the low number of positive samples in the SPR30 and its high specificity at this cut-off.

Finally the Bayesian analysis of the original model, supplemented with the SPR50 results did not produce useful outcomes (6th column in table 5). The densities were ambiguous, very much comparable with the ELISA 1.0, even though in the mixture population modeling spreadsheet (Cameron and Eamens, 2000), the SPR50 has a non-discriminating cut-off, whereas the ELISA 1.0 highly discriminates positive and negative samples. Possible reasons for the poor performances of both the EF-tests in the latent class analyses are discussed in the next paragraph.

2.2.2 Remarks and conclusions on the Bayesian analysis

The supplementation of the original latent class model (Ton-PCR, Ton-BE and Swab-PCR) as described in chapter 2 with a test based on the detection of EF in the supernatant of broth of the tonsil samples did not lead to smaller credibility intervals of the parameter estimates from the Bayesian analysis. Moreover, the Bayesian analysis only performed well when the ELISA with a cut-off at -2.1 was added as a fourth test, but when the ELISA with different cut-offs or the SPR was added either the outcomes were non-interpretable or the estimates were very different from the original analysis.

Because the bad performance of our Bayesian analysis could have been provoked by a single incompatible batch or farm, we changed the model by leaving each batch or farm out one after another. None of these model-changes improved the outcomes. Thereafter the priors for specificity of the EF-tests were changed to extremely informative, which restricted the specificity almost to 1. The same was done with the prior for the specificity of Ton-BE. Again, these interventions did not improve the outcomes in a convincing direction. There must be another reason for the untidy posteriors like a violation of the assumptions of Bayesian latent class analysis.

A Bayesian analysis of a latent class model involves at least three assumptions: (1) the tests are valid, (2) the characteristics of the tests are constant across subpopulations and (3) the tests are either conditionally independent given the disease status or dependency can be parameterized undisputedly (Enoe et al., 2000). A plausible explanation for the poor SPR and ELISA performances in our models can be that test sensitivities are very low indeed. This significant lack of sensitivity can be caused by a bacterial aspect. EF is probably not always produced in vitro due to for instance unfavorable storage or cultivation circumstances although the *epf* gene that codes for EF production is present. Samples taken from frozen tissue homogenates produce less capsular antigen as pure bacterial cultures (Serhir et al., 1993). The same is probably true with regard to EF-production, because the excretion of this protein is dependent from the optimal growth of the bacteria. In that case, the validity of the test that is supposed to detect *S. suis* 2 EF^+ carrier status of sows is low. The presence of EF in the supernatant is strictly speaking much more a measure of the in vitro circumstances of the bacterial growth.

In a general sense the different tests that are analyzed in latent class models must perform in a joint diseased and non-diseased population (Toft et al., 2005). If, for instance, diagnostic tests are directed to different aspects of a disease that are not all expressed in the same individuals of a population, at the same time or under the same circumstances, the shared underlying latent classes of positive or negative samples cannot be identified anymore in a Bayesian analysis. In our model the growth of bacteria and the subsequent EF production is possibly only optimal in a small subset of BE positive samples with so far unidentifiable biological properties.

In chapter 3, an analysis of the conditional dependence between tests in the latent class model with swab-PCR, tonsil-PCR and tonsil-BE but without the ELISA or SPR was discussed. Positive conditional dependence could not be ruled out, but was not expected to be high. However, in the Bayesian analysis of the model including ELISA or SPR conditional dependence between tests in true positive samples is much more likely considering the biological background of the tests. Alonzo and Pepe (1999) show that if test results are positively correlated in both diseased and non-diseased samples, both Se and Sp will be estimated too high. The Bayesian algorithm will strictly identify the few samples that are positive in all tests as the only true positive samples. These few samples deviate the other test parameters away from their (presumed) true characteristics. However, after the addition of the SPR30 to our original model, there is no univocal direction of alteration. Our Bayesian analysis resulted in high Sp of the SPR30, but very low specificities and sensitivities

of all other tests. This downward bias of the Se of the other tests could be due to negative conditional dependence, but that is difficult to demonstrate because of the small number of SPR30 positive samples. Interrelated with foregoing, the farm *S. suis* 2 EF^+ prevalences differ substantially from the estimates in the original model as well.

Altogether the variability of the outcomes of the Bayesian analyses suggests that identification of true positive or negative samples has become less straightforward after incorporation of the EF-tests. The biological relationship between the three tests might have given rise to violation of the statistical assumptions that constitute the frame of a Bayesian analysis of latent class models. Verification of statistical dependency between tests in a latent class model is often difficult or even impossible because of a lack of degrees of freedom in the data (Toft et al., 2005). Comparison of models for conditional dependence with the real data and simulated data can at best lead to better insight into the different inter-test correlations. Nevertheless, in our models that include an EF-test, it will be very difficult to define the structure of dependence between the tests due to the low amount of positive results from the EF-tests (Pepe and Janes, 2007).

The main conclusion concerning EF detection by SPR or ELISA is that EF-detection could be used at farm-level if the test cut-off is chosen in a way that optimizes specificity. This would allow for monitoring *S. suis* 2 EF^+ free herds. However, because many samples must be taken to generate sufficient herd-sensitivity, both the costs of sampling and the costs of the laboratory test must be extremely low. From an economic point of view as well as from the diagnostic point of view, the PCR on a tonsil-brush is currently the better choice.

Summarizing, our outcomes of the Bayesian analysis underline that biological understanding about test mechanisms and the relationships between tests is essential in a Bayesian analysis of latent class models. Statistical independency between tests is an important criterion when tests are included in latent class models. However, this statistical prerequisite for Bayesian analysis cannot be readily satisfied by incorporating tests that are statistical independent. In extremis, generation of statistical independence through biological independence can lead to non-interpretable output of the Bayesian statistics, because tests are not measuring the same entity anymore. Of course, tests must at least be valid as a measurement of the same disease in question, but a thorough inspection of the Markov chains and posteriors is indispensable in Bayesian analysis before interpretation of the outcomes. The model can be checked by fitting it to simulated data and different models for conditional dependence. Theoretically, dependency between tests can always be parameterized, but this will lead to wider credibility intervals and thus reduce the advantages of Bayesian analysis.

THE PREVENTION AND CONTROL OF STREPTOCOCCAL DISEASE AND TRANSMISSION

On many farms *S. suis* has an endemic status. Sows carrying *S. suis* strains in their tonsils are to be considered the most important source of infection for their susceptible offspring (Clifton Hadley, 1984; Robertson and Blackmore, 1989). The prevalences in weaned pigs could not be correlated with the incidence of disease at farms (Berthelot Herault et al., 2000; Clifton Hadley et al., 1984a; Clifton Hadley et al., 1984b; van Leengoed et al., 1987). At farms where *S. suis* is endemic, the prevention and control of streptococcal disease is generally based on management measurements that minimize contact between susceptible and infectious animals often combined with the use of farm-specific whole bacterin vaccines. In addition, antimicrobial drugs are applied in order to decrease the incidence and severity of disease.

Many managerial predisposing factors for the occurrence of *S. suis* meningitis in weaned pigs are described in textbooks. In the next paragraph the role of intestinal translocation as a route of infection and the implication for farm management will be discussed. Thereafter the effects of medication on the carriage and transmission of *S. suis* serotypes 2 and 9 will be demonstrated. The use of antimicrobial drugs however generates disturbance of the indigenous flora and facilitates the development of resistance in virulent strains which frustrates therapeutic protocols. Controlling the transmission of pathogenic *S. suis* serotypes by vaccination and managerial interventions alone would be an attractive alternative. Therefore some aspects that determine the success of vaccination protocols will be discussed in the next paragraphs.

1. Managerial interventions to prevent intestinal translocation of *S. suis*.

Managerial measures that predispose for the occurrence of *S. suis* meningitis in weaned pigs include stressful events like weaning, mixing of litters, change of climate and transition to solid food provision (Dee et al., 1993; Dee and Corey, 1993; Villani, 2003). Some of these events mainly promote the transmission of agents between susceptible and infectious pigs, while others mostly influence the animal health status in general including the development of *S. suis* meningitis. Although many of the pathogenic steps in the development of meningitis are still unknown, the generally accepted route of infection is through the tonsils (Gottschalk and Segura, 2000). However, bacterial translocation from the intestine to the blood could also be an important pathway shortly after weaning, considering the changes in defensive mechanisms of the gastrointestinal tract like the degree of stomach acidification, colonization resistance and the mucosal barrier functionality (Baumgart and Dignass, 2002).

We have shown that *S. suis* serotype 2 EF^+ can translocate from the gut to different organs (chapter 5). Transportation was used in our experiment to mimic stressful events for pigs that can occur around weaning at farm level (Dalin et al.,

1993). It has to be considered that in the applied infection model the tonsils and the stomach were bypassed with a canula through which a high amount of bacteria was inoculated directly in the proximal small intestines. Acting so, at least two defensive barriers were overcome: the acid stomach environment and colonization resistance in the small intestines.

The acid stomach content is an important barrier against intestinal pathogens such as *S. suis*. We showed that in a Todd Hewitt broth *S. suis* survives less than 3 hours at a pH of 5 or lower. But at a neutral pH *S. suis* survives over 10 hours (unpublished pilot study). This means that under certain circumstances *S. suis* could survive and pass the stomach. Kamphues (1990) demonstrated that after 5-8 hours of ad libitum feeding, the stomach content was not entirely homogeneous mixed, leading to large differences in acidification from under pH 3 in the fundus and pyloric region up to pH 6.8 in the cardiac region. An accidental high Calcium dose in the food (59 g Ca/kg DM) prevented acidification of the chyme in all regions (pH 5 in all stomach regions). Therefore food that buffers the acidity of the stomach, like protein rich food after weaning, possibly contributes to translocation of *S. suis* further on in the intestines.

Indigenous bacteria translocate continuously across the intact mucosal epithelium at very low rates. These bacteria are effectively killed by the host immune system before they can reach the circulation. Translocation can be promoted by increased bacterial population levels (Berg, 1992). Although in our experiment *S. suis* serotype 2 EF^+ had not colonized the intestines, it did translocate to mesenteric lymph nodes and different organs. Drum (1998) found that an abnormal content of sugar in feed probably caused *S. suis* colonization in the small intestines. This kind of dietary caused dysbacteriosis may well contribute to the importance of the intestinal route of *S. suis* serotype 2 EF^+ infection.

Finally, the intestinal mucosa itself forms an important, but variable barrier against translocation (Berg, 1992). Stressful events like transport have a negative influence on the blood flow in the superior mesenteric artery which leads to ischemia in the gastrointestinal tract (Nabuurs et al., 2001; Pargger et al., 1997; Van der Meulen et al., 2000). Ischemia enhances the catabolism of ATP and the genesis of xanthine oxidase. Upon reperfusion and accompanying reoxygenation, xanthine oxidase catalyses the reduction of O_2 to yield O_2^- and H_2O_2. These oxidants promote adherence of neutrophils to the endothelium, that then mediate microvascular damage (Grisham and Granger, 1989). This microvascular damage causes injury of the mucosal barrier dependent on the duration and level of hypoperfusion. The process is most destructive in cells that metabolize much ATP and O_2 for multiplication (crypt-cells), absorption and digestion (villous cells), which is especially the case after weaning (Niewold et al., 2000; van Beers-Schreurs and Bruininx, 2002).

Summarizing, intestinal translocation is a possible route of *S. suis* infection although many barriers have to be overcome. Several stress and food related circumstances around weaning can synergistically facilitate this. However, pathogenic *S. suis* strains need extreme circumstances in order to pass the stomach, colonize the small intestines and finally translocate injured intestinal mucosa. Probably respiratory *S. suis* infections are much more important in the pathogenesis of meningitis.

Nevertheless, at farms with severe streptococcus related disease problems after weaning, intestinal translocation might play a role as additional route of systemic infection aside the tonsils. Therefore, at such farms prevention must, besides the avoidance of infection, also be emphasized on supporting stomach function, colonization resistance and an adequate blood flow in the superior mesenteric artery to preserve the functionality of the intestinal mucosa. This can be achieved by improving the process of weaning, very similar to the prevention of *E. coli* enterotoxemia such as early habituation to solid food uptake, optimizing climate conditions and minimizing of moving and mixing of piglets (Dee et al., 1993; van Beers-Schreurs and Bruininx, 2002).

2. Antimicrobial drugs

2.1 Effect of amoxicillin on transmission of *S. suis* serotype 2

In the experiment described in chapter 4, we used both medication and vaccination in particular to stop transmission of *S. suis* 2 EF^+ between sows that carried the bacterium and their litters. The experiment focused on *S. suis* 2 EF^+, because this virulent strain was frequently isolated from diseased pigs at Dutch farms among which the two farms that provided the sows. Sows were vaccinated with a whole-cell *S. suis* 2 EF^+ vaccine at six and three weeks before farrowing. Continuous medication with amoxicillin started when the sows were placed in the isolation units around one week before farrowing. Both the sows and the piglets were sampled at the start and during the 26 day lactation period. At the end of the lactation period sows and piglets were euthanized and all tonsils were collected.

In the experimental group we did not detect any *S. suis* 2 EF^+ at the tonsils of the piglets of the seven treated carrier sows. In the control group that was without any sow-treatment, only one out of seven litters became positive (Table 6). Because of the very low incidence of infection in both groups, it was not possible to show any effect of the treatment.

At the end of the lactation period *S. suis* 2 EF^+ was not detected on the tonsils of the seven treated sows anymore in contrast with the control sows. In this experimental design it was not possible to assign the results to either medication or vaccination.

Table 6. Number of sows and piglets with *S. suis* 2 detected in the tonsil homogenates at the end of the lactation period

Group	Sow status*	Sows	Individual piglets	
			Positives	Negatives
Treatment	Free	7	0	65
	Carrier	0	0	0
Control	Free	0	0	0
	Carrier	7	9 (1 litter)	50

*All 14 sows were carriers at the start of the experiment.

2.2 Effect of amoxicillin on transmission of *S. suis* serotype 9

We were interested if *S. suis* serotype 9 (*S. suis* 9) which is frequently isolated from diseased piglets after weaning (Wisselink et al., 2000), behaved comparable to *S. suis* 2 EF^+ during lactation. Therefore we analyzed all available samples (four out of the original seven sows in both experimental groups) on *S. suis* 9 by PCR (Wisselink et al., 2002). All 8 sows were *S. suis* 9 positive at the start of the experiment.

Similar to *S. suis* 2 EF^+, at the end of the experiment *S. suis* 9 was no longer detected on the tonsils of the four treated sows in contrast to the non treated control sows. The effect on the sow carrier status was probably principally an effect of the amoxicillin treatment because the strain was amoxicillin sensitive (NCCLS, 2000), while the vaccine was aimed at *S. suis* 2 EF^+ only. Streptococcal whole bacterin vaccines probably do not provide cross-immunity (Kebede et al., 1990). However, due to the small number of animals, this difference in carriage of *S. suis* 9 between both treatments was not significant, but could still be important (Chi^2: 2,133; p= 0,144 after Yates correction (Abramson, 2004)).

Another effect of amoxicillin treatment was apparent in the number of infected piglets per litter. Although not all sows were carrier of *S. suis* 9 at the end of the experimental period, one or more piglets in each litter became positive in the tonsil as detected by PCR on the homogenate of the whole tonsil. In the litters of the four treated sows 38% of the piglets were positive (15/39), while without treatment of the sows 78% of the piglets became positive (27/32)(Table 7). Accordingly, treatment of sows seemed to be associated with less infected piglets per litter at weaning (taking intra-litter correlation into account: OR: 8.64 (CI95%:<1.24-60.1>); p=0,019).

Table 7. Number of sows and piglets with *S. suis* 9 detected in the tonsil homogenates at the end of the lactation period.

Group	Sow status*	Sows	Individual piglets	
			Positives	Negatives
Treatment	Free	4	15	24
	Carrier	0	-	-
Control	Free	1	6	2
	Carrier	3	21	3

*All eight sows were carriers at the start of the experiment.

An intermediate role of feces in the transmission of *S. suis* can be suspected, because the amoxicillin treatment did not start before arrival at the isolation units. The effect of amoxicillin on the intestinal flora will probably only become apparent after several hours. Thus *S. suis* 9 positive sows can have excreted *S. suis* 9 in the farrowing cages directly after arrival, where later on the piglets can have picked up the still viable bacterium, particularly because *S. suis* can survive about eight days in manure. This emphasizes on good cleaning and disinfection practices at farms with a high incidence of disease due to *S. suis* (Clifton Hadley and Enright, 1984).

In contrast to our results, earlier attempts by Amass et al. (1996b) and Clifton-Hadley et al. (1984a) to eliminate *S. suis* from the tonsils of weaned pigs and slaughter-pigs by medication failed. Although in our experiment (as described in chapter 4) it was very convincing that the sows and piglets were truly free of *S. suis* 2 EF^+ because of the many negative samples, it must be taken into consideration that the whole tonsils were analyzed only few hours after the last amoxicillin treatment. To be more certain that the pigs were free of *S. suis* a longer period for amoxicillin wash-out and subsequent streptococcal growth must be implemented before sampling.

2.3 Adverse effects of antimicrobial drugs

The use of antimicrobial drugs has several benefits in the treatment of streptococcal related diseases. However, antimicrobial resistance is an increasing threat for animal health as well as public health (Harbottle et al., 2006; Hendriksen et al., 2008). In general, the large amounts of drugs used especially in prevention of diseases facilitate the emergence and development of bacterial resistance (Aarestrup et al., 2008). As a consequence, it already has been shown that the use of antibiotics in livestock contributes to the increased prevalence of antibiotic-resistant bacteria that are of significance, not only in swine production but in public health as well (Mathew et al., 2007; Smith et al., 2002).

Another adverse effect of the use of antimicrobial drugs is the disturbance of the existing resident bacterial microflora at the skin, in the nasopharynx and in the intestines. The indigenous bacteria prevent the growth of pathogenic species by competing for nutrition and attachment sites. This barrier effect protects from over-

growth by species that are normally present at low numbers as well as exogenous pathogenic species (Guarner and Malagelada, 2003). In children, the indigenous flora that includes α-haemolytic streptococci, inhibits colonization by i.e. *S. pneumoniae, H. influenza* and *S. aureus*. Ghaffar (2002) showed that this inhibitory balance could be altered by antibiotics. For that reason prevention and control strategies without the use of antimicrobial drugs would be preferable.

3. Vaccination

To overcome the negative side effects of antimicrobial drugs for controlling pathogenic *S. suis* strains, vaccination would be an obvious strategy. The effect of vaccination on transmission is only examined in a few vaccination experiments at farms. Amass et al. (2000) and Torremorell et al. (1998) observed lower prevalence of the homologous strain in piglets after vaccination of the sows. Regrettably we could not draw conclusions on the effect of vaccination in our experiment described in chapter 4, because the vaccination was combined with amoxicilline treatment.

In the design of vaccination protocols many subjects have to be considered such as the serospecificity of vaccines, the immunological response at young age and the possible replacement in the population of vaccine strains by other strains. In the decision-making process, knowledge can be used from *Streptococcus pneumoniae* vaccination campaigns in human pediatric medicine. *S. suis* and *S. pneumoniae* have many features in common, such as capsular proteins, toxins and biochemical reactions in the API 20 Strep system. Because of this, the epidemiological dynamics of both streptococcal species are considered to be similar in many aspects (Brassard et al., 2004; Gottschalk et al., 1991; Gottschalk et al., 1995; Hommez et al., 1986; Segers et al., 1998; Smith et al., 1999).

S. pneumoniae is a common cause of meningitis, sepsis and pneumoniae in both young children and elderly people. The 91 known serotypes of *S. pneumoniae* are not all equally pathogenic. This is most likely due to differences in virulence factors between strains and not the result of the duration of carriage for instance (Brueggemann et al., 2003; Henrichsen, 1995). In the prevention of meningitis caused by *S. pneumoniae* in children, vaccination has become an essential tool because of the worldwide emergence of resistance to penicillin and other antibiotics in pneumococci. Pletz et al. (2008) reviewed several well-documented vaccination *S. pneumoniae* campaigns. A 23-valent-polysaccharide vaccine and a 7-valent-conjugate-vaccine effectively reduced the incidence of meningitis caused by the vaccine-included serotypes respectively in adults and children. In veterinary practice such large-scale vaccination trials are not yet performed. The effect of vaccination not only on the incidence of disease but especially on the prevalence of the vaccine-strain and non-vaccine strains at herd level or within a top-down production chain are of great interest, because this can give insight in the feasibility of eradication programmes.

3.1 Vaccine types: polysaccharide and conjugate vaccines

In public health as well as swine health, capsular polysaccharide vaccines have been widely used in vaccination protocols. The first generation of polysaccharide vaccines was not very useful in young children. The polysaccharides can not induce a B-cell-dependent immune response in young children due to their immature immune system. Progress has been made with the development of conjugate vaccines. Through attaching the polysaccharide to a highly immunogenic protein, the T-helper cell function is stimulated which subsequently activates B-cells to proliferate and produce antibodies (Pletz et al., 2008). In young piglets the same problem in efficacy of polysaccharide vaccines can occur. In pig husbandry however, the sows are vaccinated to provide protection to the piglets through maternal antibodies in the colostrum. This indirect way of (passive) immunization can already be effective against early invaders in young piglets. This means that there is no need for conjugation of capsular polysaccharide to proteins to stimulate T-helper cell function.

3.2 Serotype specificity

Streptococcal whole bacterin vaccines do probably provide protection that is limited to the homologous strain (Kebede et al., 1990). In human health, the streptococcal strains that are most frequently isolated from patients are combined in a 23-valent-polysaccharide vaccine to overcome this strain specificity. Besides that, a 7-valent-conjugate vaccine is formulated for use in children under the age of two, as described in the previous paragraph. Presently in swine health no commercial produced protective vaccine that contains a single or more *S. suis* strains is available in the Netherlands. Autogenous vaccines made of one to five strains isolated from typical ill animals are frequently used at farms with disease caused by *S. suis*. These case-specific formalin treated whole bacterin vaccines resemble in many ways the polysaccharide vaccines in human medicine and are very useful in the prevention of disease within farms. However shortcomings are reported, especially concerning vaccines that contain a *S. suis* 9 strain. Although the reasons of these fallbacks are mostly unknown, they can often be solved by isolating a pathogenic strain again, and then adding this to the vaccine.

3.3 Replacement

Every polysaccharide and conjugate streptococcal vaccine protects in particular against the strains that are added to the vaccine. In the long run diseases caused by non-vaccine serotypes can increase. The niche that arises when vaccine strains disappear in a population is filled by other strains that are possibly pathogenic as well. Replacement of vaccine-serotypes has occurred in several trials of *S. pneumoniae* conjugate vaccines (Lipsitch, 1999; Singleton et al., 2007). This might lead to ‘replacement of disease’ caused by non-vaccine serotypes. Brueggemann, et al. (2003) showed that some non-vaccine pneumococcal serotypes are highly virulent. A prom-

ising alternative type of vaccine on which much research is performed lately, is based on proteins that are associated with disease, but serotype-independent.

The use of serotype specific *S. suis* vaccines could well lead to replacement of the vaccine serotype comparable to *S. pneumoniae* replacement. Protein-subunit vaccines would provide a solution to avoid serotype-dependent replacement. Protein candidates would be suilysin, MRP, EF and fibronectin- and fibrinogen-binding protein (De Greeff et al., 2003; King et al., 2001; Staats et al., 1999). Much work still has to be done to identify a suitable combination of proteins and meet the safety regulations for mass-production (Jones et al., 2007). Anyhow, protein vaccines are expected to be cheap and therefore within reach of veterinary medicine.

3.4 Implications for *S. suis* vaccination protocols at swine farms

At swine multiplier farms vaccination protocols that make use of autogenous whole bacterins can provide an adequate management tool in the prevention of streptococcal disease. In the design of a vaccination protocol attention should be paid to the moment of infection by the strain that causes disease. *S. suis* serotypes might colonize piglets at serotype-specific ages. We detected serotype 9 in all (7/7) litters around 26 days after birth, but only one out of seven litters was colonized by *S. suis* serotype 2. Reams et al. (1993) detected serotype 2 mainly between six and 14 weeks and serotype 1 between three and ten weeks of age. Torremorell et al. (1998) could not show *S. suis* 2 tonsillar colonization before 15 days of age. The choice for maternal vaccination or administration to individual pigs depends on the age of infection by virulent *S. suis* serotypes. Serum titers of maternal derived antibodies (MDA) diminish with a minimum around seven weeks of age (Lapointe et al., 2002). To what extent these MDAs interfere with vaccination is not well documented and will not only be dependent on the vaccine formulation (Wisselink et al., 2001) but also on the capacity of the strain to elicit humoral immunity. This means that if streptococcal disease occurs late after weaning (over +/- the age of five weeks), individual weaner pig vaccination with polysaccharide formulations might be considered instead of sow vaccination.

Regular monitoring and adaptation of the vaccine composition must be performed to prevent the increase of streptococcal disease after replacement of the vaccine serotype in the population.

4. Elimination

A more appealing approach would be the total elimination of virulent strains from farms with streptococcal disease problems. After the elimination of *S. suis*, these farms are not allowed to have any external animal contacts of different *S. suis* status anymore and very strict biosecurity rules have to be applied to avoid the risk of breakdown as described by Clark et al. (1994), Fangman and Tubbs (1997). Depopulation and repopulation with sows free of virulent *S. suis* after thorough cleaning would be an obvious, but very expensive method of *S. suis* elimination. However, considering the results from chapter 4, the elimination of virulent strains could also be reached by a combination of temporary antimicrobial drugs, vaccination and segregation of age groups (or litters) especially after weaning. Especially the *S. suis* carrier sows must be treated during farrowing and lactation to diminish *S. suis* transmission to their offspring. The newborn gilts must be reared strictly separated from older sows. A field experiment in line with chapter 4, can demonstrate the feasibility of such an elimination strategy. During the trial, surveillance of different serotypes can be performed to monitor possible strain replacement and antibiotic resistance profiles.

SUMMERIZED CONCLUSIONS AND RECOMMANDATIONS

The *S. suis* 2 EF^+ PCR on tonsil-swabs of live sows is a well performing practical diagnostic tool. The estimated sensitivity and specificity of the field test are 0.63 and 0.96 respectively. Using toothbrushes instead of swabs will probably increase sensitivity and already proved to be a convenient sampling method in our transmission experiment.

Identifying the extracellular factor protein in supernatants of enriched broths from frozen samples using EF-antibodies is not a very sensitive detection method at individual pig level. Therefore this test method is only applicable in screenings with sufficient large sample sizes leading to adequate herd sensitivity.

Bayesian analysis of latent class models is a very convenient test-evaluation method when a gold standard is absent. However we experienced some complications. This was most probably caused by a violation of the assumptions of Bayesian analysis of latent class models. The validity of tests, the test-characteristics across subpopulations and the independence between test in diseased and non-diseased subjects may be difficult to check. Statisticians must not only analyze the posterior densities and Markov chains thoroughly, but they must also make use of complementary biological knowledge of the various diagnostic test systems. The model can then be verified by fitting it to simulated data and different models for conditional dependence. But with the current methods, it will not always be without any trouble to find the reasons why some models perform badly. Model-checks that are easy to execute are needed to make Bayesian analysis of latent class models better accessible for use in veterinary science.

Prevention and control of streptococcal disease is currently a continuous task in pig farm management, because carriers (sows) will always be present on farms. Probably oronasal *S. suis* infections are most important in the pathogenesis of meningitis. Nevertheless, at farms with severe streptococcus related disease problems after weaning, intestinal translocation might play a role as additional route of systemic infection. At such farms emphasis must be put on early habituation to solid food uptake and avoidance of stressful events to preserve the barrier functions of the intestinal tract.

An intervention at farm level with antimicrobial drugs to control outbreaks of *S. suis* related meningitis must, although often effective, only be temporal, because of the threat for public health of antimicrobial resistance against drugs in livestock, and the disturbance of bacterial balance (colonization resistance) in the individual pigs. This forces to focus research on vaccination and elimination of pathogenic streptococcal strains.

S. suis vaccination protocols are nowadays mainly performed with autogenous farm specific vaccines made of streptococcal strains isolated from diseased animals. A more uniform strategy that can be used on different farms would be preferable. To achieve this, the effect of vaccination should not be strictly limited to the vaccine serotype. The formulation of serotype independent commercial vaccines seems essential in the prevention and control of streptococcal diseases. Therefore, further research on serotype independent virulence markers of *S. suis* is indicated, as stated before in the thesis of de Greeff (2002). Furthermore insight in the moment of infection with pathogenic strains and their behaviour in herds are needed to design vaccination protocols with such serotype independent vaccines.

Strong arguments that support specific elimination strategies are not available. On basis of our results, an intervention using temporally medication as well as vaccination seems a feasible approach. With the use of amoxicilline medication and *S. suis* 2 EF^+ vaccination we interrupted vertical transmission of *S. suis* 2 EF^+ from carrier sows to their litters completely. However, little is known about the possible replacement of vaccination strains at herd level and the effects of antibiotics on the defensive bacterial balance of individual pigs.

The current conventional pig husbandry systems are associated with outbreaks of meningitis caused by *S. suis* comparable to *S. pneumoniae* meningitis outbreaks in healthy young children that visit day-care centres. Both the decline of maternally derived antibodies and a high pressure on other defence systems by stress related events, combined with a high risk of infection due to mixing of infectious and susceptible individuals, favour large outbreaks of disease. More animal friendly farming systems with lower stock densities, less mixing and strictly spatial separation could potentially reduce the risk of outbreaks. Incidental cases of meningitis, however, will remain because of the persistence of carriers of pathogenic *S. suis* strains.

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Summary

Detection and transmission of extracellular factor producing *Streptococcus suis* serotype 2 strains in pigs

INTRODUCTION

Streptococcus suis has been implicated in the etiology of meningitis, arthritis, pneumonia, septicemia, endocarditis, polyserositis and sudden death in pigs. The virulent extracellular factor-positive strains of *S. suis* serotype 2 (*S. suis* 2 EF^+) have frequently been isolated from diseased pigs in many European countries as well as in the USA and Australia. Sows carrying *S. suis* strains in their tonsils are considered the most important source of infection for their susceptible offspring, which can become infected very early in their lives with different *S. suis* serotypes including virulent strains. In addition, *S. suis* infection is a zoönosis that has repeatedly been reported in pig-rearing and pork-consuming countries.

DETECTION OF *STREPTOCOCCUS SUIS*

To be able to perform transmission studies, a well-defined diagnostic method was needed. We estimated the sensitivity and specificity of a polymerase chain reaction assay (PCR) on swabs taken from live sows. This PCR was developed by Wisselink and others (1999) for the detection of EF-positive *S. suis* serotype 2 strains. The outcomes of three tests, the PCR on tonsillar swabs (Swab-PCR), the PCR on the whole tonsils of the same sows removed after euthanasia (Tonsil-PCR) and the bacterial examination of these whole tonsils (Tonsil-BE), were evaluated in a Bayesian analysis using the WinBUGS application formulated by Spiegelhalter and others.

The estimated sensitivity of Swab-PCR was 0.63 (<0.52, 0.74>) and the sensitivity of Tonsil-BE 0.65 (<0.51; 0.76>). The Tonsil-PCR had a higher sensitivity of 0.88 (<0.75; 0.96>). The specificities of all tests were around 0.96. It was concluded that, as expected, the removed whole tonsil sample is the most sensitive specimen to detect carrier sows. However, this method is not usable to select animals alive or monitor *S. suis* carrier statuses during prolonged periods of time. For that purpose swabs are a good alternative, if sampling is repeated. The sensitivity can be further increased by using a slightly more invasive technique like tooth-brushes.

The credibility intervals of the sensitivities of all three tests were larger than 0.2. To obtain smaller intervals, more valid and conditionally independent tests can be incorporated into Bayesian analysis. Accordingly we tested all whole tonsil samples again in an Enzyme-Linked Immuno Sorbent Assay (ELISA) and a Surface Plasmon Resonance technique (SPR). These tests are based on the detection of the Extracellular Factor-protein in the supernatants of *S. suis* 2 EF^+ cultures and were expected to be conditionally independent from the other tests that were based on the presence of DNA of the bacterium (PCR) or growth of the bacterium (BE). The outcomes however were not much better than the Bayesian estimates and credibility intervals of sensitivities and specificities derived from the original model with only three tests.

BAYESIAN ANALYSIS

Bayesian analysis of latent class models is a very convenient test-evaluation method when a gold standard is absent. The basic principle of the Bayesian approach is that every valid test (a test that performs better than chance) correctly classifies a proportion of the true positive and true negative test-samples. Because these proportions usually do not completely overlap for different tests, an iterative procedure can generate sensitivity and specificity from a latent class model composed of the new test results combined with a priori defined probability distributions based on previous studies carried out on the applied tests or on comparable tests. When performing a Bayesian analysis of a latent class model the tests must be independent given the true disease status and the tests must be valid as measure of disease.

We did some work to assess the degree of conditional dependences between the three tests, Swab-PCR, Tonsil-PCR and Tonsil-BE, in the original model. Several different priors for conditional dependence between tests, ranging from fairly uninformative to quite informative, were used to estimate the percentages of change in sensitivity and specificity after adding a new test to the model. The percentages increase in specificity for truly negatives varied between 0.1 % en 0.6 % suggesting low conditional dependence. For true positives there was an indication for moderate conditional dependence particularly between Tonsil-PCR and Tonsil-BE (6.8 %).

When results of the ELISA or SPR at different cut-offs were added to the original model with three tests, at some cut-offs of these continuous EF-tests the Bayesian analyses produced non-interpretable output. Explanations can be that the sensitivities of the ELISA and SPR are very low indeed or that the production of EF is not a good measure for the presence of *S. suis* bacteria at the tonsil. This can lead to high variability in the outcomes of the Bayesian analyses, because identification of true positive or negative samples has become less straightforward after incorporation of an EF-tests to the model.

A thorough inspection of the Markov chains and posterior distributions is indispensable in Bayesian analysis before interpretation of the outcomes. The model can be checked by fitting it to simulated data and different models for conditional dependence. Theoretically, dependency between tests can always be parameterized, but this will lead to wider credibility intervals and thus reduce the advantages of Bayesian analysis. Our experimental outcomes underline once again that understanding about the biology of test mechanisms and the relationships between tests is highly required in a Bayesian analysis of latent class models.

PREVENTION AND CONTROL

Managerial interventions

The generally accepted route of *S. suis* infection is through the tonsils and respiratory tract. However, bacterial translocation from the intestine to the blood and organs could also be an important pathway shortly after weaning, considering the changes in defensive mechanisms of the gastrointestinal tract like the degree of stomach acidification, colonization resistance in the intestines and the mucosal barrier functionality.

To investigate the possibility of *S. suis* 2 EF^+ translocation, 24 weaned pigs were randomly allocated over individual cages to prevent cross infection. The upper respiratory tract was excluded as infection route by means of a canula placed in the proximal jejunum. To induce stress, 18 animals were transported in individual cages during one hour in a small truck over a meandrous road. Thereafter 12 of these pigs and the six pigs that were not transported, were inoculated through the canula with a high amount of *S. suis* 2 EF^+. The other six transported pigs served as controls. After euthanasia, in three animals *S. suis* 2 EF^+ was re-isolated from organs, indicating intestinal bacterial translocation. This was in contrast to four non-transported and four growth-medium inoculated animals where *S. suis* 2 EF^+ was not cultured. This way we have shown that *S. suis* 2 EF^+ can translocate from the gut to different organs although many natural barriers have been surpassed in this experiment.

Pathogenic *S. suis* strains need extreme circumstances in order to pass the stomach, colonize the small intestines and finally translocate injured intestinal mucosa. At farms with severe streptococcus related disease outbreaks after weaning, several stress and food related circumstances around weaning can synergistically have facilitated intestinal translocation. Therefore, at such farms prevention must emphasize on improving the process of weaning, such as early habituation to solid food uptake, optimizing climate conditions and minimizing of moving and mixing of piglets.

Pharmacological intervention & vaccination

We examined the effect of a combination of medication and vaccination of sows on the *S. suis* 2 EF^+ carrier status of the sows and their offspring. From two herds, 14 pregnant *S. suis* 2 EF^+ positive sows were randomly assigned to be treated with amoxicillin combined with a killed *S. suis* 2 EF^+ whole-cell vaccine or to be left untreated as controls. Two weeks before parturition, the sows were housed in individual isolation farrowing rooms, and from then on the sow and later her litter were sampled by using tonsil brushes and tonsil swabs. Approximately 27 days postpartum, the sows and their piglets were euthanized and their tonsils were collected and analyzed by PCR. No *S. suis* 2 EF^+ could be detected in the tonsils of the seven treated sows, whereas the tonsils of all seven untreated sows remained positive. However, only one of the litters of these untreated sows became infected. This showed that the

incidence of vertical transmission of *S. suis* 2 EF^+ in farrowing pens is low. None of the litters of the treated sows became infected.

Similar to *S. suis* 2 EF^+, at the end of the experiment *S. suis* serotype 9 was no longer detected on the tonsils of four treated sows in contrast to non treated control sows (n.s.; p= 0,144). The effect on the sow *S. suis* serotype 9 carrier status was probably principally an effect of the amoxicillin treatment because the *S. suis* serotype 9 strain was amoxicillin sensitive, while the vaccine was aimed at *S. suis* 2 EF^+ only. Streptococcal whole bacterin vaccines are assumed not to provide cross-immunity.

An intervention at farm level with antimicrobial drugs to control outbreaks of *S. suis* related meningitis must only be temporal, because of the threat for public health of antimicrobial resistance against drugs in livestock, and the disturbance of bacterial balance (colonization resistance) in the individual treated pigs. To overcome the negative side effects of antimicrobial drugs for controlling pathogenic *S. suis* strains, vaccination or even elimination would be obvious strategies.

At swine multiplier farms vaccination protocols that make use of autogenous *S. suis* serotype 2 whole bacterins can provide an adequate tool in the prevention of streptococcal disease. However, a more uniform strategy that can be used on different farms independent of the *S. suis* serotype would be preferable. Until then, regular monitoring and adaptation of the vaccine composition must be performed to prevent the increase of streptococcal disease because of replacement of the vaccine serotype in the population by other so far less prevalent strains.

CONCLUDING

The current conventional pig husbandry systems are associated with outbreaks of meningitis caused by *S. suis*. After weaning, the combination of a decline of maternally derived antibodies and a high risk of infection due to mixing of infectious and susceptible individuals, favors large outbreaks of disease. Farming systems with lower stock densities, less mixing and strictly spatial separation of age groups can potentially reduce the risk of outbreaks. Vaccination strategies can support the control of disease outbreaks. Nevertheless, incidental cases of meningitis will remain because of the persistence of carriers of pathogenic *S. suis* strains.

Samenvatting

De detectie en overdracht van extracellulaire factor producerende *Streptococcus suis* serotype 2 stammen in varkens

INTRODUCTIE

Streptococcus suis (*S. suis*) is betrokken bij het ontstaan van meningitis, artritis, pneumonie, septicemie, endocarditis, polyserositis en bij plotselinge sterfte van varkens. De ziekteverwekkende extracellulaire factor-positieve stammen van *S. suis* serotype 2 (*S. suis* 2 EF^+) zijn frequent geïsoleerd uit zieke varkens in vele Europese landen evenals in de Verenigde Staten en Australië. Zeugen die drager zijn van *S. suis* stammen op hun tonsillen worden algemeen beschouwd als de belangrijkste bron van infectie voor hun vatbare toom biggen. Deze biggen kunnen al vroeg in hun leven geïnfecteerd worden met verschillende *S. suis* serotypes waaronder pathogene stammen. *S. suis* infectie is bovendien een zoönose die regelmatig wordt gerapporteerd in landen waar varkens worden gefokt en varkensvlees wordt geconsumeerd.

DETECTIE VAN *STREPTOCOCCUS SUIS*

Om transmissie studies te kunnen verrichten was een goed gedefinieerde diagnostische test vereist. We hebben de sensitiviteit and specificiteit bepaald van een Polymerase Chain Reaction (PCR) op swabs genomen bij levende zeugen. Deze PCR was ontwikkeld door Wisselink e.a. (1999) voor de detectie van EF-positieve *S. suis* 2 EF^+ stammen. De uitkomsten van deze test op tonsil swabs (Swab-PCR) werd samen met de PCR op de hele tonsillen van dezelfde zeugen uitgenomen na euthanasie (Tonsil-PCR) en van het bacterieel onderzoek van deze tonsillen (Tonsil-BE), geëvalueerd in een Bayesiaanse analyse gebruikmakend van het WinBUGS programma geformuleerd door Speigelhalter e.a.

De berekende sensitiviteit van de Swab-PCR was 0.63 (<0.52, 0.74>) en de sensitiviteit van de Tonsil-BE was 0.65 (<0.51; 0.76>). De Tonsil-PCR had een hogere sensitiviteit van 0.88 (<0.75; 0.96>). De specificiteit van alle testen waren rond de 0.96. Geconcludeerd werd dat, zoals verwacht, de uitgenomen hele tonsil het meest gevoelige monster is om *S. suis*-drager zeugen mee op te sporen. Deze procedure is echter niet bruikbaar om levende dieren te selecteren of de *S. suis* infectiestatus gedurende langere tijd te monitoren. Voor dit soort onderzoeken zijn swabs een goed alternatief, vooral als het bemonsteren wordt herhaald. De sensitiviteit kan verder worden verhoogd door een iets invasievere bemonsteringsmethode te gebruiken zoals tandenborstels.

De Bayesiaanse betrouwbaarheidsintervallen van de sensitiviteiten van alle drie de testen waren groter dan 0.2. Om deze intervallen te verkleinen kunnen meer valide en voorwaardelijk onafhankelijke testen toegevoegd worden aan de Bayesiaanse analyse. Dienovereenkomstig hebben we alle tonsil monsters opnieuw geanalyseerd, maar nu met een Enzyme-Linked Immuno Sorbent Assay (ELISA) en een Surface Plasmon Resonance techniek (SPR). Deze testen zijn gebaseerd op de detectie van het Extracellulair Factor-eiwit in de supernatanten van de *S. suis* 2 EF^+ bacterie culturen en daarom zeer waarschijnlijk voorwaardelijk onafhankelijk van de andere testen die gebaseerd zijn op de aanwezigheid van het DNA van de bacterie (PCR) of

groei van de bacterie (BO). De uitkomsten waren echter niet beter dan de hierboven genoemde Bayesiaanse schatters en betrouwbaarheidsintervallen afgeleid van het originele model met slechts drie testen.

BAYESIAANSE ANALYSE

Bayesiaanse analyse van latente klassen modellen is een gemakkelijk toepasbare procedure om testen te evalueren als er geen gouden standaard voorhanden is. Het grondbeginsel van de Bayesiaanse benadering is dat elke geldige test (een test die beter presteert dan op basis van kans alleen) een gedeelte van de werkelijk positieve en negatieve monsters juist classificeert. Omdat voor verschillende testen deze fracties meestal niet volledig overlappen, kan een zich herhalend proces de sensitiviteiten en specificiteiten van meerdere testen genereren uit een latente klassen model. Daarin worden de testresultaten gecombineerd met vooraf gedefinieerde kansverdelingen voor de parameters gebaseerd op voorgaande experimenten die uitgevoerd zijn met de testen in kwestie of vergelijkbare testen. Een voorwaarde voor het uitvoeren van een Bayesiaanse analyse van latente klassen modellen is wel dat de testen onafhankelijk zijn gegeven de werkelijke ziekte status en dat de testen geldig zijn als maatstaf voor de ziekte.

We hebben berekeningen gedaan om de mate van voorwaardelijke afhankelijkheid te bepalen tussen de drie testen, Swab-PCR, Tonsil-PCR en Tonsil-BE, in het originele model. De percentages verandering van sensitiviteit en specificiteit na toevoeging van een test aan het model zijn berekend gebruikmakend van verschillende a priori kansverdelingen voor voorwaardelijke afhankelijkheid tussen testen, variërend van niet informatief tot zeer informatief. De afhankelijkheid tussen testen voor werkelijk negatieve monsters was gering. De percentages toename in specificiteit varieerden tussen 0.1 % en 0.6 %. Voor de werkelijke positieve monsters was er een indicatie voor enige voorwaardelijke afhankelijkheid, voornamelijk tussen Tonsil-PCR en Tonsil-BE (6.8 %).

Wanneer resultaten van ELISA of SPR met verschillende afkapwaarden aan het originele model met drie testen werden toegevoegd, produceerde de Bayesiaanse analyse op enkele afkapwaarden van deze continue EF-testen niet interpreteerbare kansverdelingen. Een verklaring hiervoor kan zijn dat de sensitiviteit van de ELISA en SPR daadwerkelijk heel laag zijn of dat de productie van EF geen goede maat is voor de aanwezigheid van *S. suis* bacteriën op de tonsil. Dit kan leiden tot een grote variabiliteit in de uitkomsten van de Bayesiaanse analyse, omdat de identificatie van de werkelijk positieve of negatieve monsters minder vanzelfsprekend is geworden na toevoeging van een EF-test aan het model.

Een diepgaande inspectie van de Markov-ketens en de kansverdelingen is onontbeerlijk bij een Bayesiaanse analyse voordat de uitkomsten geïnterpreteerd worden. Het model kan worden gecontroleerd door het te vergelijken met gesimuleerde data en verschillende modellen voor voorwaardelijke afhankelijkheid. Theoretisch kan afhankelijkheid tussen testen altijd geparametriseerd worden, maar dat zal leiden tot bredere geloofwaardigheidsintervallen en daarmee worden de voordelen van Bayesiaanse ana-

lyse ingeperkt. Onze analyse-uitkomsten benadrukken eens te meer dat kennis van de biologie van testmechanismen en de verbanden tussen testen vereist is bij een Bayesiaanse analyse van latente klassen modellen.

PREVENTIE EN BEHEERSING

Maatregelen in de bedrijfsvoering

De algemeen erkende *S. suis* infectie route is via de tonsillen en ademhalingswegen. Bacteriële translocatie vanuit het spijsverteringskanaal naar het bloed en organen kan echter van belang zijn als infectieroute, gezien de veranderingen in de beschermingsbarrières van het maagdarmkanaal kort na het spenen zoals de zuurgraad van de maag, de kolonisatie resistentie in de darm en de functionaliteit van de darmmucosa.

Om de mogelijkheid van *S. suis* 2 EF^+ translocatie te onderzoeken werden 24 gespeende biggen willekeurig geplaatst in individuele hokken om onderlinge besmetting te voorkomen. De voorste luchtwegen werden uitgesloten als infectieroute door een canule te gebruiken die in het proximale jejunum was geplaatst. Om stress op te wekken werden 18 dieren gedurende een uur getransporteerd in individuele hokken in een veewagen over een bochtige route. Daarna werden 12 van deze biggen en zes biggen die niet waren getransporteerd, door de canule geïnoculeerd met een grote hoeveelheid *S. suis* 2 EF^+. De andere zes getransporteerde dieren fungeerden als controledieren. Na euthanasie werd uit de organen van drie dieren *S. suis* 2 EF^+ gekweekt, hetgeen duidde op intestinale bacteriële translocatie. Dit was in contrast met de vier niet getransporteerde dieren en vier dieren die alleen het bacteriële groeimedium toegediend hadden gekregen, waaruit geen *S. suis* 2 EF^+ gekweekt werd. Hiermee is aangetoond dat *S. suis* 2 EF^+ kan transloceren van het maagdarmkanaal naar verschillende organen hoewel veel natuurlijke barrières zijn omzeild in dit experiment.

Pathogene *S. suis* stammen hebben extreme omstandigheden nodig om de maag te kunnen passeren, de darmen te koloniseren en tenslotte de (beschadigde) darmwand te penetreren. Op varkensbedrijven met ernstige aan Streptococcen gerelateerde ziekte-uitbraken na het spenen, kunnen diverse stress en voedingsgerelateerde omstandigheden rond het spenen synergistisch de intestinale translocatie vergemakkelijkt hebben. Daarom moet op zulke bedrijven de ziektepreventie gericht worden op verbetering van het speenproces, zoals vroege gewenning aan de opname van vast voedsel, optimale klimaatomstandigheden en het minimaliseren van verplaatsing en mengen van tomen.

Maatregelen met medicijnen en vaccinatie

We onderzochten het effect van een combinatie van medicatie en vaccinatie van zeugen op de *S. suis* 2 EF^+ drager-status van de zeugen en hun tomen biggen. Uit twee koppels werden 14 drachtige *S. suis* 2 EF^+ positieve zeugen willekeurig toegewezen om behandeld te worden met amoxicilline en een afgedood *S. suis* 2 EF^+ vaccin of om onbehandeld te blijven als controledieren. Twee weken voor het werpen zijn de zeu-

gen gehuisvest in individuele kraamhokken en vanaf dat moment werden de zeug en later haar biggen bemonsterd met tandenborstels en swabs. Ongeveer 27 dagen postpartum, werden de zeugen en hun tomen geëuthanaseerd en de tonsillen uitgenomen en geanalyseerd met de PCR. Er werd geen *S. suis* 2 EF^+ gevonden in de tonsillen van de 7 behandelde zeugen, terwijl de tonsillen van de onbehandelde positief bleven. Echter slechts één van de tomen van deze onbehandelde zeugen werd geïnfecteerd. Dit laat zien dat de verticale overdracht van *S. suis* 2 EF^+ in het kraamhok niet vaak voorkomt. Geen enkele big in de tomen van de behandelde zeugen werd geïnfecteerd.

Vergelijkbaar met *S. suis* 2 EF^+ aan het einde van het experiment, kon *S. suis* serotype 9 niet aangetoond worden op de tonsillen van vier behandelde zeugen, in contrast met de onbehandelde controledieren (n.s.; p= 0,144). Het effect op de dragerstatus van de zeugen was waarschijnlijk voornamelijk een effect van de amoxicilline behandeling, want de *S. suis* serotype 9 stam was amoxicilline gevoelig, terwijl het vaccin uitsluitend gericht was tegen *S. suis* 2 EF^+. Dit type vaccines geven zover bekend geen kruisimmuniteit tegen andere serotypes.

Een maatregel op bedrijfsniveau met antibacteriële medicijnen om uitbraken van hersenvliesontsteking ten gevolge van *S. suis* te beheersen moet slechts tijdelijk zijn, vanwege de verstoring van de bacteriële balans (kolonisatie resistentie) in de individuele behandelde dieren en de dreiging voor de volksgezondheid door bacteriële resistentie tegen de gebuikte middelen. Om deze negatieve bijwerkingen te overwinnen zijn vaccinatie of zelfs eliminatie van pathogene *S. suis* stammen meer voor de hand liggende strategieën.

Op varkensvermeerderingsbedrijven kunnen vaccinatie protocollen die gebruik maken van autogene vaccins van afgedode *S. suis* serotype 2 stammen een afdoende maatregel zijn bij het voorkomen van ziekte-uitbraken door *S. suis*. Maar, een meer uniforme strategie die onafhankelijk van het *S. suis* serotype kan worden gebruikt op meerdere varkensbedrijven zou de voorkeur hebben. Totdat deze er is, moet het *S. suis* dragerschap regelmatig gecontroleerd worden en eventueel de vaccinsamenstelling worden aangepast. Dit is om te voorkomen dat ziekte weer toeneemt door andere tot dan toe minder voorkomende *S. suis* stammen, die in de populatie de plaats innemen van de vaccinstammen.

AFSLUITEND

In de huidige conventionele varkenshouderijsystemen komen uitbraken van hersenvliesontsteking ten gevolge van *S. suis* vaak voor. Na het spenen is de combinatie van afnemende maternale immuniteit en een hoog risico van infectie door het mengen van tomen met infectieuze en gevoelige dieren gunstig voor grote ziekte-uitbraken. Houderijsystemen met meer ruimte per dier, minder mengen en strikte ruimtelijke scheiding van leeftijdsgroepen kunnen in potentie het risico van uitbraken verlagen. Vaccinatiestrategieën kunnen de ziektepreventie ondersteunen. Incidentele gevallen van hersenvliesontsteking zullen niettemin blijven bestaan vanwege de persistentie van dragers van pathogene *S. suis* stammen op de bedrijven.

Dankwoord

DANKWOORD

Na jarenlang bijna dagelijks op één of andere manier, meer of minder intensief met het uitwerken van dit promotieonderzoek bezig geweest te zijn, is het af! Dank aan de velen die me geholpen hebben met de arbeidsintensieve experimenten en hersenkrakende analyses.

Irene, Suzanne en Okke, jullie zijn echt geweldig lief geweest. Alle ruimte om te schrijven heb ik gekregen inclusief de positieve aanmoedigingen. Irene, je hebt me ook wel eens van mijn werkkamer weggesleurd. En je had gelijk, de effectiviteit van schrijven daalde na lange werkdagen volgens een vreemde onnatuurlijke curve. Daarom is dit laatste hoofdstuk 's ochtends geschreven. Jullie hebben me formidabel geholpen door het hele proces heen, wetende dat ik eenmaal aan het proefschrift begonnen, niet tevreden zou zijn met afgeraffeld broddelwerk.

Jos, je hebt me vanaf de eerste ideeën bijgestaan. De droom om streptococcen problemen op varkensbedrijven de wereld uit te helpen, heeft tot veel onderzoeksplannen geleid. Je commentaren waren kritisch, maar doordacht. Je zorgde altijd voor verdieping en nieuwe inzichten. Gemakzucht was je daarbij vreemd. Elke alinea heb je binnen zeer korte tijd doorvorst en geretourneerd voorzien van zinnige vragen. Je hebt me daarmee behoed voor zeperds. Dank voor je geweldige inzet en geduld. Arjan, lastige knopen heb je helpen doorhakken. Soms hielp het om een flink eind hard te lopen om een oplossing in de voortgang van onderzoek te bedenken. Jij hebt de vaart er altijd in gehouden met soms pittige deadlines. Je heldere analyses van complexe data waren om jaloers op te zijn. Dank voor je gestructureerde en vriendschappelijke benadering van een lastig onderzoeksproject. Mirjam, realistische positief heb je elk wetenschappelijk, logistiek of 'domweg' mentaal probleem helpen tackelen. Zonder opportunistisch te zijn zag je altijd mogelijkheden voor verbetering. Bij jou zijn de (thee)glazen gedurende de hele periode meer dan half vol geweest. Het was prettig bij je binnenlopen met nieuwe resultaten, maar ook als er geen prettig nieuws was. Dank voor de uitzonderlijke coaching.

Henk, *Streptococcus suis* man van het eerste uur. In het verlengde van jouw werk heb ik mogen doorwerken. Je hebt me daarin alles behalve laten zwemmen. Je betrokkenheid was een geweldige ondersteuning. Mijn exemplaar van jouw proefschrift ligt uit elkaar door het vele lezen. Uiteindelijk heb je me tot aan mijn laatste experiment met de SPR techniek bijgestaan. Heel veel dank voor je niet aflatende meedenken en meehelpen. Hilde, van het begin af aan was je betrokken bij het experiment waarmee we de *Streptococcus suis* PCR in het veld hebben gekarakteriseerd. Je eigen onderzoek op dit gebied is van wereldfaam en met je kennis heb je me heel wat tips gegeven en ook woeste ideeën snel naar het rijk der fabelen kunnen verwijzen. Jammer dat de afstand tussen onze instituten het op den duur lastiger maakte regelmatige discussies te voeren. Theo, Jan en Norbert, de wetenschappelijk uitgebreid doordachte plannen vroegen om ingenieuze technische oplossingen. Soms was het net een knutselclub, maar uiteindelijk hebben we met een schijnbaar simpel slangetje de Streptococcen daar gekregen waar we wilden. Norbert, jij hebt de aangerichte schade vakkundig beoordeeld en de Streptococcen getracht weer uit de big-

gen te halen, zodat we daarna op het lab, wederom samen met Henk, ook weer wat uurtjes zoet waren. Dank voor deze leerzame prettige tijd.

Bas Engel, je hebt echt reusachtig geholpen met de opzet en analyse van het testvalidatie onderzoek. Het begon met het WinBUGS clubje op het ID-Lelystad waar je nuchtere kijk op Bayesiaanse statistiek me over de streep trok om deze technieken te gaan gebruiken. Dat niet alles van een leien dakje liep heeft vele uurtjes denkwerk gekost, maar ook een artikel opgeleverd. En ook daarna hebben we nog heel wat leuke gesprekken gehad over de grenzen aan Bayesiaanse statistiek.

Hans, ex-kamergenoot, enorme mentale en fysieke ondersteuner, collega maar nog veel meer, je bent er de hele periode geweest en hebt alle fasen meegemaakt. Je hebt me letterlijk met raad en daad bijgestaan. Dank voor je reken- en puzzelwerk en het maken van de grafieken. Dank voor je gezonde kijk op zaken waarmee menig scherp kantje ineens gereduceerd werd tot een simpel hobbeltje dat eenvoudig glad te strijken viel. Ik hoop nog lang met je te kunnen samenwerken.

Jos van Putten, je opmerking "misschien staat het gen wel uit", lijkt zo simpel maar is zo aannemelijk gezien de resultaten die we kregen. Leo, jouw ervaringen met *Streptococcus suis* hebben tot vele mogelijke onderzoeksideeën geleid. Het was leuk en leerzaam om met jou hierover te filosoferen. Hetty, wat een tegenvaller hadden we met het indienen van zo'n mooi project. Jij had alles klaarstaan en het kon niet meer stuk. Best wel leerzaam achteraf, maar toch. Aldert, je vindingrijkheid heeft een nieuwe test opgeleverd. Helaas is het proces nog erg bewerkelijk, maar de test heeft zondermeer bijgedragen tot inzicht in het evalueren van testmethoden. Dank voor je inspirerende bijdrage. Len, Rob, Gertie en Betty dank voor het begeleiden van dit pionierswerk.

Collega's bij de afdeling varkensgezondheidszorg, Arie, Bas, Gerdien, Jacomijn, Janneke, Manon, Marieke, Marion, Petra, Susanne, Suzanne, Tijs en Wikke heel erg bedankt. Jullie hebben stilletjes heel wat extra werk gedaan omdat die promovendus zo nodig een onderzoeksplan wilde indienen of uitvoeren. Jullie zijn zeldzame teamgenoten om mee te werken. Jacomijn, je energie heeft de vaart in het experiment in de infectiestal gehouden. Voeren, bemonsteren, kweken, uitpipetteren, stickeren en opbergen, met jou erbij was het nooit saai. Dank voor je tomeloze inzet en gezellige uurtjes. Kamergenoten: Wikke, Lenny, Hans, en nu Maarten, jullie weten waar een promovendus tegenaan loopt. Bedankt voor de schertsende opmerkingen, bedankt voor de humor, bedankt voor het delen van de ups en downs.

Bij het uitvoeren van de experimenten, de noeste arbeid, ben ik grandioos geholpen. Dank aan collega's Onno Schreurs, Piet Dirven en Roel Sneepers voor het vinden van bedrijven die mee wilden doen. Zonder jullie en de onvoorwaardelijke positieve medewerking van de veehouders Arné van den Broek, Pieter de Vet, Geert Houben en Coen Wijgergangs hadden we de huidige kennis niet kunnen vergaren. De zeugen van 250 kg werden vervolgens netjes klaargezet zodat ik de keelmonsters kon nemen. Dank voor dit bloed- zweet- en tranenwerk en ook voor het hokken bouwen, dieren voeren en bemonsteren in de andere experimenten Arie, Dick, Bart, Bert, Nico, Nico, Staas, Paul, Wim, Sikko, Jan, Paulco, Teus, Irene, Piet, Freek, Gerrit, Hans, Frans, Nordine, Jan en Jan. Met jullie ondersteuning is het uitvoeren van de experimenten een erg leuke tijd geweest. Met de monsters is er daarna met

hulp van Ali en Peter hard gewerkt in het laboratorium door studenten die inmiddels collega's zijn: Margrit, Bas, Linda, Kim, Jolanda, Ard, Marcel, Jobke, Rik, Jan, Cindy, Chantal, bedankt.

Pa, jouw belangstelling en doorzettingsvermogen heeft zijn uitwerking niet gemist. Hoewel je de verdediging niet meer mee kan maken, weet ik hoe trots je bent. Ik zal je missen op de eerste rij. Lieve ma, eindelijk kan je het 'boekje' lezen. Je hoeft niet meer te vragen wanneer ik klaar ben met het schrijven. Het is klaar en dat gaan we vieren! En, Henk, Adje en Erica, geweldig bedankt voor de altijd openstaande deur en de warmhartige belangstelling.

Ten slotte, gezinnetje, het behalen van een deadline betekende voor jullie soms de verlossing van een humeurige huisgenoot met een kort lontje. Gelukkig hebben we ook veel tijd gehad voor prachtige vakanties en vele uurtjes langs het hockeyveld. Irene, Suzanne en Okke, jullie zijn super! Er is niets leuker dan met jullie dollen en gekkigheid uithalen. Dus, let the fun commence!

Curriculum Vitae

CURRICULUM VITAE

Bas Swildens werd geboren op 10 februari 1960 in Utrecht. Na het behalen van het VWO-diploma aan de Rijksscholengemeenschap in Amersfoort werd hij vier keer uitgeloot voor de studie Diergeneeskunde. In deze periode heeft hij naast de propedeuse Nederlandse Taal en Letterkunde alle diploma's als professioneel hockeytrainer behaald. Begin juni 1994 is hij afgestudeerd in de richting landbouwhuisdieren en dezelfde zomer aan het werk gegaan in de buitenpraktijk van de Faculteit. Aan het einde van het jaar is hij aangesteld bij de vakgroep Bedrijfsdiergeneeskunde en Voortplanting. Naast het geven van onderwijs heeft hij regelmatig assistentie verleend bij onderzoeken aan varkens, om in 2001 onder de begeleiding van prof. dr. Verheijden zelf promotieonderzoek te starten. Na zijn promotie blijft hij werkzaam bij het departement Gezondheidszorg Landbouwhuisdieren van de Faculteit Diergeneeskunde te Utrecht.