

**DUTCH SOCIETY FOR VETERINARY
EPIDEMIOLOGY AND ECONOMICS
*VEEC***



Universiteit Utrecht



**EPIDEMIOLOGY OF ANTIBACTERIAL
RESISTANCE**

BOOK OF ABSTRACTS

**UTRECHT, THE NETHERLANDS
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**DUTCH SOCIETY FOR VETERINARY
EPIDEMIOLOGY AND ECONOMICS**
VEEC

EPIDEMIOLOGY OF ANTIBACTERIAL RESISTANCE

Book of abstracts 24th Annual Meeting held on 22 November 2011
Faculty of Veterinary Medicine, Utrecht University

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PREFACE

Thank you for attending the 24th annual meeting of the Dutch Society for Veterinary Epidemiology and Economics (VEEC), organized by the Faculty of Veterinary Medicine of Utrecht University.

This year's topic of the morning session is "Epidemiology of Antibacterial Resistance". Antibacterial resistance is one of the big challenges facing Veterinary Medicine at current. More and more, antibiotic use has become a management measure to prevent disease in production animals instead of a therapeutic measure. As a consequence, we have seen a strong rise of antibacterial resistance as demonstrated by the emergence of MRSA and ESBL. Moreover, resistant strains circulating among animals have been associated with those of humans. Medical, societal and political concern has resulted in a strong pressure to reduce routine antibiotic use in production animals in order to halt antibiotic resistance. However, is the solution to the problem as straightforward that we only need to reduce antibiotic use in farm animals and that the problem will disappear? Many gaps in our knowledge still exist related to the association between antibacterial resistance in humans and in animals and the epidemiology of antibacterial resistance in animal populations. So I am really proud to have scientists working at the front line of this epidemiological arena here to help us answering these questions.

In the afternoon program, which has a variety of interesting subjects, Dutch scientists will present their work in short presentations.

Next year, our society, together with our Flemish sister society, will host the Symposium of the International Society of Veterinary Epidemiology and Economics (ISVEE) in Maastricht from 20-24 August. We hope and expect that many of you will attend this congress, because it is an excellent opportunity to meet veterinary epidemiologists and economists from all over the world and learn about their latest scientific findings. The call for abstracts has been opened already. Please keep your eyes on www.ISVEE13.org to keep track on the further developments. Moreover, during ISVEE on Wednesday August 22 we will celebrate our 5th lustrum in Maastricht and I hope to meet you all there.

For now I wish you an inspiring meeting.

Arjan Stegeman
President VEEC

24^e studiedag van de VEEC – 22 november 2011

Epidemiologie van antibacteriële resistentie - Epidemiology of antibacterial resistance
Faculteit Diergeneeskunde, Collegezaal Departement Gezondheidszorg Paard, Yalelaan 112, Utrecht

- 9.30 Opening
- 9.35 Association between antibiotic resistance in hospitals and animals
- Prof. Dr. Marc Bonten (Universitair Medisch Centrum Utrecht)
- 10.20 Modelling antibiotic resistance in farm animal populations
- Dr. Christina Lanzas (University of Tennessee)
- 11.05 Break
- 11.30 Epidemiology of MRSA in pigs
- Dr. Els Broens (Klinische Infectiologie, Faculteit Diergeneeskunde)
- 12.00 Epidemiology of MRSA in veal calves
- Dr. Haitske Graveland (IRAS, Universiteit Utrecht)
- 12.30 ESBL-producing bacteria in broilers: From model to experiment and back again
- Dr. Egil Fischer (ECD, CVI van Wageningen UR)
- 13.00 Lunch
- 14.00 Comparison of ESBL contamination in organic and conventional retail chicken
- Dr. Maurine A Leverstein – van Hall (Universitair Medisch Centrum Utrecht)
- 14.20 MRSA in pig farms: a prospective cohort study
- Drs. Brigitte van Cleef (Epidemiologie en Surveillance unit, RIVM)
- 14.40 Prognosis in canine idiopathic immune-mediated haemolytic anaemia
- Dr. Christine Piek (Departement Geneeskunde van Gezelschapsdieren, Faculteit Diergeneeskunde)
- 15.00 Seroprevalence and risk factors for *Toxoplasma gondii* infection in domestic cats in The Netherlands
- Dr. Marieke Opsteegh (Centrum voor Infectieziektenbestrijding, RIVM)
- 15.20 Break
- 15.50 Cost analysis of various Low Pathogenic Avian Influenza surveillance systems in the Dutch layer sector
- Niels Rutten (Bedrijfseconomie, Wageningen UR)
- 16.10 A Sero-surveillance programme for early detection of Low Pathogenic Avian Influenza outbreaks in layer chickens
- Jose Luis Gonzales (Utrecht University and CVI of Wageningen UR)
- 16.30 Estimating test characteristics of somatic cell count for detection of *Staph. aureus*-infected dairy goats using latent class analysis
– Gerrit Koop (Departement Gezondheidszorg Landbouwhuisdieren, Faculteit Diergeneeskunde)
- 16.50 VEEC housekeeping meeting

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Modeling antimicrobial resistance in farm animal populations

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Introduction

Antimicrobial drug resistance is an ever-increasing threat for human and veterinary medicine. Medicine has confronted the recurrent evolution of resistance to virtually all available antimicrobial drugs since they first started to be widely applied in the 1950's. Resistance is evident wherever antimicrobial drugs are used, on farms and in communities and hospitals. We often conceptualize the problem of antimicrobial resistance as three different processes: emergence, dissemination, and persistence. The rate of emergence of antimicrobial resistant bacteria is determined by the rates of *de novo* mutation and acquisition of novel resistant determinants by horizontal gene transfer (Boerlin and Reid-Smith 2008). Dissemination of resistance takes place at different organizational levels (e.g. bacteria and host populations), as well as across temporal and spatial scales. Transmission of successful resistant clones among hosts, movement of animals, antimicrobial selection pressure, and microbial interactions are few of the underlying mechanisms behind antimicrobial resistance dissemination. Persistence of antimicrobial resistance in the absence of antimicrobial pressure is dependent on the associated fitness cost, compensatory evolution to overcome the fitness cost, and the genetic co-selection among other factors (Andersson and Hughes 2010). The mechanisms behind these general three processes are complex and interactive. Mathematical modeling is an important tool to understand the mechanisms underlying antimicrobial resistance, guide the surveillance and design of control strategies against antimicrobial resistance. In humans, mathematical models have played central role in understanding and controlling hospital- and community-acquired infections with resistant bacteria (Temime et al. 2008). Surprisingly, there has been very limited use of mathematical models to understand antimicrobial resistance in farm animals. In this presentation, we describe the use of mathematical models to address the dissemination and persistence of antimicrobial resistance in farm animal populations. We present an overview of the available modeling frameworks, and we address the main model characteristics and assumptions to consider when building models for antimicrobial resistance. Our main examples are drawn from the modeling of antimicrobial resistance in zoonotic enteric pathogens, such as multidrug-resistant (MDR) *Salmonella*.

Modeling frameworks for antimicrobial resistance

The two most common frameworks for modeling antimicrobial resistance are population genetics models (Levin et al. 1997, Johnsen et al. 2011) and population biology models (Lanzas et al. 2011). Population genetics models describe how variants of a gene (alleles) change in frequency overtime, and have been mostly applied to understand the emergence and long-term persistence of resistance. Population biology models describe explicitly the transmission of resistance either at the bacteria level or the host level (i.e., epidemiological models). Table 1 presents an overview of the use of population biology models to address antimicrobial resistance. In this presentation, our main focus is population biology models.

Table 1. Mathematical models of antimicrobial resistance

Organizational Level	Example of research questions	Model elements	Most common mathematical framework
Within-host	Emergence and dissemination of resistance during treatment	Population dynamics of sensitive and resistant bacteria Pharmacokinetic and pharmacodynamic models to characterize selective pressure	Ordinary differential equations (Mutation as random event)
Between-host	Transmission of resistant strains Control strategies	Epidemiological models	Ordinary differential equations Difference equations Continuous or discrete-time Markov chains
Between populations of hosts	Spatial spread across populations	Metapopulation models Animal movement Environmental transmission	Stochastic metapopulations Network, individual-based models

Modeling antimicrobial resistance dissemination

Many drug-resistant microorganisms disseminate by a combination of clonal spread and antimicrobial selective pressure. Epidemiological models have been the most widely approach used in modeling the spread of resistant strains among host populations. Epidemiological models, also called susceptible-infectious-recovered (SIR) type models classify the host population according to its epidemiological status. Susceptible animals are those not infected, but which may become infected later. Infectious animals are infected animals that shed the pathogen, and therefore can infect other animals. Recovered animals have immunity against the pathogen. The choice of which transition states and the mathematical framework to use (e.g. differential equations or continuous Markov chain models) in a model depends on the host-pathogen being modeled and the purpose of the model. An important application of the epidemiological models is to estimate the basic reproduction number (R_0) (Heffernan et al. 2005). The basic reproduction number is the expected number of secondary cases produced by a typical infected individual during its infectious period in a completely susceptible population (Anderson and May 1992). The basic reproduction number is a threshold quantity because if R_0 is greater than one, on average one case leads to more than one secondary case, and therefore, the number of cases will grow in the population, and if R_0 is less than one, on average one case leads to less than one secondary case, and therefore, the infection will die out in the population. The basic reproduction number is also interpreted as the epidemiological fitness of a strain (Luciani et al. 2009). The acquisition of antimicrobial resistance by the bacteria is often assumed to cause a decrease in the strain fitness that may cause a reduction either in the transmissibility and/or the duration of infection, and therefore a reduction on its R_0 in the absence of antimicrobial selective pressure. However, some resistant clones can be highly transmissible due to reasons unrelated to the carriage of resistance (Davis et al. 2002). Overall there is a scarcity of data regarding the R_0 of resistant strains of important zoonotic pathogens. The basic reproduction number has been estimated for some drug resistant strains in field conditions, for example, the R_0 for a multidrug-resistant (MDR) *Salmonella* Newport in a calf-raising operation was estimated to be 2.4 (Lanzas et al. 2008).

In order to understand the interplay between clonal dissemination and antimicrobial selection pressure, we need to expand our epidemiological models to consider the effects of the interaction between sensitive and resistant strains, and the selection pressure of antimicrobial drugs. Mathematical models describing interaction between drug resistance and sensitive strain dynamics can consider different interacting mechanisms (in the presence and

absence of antimicrobial use). Figure 1 presents the structure of three simple models with different assumptions regarding the interaction between resistant and sensitive strains.

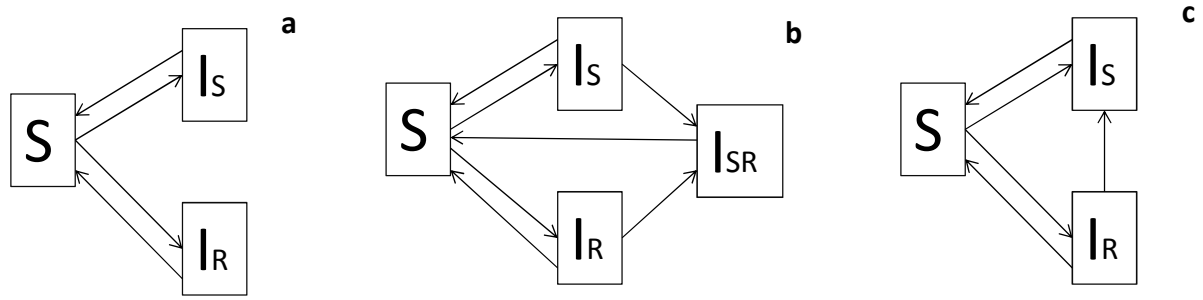


Figure 1. Model flow charts for two strain models (S: susceptible, I_S: sensitive strain, I_R: resistant strain)

In the simplest case (Fig.1, a), the host may be infected at a given time with one strain or another, but not both. This simple model generates competitive exclusion (only the strain with the greatest fit remains). In the second case (Fig.1,b), sensitive and resistant strains can co-infect hosts. The proportion of resistant and sensitive strains in the co-infected individuals depends on the level of competition between the strains within the host. In the third case (Fig.1,c), super-infection takes places. The biological cost of the resistance may allow sensitive strains to displace resistant strains. Individuals can convert directly from resistant to sensitive without clearing the infection. Antimicrobial use can modify the interaction between sensitive and resistant-strains during treatment at the host population level in several ways:

- a) Under treatment, the recovery of hosts that are infected with the sensitive-strain is assumed to be faster than the recovery of resistant strain infected hosts.
- b) Transmission from the infectious individuals carrying the sensitive strain may be less efficient than transmission from individuals carrying resistant strains.
- c) Susceptible individuals can be protected from infection by sensitive strains.
- d) Under antimicrobial treatment, resistant strain can infect individuals carrying sensitive strains without previous clearance (i.e., super-infection with resistant strains).
- e) Novel resistance may appear through mutation or horizontal transfer. Individuals infected with the sensitive strain can change their status to ‘infected with resistant strain’.

Abatih et al. (2009) considered several of these mechanisms to evaluate the effect of antimicrobial usage on the transmission of antimicrobial resistant bacteria among pigs. They concluded that control measures that reduce the transmission rate or increase the spontaneous clear-out rate for resistant bacteria would reduce the proportion of pigs with drug-resistant bacteria prior to transport to slaughter (Abatih et al. 2009). What mechanisms should be included in the models depends on the pathogen-antimicrobial drug combination, antimicrobial drug use and host population. Experimental transmission studies comparing resistant and sensitive strains would be of great value to assess the impact of resistance on transmission, and assist modeling assumptions regarding the interaction of sensitive and resistant strains. In the absence of data on strain competition at the epidemiological level, understanding the within-host competition between strains can provide some guidance. Mathematical models can specifically address the emergence and dissemination of antimicrobial resistance at the bacteria level during the use of antimicrobial treatments. Models that combine pharmacokinetics and pharmacodynamics with bacteria population dynamics can predict the effect of antimicrobial selection pressure on antimicrobial resistance dissemination (Lipsitch and Levin 1997). Through bacteria growth, clonal spread of resistant bacteria takes place. Horizontal gene transfer can be modeled as a contagious process (Lili et al. 2007). The term that describes how sensitive bacteria acquire the plasmid by contact with resistant bacteria is similar to the transmission term use for the *SIR*

models; $\beta \frac{N_s N_r}{N}$, where β is the conjugate rate and, N_s, N_r , and N are, the sensitive, resistant

and total bacteria populations, respectively. Most antimicrobial resistance mechanisms are associated with a fitness cost (α) that is typically observed as a reduced bacterial growth rate (r), and can be included in the model

by assuming that the growth of the resistant bacteria is proportional to the growth of the sensitive bacteria times one minus the cost of the fitness ($r(1-\alpha)N_r$). As an example, we evaluated the effect of exposure to cephalosporins (intramuscular administration of ceftiofur following label recommendations, 2.2 mg/kg/day, for five days) on the gastrointestinal *Escherichia coli* population (as representative of gut bacteria) in cattle. The model indicated that the presence of residual levels of ceftiofur in the gastrointestinal tract favored the selection and dissemination of ceftiofur-resistant bacteria during treatment. Following the last dose of ceftiofur on day 5, the model predicted an increase in resistant bacteria during treatment, but sensitive bacteria reached pre-treatment levels by day 7 after the beginning of the treatment, suggesting a potential for super-infection with sensitive strains after treatment. Within host models can be expanded at the population level, by considering hosts and other environments as habitat patches for bacteria. This concept results in metapopulation models in which the movement of bacteria and growth within the different habitats is considered and different selective pressures on bacteria can be evaluated (Ayscue et al. 2009). Within-host models can also be integrated with epidemiological model to create models that address explicitly the population dynamics of the pathogen at the two scales (Chen et al., in preparation).

On modeling persistence of antimicrobial resistance strains in herds

Despite R_0 greater than 1, pathogens can go extinct in the herd, with only a few infected animals as a final outbreak size due to stochasticity in transmission. Addressing the persistence of resistant strains in a herd requires the use of stochastic models. After the introduction of the pathogen in a herd, the persistence of the pathogen in the facilities is limited, and often positively correlated with herd size (Lanzas et al. 2010b). Empirically herd size and hospital size in humans have been associated with the level of resistance (Bhavnani et al. 2003). Time to extinction is largely affected by herd size. Imports of infectious individuals can further contribute to the persistence of successful resistant clones. For example, in a longitudinal study on the introduction of MDR *Salmonella* strains in commercial dairy farms in Washington state, the average rate of new MDR *Salmonella* strain introduction was 0.9 per herd-year (Adhikari et al. 2009a). Understanding the patterns of inter-herd transmission is necessary to assess the persistence of multidrug strains in the overall population (metapopulation). For dairy farms, this inter-herd transmission can be driven by the interaction among herds and off-calf raising facilities. As dairy farms become larger, the use of off-site calf raising is becoming increasingly common (Wolf 2003). About 1 in 10 dairy operations raised some dairy heifers off-site (USDA 2007). Off-site calf raising has been associated with the dissemination of multi-drug resistance strains of *Salmonella* (Hegde et al. 2005, Adhikari et al. 2009b). A stochastic metapopulation model that includes a calf-raising operation and the herds sending the calves to the facility indicates that the inter-herd movement of infected animals and the commingling at the calf-raising facility can considerably increase the persistence of MDR *Salmonella* in the overall population (Lanzas, unpublished).

Long-term persistence of antimicrobial resistance is often predicted by population genetics models. The fitting of surveillance data on glycopeptides-resistant *Enterococcus faecium* to a population genetic model indicated that biological cost of the resistance decreased overtime (Johnsen et al. 2011). Even when the biological cost of antimicrobial resistance is initially high, the resistance determinants may persist for decades after a complete cessation of antimicrobial consumption in farm animals due to compensatory evolution. This suggests the need to apply intervention strategies beyond antimicrobial stewardship.

Conclusions

Mathematical modeling provides a framework to integrate information regarding the transmission and control of antimicrobial resistance. Model building of models of antimicrobial resistance dissemination requires addressing specifically whether we consider strain competition, and how antimicrobial drug use modifies that interaction. Linking mathematical models with data is necessary to understand what assumptions regarding antimicrobial resistance transmission hold true and under what conditions model predictions are valid (Lanzas et al. 2010a).

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Epidemiology of MRSA in pigs

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In 2004, an association between human carriage of methicillin resistant *Staphylococcus aureus* (MRSA) and contact with pigs was found. To assess the implications of this finding for veterinary and public health more insight into the prevalence, risk factors and transmission dynamics of this so-called livestock-associated (LA-)MRSA was needed. Therefore, field and experimental studies were conducted in pig and human populations of which the results are presented in this thesis. First, observational studies on pig farms were performed to estimate the prevalence of MRSA positive herds, and to identify factors associated with LA-MRSA in pig herds. It was shown that LA-MRSA was present in the majority, i.e. ~70%, of Dutch pig herds and that the prevalence increased over time. Larger herds were more often found LA-MRSA positive than smaller herds, and transmission was shown to occur by animal trade. From all this, it was concluded that LA-MRSA has become endemic in the Dutch pig population. Secondly, studies on LA-MRSA in pigs, the environment and personnel in pig slaughterhouses were performed. In pigs, a clear increase in LA-MRSA positive pigs from 0 to 60% was shown in the time period between loading at the farm and stunning at the slaughterhouse. This indicated a very rapid transmission of LA-MRSA between pigs through direct contact or through contact with a contaminated environment. An increase in LA-MRSA positive environmental samples taken in the slaughterhouse was found during the working day. In personnel, LA-MRSA prevalence was 6% and working with live pigs was the single most important factor for being positive; personnel not working with pigs or working only with dead pigs were all LA-MRSA negative. Thirdly, transmission of LA-MRSA within herds was studied longitudinally both in an experimental setting and also in 6 pig herds. Transmission rates and the factors affecting these rates were determined. The results of both studies indicated that LA-MRSA is able to spread easily and persist in pig populations, resulting in an endemic situation. Use of selective antimicrobials has a positive effect on the transmission rate of LA-MRSA, but transmission occurs even without use of antimicrobials. The key to limiting LA-MRSA transmission from pigs to humans is to eliminate the source, i.e. eradicate LA-MRSA from pig herds, and a combination of different intervention strategies controlling both within- and between-herd transmission will be needed to achieve this.

Epidemiology of MRSA in veal calves

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Traditionally Methicillin-resistant *Staphylococcus aureus* (MRSA) has been considered as a hospital-associated pathogen (HA-MRSA). However, since 2004, MRSA has been found to be emerging in livestock (LA-MRSA), particularly pigs and veal calves. MRSA in livestock, predominantly belongs to Clonal Complex (CC) 398. Animals have the capacity to act as reservoirs of MRSA, and potentially transmit this bacterium to humans in close contact with MRSA colonized animals. Human infections with MRSA are associated with increased morbidity and mortality, length of hospitalization and health care costs. However, the public health consequences and risk factors for MRSA carriage of CC398 are currently unknown. This thesis focuses on MRSA in veal calf farming. The main aim of this thesis is to investigate associations between determinants and ST398 MRSA carriage in both humans and veal calves and their inter-relationship. Furthermore the persistence and dynamics of MRSA carriage in both human and veal calves were quantified.

The sudden increasing emergence of MRSA in animals raised questions about the possible public health threats. Therefore there was need for further research. Risk factors for both veal calf and human ST398 carriage were investigated in a cross-sectional study. Randomly selected, 102 veal calf farms participated in this study. Specific attention was given to the presence of MRSA among veal farmers, their family members and their animals. We demonstrated a direct relationship between human and animal MRSA carriage. A positive association between human MRSA carriage and the number of MRSA positive calves on the farm was demonstrated. Furthermore, we observed that calves were more often MRSA carrier when treated with antibiotics. Farm hygiene however, was associated with a lower prevalence of MRSA in calves.

A refined and subsector stratified analysis of determinants for developing intervention strategies to control LA-MRSA on veal calf farms and in veal calves demonstrated again that antimicrobial use in calves was positively associated with MRSA carriage in both white and rose veal calves. This finding further emphasize the need for prudent use of antimicrobials. However, several age-related- and farm management factors seemed to be correlated with use of antimicrobials in calves and thereby complicate the interpretation of the results. These factors needs further exploration and study in experimental designs or intervention studies.

MRSA occurrence and dynamics in veal calves were investigated longitudinally. Determinants associated with MRSA carriage, such as environmental exposure and antimicrobial use, were explored. In addition, the reliability and reproducibility of nasal samples in veal calves to establish MRSA status were investigated, as well as the additional value of rectal samples. MRSA prevalence and MRSA air loads in stables rapidly increased during the production cycle, especially after releasing calves from their individual houses, but not simultaneously with or directly after treatment with antimicrobials. This suggests that antimicrobial use is not necessarily the main condition for MRSA transmission in veal calves but other factors seemed to determine transmission as well. MRSA in calves was present both nasally and rectally. Relatively more positive rectal samples were found in the first 6 weeks. Therefore, we hypothesise that rectal MRSA carriage contributes to a higher environmental MRSA load and thereby influences MRSA spread into the population.

Human persistence and dynamics of MRSA ST398 was investigated after both short and long term exposure to MRSA positive animals. LA-MRSA acquisition after short term (up to 3 hours per day maximally) occupational exposure is frequent. However, the majority of people, who acquire LA-MRSA during occupational exposure, test negative for MRSA again within 24 hours. In farmers, who are long term exposed to MRSA positive animals, the presence of LA-MRSA is strongly animal-exposure related. During absence of animal contact, MRSA prevalence decreased rapidly, which suggests that LA-MRSA is a poor persistent colonizer in most humans. The study suggests that highly exposed people could be a source for MRSA for lower exposed family members.

The findings in this thesis provide important insights that add to our understanding of LA-MRSA carriage in animals and humans. Antimicrobial use in calves is associated with higher MRSA prevalence. However, future research should be focussed on the quantitative contribution of specific antibiotic classes or dosages to MRSA occurrence livestock environments. In addition, to reduce MRSA occurrence in veal farming, optimizing the complex structure of the veal calf production chain needs specific attention.

Our data point out that exposure plays a major role in MRSA carriage in humans but it seemed that MRSA of ST398 is not a persistent colonizer in humans. This is in line with other studies in which a low nosocomial

transmission rate of ST398 MRSA is demonstrated. These findings implicated that control measures as described in the Search and Destroy policy could be less stringent. However, the exact ST398 related disease risk for farmers is still unclear and needs further exploration. The ongoing evolution and development of ST398 MRSA suggests that adaptation to the human host might be happening and therefore close monitoring of its evolution and surveillance (including prevalence, disease risk and molecular characterization) over time will be required.

ESBL-producing bacteria in broilers; from model to experiment and back again

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Extended Spectrum BetaLactamases (ESBL) are enzymes capable of hydrolysing certain antibiotics including cephalosporins and penicillins. In the Netherlands most chicken meat is contaminated with ESBL producing bacteria and 1 out of 5 human isolates are closely related to chicken isolates. The presence of ESBL producing bacteria on broilers poses a public health problem.

Genes coding for ESBLs are carried by plasmids. These plasmids are extra-chromosomal circular pieces of DNA. Often multiple copies of the plasmid are present in a bacterium cell and after cell-division both daughter cells will carry the plasmid. An important property of these plasmids is that they can be horizontally transferred to other bacteria, both inter- and intraspecific. In this case the donor will provide a plasmid to the recipient, without plasmid, which will create a new type of bacterium, the transconjugant.

Here the preamble of the PEDEP study and its first results are presented. The main objectives of the study are (1) to explain the widespread occurrence of the IncI1/CTX-M-1 –positive isolates and to understand **under what circumstances** it can be **successful** (2) with the results of this project we intend to identify determinants [...] that may help to define **targeted interventions** for the distribution of this plasmid.

The study is an interaction between mathematical modelling and experimental research. We started with simplest model for the interaction between donor, recipient and transconjugant in the gut of a chicken. Using standard ecological models we created hypotheses which were tested *in vitro*.

We found that the donor, recipient and transconjugant did not differ in any of their life history parameters. However, a quantitative difference was found between the estimate for the conjugation rate *in vitro* and an estimate from a previous *in vivo* experiment. This difference results in a qualitative different outcome, where *in vivo* the recipient is replaced by the transconjugant, this is not observed *in vitro*.

Comparison of ESBL contamination in organic and conventional retail chicken meat.

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Abstract

Contamination of retail chicken meat by ESBL producing bacteria likely contributes to the increasing incidence of infections with these bacteria in humans. This study aimed to compare between organic and conventional retail chicken meat samples the prevalence and load of ESBL positive isolates, the distribution of ESBL genes, strain genotypes and degree of co-resistance. In 2010, 98 raw chicken breasts were purchased in 12 stores in the Netherlands (n=60 conventional; n=38 organic). Prevalence of ESBL producing micro-organisms was 100% on conventional and 84% on organic samples ($p<0.001$). Median loads of ESBL producing micro-organisms were 80 (range <20-1360) in conventional, and <20 (range 0-260) CFU/25 gram in organic samples ($p=0.001$). The distribution of ESBL genes in conventional samples and organic samples was 42% versus 56%, respectively (N.S.), for CTX-M-1, 20% versus 42% (N.S.) for TEM-52, and 23% versus 3% ($p<0.001$) for SHV-12. CTX-M-2 (7%), SHV-2 (5%) and TEM-20 (3%) were exclusively found in conventional samples. Co-resistance rates of ESBL positive isolates were not different between conventional and organic samples (co-trimoxazole 56%, ciprofloxacin 14%, and tobramycin 2%), except for tetracycline, 73% and 46%, respectively, $p<0.001$). Six of 14 conventional meat samples harbored 4 MLST types also reported in humans and 5 of 10 organic samples harbored 3 MLST types also reported in humans (2 ST10, 2 ST23, ST354). In conclusion, the majority of organic chicken meat samples were also contaminated with ESBL producing *E. coli*, and the ESBL genes and strain types were largely the same as in conventional meat samples.

MRSA in pig farms: a prospective cohort study

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Introduction

Livestock associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is a known and prevalent bacterium related to livestock farming. Little is known about the dynamics of carriage over time in pig farmers and their household members.

The aim of this study was to determine the prevalence, determinants and dynamics of human carriage of LA-MRSA in pig farms.

Methods

In this prospective cohort study in 50 pig farms in The Netherlands, questionnaires and samples were taken from pig farmers, their employees and household members. Human nasal samples and environmental samples were collected on 6 time points in 12 months. At the start and at the end of the study, throat samples were taken as well. Persistent carriers were defined as persons with 100% of nasal samples positive for MRSA, non-carriers had no MRSA and intermittent carriers had at least one positive sample. A preliminary analysis will be presented here.

Results

In total, 281 persons participated in the study. Forty-eight persons were persistent MRSA carriers (48/281=17%): 4 household members (4/149=3%) and 44 farmers/employees (44/132=33%). Furthermore, 87 (87/281=31%) intermittent carriers and 146 (146/281=52%) non-carriers were found. Sixty-seven percent (33/49) of the residences and 80% (40/50) of the stables harbored MRSA.

Multivariate multilevel regression analysis showed that working in the stables for ≥ 50 hours per week (OR=6.4, 95% CI 1.7 - 24.5), no recent hospital admission (OR=21.9, 95% CI 1.4 - 333.3) and high amounts of cfu of MRSA nasally (OR=93.1 for >2.0 cfu compared to 0 cfu, 95% CI 19.6 - 441.9) were determinants for persistent carriage.

Conclusions

Persistent MRSA nasal carriage is common in persons working at pig farms and strongly relates to the duration of stay in the stables. It is unclear if this is caused by direct contact with the animals or by the inhalation of contaminated dust. LA-MRSA is able to spread from person to person, albeit not to great extent, suggested by the relatively low prevalence of persistent carriage in household members.

Prognosis in canine idiopathic immune-mediated haemolytic anaemia

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Introduction

Idiopathic immune-mediated haemolytic anaemia (IMHA), characterized by antibody-mediated red cell destruction, is recognized as one of the most frequently occurring immune-mediated diseases in the dog. Idiopathic IMHA is diagnosed based on a Coombs' positive haemolytic anaemia, the presence of spherocytes, and exclusion of infectious or neoplastic disorders, vaccination, and medication that can cause a secondary IMHA. The presentation of a dog diagnosed with idiopathic IMHA varies from a mild anaemia to a severe haemolytic crisis with a mortality of 20 % - 70 % that mainly occurs in the first 2 weeks post diagnosis. The central theme of this study was to analyze clinical, therapeutic, and pathophysiological factors that contribute to disease outcome in dogs with idiopathic IMHA (1).

Retrospective studies report a poor outcome in dogs with autoagglutination, non-regenerative or severe anaemia, thrombocytopenia, severe leucocytosis, high plasma bilirubin concentration, or increased prothrombin time. Not every dog diagnosed with idiopathic IMHA displays all these characteristics, however, and it is unclear if these characteristics are part of the process of antibody-mediated red cell destruction or that in fact they result from independent pathways or pathophysiological processes secondary to the haemolysis. Lifelong immunosuppression has been recommended but evidence for this recommendation is lacking. Therefore, a study was devised to estimate survival time in dogs with idiopathic IMHA and to explore which clinical and laboratory characteristics determine the probability of survival and in addition to determine if immunosuppression for three months is sufficient to maintain remission of idiopathic IMHA (2).

Immunomodulation is the mainstay of treatment in IMHA and may be combined with whole blood or packed red cell transfusions and anticoagulation. It has been suggested to combine glucocorticoids with other immunosuppressive agents of cytotoxic drugs if clinical condition worsens, if side effects of glucocorticoids are unacceptable, or as part of standard treatment protocols. Azathioprine, a thiopurine analog, is a cytotoxic drug that interferes with DNA synthesis by competition with adenosine. It has been reported to have a synergistic effect with prednisolone and if used in combination the prednisolone dose may be reduced. A beneficial effect of treatment with azathioprine in idiopathic IMHA has been reported in two retrospective studies. However, the duration of azathioprine therapy in one study must be judged suboptimal since the clinical effect of azathioprine may be expected after at least 11 days of treatment. In the other study the efficacy of azathioprine alone could not be determined since dogs were treated with cyclophosphamide on top of azathioprine and prednisolone. Therefore, a study was devised to assess the additional beneficial therapeutic effect of azathioprine compared to prednisolone therapy in dogs with idiopathic IMHA (3).

The laboratory diagnosis of IMHA rests upon the demonstration of an immune-mediated mechanism for the haemolysis. The direct agglutination test (DAT) demonstrates the presence of anti-erythrocyte antibodies by incubating a suspension of washed patient erythrocytes with polyvalent or monovalent antisera specific for dog immunoglobulin or complement. There is a lack of uniformity between laboratory procedures for the DAT. Instead of testing only a few dilutions testing of more dilutional steps in a microtitre tray has been advocated to result in less false negative results due to the prozone effect. Incubation is generally performed at 37°C but some laboratories also incubate at 4°C, a procedure that is debated by others. The monovalent DAT is suggested to have a higher sensitivity than the polyvalent DAT and the sensitivity and specificity of reagents from different manufacturers differ considerably. A gel-based polyvalent DAT has been developed that offers the possibility to standardize anti-erythrocyte antibody testing between laboratories. To assess the performance of this gel-based DAT in comparison to the traditional DAT based on erythrocyte agglutination and to assess the usefulness of this gel-test as a diagnostic tool in the diagnosis of IMHA a study was devised (1).

Abundant evidence has been presented for a state of hypercoagulability during the hospitalization period in dogs with idiopathic IMHA. Post-mortem examinations of dogs with idiopathic IMHA demonstrate the presence of

thromboembolisms in many organs. Leucocytosis and a left shift are present in the majority of dogs with IMHA and increased leucocyte counts have been associated with moderate to marked tissue damage. In dogs with IMHA changes in acute phase protein concentrations have been measured fitting the presence of an acute phase response. Interleukin-8 (IL-8) is one of the cytokines that is increased in the acute phase response. It is a major chemotaxin for leucocytes and orchestrates the margination and extravasation process of leucocytes through increases in selectin expression on endothelial cells. Leucocytes, in particular monocytes, play an important role in thrombogenesis by producing tissue factor (TF) which initiates the extrinsic pathway of coagulation. It was hypothesized that dogs with idiopathic IMHA have increased blood levels of IL-8 and TF. To validate reference genes for future quantitative RT-PCR studies in idiopathic IMHA in canine whole blood was studied. To assess the contribution of whole blood gene expressions of TF and IL-8 to the inflammatory response and the coagulation abnormalities in dogs with idiopathic IMHA another study was devised (4).

In an overview of the current state of evidence in the canine IMAH the study gives an additional review of literature and critical evaluation on the diagnostic tests and results of therapy. This part was motivated by a striking example: The mortality rate in canine idiopathic IMHA has not decreased since the earliest publications. Azathioprine, cyclophosphamide, other immunomodulators, and heparin are used often in the treatment of canine idiopathic IMHA despite the fact that evidence of their efficacy is lacking (5).

Discussion

Mortality percentages in canine idiopathic IMHA have been reported between 20 % - 70 % with most deaths in the first two weeks after. Calculations of survival times were performed in three different cohorts of dogs with idiopathic IMHA. Respectively, in the cohort of 149 dogs treated with prednisolone and azathioprine (AP protocol) the half year survival was 72.6% (95% CI: 64.9 – 81.3%) (3), in the cohort of 73 dogs treated with prednisolone (P protocol) the 1-year survival was 64% (95% CI: 54 – 77%) (2), and in the cohort of 24 dogs treated with prednisolone the half year survival was 75% (95% CI: 59.5 – 94.5%)(4).

Eighty percent of dogs with idiopathic IMHA in the cohort treated with azathioprine and prednisolone (n=149) had a leucocytosis and a left shift at presentation which is similar to the findings in the cohort treated with prednisolone alone (n=73) (2, 4). The cohort of 24 dogs had significantly increased leucocytes, band neutrophils, and monocytes. The summary of CBC data from other studies on canine idiopathic IMHA supports the conclusion that an inflammatory response consisting of a pronounced leucocytosis and a left shift are common laboratory features at presentation (5).

The literature survey suggests that as many as half of the deaths in idiopathic IMHA are related to thromboembolisms. From the summary of clinical observational studies of dogs with idiopathic IMHA it can be concluded that up to 50% of dogs with idiopathic IMHA present with abnormalities in coagulation parameters suggesting the presence of disseminated intravascular coagulation (DIC). This is confirmed by the results for the coagulation times, fibrinogen concentration, and thrombocyte counts (5). The prothrombin time and activated partial thromboplastin time were increased in respectively 46% and 67% (n=98), and a decreased fibrinogen concentration and a thrombocyte count below $50 \times 10^9/l$ were found in respectively 18% (n=96) and 25% (n=148) of the cohort of dogs with idiopathic IMHA treated with azathioprine and prednisolone (3). These coagulation tests and thrombocyte count results were not significantly different from those found in the cohort of dogs with idiopathic IMHA treated with prednisolone. Decreases in individual coagulation factor activities fitting DIC were found in the cohort of 24 dogs with idiopathic IMHA as well. A low mean platelet content (MPC), indicative of platelet activation, was found in the group of dogs diagnosed with DIC and in the group with idiopathic IMHA. In addition, large platelets, characterized by an increase in mean platelet volume (MPV) and mean platelet mass (MPM) were found in the dogs with idiopathic IMHA, which most likely results from an increase in platelet production rate(4). It has been reported that large platelets are associated with an increased haemostatic capacity. In conclusion, the dogs with idiopathic IMHA have decreased MPC and increased MPV and MPM reflecting a high platelet turnover due to the continuous platelet activation that occurs in DIC.

Uni- and multivariate analysis with the aim to identify prognostic variables and their relationship has been performed in 3 different cohorts of dogs with idiopathic IMHA. The finding of increased plasma urea or creatinine concentration, the presence of icterus, the presence of increased band neutrophils, increased monocyte counts, thrombocytopenia, and prolonged APTT as prognostic variables in the respective multivariate models indicates that parameters that signal the presence of renal failure, liver failure, inflammation, and DIC, alone, or in combination are robust independent predictors of mortality(2-4). The finding of the same or related prognostic factors by other authors supports this conclusion (5).

Hypoxia is a risk factor found by some, but could not be confirmed in the univariate analyses. Nevertheless, a pathology study that established a positive relation between the presence of hypoxic necrosis, especially in the liver, and increased leucocyte counts and a study that related increased duration of hyperlactemia to mortality both support that anaemia may be central in the development of a high mortality risk. Oxygen delivery was impaired in a canine isovolemic anaemia model below a haematocrit of 10%. The fact that icterus and failing liver functions form an important risk factor is in agreement with hypoxia as risk factor, since the failing liver functions can be entirely attributed to centrilobular hypoxic hepatocyte necrosis resulting from severe anaemia.

The establishment of both the inflammatory response and the thrombotic tendency as major independent factors that determine outcome in dogs with idiopathic IMHA was the immediate cause to investigate the possible underlying shared pathophysiological mechanisms. Tissue factor (TF) expression by inflammatory cells, especially monocytes has been reported as a link between inflammation and coagulation. This occurs mainly through the NF- κ B signaling pathway and leads to increases in cytokines such as IL-8 which is a major chemotaxin for leucocytes. It was hypothesized that blood levels of TF and IL-8 in dogs with idiopathic IMHA are increased due to increased expression by inflammatory cells. Total leucocyte counts and band neutrophils are increased in 80% of dogs with idiopathic IMHA at presentation and further increase during the hospitalisation period. The high leucocyte turnover suggests a continuing production of IL-8. Similarly, to explain a thrombotic tendency a continuous intravascular source for TF must be present. Therefore we choose to measure TF and IL-8 gene expressions during the hospitalisation period by quantitative RT-PCR. Gene expressions were measured in whole blood since isolation procedures of leucocytes may cause up regulation of cytokine expressions (6).

One of the solutions to control for the internal variation that affects the outcome of the quantitative RT-PCR reaction is the use of reference genes as an internal standard. Reference genes are selected based on the supposition that their expression is stable in all cells regardless of the tissue or individual used in the study. The suitability of nine frequently used canine genes as reference genes for quantitative RT-PCR in whole blood was investigated. The analysis revealed that white blood cell count and disease category had a statistically significant effect on the expression of the potential reference genes in canine whole blood. Two software applications were used to select the potential reference genes that had the most stable expression and the number that was needed to provide an optimal normaliser within the experimental setting. Normfinder selects those reference genes that have the most stable expression between groups and GeNorm selects the genes with the least variation in individual samples. It was concluded that multiple reference genes are necessary to provide a stable normaliser for quantitative RT-PCR in canine whole blood samples and, since expression may be influenced by the experimental conditions, that it is necessary to assess the stability of expression for each experimental situation anew (6).

The hypothesis was rejected that the presence of hypercoagulability and an inflammatory response in dogs with idiopathic IMHA is due to increased expression of IL-8 and TF by monocytes (4). Our study demonstrated that whole blood TF expression was increased but IL-8 expression was not significantly different from the IL-8 expression in healthy dogs and significantly lower than in the groups with systemic disease, neoplasia and DIC, and sepsis. The decreases in coagulation factors FII, FV, FVII, FIX, FXI, FXII confirmed the presence of DIC in many of the dogs with idiopathic IMHA. The high FVIII and fibrinogen activity in the dogs with idiopathic IMHA suggested an acute phase response. Much evidence has been reported in the literature for the presence of an acute phase response in dogs with idiopathic IMHA. Increased leucocyte counts and turnover have been related to severity of centrilobular hepatocyte necrosis. In the first part of the acute phase response macrophages activated by liver hypoxia release mediators such as interleukin-1 and tumor necrosis factor which is followed by the release of IL-8 and monocyte chemoattractant protein by local fibroblasts and endothelial cells. We identified an increased monocyte count as independent negative prognostic parameter in the multivariate model predicting death in dogs with idiopathic IMHA. In a recent study monocyte count in dogs with idiopathic IMHA was not identified as a prognostic factor. But serum cytokine concentrations related to monocyte recruitment (monocyte chemoattractant protein-1, granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 15 and interleukin-18) were increased, and two of them, monocyte chemoattractant protein -1 and interleukin-18, were independently associated with higher mortality. And similar to our findings, the results for the serum IL-8 concentration (median 2.6 μ g/l, range (1.2–32.0), n=20) in this study were not significantly different from the healthy controls (median 1.6 μ g/l, range (0.6–5.4), n=6).

Whole blood TF expression was increased in dogs with idiopathic IMHA and thus contributes to the consumptive coagulopathy documented in dogs with idiopathic IMHA. TF expression in monocytes is regulated via the NF- κ B signaling pathway and activation of this pathway is expected to result in increased expression of IL-8 as well. Since IL-8 expression in dogs with idiopathic IMHA was not increased, our results suggest that another source than blood monocytes is responsible for the increased whole blood TF expression. Platelets have

been reported to express TF but not IL-8 and may be the alternative source of TF expression in our study. This is supported by the fact that p-selectin, a platelet activation marker was elevated in dogs with idiopathic IMHA.

The summarized evidence that has been gathered from the literature on canine idiopathic IMHA (5) demonstrates that research results supporting the use of immunomodulators in addition to glucocorticoids are lacking, despite the efforts that have been made in observational studies and randomized clinical trials. The analysis of the prerequisites of such trials with regard to inclusion criteria suggests that it may be advantageous to categorize dogs with idiopathic IMHA based on their probability of survival in addition to the randomisation procedure. The estimates of the effect size in power analyses to estimate sample sizes necessary for clinical trials in canine idiopathic IMHA may have been too optimistic and led to false negative outcomes. An example of a power analysis shows that larger sample sizes are needed than hitherto used in the literature. In fact, our retrospective cohort study had respectively 149 dogs in the AP protocol group and 73 dogs in the P protocol group and is one of the largest studies comparing treatment in canine idiopathic IMHA (3). This study is an observational study using an historical control group, however, and not a randomized controlled trial.

Observational studies may be more suited than controlled randomized trials to establish the required duration of immunosuppression, the natural history of the disease studied, and to detect occasionally occurring side effects. We were able to demonstrate in the retrospective cohorts of dogs with idiopathic IMHA that in contrast with general recommendations that include lifelong immunosuppression, an immunosuppression with prednisolone alone or in combination with azathioprine for 3 months is sufficient to obtain remission of idiopathic IMHA. Side-effects due to azathioprine were observed in 8% of dogs (n=222). It was established that recurrences of a haemolytic crisis may occur in at least 10% of the dogs with idiopathic IMHA (n=222). A lack of additional therapeutic effect of azathioprine in the cohort of dogs with idiopathic IMHA treated with azathioprine and prednisolone in comparison to the cohort treated with prednisolone only was reported. The use of the fact that historical controls may have resulted in confounding due to improvement of supportive care within the time span of both cohorts was discussed (3).

It was concluded that a mortality risk based classification of dogs with idiopathic IMHA is needed to ensure that dogs that enter a trial have similar mortality risks. Indeed, the survival probabilities between the treatment arms may have differed in study since the dogs in the trial arm treated with azathioprine and prednisolone had lower thrombocyte counts and longer duration of clinical signs which may have obscured an additional treatment effect of the azathioprine. Therefore a blinded randomized clinical trial is still needed to establish the true effect of azathioprine. In view of the expected slow onset of azathioprine it is unlikely that a benefit of azathioprine can be discerned in the first 1-2 weeks after the start of treatment. Therefore such a trial should be conducted in the subset of dogs with idiopathic IMHA that are likely to survive the initial hazardous hospitalization period (5).

It was concluded that progress in treatment improvements in canine idiopathic IMHA is slow and that multicenter trials may be the only solution to obtain study groups with enough power to find differences in outcome due to treatment. The datasets of the cohorts of dogs with idiopathic IMHA, and other previously reported data sets, may be utilized to provide the basics for a mortality risk based scoring system. Such a system, however, should be validated in a prospective study that preferably incorporates data from dogs from different research groups working on canine idiopathic IMHA to ensure that the resulting scoring system is properly validated for general application (5).

Universal agreement on the diagnostic criteria for canine idiopathic IMHA will be a necessary prerequisite for the initiation of such multicenter trials. The use of similar inclusion and exclusion criteria in the studies on canine idiopathic IMHA summarized in [17] predicts that criteria that researchers agree upon will consist of the presence of moderate to marked anaemia, and diagnostic procedures that ensure exclusion of pathophysiological routes of development of anaemia other than haemolysis.

However, ultimately, the diagnosis of immune-mediated haemolysis depends upon demonstration of anti-erythrocyte antibodies which is most commonly done by the conventional direct agglutination test. The execution of the DAT is poorly standardized and source for much debate. It was shown that the results of a fast polyvalent gel-based DAT agreed very well with the results of the conventional DAT as it is performed in two veterinary university clinic laboratories specialized in haematology. Since a gel-based DAT may be commercially produced these results are encouraging with regard to future standardisation of diagnostic testing in canine idiopathic IMHA. A potential additional advantage may be that the gel-based DAT was less often positive in secondary IMHA (1).

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Seroprevalence and risk factors for *Toxoplasma gondii* infection in domestic cats in The Netherlands

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Toxoplasma gondii belongs to the most important foodborne pathogens in the Netherlands. As definitive hosts, cats play an important role in the epidemiology of *T. gondii* and therefore measures aimed at preventing cat infection could be very effective in decreasing both oocyst- and tissue cyst acquired infections in humans. To determine the seroprevalence and risk factors for *T. gondii* infection in Dutch domestic cats, serum samples of 450 cats were tested for *T. gondii* antibodies by indirect ELISA.

The seroprevalence was estimated at 18.2% (95% CI: 16.6-20.0%) by binary mixture analysis; and showed a decrease with age in very young cats, an increase up to about 4 years old, and ranged between 20 and 30% thereafter. Besides age, hunting (OR 4.1), presence of a dog in the household (OR 2.1), former stray cat (OR 3.3) and, at $p=0.056$, feeding of raw meat (OR 2.7) were identified as risk factors by multivariable logistic regression analysis. Next, the fitted distributions from the binary mixture analysis were used to estimate the probability of being positive for each individual cat. This probability ($P(\text{pos} | X=x)$) is a more informative test result than a score based on a cut-off value because it takes into account the sensitivity and specificity of the test. These probabilities were subsequently used to estimate the prevalence differences corrected for confounding by other factors in a linear regression model, and the prevalence differences were used to calculate the population attributable fraction for each risk factor. Hunting contributed most to the *T. gondii* seroprevalence in the sampled population (35%).

In conclusion, *Toxoplasma gondii* infection is quite common in Dutch domestic cats, with hunting as the most important risk of infection, and feeding of raw meat as the easiest to prevent. Because all *T. gondii* infections in intermediate hosts have a cat shedding oocysts as starting point of the cycle, controlling these risks might eventually reduce human toxoplasmosis in The Netherlands.

Cost of surveillance systems for low pathogenic avian influenza in the Dutch layer sector

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Introduction

Recently, an alternative LPAI surveillance system based on egg sampling instead of blood sampling is investigated for layer farms [1]. In this system variation in number of eggs sampled and variation in sampling frequency are studied in relation to the probability of detection of an AI virus infection.

Although the effectiveness of the LPAI surveillance system using eggs as commodity might equal the system that uses blood samples, there are some advantages and disadvantages. The advantage of the egg surveillance is that the sampling of eggs is flexible as it can be done at farm level or at the level of packing stations where eggs of multiple farms are delivered. Even more, egg sampling is more desirable in the perspective of animal welfare as the hens do not have to be distressed and sampled in an invasive manner anymore. On the other hand, egg-sampling has disadvantages at the laboratory level. Preparing egg-yolk samples is technically more complex and more time consuming, hence it might be that processing yolk samples is more expensive than the processing of serum samples. Using egg as sample material produces more high risk waste (i.e. more waste processing cost) in the laboratory than using serum samples. Most advantages and disadvantages can be reflected in monetary terms, which enhances the decision making [2].

For policy makers it is necessary to have a comparison of the costs and benefits of different surveillance systems. This study will present the differences in cost between surveillance systems that originate from the before-mentioned advantages and disadvantages. Because alternative surveillance system should have a comparable probability of detecting LPAI virus introductions (i.e. an equal benefit), a cost minimization analysis is used, where the system with the lowest cost is preferred [3]. Thus, the objective of this study is to perform a cost minimization analysis of various LPAI surveillance systems for Dutch layer farms based either on blood or egg sampling with equal probability of detecting a LPAI virus introduction.

Material & Method

Alternative surveillance systems

The various LPAI surveillance systems evaluated are summarized in Table 1. They can differ in six aspects: i) sample material: blood and/or eggs, ii) location of sampling: layer farm and/or packing station, iii) sampling frequency, iv) number of samples obtained per farm, v) location of sample preparation: central laboratory and/or packing station, and vi) the number of farms with a high or low risk to induce an epidemic in case of an outbreak. Acronyms are used to describe the different systems. The first part represents the sampling material: B for blood and E for eggs. The second part represents the sampling location: F for layer farm and P for packing station. The third part represents the location where the sampling is prepared: L for central laboratory and P for packing station. For example, the current surveillance system is represented by B/F/L meaning that blood samples are taken at the farm and sent to a central laboratory for preparation (and the test).

Table 1: Overview of the evaluated LPAIv surveillance systems

Surveillance system	Sampling						Sample preparation				
	Material		Location		Frequency/yr		Number		Location		Method
	Blood	Eggs	Layer farm	Packing station	Indoor farms	Outdoor farms	Indoor farms	Outdoor farms	Central lab	Packing station	Robot Hand
B/F/L	X		X		1	4	30	30	X		X
E/F/L		X	X		1	4	35	35	X		X
E/P/L		X		X	1	4	35	35	X		X
E/P/P		X		X	1	4	35	35		X	X
E/P/LP		X		X	1	4	35	35	X	^a X	^b X
E/FP/LP		X	X	^c X	^d 1	4	35	35	X	X	X
BE/FP1/L	X ^e	X ^f	X	X	1	4	30	35	X		X
BE/FP2/L	X ^e	X ^f	X	^c X	^d 1	4	30	35	X		X

^aSmall packing stations send in eggs

^bLarge packing station have a robot

^cFarms that deliver to small packing stations send in eggs

^dLarge packing stations have a robot

^eBlood samples for conventional farms

^f Egg samples for outdoor ranging farms

Sampling

Blood samples for AI surveillance are taken by a licenced veterinarian (as required by the Dutch authorities) at the layer farms. Eggs are collected at either layer farms and/or packing stations depending on the surveillance system. It is assumed that the egg collection can be performed by the farmer and/or a worker of the packing station as eggs are easy to collect and to trace by the official printed date and unique farmcode on the egg.

The frequency of sampling depends on farm type and sampling material (blood or eggs). For indoor farms the sampling frequency is once a year for both blood samples and eggs, whereas for outdoor farms the sampling frequency is once every 90 days both blood samples and eggs. The number of samples is 30 blood samples or 35 egg samples per sampling for all farm types. The number of egg samples is corrected for the lower egg production due to a possible LPAI infection [4]. Samples are tested for the presence of antibodies against AI viruses using a commercial ELISA test kit. A study evaluating the performance of the ELISA test using egg-yolk and serum samples showed that the sensitivity (conditional on true serological conversion of infected chickens) and specificity is practically the same with both type of samples [1].

Sampling preparation

Next a sample is taken of the serum by a robot from the vial. This sample is transferred to an ELISA-plate and diluted to a concentration appropriate for testing. Next, the sample is tested using an automated ELISA procedure.

The sample preparation of eggs is different from that of blood. Trays of eggs are handled by a specialised robot: a needle guided by a robot penetrates the egg-shell, the egg white and finally the egg-yolk. Then a yolk sample is taken and transferred to an ELISA-plate and diluted to a concentration appropriate for testing [1]. Next, the sample is tested using an automated ELISA procedure.

Cost calculations

The total cost of surveillance system i (TC_i) include costs related to the following activities: sampling (i.e. SB_i for blood and SE_i for eggs), sample preparation (SPB_i for blood and SPE_i for eggs), testing ($Test_i$), waste processing (WP_i), transport to the central laboratory ($Trans_i$), communication to the farmer (COM_i) and confirmation testing for false positive results (CT_i):

$$TC_i = SB_i + SE_i + SPB_i + SPE_i + Test_i + WP_i + Trans_i + COM_i + CT_i \quad (1)$$

Sensitivity analysis

A sensitivity analysis was performed to assess and identify the inputs that influence the net benefits the most. Each individual input was changed with +10% and -10% and the total net benefit was calculated. This analysis was carried out using the add-in software TopRank 5.5 for Excel of Palisade Decision Tools[5].

Results

Total cost of the surveillance systems

Figure 1 shows that the E/P/P system is most expensive (i.e. € 2,354,632). This was expected as in the E/P/P system it was assumed that all packing stations would have a sample preparation robot for eggs which are very costly.

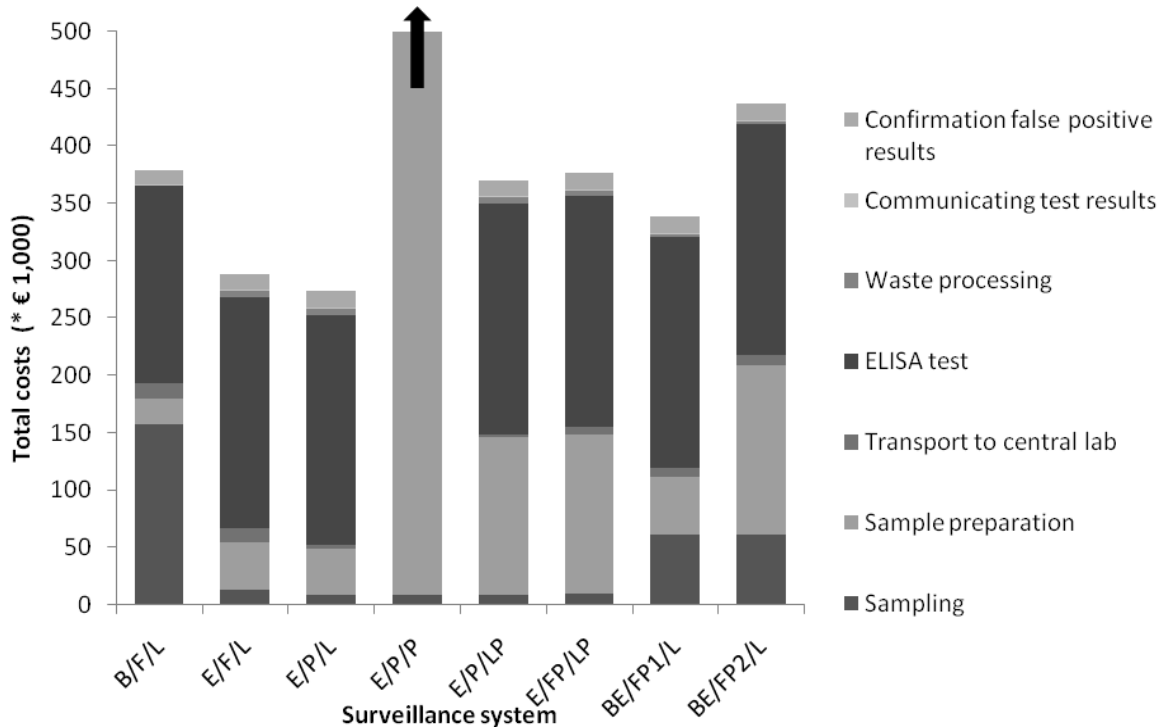


Figure 1: Total net cost of various surveillance systems were the activities are differentiated.

The lowest costs are found for the E/P/L system (i.e. € 273,393). This is caused by lower costs for sampling which was partially offset by higher costs of sample preparation. Compared to the E/P/L system the other systems are more costly: 39% for B/F/L, 6% for E/F/L, 761% for E/P/P, 36% for E/P/LP, 38% for E/FP/LP, 24% for BE/FP1/L and 60% for BE/FP2/L. It was expected that the egg surveillance systems would be cheaper than the B/F/L system. Sampling eggs at a packing station seemed more efficient and therefore total cost for E/P/L are lower than other systems based on egg samples.

Cost of different activities

Table 3 shows that sampling cost of system B/F/L is 11 - 20 times higher than the cost of systems E/F/L and E/P/L. By contrast, the cost for sample preparation of systems E/F/L and E/P/L are less than 2 times higher than that of B/F/L. The high rates charged by the veterinarian for blood sampling causes the high cost for sampling in the B/F/L system. The higher purchase price and lower capacity of a robot for egg-preparation causes the high cost for sample preparation in the E/F/L and E/P/L systems. For B/F/L, ELISA test costs are lower than in the other systems, which is caused by the lower sample sizes needed in the B/F/L system (30 blood samples per farm per sampling) compared to the other systems (35 egg samples per farm per sampling).

Table 2: Costs of LPAI surveillance systems

Activity within surveillance system	Cost for each activity and total cost of various surveillance systems					
	B/F/L	E/F/L	E/P/L	E/P/P	E/P/LP	E/FP/LP
Sampling	157,609	13,628	8,116	8,116	8,116	10,286
Sample preparation	22,524	40,358	40,358	2,111,808	137,838	137,838
Transport to central lab	13,053	13,053	3,263	13,053	2,945	6,800
ELISA test	172,398	201,131	201,131	201,131	201,131	201,131
Waste processing	-	5,415	5,415	5,415	5,415	5,415
Communicating test results	967	967	967	967	967	967
Confirmation false positive results	12,124	14,144	14,144	14,144	14,144	14,144
Total Costs	378,674	288,695	273,393	2,354,632	370,554	376,580

Discussion

The objective of this study was to perform a cost analysis of various LPAI surveillance systems for Dutch layer farms based either on blood or egg sampling. It can be concluded that the systems E/F/L and E/P/L have lower total cost than the current B/F/L system. The other systems are less efficient and therefore more expensive. The difference in total cost between the B/F/L system and the EFL and EPL systems are -€ 89,979 and -€ 105,281. For policy makers matters of animal welfare and vulnerability to fraud will also be an issue. The sampling frequency and the sample size are the most influential inputs of the economic model. The reason for their importance is that these inputs determine the amount of work in every activity of a surveillance system. The E/P/L system seems interesting in this perspective because packing stations are independent of farmers and all eggs are printed with a unique identification number when they arrive at a packing station. The most influential inputs are of a chosen value based on epidemiological efficiency of the surveillance systems or the current situation. Therefore the values are certain, for decision makers it is however important to know that the test price is an important factor in the total cost of a surveillance system. In recent year the number of outdoor farms increased in the Dutch layer sector. It seems reasonable to assume that this trend will continue in the coming years and thus increase total cost of any AI surveillance system. The high rate a veterinarian charge for sampling blood is an important factor in the costs of the blood surveillance system. However these costs should be considered to be sunk costs (i.e. cost that are incurred and cannot be recovered) in the case of a combined surveillance. Therefore the use of eggs will not eliminate these costs. When the use of eggs could

eliminate all costs for blood sampling the differences in total cost between B/F/L, E/F/L and E/P/L are likely to disappear.

Future research

The current economic model can be used to calculate cost of surveillance programmes with a different aim from the programme here studied. For example, this economic model could be of interest for an early detection programme that allows rapid detection of a LPAI viruses introduction in poultry, and therefore reduces the probability of both spread of the LPAI viruses to other farms and mutation of the LPAI viruses to a HPAI viruses. Such surveillance programme would require higher sampling frequency and sampling size than the programme studied here and therefore this model can be used to evaluate the economic impact of increased sampling either of sera or egg samples.

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A Sero-surveillance programme for early detection of Low Pathogenic Avian Influenza outbreaks in layer chickens.

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Current knowledge does not offer an estimate of the rate at which low pathogenic avian influenza virus (LPAIV) of the H5 and H7 subtypes mutate to their highly pathogenic form (HPAIV) when infecting poultry. Such a mutation can already take place in the first infected flock; hence early detection and control of LPAIV outbreaks will reduce the probability of pathogenicity mutations and occurrence of large epidemics in affected countries. The objective of this study was to develop a model for the design and evaluation of serological-surveillance programmes aiming at early detection of LPAIV infections in layer chicken flocks. Here, early detection is defined by the detection and culling of an infected flock before the infection spreads to (on average) more than 1 other flock (between flock reproduction ratio " R_f " < 1), hence large epidemics cannot occur. We developed a mathematical model that investigates the required sample size and sampling frequency for early detection by taking into account the LPAIV within- and between-flock infection dynamics as well as the diagnostic performance of the serological test used for surveillance. Since the programme is for layer flocks, it also explored the use of eggs, as an alternative to sera, as sample commodity. The model was applied to evaluate and refine the current Dutch serological-surveillance programme, which presently aims at gaining insight into the LPAIV introduction rate. LPAIV transmission-risk maps were constructed and used to target a risk-based surveillance strategy. The results of our study show that in areas of expected high risk of transmission the Dutch programme would usually not detect an initial outbreak before transmission to other farm has taken place. Increasing sampling frequency and/or sample size would improve the probability of early detection in these areas. However, increasing sampling frequency has animal welfare and economic consequences; hence the use of egg-samples instead of sera, based on the results of this study, is a useful and effective alternative for surveillance of LPAIV in layer flocks. The model here presented is a tool that can be used to design new surveillance programmes or refine existing ones.

Estimating test characteristics of somatic cell count for detection of *Staph. aureus*-infected dairy goats using latent class analysis

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The aim of this study was to estimate test properties of composite somatic cell count (cSCC) to detect subclinically *Staphylococcus aureus*-infected dairy goats. We collected samples from 384 animals in 4 herds for bacteriological culture and cSCC on three occasions in lactation: at early lactation, peak, and late lactation. Latent class models were used to estimate test properties of cSCC and bacteriological culture in the absence of a 'gold standard' reference test under the assumption that both tests detect *Staph. aureus* intramammary infection. Estimates for test properties of cSCC in early lactation at a cut-off value of $1,500 \times 10^3$ cells/mL were 0.90 for sensitivity and 0.95 for specificity, making cSCC a useful screening tool for detection of *Staph. aureus*. The sensitivity of bacteriological culture was estimated to be very low in the latent class models and the models suggested that the true prevalence of *Staph. aureus* in dairy goat herds is much higher than what is commonly reported based on bacteriological culture. This implies that intramammary infection by *Staph. aureus* may be an underestimated problem in dairy goat herds, and that cSCC can be used to diagnose infected animals.