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Elements of survey design and analysis regarding endemic infections in (Belgian) livestock

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LIST OF ABBREVIATIONS

AP animal apparent (measured) prevalence, animal seroprevalence
BoHV-1 bovine herpesvirus 1
CI confidence interval
ELISA enzyme-linked immunosorbent assay
EU European union
GEE generalised estimating equations
HAP herd apparent (measured) prevalence, herd seroprevalence
HP herd true prevalence, herd prevalence (proportion of infected herds)
HPV herd predictive value
HSe herd sensitivity
HSp herd specificity
IHD infected herd detectability
Map *Mycobacterium avium* subsp. *paratuberculosis*
OD optical density
OR odds ratio
ρ p-value
P animal true prevalence, animal prevalence, prevalence (proportion of infected animals)
PI post inoculation
Pr probability
PRV pseudorabies virus
PTB bovine paratuberculosis
PV* positive predictive value
PV- negative predictive value
ρ intra-cluster correlation coefficient
σ² variance
SD standard deviation
SE standard error (of an estimate)
Se diagnostic test sensitivity
Sp diagnostic test specificity
WHAP within-herd apparent (measured) prevalence, within-herd seroprevalence
WHP within-herd true prevalence, within-herd prevalence (average proportion of infected animals within a positive herd, weighted per herd size)
CHAPTER 1:
INTRODUCTION
INTRODUCTION

The globalisation of trade of animals and animal products exerts a strong pressure on animal disease management. The World Trade Organisation Sanitary and Phyto sanitary Agreement (SPS Agreement) sets out the basic rules for food safety, and animal and plant health standards. The SPS Agreement proposes measures that are science-based and that follow international standards laid out in the International Animal Health Code of the Office International des Epizooties (O.I.E., 1999). It has ratified epidemiology’s role, placing it at the centre of animal health trade-related decisions. An epidemiological key notion contained in the SPS Agreement is risk analysis, the cornerstone of which is risk assessment that generates data by comprehensive surveillance systems with a sound epidemiological design (Zepe da et al., 2001). Surveys are such data generating tools that provide animal health managers with information about the animal health status. The information produced by these surveys is to meet reporting requirements of international organisations (e.g. O.I.E.), to demonstrate disease status to trading partners, to determine the level and location of diseases, and to plan, implement and monitor disease control programs.

Animal health managers can only make sound decisions regarding animal health trade when adequate (accurate) information is available to them. This means that there is an increasing need for quantitative data and for proper analysis and interpretation of these data. With the aim to provide animal health managers with adequate (accurate) information about the animal health status, two large single-test animal disease surveys were carried out in Belgium, in the past decade. They investigated three pathogens of the B List of the O.I.E.’s International Animal Health Code: pseudorabies (Aujeszky’s disease) virus, bovine herpesvirus 1 (infectious bovine rhinotracheitis virus), and the bacterium \textit{Mycobacterium avium} subsp. \textit{paratuberculosis}. Before the start of these surveys, hardly any information existed about the analysis and interpretation of surveys regarding veterinary infections, which questioned the accuracy of the information they provided. Did these two surveys provide animal health managers with adequate (accurate) information to argue sound animal health trade-related decisions? The major aim of this thesis was thus to evaluate (analyse) the epidemiological usefulness of the diagnostic information obtained by large single-test animal disease surveys.

REFERENCES

CHAPTER 2:
AIMS OF THE STUDY
AIMS OF THE STUDY

The globalisation of trade in animals and animal products has resulted in efforts to improve and control the analytical and diagnostic quality of tests. However, little attention has been given to the post-analytical phase of the diagnostic process (which includes data management, analysis and interpretation). For infectious diseases, serological tests are used frequently to determine the disease state. Therefore, statements will often refer to the serological rather than to the unknown true disease state of animals (Greiner and Gardner, 2000). In the veterinary literature, the uncertainty associated with the diagnosis (test misclassification bias) has received only little coverage. Consequently, the accuracy of the diagnostic information obtained by single-test animal disease surveys may be questioned. Sound animal health decisions require adequate (accurate) information. Thus, one of the major challenges in the interpretation of these serodiagnostic data is the need to adjust test results for misclassification.

Two large-scale single-test animal disease surveys were conducted in Belgium in the past decade. A first survey was performed in 1996 in 553 pig herds, whereas the second survey, in 1998, involved 556 cattle herds. These two field studies investigated epidemiological aspects of three pathogens of the B List of the O.I.E.’s International Animal Health Code. The major aim of this thesis was to evaluate the overall epidemiological usefulness of these large-scale single-test surveys as information generators to study occurrence of disease. More specifically, the impact of diagnostic uncertainty on the interpretation of prevalence estimation and risk assessment was explored.

First, the herd-level apparent prevalence (seroprevalence) of pseudorabies virus (PRV) in the Belgian pig population, and of bovine herpesvirus 1 (BoHV-1) and Mycobacterium avium subsp. paratuberculosis in the Belgian cattle population were estimated. Also the apparent prevalence of BoHV-1 and Map at animal-level, and within-herd, were assessed. To explore the impact of diagnostic uncertainty on the interpretation of these seroprevalence figures, true prevalence was estimated using test misclassification bias adjustment methodology (Chapter 5.1, 5.2 and 5.3). Second, risk factors associated with PRV apparent herd-level prevalence (Chapter 5.1), and with BoHV-1 apparent animal-level prevalence were investigated (Chapter 6.1, and 6.2). To evaluate the impact of diagnostic uncertainty on the interpretation of the latter BoHV-1 risk assessment, a sensitivity analysis was implemented (Chapter 6.3). A last aim of the second survey was to create a bovine serum bank for further survey research objectives.

REFERENCES

CHAPTER 3:

ELEMENTS OF SURVEY DESIGN AND ANALYSIS: BASIC NOTIONS
ELEMENTS OF SURVEY DESIGN AND ANALYSIS: BASIC NOTIONS

The main goal of this chapter is to briefly review the basic methodology of surveys conducted with the aim of obtaining information about the occurrence of infections in animal populations. Only a selection of the existing literature was performed. All the principles and concepts discussed are covered in numerous textbooks on survey methodology (Cochran, 1977; Foreman, 1991; Fowler, Jr., 1993; Litwin, 1995; Levy and Lemeshow, 1999). It must be noted that considerable confusion exists with regard to terminology and an attempt has been made to develop a uniform framework of definitions. Levy and Lemeshow (1999) were taken as standard reference, if the different sources were in patent contradiction. A reference epidemiological textbook was Rothman (1986).

Before the start of the surveys that are covered by this thesis, only little information existed about the analysis and interpretation of surveys regarding veterinary infections. Aspects of survey analysis are developed throughout the thesis, and are highlighted in chapter 7. Therefore, in the present chapter, mainly relevant design aspects get the focus.

SAMPLE SURVEYS

Politicians, marketing departments of companies, and many others, constantly need information on characteristics of populations. Survey research methodology can also be applied to obtain information about a health characteristic in an animal population. The investigation of a subset instead of the complete population is attractive because it is less labour-intensive, quicker and less costly (Noordhuizen et al., 2001). Also, the laboratory capacity needed is reduced. Moreover, if a survey is properly designed and conducted, it can provide valid and precise results (Thrusfield, 1997). More formally, a sample survey may be defined as a study involving a sample (subset) of individuals (elements) selected from a larger, well-delineated population.

SURVEY OBJECTIVES

Surveys belong to a larger class of non-experimental studies generally given the name ‘observational studies’ because the researcher observes events without intervention (experimentation). In observational research, the researcher has no control over assignment of subjects to groups. Most sample surveys can be put in the class of observational studies known as ‘cross-sectional studies’. The essence of a cross-sectional study is the contemporaneous classification of individuals with respect to both exposure and disease (Rothman, 1986). Their
sampling protocol used implies that individuals are sampled without considering health and exposure status beforehand. Individuals are tested for the presence of the disease under study and for the exposure status at the same time. Technically, cross-sectional studies thus provide a snapshot of events at a particular time. The point of time may range from an instant (‘at the time of sampling’) to longer periods (such as ‘during the past year’), although all are treated as static, point-in-time events. This type of study is often known as a descriptive survey. Its main objective is that of estimating the mean level of some characteristics in a defined population (Levy and Lemeshow, 1999), including a measure of the precision for those estimates.

A secondary objective of surveys often is the measurement of the relationship between two or more variables measured at the same point in time. The main focus of such research is to test a hypothesis (Noordhuizen et al., 2001) concerning the association between a set of independent variables (e.g., exposure to suspected causal risk factors) and a dependent variable (e.g., disease prevalence). These are analytical surveys. In veterinary epidemiology, analytical surveys are frequently used to screen for potential risk factors. Risk factors are factors that are associated with an increased likelihood of an event occurring (e.g. disease). But, a significant statistical association does not automatically signify causality. When the independent variable is a non-permanent factor, one can never be sure whether the factor status is influencing disease occurrence or vice versa (Martin et al., 1987). In these cases, cross-sectional studies are less suitable for identifying relationships between variable or transient exposures and the disease of interest, because the current exposure information might be too recent to be etiologically meaningful. Also, the exposure was to be started before the onset of the disease. On the contrary, when exposures have been constant throughout the individual’s life (like blood group, breed, sex and sometimes housing system), association may signify causality because such factors cannot be altered by the passage of time or by the presence or absence of disease (Martin et al., 1987). For such subject characteristics, current information is as useful as any (Rothman, 1986).

In cases where the cause-effect relationship is not clear, the validity of the presumed temporal relationships must be based on our understanding of the mechanisms of disease, i.e. biological plausibility. Epidemiologists must resist an automatic temptation to regard all statistically significant associations between factors and disease as causal associations because, for various reasons, they may not be. The same remarks are of course valid for absence of association. This illustrates the mutual dependency of epidemiological and mechanistic (or basic) research. Epidemiological studies cannot prove with certainty that a cause-effect relationship exists, only that an association exists. Research on mechanisms of disease provides the biological basis for believing that associations are, in fact, causal. Likewise, information derived from research on mechanisms of disease cannot assume that a particular phenomenon behaves in nature as it does in the laboratory. For this, epidemiological studies must be conducted (Smith, 1995).
Thus, not all factors that are statistically associated with a disease are necessarily causal. Risk factors therefore may be either causal or non-causal. Some authors reserve ‘risk factor’ exclusively for causal factors, and use ‘risk indicator’ or ‘risk marker’ to describe both causally and non-causally associated factors (Last, 1988). Knowledge of risk factors is useful in identifying populations towards which veterinary attention should be directed.

THE POPULATION AND THE SAMPLE

The population (target population) is the entire set of individuals to which findings of the survey are to be extrapolated. The description of the population at risk of developing the disease is an essential concept in epidemiological thinking, as nicely phrased by Stewart (1970): “Epidemiology is the science of denominators and, as such, is the rational counterbalance of clinical training which tends to be preoccupied with numerators. The introduction of the denominator was about as important in medical thinking as the invention of the wheel, and equally revolutionary. The denominator is our foundation for rates and hence for our sense of proportion and priorities. Denominators are dull but indispensable whenever or wherever we try to draw conclusions about distributions, differences, and dividends, fiscally, socially, or medically.”

The objectives of a survey include estimation of certain values of the distribution of a specified variable (characteristic) in a population. These values for a population are called parameters, and for a given population a parameter is constant and almost always unknown. When the characteristic being measured represents the presence or absence of some dichotomous attribute (e.g. serological status regarding an infection), notably \( X_i = 1 \) (specified attribute present in element \( i \)) and \( X_i = 0 \) (the specified attribute absent in element \( i \)), the population total is:

\[
X = \sum_{i=1}^{N} X_i.
\]

It represents the total number or elements having the specified attribute. The population mean is the proportion of elementary units in the population having the attribute:

\[
P_X = \frac{X}{N}
\]

where, in the case of status of animals regarding an infection:

- \( P_X \) = the population proportion of infected animals
- \( X \) = the total number of infected animals in the population
Basic notions of survey design and analysis

\[ N = \text{the number of animals in the population} \]

In a sample survey these characteristics of interest are observed or measured on each of the sampled elements. In veterinary surveys these elements are usually individual animals, but they can also be individual lots of animal feed. The measurements are then aggregated over all individuals in the sample to obtain summary statistics (e.g., means, proportions, totals) for the sample. It is from the summary statistics of the sample that extrapolations can be made concerning the entire population. Examples of sample surveys in veterinary epidemiology are numerous. There are the examinations of a sample of animals regarding their serological status to specific infections, which are called serosurveys. When all the individuals in a population are selected for measurement, the study is called a census (screening).

In a sample survey, the major statistical components are referred to as the sample design and include both the sampling plan and the estimation procedures. The sampling plan is the methodology used for selecting the sample from the population. Before a sample is taken, constructing a list must identify members of the population. This list from which the sample can be selected is called the sampling frame. It should have the property that every element in the population has some chance of being selected in the sample by whatever method is used to select elements from the sampling frame. Each member of the sampling frame is a sampling unit. The estimation procedures are the algorithms or formulas used for obtaining estimates of population values from the sample data and for estimating the reliability of these population estimates.

THE POPULATION PARAMETER PREVALENCE

When a dichotomous characteristic represents the presence or absence of clinical disease or infection, the variable of interest is the prevalence (P), which is a population parameter. In this context, it is useful to think of each individual as being in one of two ‘states’: disease-free or diseased. The prevalence is a static measure describing the number of instances of disease (cases) or related attributes (e.g. infection or presence of antibodies) in a known population, at a designated time, without distinction between old and new cases. At the individual-animal level the probability to be diseased is:

\[ P = \Pr(D +) \]

Thus, the primary objectives of a sample survey are to take a sample from a well-defined population and to estimate population parameters from that sample. The estimation of the population parameter prevalence is the aim of descriptive surveys. The standard for presenting the
results of such a diagnostic evaluation study is a 2x2 contingency table where TP (true positives), FP (false positives), FN (false negatives) and TN (true negatives) represent observed frequencies of the four possible decision fractions (Table 1).

\[
\begin{array}{c|cc|c}
\text{True disease status} & \text{Yes} & \text{No} & \text{Total} \\
\hline
\text{Test} + & \text{TP} & \text{FP} & \text{TP + FP} \\
- & \text{FN} & \text{TN} & \text{FN + TN} \\
\hline
\text{Total} & \text{TP + FN} & \text{FP + TN} & \text{N}
\end{array}
\]

The prevalence can be estimated from the data from such a 2x2 table as:

\[
\hat{p} = \frac{(\text{TP + FN})}{\text{N}}
\]

The fraction (TP+FN)/N would be denoted as \( \hat{p} \) to clarify that this sample estimate is not the same as the population parameter.

**Animal-level, within-herd, and herd-level prevalence**

Individual (elementary) units are the usual analytic currency of epidemiology (Susser, 1994). Although veterinary epidemiological research is conducted at the level of the population, the sampling units of the population may be animals, herds, farms, regions, or countries (Noordhuizen et al., 2001). Prevalence may be calculated in relation to these different units (Thrusfield, 1997). Since there are two (or more) levels of sampling, we define also two (or more) levels of prevalence (Donald, 1993). The following prevalence parameters can be defined. First, the animal prevalence (prevalence) is the proportion of diseased animals. Second, the within-herd prevalence is the proportion of diseased animals within an infected herd. If more than one herd is considered, and infected, the within-herd prevalence can be summarized as a weighted (according to herd size) average or median of the individual within-herd prevalence. Third, the herd prevalence is the proportion of herds detected as being infected.

As opposed to extensive stock breeding systems where animal populations are considered to be homogeneously mixed, animals in intensive husbandry systems are kept in farms or herds (clusters) and are heterogeneously mixed. In the latter breeding system, the infection prevalence
may be quite different between herds, since diseases have a strong tendency to focus in clustered populations. Therefore, it is the herd that is of epidemiological importance in terms of the transmission and maintenance of infection and therefore of disease control and eradication (Thrusfield, 1997; Toma et al., 2001). Thus, the proportion of diseased herds must be estimated to take into account the possible clustering of disease in the population (Donald, 1993). On the other hand, estimating only herd prevalence will not reflect the level of disease in the global population of animals. Thus the animal prevalence has also to be estimated.

In literature, terminology regarding these prevalence parameters is not uniform. For example, in a PRV risk factors study of Maes et al. (1999), the herd-level prevalence matches the above given within-herd prevalence definition. The cluster prevalence parameter described by Thrusfield (1997), p. 184, matches the within-herd prevalence definition. Evers and Nauta (2001) model an animal-level prevalence parameter within an infected group. This definition matches the above given within-herd prevalence.

**ACCURACY OF DIFFERENT SAMPLING PLANS**

Every epidemiological study should be viewed as a measurement exercise. Its overall goal is accuracy in measurement, i.e. estimating the value of the parameter that is the object of measurement with little error (Rothman, 1986). The accuracy of an estimator refers to how far away a particular value of the estimate is, on average, from the true value of the parameter being measured. It is generally evaluated on the basis of its mean square error (MSE). The smaller the MSE of an estimate, the greater is its accuracy.

Lack of accuracy in estimation of population values are due to errors arising from both sampling design and measurement problems. Both sources of error may be classified as either random or systematic. The principles of survey design emerge from consideration of approaches to reducing both random and systematic errors. Any interpretation of survey findings should take both types of errors into consideration (Levy and Lemeshow, 1999).

**Random error**

The reliability (lack of random error, lack of variance) of an estimated population characteristic refers to how reproducible the estimator is over repetitions of the process yielding the estimator. It depends on how well the measurements were made (quality of the data). If we assume that there is no measurement error in the survey, then the reliability of an estimator can be stated in terms of its sample variance or, equivalently, its standard error, which is the standard deviation of the sampling distribution. The sampling distribution is the distribution of sample
estimates that would be formed if an infinite number of samples of a given size were drawn. The smaller the standard error of an estimator, the greater is its reliability. Appreciation of the variance (spread, dispersion, noise) is one of the most important concepts in statistics.

Random error can be reduced (controlled) by increasing the sample size. More samples means more information, and thus a higher precision: it increases the statistical power of the study. To allow calculation of the sample size, basically three important choices have to be made: the expected (a priori) prevalence of disease, the allowable error in estimate of prevalence, and a confidence level. For further information, the reader is referred to the textbooks referenced in the beginning of this chapter.

A major reason for using probability-sampling methods is to allow the use of a variety of statistical tools to estimate the reliability (precision) of sample estimates. That is, if our sampling plan uses probability sampling, mathematical insight can be gained into the confidence we may have in an estimate. All statistical analyses of data are based on probability models, even though the models may not be explicit. A probability sample has the characteristic that every element in the population has a known probability between zero and one (both limits not included) of being included in the sample. Non-probability sampling (subjective sampling), on the other hand, does not have this feature, and the user has no firm method of evaluating the precision of the resulting estimates.

The choice of a sampling strategy rests in part on feasibility and costs, but it also involves the precision of sample estimates, since all different sampling plans need appropriate methods of estimating population characteristics. That is, the design of the sampling plan affects the estimates of sampling error for a sample of a given size.

**Simple random sampling**

Simple random samples are the cornerstones of sample surveys. Using this sampling scheme, every subject has a known probability between zero and one of being included in the sample. The term 'simple' in a simple random sample indicates that the selection probability is equal for all subjects. Operationally, drawing a simple random sample requires an up-to-date numbered list of the animals in the population. A random sample may be selected by numbering each animal in the population (such as by ear-tag numbers). Then a computer, a table of random numbers, or some other generator of random numbers would be used to produce the different numbers in the same range. The animals corresponding to the numbers chosen would constitute a simple random sample of that population.

The most common kind of mean calculated from a sample survey is probably a proportion, that is, the percentage of a sample that has a certain characteristic or gives a certain response (e.g.
prevalence of disease). Because \( \frac{p(1-p)}{n} \) is the variance of a proportion \( p \), the SE of a proportion \( p \) is:

\[
SE = \sqrt{\frac{p(1-p)}{n}}
\]

where:
- \( p \) = the sample proportion having a certain characteristic
- \( n \) = sample size

This formula is based on the binomial law, and is valid when the population is considered as infinite or when the sample fraction \( f = \frac{n}{N} \) does not exceed 0.05. When using the binomial approximation one further assumes that \( N \) remains almost constant, as does the probability to select a diseased element from that population: \( P = \Pr(D+) \). In this context we can define the population parameter prevalence as the mean of binomial sampling distribution, which is the same as the Bernoulli-trial probability \( p \).

When the population is of limited size, or when the sample fraction \( f = \frac{n}{N} \) exceeds 0.05, the population size can no longer be considered as constant. Drawing and testing \( n \) animals from such a population no longer constitutes \( n \) independent trials: when selecting the first animal, the probability of drawing a diseased animal is:

\[
\frac{d}{N}
\]

where:
- \( d \) = the number of diseased animals in the population (\( d = P \times N \))

But with each diseased element drawn, \( d \) is decreased by one. \( N \) also decreases with every animal drawn (Cameron and Baldock, 1998). As a consequence, the probability to select a diseased animal may vary, which makes the binomial approximation no more to be allowed. The hypergeometric distribution will be used to describe the number of diseased elements in such a sample.

**Systematic sampling**

Unless a list is short, has all units pre-numbered, or is computerized so that it can be numbered easily, drawing a simple random sample as described above can be laborious. With most
lists, there is a way to use a variation called systematic sampling that will have precision equivalent to a simple random sample and will be mechanically easier to create. When drawing a systematic sample from a list, the researcher first determines the number of entries on the list and the number of elements from the list that are to be selected. Dividing the latter by the former will produce a fraction. If a sample of $l/n$ of the population were required, we would start at a randomly selected animal in the first $n$ animals, and thereafter sample every $n^{th}$ animal. Care must be taken in systematic sampling that, if cyclic fluctuations exist in the population, the sample interval does not coincide with the fluctuation interval.

**Stratified sampling**

When a simple random sample is drawn, each new selection is independent of any selections that came before. As a result of this process, any of the characteristics of the sample may, by chance, differ somewhat from the population from which it is drawn. This could be the case when the sample of animals to be selected is very small in comparison with the total population. Then it is quite likely that a simple random sample selected from the total population, could, by chance, severely under-represent some herds.

It is not uncommon, however, for at least a few characteristics of a population to be identifiable at the time of sampling. When that is the case, and there are stratum specific lists, there is the possibility of structuring the sampling process to reduce the normal sampling variation, thereby producing a sample that is more likely to reflect the total population than a simple random sample. The process by which this is done is called stratification. Stratified sampling ensures that each stratum (group) in the population is adequately represented. The variance of stratified samples is minimal and smaller compared to simple random samples. But stratification only increases the precision of estimates of variables that are related to the stratification variables. However, the analysis of the results is more complex. If a number of samples in every stratum is taken that is proportional to the total sample size, we have a proportional allocation scheme.

**Cluster sampling and multi-stage sample**

When there is no adequate list of the individuals in a population or when the cost of random sampling is prohibitive, a strategy is needed for linking the population members to some kind of grouping for purposes of sampling. In other words, in absence of a direct sampling source at the level of the individuals, clusters (sources, listing units, enumeration units, groupings, aggregates, composite sampling units) are chosen at random or systematically, and sampled as a first stage. Thus, initially, no control is exercised over membership of each group. After the selection of the
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groups, lists are made of individual members of the selected groups, with possibly a further selection from the created list at the second (or later) stage of sampling. This particular sampling design specifying that the sampling be performed in two or three stages is called a *multistage sampling design*. The sampling units for the first stage are generally called *primary sampling units*.

For example, in a pig disease sample survey, it is conceivable that a list of all pigs herds is available or at least can be constructed without great difficulty or expense. If this list is available, a sample of pig herds can be drawn, and those pigs in the pig herds are taken as the sample elementary units. The pig herds are the enumeration units. These units can be geographic, temporal, or spatial in nature (Levy and Lemeshow, 1999). A random sample of farms on which all relevant animals are tested would be a one-stage cluster sample.

In intensive animal husbandry the animal population is clustered, since animals are kept in farms or herds (clusters), rather than on an individual basis. Sub-clusters can be pens, compartments, or flocks. In clustered populations disease agents are generally not evenly distributed throughout the population (Rothman, 1990), but have a strong tendency to focus. Susceptibility of animals may also differ because of possible intra-cluster correlating factors or variability of the influence of risk factors between clusters (Donald, 1993). Animals within such clusters share common characteristics such as nutrition, housing, and exposure (e.g. contacts) to infectious agents. Therefore, animals within clusters can be considered as a homogeneously mixed population. Exposure to infectious agents probably results in a more homogeneous serological status of animals within clusters. A measure for agreement in status between animals within a cluster is given by the intra-cluster correlation coefficient (Donald and Donner, 1987). Indeed, infection incidence within a cluster is usually a positive feedback system: compared to other population members, animals within clusters have a higher chance to become infected once the infection is introduced in the herd. This interrelatedness of incidence and prevalence is the very property that defines infectious diseases (Koopman et al., 1991; Susser, 1994; De Jong, 1995). Consequently it cannot be assumed that animals within clusters are independent units (Schukken et al., 1991; Elbers and Schukken, 1995).

The within-cluster correlation of responses induces extra variation between clusters (heterogeneity, over-dispersion). This extra variation (so-called ‘extra-binomial variation’) must be taken into account in statistical analyses. For classical statistical tests, which assume that data have been obtained by simple random sampling, this extra variation is considered a nuisance.

**Systematic error**

The *validity* of an estimated population characteristic refers to how the mean of the estimator over repetitions of the process yielding the estimate, differs from the true value of the
parameter being estimated. It depends on how well the sample was chosen. If we assume that there is no measurement error, the validity of an estimator can be evaluated by examining the bias of the estimator. The smaller the bias, the greater is the validity. An unbiased estimator yields, on average, estimates without systematic errors. Bias is of major concern in observational studies. It is the consequence of flawed research design, leading to selection of a non-representative sample of the population and to a systematically wrong (biased) estimate.

All sources of bias can be eliminated (controlled) through randomisation, except accidental bias. Thus, without proper randomisation, we cannot validly extrapolate from the sample to make inferences about the population it is supposed to represent. Validity has two components: external and internal validity.

**External validity**

External validity (generalisibility) is the validity of the inferences as they pertain to subjects outside the population. The process of generalising beyond a set of observations requires a judgment about what features of the observations may be extrapolated. Such judgments require an understanding of which conditions are relevant and which are irrelevant to the generalisation. Reichenbach (1965, referred to by Rothman, 1986) nicely described the essence of scientific generalisation:

“The essence of knowledge is generalisation. That rubbing wood in a certain way can produce fire is a knowledge derived by generalisation from individual experiences; the statement means that rubbing wood in this way will always produce fire. The art of discovery is therefore the art of correct generalisation. What is irrelevant, such as the particular shape or size of the piece of wood used, is to be excluded from the generalisation; what is relevant, for example, the dryness of the wood, is to be included in it. The meaning of the term relevant can thus be defined: that is relevant which must be mentioned for the generalisation to be valid. The separation of relevant from irrelevant factors is the beginning of knowledge”.

**Internal validity**

Internal validity is the validity of the inferences drawn as they pertain to the actual population members. It is obtained by good survey design (e.g. randomization). Dozens of possible types of biases can detract from internal validity (Sackett, 1979). The distinction among these biases is occasionally difficult to make, but three general types are classically identified: selection bias, information bias, and confounding. A useful practical distinction between confounding and other biases is to consider a bias confounding if it can be controlled in the data analysis. A fourth
systematic error, test misclassification bias that is focussed on in this thesis, is reviewed in a separate section.

Selection bias

The bias of the estimation of effect in this case is the result of inadequate selection of the sampling units. The result is non-representation of the sample with regard to the (target) population. For example, non-response can cause this bias. More important than reporting a ‘response percentage’ is the documentation of the non-response. Is it related to administrative problems or are there disease reasons?

Information bias

Bias in evaluating an effect can occur from errors in obtaining the needed information from groups to be compared. Information bias can occur whenever there are errors in the classification of subjects, but the consequences of the bias are different depending on whether the classification error on one axis of classification (either exposure or disease) is independent of the classification on the other axis. The existence of classification errors that are not independent of the other axis is referred to as differential misclassification, whereas the existence of classification errors either exposure or disease that are independent of the other axis is considered non-differential misclassification.

Information bias is evitable by correct study design, namely by ensuring that every subject has the same screening test. However, tests are not perfect, and these test misclassification biases have also to be appreciated (see further).

Confounding

The concept of confounding is a central one in epidemiology. Although confounding also occurs in experimental research, it is a considerably more important issue in non-experimental research (Rothman, 1986). Confounding is the effect of an extraneous variable that can wholly or partly account for an apparent association between variables (Thrusfield, 1997). Confounding can produce a spurious association between study variables, or can mask a real association. As a result, the estimation of the effect of studied risk factors is biased. A variable that confounds is called a confounding variable or confounder. Such a variable is distributed non-randomly, meaning that it is positively or negatively correlated with the explanatory and response variables that are being studied. In other words: the exposed and unexposed populations differ in a systematic way with respect to relevant covariates. A confounding variable must be:

- a risk factor for the disease. The potential confounding variable need not be an actual cause of the disease, but serves as markers for the cause of the disease or the diagnosis of the disease.
Besides the association between the potential confounder and the exposure that may be a cause of the disease, a confounding variable should be a risk factor for disease, even among non-exposed individuals under study, even after controlling for other known confounders.

- associated with the exposure under study in the population from which the cases derive.
- not an intermediate step in the causal path between the exposure and the disease. This criterion requires information outside the data. The investigator must decide whether the causal mechanism that might follow from exposure to disease would include the potentially confounding factor as an intermediate step. If so, the variable is not a confounder.

Confounding is a nuisance and must be controlled (removed). Either we take it into account while designing the study (e.g. randomisation, matching, blocking), or we adjust for it in the analysis, but then we need to have collected the data.

The association measure (e.g. the odds ratio) relating exposure to disease does not alter when one considers different values of a confounding variable. This is exactly the reason why we are interested in confounders. On the contrary, when the measure of association between a variable A varies with the level of another variable B, then variable B is described as an effect modifier. In statistics, this effect is called interaction. Effect modification relates to a biological phenomenon. It has to do with details of the relation of interest and therefore effect modification is an aspect of the study object that has to be fully described.

**TEST MISCLASSIFICATION BIAS (BIASED DISEASE STATUS ASSESSMENT)**

For infectious diseases, diagnostic tests are used frequently to determine the disease state. Indeed, many common viral and bacterial infections induce antibodies that can be used as markers of previous infection. Many of these correlate well with protective immunity and are life-long. Therefore, statements will often refer to the serological rather than to the unknown true state of animals. One of the major challenges in the interpretation of these serodiagnostic data is the need to ‘adjust’ test results for misclassification. In the veterinary literature, the uncertainty associated with the diagnosis (test misclassification bias) has received only little coverage. Indeed, in the evaluation of diagnostic tests for infectious diseases in the biomedical literature, primarily laboratory (analytical) aspects of test validation get the focus, as exemplified in an issue of the O.I.E. Scientific and Technical Review (Anonymous, 1998). But once an analytically optimal test is available, it should undergo field (epidemiological) evaluation to demonstrate its relative merits in terms of its diagnostic performance characteristics (Jacobson, 1998). Two of these attributes, the diagnostic sensitivity (Se) and specificity (Sp) are classically defined as intrinsic characteristics of the test (Thrusfield, 1986; Jacobson, 1991; Martin et al., 1992). The diagnostic sensitivity and specificity are usually determined for a specific test and a specific disease or infection. They can
be determined from data that describe the results of the test on one population, all of whom are known to be disease free and a second population, all of whom are known to have the disease of interest. The existence or non-existence of the particular disease in the individuals of the two populations is based on a ‘definitive’ or ‘gold standard’ method, which is error free. If an individual has a specific disease, *diagnostic sensitivity* indicates the probability that the patient will be positive for the particular test:

\[
Se = \Pr(T | D +)
\]

It can be estimated as the relative frequency of positive test results in infected individuals (see Table 1):

\[
\hat{Se} = \frac{TP}{TP + FN}
\]

If an individual is free of a specific disease, *diagnostic specificity* indicates the probability that the subject will be negative for a certain test:

\[
Sp = \Pr(T | D -)
\]

It can be estimated as the relative frequency of negative test results in non-infected individuals (see Table 1):

\[
\hat{Sp} = \frac{TN}{FP + TN}
\]

Because of limitations in current diagnostic test technology, it has been impossible to devise the perfect test, which would predict the infection status of subjects with total accuracy. Consequently, nearly all tests have non-perfect Se and Sp. The false negative rate for a test equals 1 minus the Se, whereas the false positive rate for a test is 1 minus the Sp. There are numerous reasons for lack of Se leading to false negative test results: natural or induced tolerance (animals that do not produce antibodies in contact with a specific antigen), improper timing (sampling during the serological ‘silent’ period after infection, or a period close to parturition), non-specific inhibitors, blocking antibodies, immunosuppression, laboratory errors, etc… However, many more biological variables may contribute to lack of Sp leading to false positive results (cross-reactive
antibodies to many other agents, non-specific reactions, true exposures unrelated to the present disease status – maternal antibodies, vaccination, previous exposure -, laboratory errors, etc…) than to false negative results, since animals generally develop antibody responses towards for most but not all pathogens, and thus are not falsely negative (Schrijver and Kramps, 1998).

**Test misclassification bias adjustment at the animal-level**

Test misclassification bias should be adjusted for. Using multiple tests it is possible, under certain conditions, to estimate error parameters such as Se, Sp, relative risk, or predictive value, even though no gold standard is available. The parameter estimates are obtained by modelling the data, using maximum likelihood, with or without some constraints. The models recognize that the true classification of an individual is unknown, and so are sometimes referred to as ‘latent class’ models. The latent class approach provides a unified framework for various methods, characterising each by the number of (sub-) populations that are sampled, and the number of observations (tests) made on each individual. Both numbers together determine the number of cross-classifications into which the data are grouped, and hence the number of degrees of freedom that are available for parameter estimation. Thus, the number of degrees of freedom is implied by the sampling design. Data sets with three or more observations (tests) per individual lead directly to estimates of the misclassification rates, subject to some simple assumptions (Walter and Irwig, 1988).

If there are too many parameters to be estimated from the available degrees of freedom (parameter redundancy, over-parameterised model, over-specified model), constraints must be applied to render the model identifiable. For instance one may regard some of the parameters as known constants. This is the case in single-test sample surveys. These designs imply an over-parameterised model if estimation of the (true) prevalence (P) and Se and Sp is intended. Indeed, animals from a single source population were classified by a single test as positive or negative. This is the apparent or measured prevalence (seroprevalence) (AP). The apparent prevalence denotes the probability of an animal to have a positive test result:

\[ AP = Pr(T +) \]

This probability can be estimated from the data of the 2x2 table (Table 1) as:

\[ AP = \frac{TP + FP}{N} \]
The AP depends on the Se and the Sp of the test used in the survey, and on the true prevalence of the infections in the population. This dependence can be deduced from the cross-classification in Table 2 that is constructed starting with the probabilities that are in bold. With N equal to 1, the proportion of diseased is equal to the prevalence, the proportion that is truly diseased and test-positive is equal to the product of the Se and the true prevalence and the proportion that is truly disease-free and test-negative equals the product of the Sp and the proportion of disease-free animals. The other proportions like the apparent prevalence can be obtained by summation or subtraction.

Table 2 Relation between diagnostic sensitivity, specificity and prevalence

<table>
<thead>
<tr>
<th>Truly diseased</th>
<th>Yes</th>
<th>No</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test result</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Se^a * P</td>
<td>(1 - Sp) * (1 - P)</td>
<td>Se * P + (1 - Sp) * (1 - P)</td>
<td></td>
</tr>
<tr>
<td>- (1 - Se) * P</td>
<td>Sp^b * (1 - P)</td>
<td>(1 - Se) * P + Sp * (1 - P)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>P^c</td>
<td>1 - P</td>
<td>1</td>
</tr>
</tbody>
</table>

^a Diagnostic sensitivity
^b Diagnostic specificity
^c Prevalence

Regarding the total number of individuals observed as fixed, the number of test positives determines the number of test negatives, and vice versa; thus only 1 degree of freedom is available, whereas there are three parameters to be estimated (the prevalence, the Se and Sp). Thus, two constraints must be applied to render the model identifiable, if parameter estimation is intended. A common option in standard statistical practice is to impose the two constraints by regarding the Se and the Sp as known, and then to estimate the prevalence. Assuming these constraints it is simple to correct the apparent prevalence for the imperfect Se and Sp:

\[ P = \frac{AP + Sp - 1}{Se + Sp - 1} \] (Marchevsky, 1974; Rogan and Gladen, 1978)

From Table 2 also predictive values can be estimated. A predictive value is a population characteristic because it depends on the prevalence of disease. It is useful when one is interested in the likelihood of a correct classification of individual subjects in a specified population. The
positive predictive value ($PV^+$) is the conditional (post-test) probability that an individual tested as positive is actually truly diseased:

$$PV^+ = Pr(D \mid T^+)$$

Or, $PV^+$ is the fraction of positive test results that are truly diseased individuals. The positive predictive value is established using (Table 2):

$$PV^+ = \frac{Pr(T^+ \mid D)}{Pr(T^+)}$$

( Rogan and Gladen, 1978)

If a disease has a low prevalence, the $PV^+$ will largely depend on the Sp. The negative predictive value ($PV^-$) is the conditional (post-test) probability that an individual tested as negative is actually truly non-diseased:

$$PV^- = Pr(D \mid T^-)$$

Or, $PV^-$ is the fraction of negative test results that are truly non-diseased individuals. The negative predictive value is established using (Table 2):

$$PV^- = \frac{Pr(T^- \mid D)}{Pr(T^-)}$$

( Rogan and Gladen, 1978)

Sensitivity and specificity change as the decision level (test cut-off level) varies. The more analytically sensitive an assay is, the less specific it becomes. For example, if detection of every true-positive is required, then specificity will be lowered because of an increased number of false-positive results. Similarly, when high specificity is required, tests become less sensitive. The lower sensitivity is attributable to a high incidence of false-negative results (Golden, 1991).

**Test misclassification bias adjustment at the herd-level**

As mentioned no standard sampling design exists that accommodates the three different typical objectives of veterinary surveys, i.e. estimation of animal-, herd- and within-herd prevalence. In the following we concentrate on the estimation of the true herd prevalence (aggregate testing, herd-level interpretation of tests, herd testing). Estimation of the herd true
prevalence requires first an estimation of the herd-level sensitivity (Hse) and the herd-level specificity (HSp).

**Herd sensitivity**

First, a herd test has to be defined, which is a test at the aggregate level. Herd will be classified as test positive or negative, based on decision rules to summarise the individual tests (taking account of the misclassification bias of the survey test) and on rules concerning the uncertainty due to sampling of animals from the herd. A threshold value (c) is required that denotes the maximum number of test positive animals that are accepted for the diagnosis of ‘no infection’ on the herd-level. Classically, this value for c is considered to be null. In this situation, a single test positive animal would result in a classification of the herd as infected. This null threshold is useful when tests are very specific. As to (Martin et al., 1992; Jordan and McEwen, 1998) a herd is classified diseased if the number of positive results in the sample equals or exceeds c and it is classified non-diseased if this number is less than c (e.g. c=1).

Abstraction made of the herds, one can deduce from Table 2 that the probability of a positive test result (apparent animal prevalence) is given as:

\[ AP = \Pr(T+) = Se \cdot P + (1 - Sp)(1 - P) \]

If none of the animals in the sample is infected (P=0) the probability of a (false-) positive result is \( AP = 1 - Sp \). If all animals in the sample are infected (P=1) the probability of a (true-) positive result was \( AP = Se \).

When testing a herd to determine its health status, the number of reactors gives also the within-herd apparent prevalence.

\[ WHAP = \Pr(T+) = Se \cdot WHP + (1 - Sp)(1 - WHP) \]

where:

- \( WHAP \) = the within-herd apparent prevalence
- \( WHP \) = the within-herd true prevalence

At this point it is important to mention that the WHAP increases directly with Se, but decreases as Sp increases. It depends also on sample size.

The HSe is defined as the probability of a truly diseased herd to be classified as diseased by the test. Thus, HSe is the proportion of diseased herds in which the number of reactors meets or
exceeds the specified cut-point number of reactors (Martin et al., 1992). If one assumes that some animals of the herd are truly infected (P>0), then the Hse, in case c=1, is the probability of finding more than 1 animal test positive and is given as:

\[ Hse = 1 - (1 - AP)^n \]

**Herd specificity**

The same deduction can be made for test negative results. From Table 2 it follows that the probability of a negative test result is given as:

\[ 1 - AP = Pr(T^-) = (1 - Se)P + Sp(1 - P) \]

If none of the animals in the sample is infected (P=0) the probability of a (true-) negative result is 1-AP=Sp. If all animals in the sample are infected (P=1) the probability of a (false-) negative result is 1-AP=1-Se. A herd is usually considered as non-diseased if all animals in that herd are disease free. The HSp is the probability of a truly non-diseased herd to be classified as non-diseased with the test (only negative results). But again if the health status is defined in relation with cut-point number of reactors, HSp is also the proportion of non-diseased herds in which the number of positive reactors is below the specified cut-point (Martin et al., 1992).

If one assumes that no animal of the herd is truly infected (P=0; 1-AP)=Sp), then the HSp, in case c=1, is the probability of finding less than 1 animal test positive, and is given as:

\[ HSp = (Sp)^n \]

**Herd prevalence**

The herd true prevalence is calculated using the formula for P at the animal-level in which number of herds replaces the number of animals.

(Stewart, 1970)
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CHAPTER 4:
INFECTIONS WITH PSEUDORABIES VIRUS, BOVINE HERPESVIRUS 1, AND
*Mycobacterium avium* subsp. *paratuberculosis*:
INTRODUCTION
INFECTIONS WITH PSEUDORABIES VIRUS, BOVINE HERPESVIRUS 1, AND MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS: INTRODUCTION

This chapter provides a general introduction into aspects of infection with pseudorabies virus (PRV), bovine herpesvirus 1 (BoHV-1), and Mycobacterium avium subsp. paratuberculosis (Map). The most important data on their distribution, pathogenesis, immunity, transmission, diagnosis, control and eradication are highlighted, since these aspects are important for a good understanding of this thesis.

INFECTION WITH PSEUDORABIES VIRUS

Pseudorabies or Aujeszky’s disease is a serious illness of pigs causing significant economic losses in pig industry. The causative agent is a member of the alpha herpesvirus subfamily of the herpesviruses (reviewed by Roizman, 1996), and is designated Suid Herpesvirus 1 (SHV-1; (Roizman et al., 1992)). Synonyms are pseudorabies virus (PRV) and Aujeszky’s disease virus.

Distribution

Since 1960 pseudorabies has emerged as an important disease in most areas of the world where swine are raised. One commonly cited reason for the apparent increase in disease severity, prevalence, and worldwide distribution is the dramatic change in swine management systems during the past 30 years. The advent of total confinement systems with large numbers of animals and the incorporation of continuous farrowing has created an environment that facilitates the maintenance and spread of a virus within herds (Kluge et al., 1992). In Belgium, sporadic outbreaks due to PRV in pigs were reported before 1975 (Pensaert et al., 1987). Then, a severe epidemic was recorded, after which the number of outbreaks decreased again. In 1983-1984, a second peak of cases was diagnosed. Thereafter, PRV became endemic in the dense pig population. Maes et al. (1999) organized a regional sample survey in 1994 and estimated the PRV herd seroprevalence in farrow-to-finish herds to be 76 %. At the national level the Belgian PRV prevalence was unknown before the work of this thesis begun.

Pathogenesis and immunity

Under natural conditions, pigs take PRV up by the oronasal route. The primary site of replication of PRV is situated in the respiratory tract, including nasal cavity, tonsils, pharynx and lungs (Sabo et al., 1969; Baskerville, 1973; Miry and Pensaert, 1989). The extent of replication at
the different localisations depends on the amount of virus in the inoculum and on the route of inoculation. The replication leads to virus excretion and infection transmission to non-immune as well as to immune hosts. Next, PRV spreads to distant secondary replication sites via lymph, blood and nerves (Sabo et al., 1969). The viraemia occurs both under a cell-free and cell-associated form (with monocytes as most susceptible for PRV infection) (Nauwynck and Pensaert, 1995). Secondary replication sites are the central nervous system, draining lymph nodes, olfactory bulb, medulla, spleen, kidney and the genital organs. Virus replication at secondary sites is less extensive than in the primary sites and is clearly reduced with the onset of the immune response (Wittmann et al., 1980). Despite the presence of humoral and cellular immunity, virus can be isolated from the tonsils up to 18 days PI (Sabo, 1969) and from nasal swabs up to 13 days PI (McFerran and Dow, 1964; Pensaert et al., 1990; Vannier et al., 1990).

Immunity can be established after a natural infection or after vaccination. The PRV-specific immune response starts from 7 days PI. It includes the effects mediated by PRV-specific cytotoxic T-lymphocytes (Martin and Wardley, 1987; Zuckermann et al., 1990) and PRV-specific antibodies (Sabo et al., 1969). The course of titers of the different antibody isotypes IgM, IgA, IgG1 and IgG2 antibodies in serum, saliva and tears have been studied by means of ELISA (Roda et al., 1987; Kimman et al., 1992). IgM and IgA are detected in all fluids and their titers follow a similar course. IgG1 and IgG2 are detected only in serum. Antibodies of the IgM class are first demonstrated at about 7 days PI and reach maximum titers at about 10 days PI. Antibodies of the IgA class are first detected at about 10 days PI and reach maximum titers at about 13 days PI. This isotype, however, was not detected in all pigs. IgG1 and IgG2 are first found in serum at about 10 and 15 days PI respectively and reach maximum titers at about 19 and 22 days PI respectively. The titers persist for several months to years (Pensaert and Kluge, 1989).

Immunity after PRV-infection induces a short virological but a long clinical protection. Upon re-infection of pigs one month after the primary infection, PRV replicates in a restricted way in the deeper airways (alveoli and alveolar macrophages) but not in the upper airways. A time interval of 3 months between two subsequent PRV-infections resulted in virus replication of both the upper and lower respiratory tract (Sabo and Blaskovic, 1970; Miry and Pensaert, 1989). Protection against PRV after vaccination is less efficient compared to the protection induced by natural infection. Re-infection at one month after vaccination (with an inactivated vaccine parenterally or an attenuated vaccine intranasally) results not only in PRV replication in the lungs, but also in the pharynx, tonsils and nasal cavity (Wittmann et al., 1980; Miry and Pensaert, 1989).

PRV causes different clinical signs due to its broad spectrum of cell tropism. In pigs, there is an age-dependent variation in the severity of the disease and in the clinical symptoms. Clinical signs also depend on the virulence of the virus strain and the infectious dose. Comprehensive
reviews can be found elsewhere (Baskerville et al., 1973; Akkermans, 1976; Wittmann and Rziha, 1989; Kluge et al., 1992).

A hallmark of herpesvirus infection is the lifelong persistence of the virus in the infected organism, which is designated viral latency. During latency, the virus hides intra-cellularly in a disassembled form and is not cleared by the immune system nor detected by conventional virological assay procedures. In alphaherpesviruses, latency is mostly established and maintained in neuronal tissues, notably in the trigeminal ganglion. Latency is a biological property of great epidemiological importance that is shared by all herpesviruses examined to date (Honess and Watson, 1977; Engels and Ackermann, 1996; Fields et al., 1996). The latent viruses (latent persistent infections) represent a long-term reservoir that may play a role upon reactivation. Then, carrier animals, i.e. seemingly healthy animals with an unapparent infection, are able to re-excrete and transmit the virus to non-immune as well as to immune hosts (Engels and Ackermann, 1996). Latency thus allows herpesviruses to persist in a closed herd without introduction of exogenous virus.

Pigs of any age that survive the acute infection phase of PRV thus become latently infected and are considered to be lifelong carriers of potentially infective virus (Rziha et al., 1982). A characteristic pattern of PRV infection in a breeding herd follows a sigmoid curve, showing high prevalence that declines with turnover of the breeding herd, followed by a rise as viral transmission is reinitiated, followed again by a decline in prevalence (Kluge et al., 1992). Latent PRV may be reactivated spontaneously and detected under field conditions or experimentally after a corticoid treatment (van Oirschot and Gielkens, 1984a; van Oirschot and Gielkens, 1984b; Mengeling, 1989; Cowen et al., 1990; Schoenbaum et al., 1990). The responsible triggering mechanism for PRV reactivation and transportation back to the original portal of entry where it is available for re-excretion and transmission to other susceptible hosts, are farrowing, crowding, and transport (Davies and Beran, 1980). The virus is re-excreted even in the presence of antibodies.

**Transmission**

Pigs are the natural host in which the virus circulates (Mettenleiter, 1996). Other mammals, except higher primates, may become infected when they are exposed to high quantities of PRV excreted by PRV-infected pigs or present in organic material coming from PRV-infected pigs. The infection in these animal species has a fatal course. Since infected non-porcine animals usually die without having shed PRV, they are of minor importance for virus transmission to contact animals and are not considered to be virus reservoirs.

Introduction of PRV onto a breeding farm most often occurs by way of virus-excreting stock. However, virus-carrying persons or objects may also be responsible for introducing the virus. PRV infection spreads rapidly in closely confined populations, because large quantities of
Introduction of infections with PRV, BoHV-1 and Map

virus are excreted in respiratory and other secretions of infected animals. The degree of spread depends on the herd immunity status. PRV is usually transmitted among swine within a herd by direct contact from animal to animal (nose-to-nose contact). Also, PRV can be transmitted between animals via the venereal route or by the use of semen in artificial insemination (Philpott, 1993), or by transplacental transmission. Indirect transmission is common by inhalation of aerosolised virus, or drinking of water contaminated with PRV. As already mentioned, carrier animals are able to re-excrete and transmit the virus to non-immune as well as to immune hosts (Engels and Ackermann, 1996).

**Diagnosis**

Mass diagnosis of PRV infection is made indirectly by detecting a serological response in the live animal. Numerous tests have been used to this aim. Up to now, antibodies are most frequently demonstrated by virus neutralisation (serum neutralization test) or enzyme linked immunosorbent assay (ELISA). Serologic testing consistently detects pigs in the latent phase of PRV infection (White et al., 1996). These tests do not allow differentiation between PRV-infected and PRV-vaccinated swine. In contrast, gE-competitive differential ELISA (van Oirschot et al., 1988) (see further) are able to discriminate between antibodies induced by an infection with a PRV field strain and antibodies induced after vaccination with a PRV marker vaccine that lacks gE. (In a competitive ELISA, the test samples are mixed with conjugated virus-specific antibodies, and this mixture is then added to the antigen (Schrijver and Kramps, 1998).)

Virus isolation and detection of viral antigens (e.g. immunofluorescence tests) or identification of PRV genomes by polymerase chain reaction (PCR) are techniques that can identify infected animals, but they are only useful during the acute periods of infection in which viral shedding occurs. Virus isolation can be tempted from living animals’ secretions from affected tissues, e.g. from the nose, eyes, vagina, etc… Virus isolation can also be tempted post-mortem from brain, tonsil, and lung.

**Control - eradication**

An important international event has been the realisation of the European Single Market since 1993. This means that there should be no more restrictions in trade within the European Union (EU) with live animals. Unfortunately, this is not the case, since differences in the animal health status of member states create a problem. Relevant EU laws leave the option for Member States with a high animal health situation to demand additional guarantees in trade in livestock regarding individual epidemic diseases. These additional health guarantees enable these Member States to give their animal populations particular protection from certain epidemic diseases, like pseudorabies. This situation renders trade in live animals and animal products (e.g. embryos or
sperm) with these Member States difficult. Such restrictions are incompatible with the principle of free trade within the EU, and its member states are therefore encouraged to eradicate PRV and BoHV-1. This policy puts high pressure on PRV infected countries that export pigs. These countries may suffer considerable financial losses from restrictive regulations imposed by importing countries or from competition with PRV-free member states.

Eradication of PRV in swine in countries where these infections are highly prevalent, is only economically feasible by first reducing the prevalence, through mass vaccination, eventually followed by test and removal procedures of infected swine. In the past, vaccination was widely practised in endemically infected regions to control clinical episodes of pseudorabies. Apart from reducing clinical signs, vaccination does increase the virus dose needed to establish an infection (Wittmann et al., 1982; Pensaert et al., 1990) and decreases the level and duration of virus excretion after infection (De Leeuw and van Oirschot, 1985). This reduced susceptibility and infectiousness lowers the amount of circulating virus and can diminish the transmission of PRV among pigs and herds under field conditions. However, the use of conventional vaccines interferes with standard serological diagnosis used to assess PRV exposure following vaccination, and with the determination of the true prevalence of infection. Therefore, in PRV eradication programmes, all animals, vaccinated with conventional vaccines, must be regarded as infected. This major hindrance was lifted with the discovery of marker vaccines, which lack a non-essential protein (mostly glycoprotein E, gE). Marker vaccines do not induce antibodies against the deleted protein, whereas wild-type PRV strains do (Mettenleiter et al., 1985; van Oirschot et al., 1996). Subsequently, discriminatory serological tests were developed that detect the presence or absence of anti-gE antibodies in the animal (van Oirschot et al., 1986; van Oirschot et al., 1990). Thus, implementation of vaccination in PRV eradication campaigns became feasible, e.g. in the Netherlands (Stegeman, 1995), because now infected pigs could be traced in populations that have been vaccinated with these vaccines. In Belgium, a PRV control and eradication programme has been implemented since 1993. In the northern part of the country, an intensive mass-vaccination programme (vaccination with marker vaccines) was installed to this end. In 1999, this area-wide vaccination became also obligatory in the southern part of the country.
INFECTION WITH BOVINE HERPESVIRUS 1

Bovine herpesvirus 1 (BoHV-1) also belongs to the alphaherpesviruses. BoHV-1 causes infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis, and infectious pustular balanoposthitis (IPB) (Kahrs, 1977). Cattle are the principal reservoir of BoHV-1.

Distribution

BoHV-1 has a worldwide distribution that parallels the distribution of domestic cattle. It is indigenous on all continents (Straub, 1990). Serological surveys in different countries have demonstrated BoHV-1 antibodies in numerous other ruminant species: goats, swine, and water buffalo (Kahrs, 1977), and in a variety of wild ruminants throughout the world (Gibbs and Rweyemamy, 1977). Clinical outbreaks due to infection with BoHV-1 spread in Belgium since 1971-1972. Consecutively, BoHV-1 became endemic (Van Malderen et al., 1987). Regional BoHV-1 surveys in Belgian mixed and beef herds estimated the herd seroprevalence to be 62% in 1986 (Van Malderen et al., 1987), whereas the individual-animal and the within-herd seroprevalence were estimated to be 64% and 51%, respectively, in 1995 (Lemaire et al., 1998). The national BoHV-1 prevalence in Belgian cattle was unknown before the work of this thesis begun.

Pathogenesis and immunity

Usually cattle get infected with BoHV-1 through airborne transmission. The virus locally replicate to high titers in mucous membranes of the upper respiratory tract and in the tonsils. This replication leads to virus excretion and infection transmission to non-immune as well as to immune hosts. During primary infection, cattle shed virus from nasal and ocular fluids in virus titers up to $10^8$ TCID$_{50}$, approximately during 14 days (Kaashoek et al., 1996). The primary virus replication in the upper respiratory tract causes the first, respiratory symptoms, like rhinitis and tracheitis in BoHV-1 infected cattle (Kahrs, 1977). Progression of the BoHV-1 infection to the lower respiratory tract causes more severe respiratory disease symptoms. But, many infections run a subclinical course (van Oirschot et al., 1993) and go unobserved. The incubation period varies from two to six days depending on the dose, the route of inoculation and the criteria for indicating the onset of disease. Next, systemic spread is achieved by invasion of lymph nodes and lymph vessels, followed by a lymphocyte-associated viraemia (Nyaga and McKercher, 1980; Nauwynck, 1993).

Where natural mating is practised, genital infection occurs. Genital infection can lead to infectious pustular vulvovaginitis (IPV) or infectious pustular balanoposthitis (IPB). Virus is excreted in reproductive secretions.
Babiuk et al. (1996) reviewed the immunology of infection with BoHV-1. An infection with BoHV-1 normally elicits an antibody response and a cell-mediated immune response within 7-10 days (Davies and Carmichael, 1973; Kramps et al., 1994). After infection with BoHV-1 cattle develop antibodies, which may persist for at least 5.5 years (Chow, 1972). The mechanism is thought to be reactivations from latency that cause re-exposure to antigen, which results in increase of antibody titer and partially explains the persistence of humoral antibody for the life of some animals (Kahrs, 1977). Thus, the immune response is presumed to persist for life, although it may fall below the detection limit of some tests. Any animal with antibodies against BoHV-1 must be considered to be a potential carrier and may shed the virus spontaneously (Davies and Duncan, 1974). Most, not all, latently infected cattle possess antibodies (Kahrs, 1977). Seronegative carriers exist (Hage et al., 1998). These are false-negative young stock (calves) infected under maternal immunity. Maternal antibodies are transferred via colostrum to the young calf, which is consequently protected against BoHV-1 - induced disease (Mechor et al., 1987), and which becomes seropositive, although not infected with BoHV-1. As calves can retain maternal antibodies for up to nine months (Brar et al., 1978), it is not easy to predict when a calf with circulating maternal antibodies against BoHV-1 will become seronegative to BoHV-1.

After replication in nasal mucosae, BoHV-1 is transported along axons of nerves and becomes latent in peripheral sensory ganglia, where it remains during the lifetime of the animal (Ackermann et al., 1982; Ackermann and Wyler, 1984). Like PRV in pigs, a hallmark of infection of cattle with BoHV-1 is its lifelong persistence in the infected organism, which is designated viral latency (see before). Cattle of any age that survive the acute infection phase of BoHV-1 become latently infected and are considered to be lifelong carriers of potentially infective virus. Then, carrier animals, i.e. seemingly healthy animals with an unapparent infection, are able to re-excrete and transmit the virus to non-immune as well as to immune hosts (Engels and Ackermann, 1996).

BoHV-1 latency was first defined by Snowdon (1965) who recovered virus intermittently from cattle for up to 578 days. It was further documented by (Pastoret et al., 1979; Ackermann et al., 1982; Homan and Easterday, 1983; Ackermann and Wyler, 1984; van Engelenburg et al., 1995a; van Engelenburg et al., 1995b). Virus reactivation and re-excretion is triggered by several stimuli, including stress that may occur at variable intervals during a lifetime with or without clinical signs of disease (Bitsch, 1973; Pastoret et al., 1982; Thiry et al., 1985; Pastoret et al., 1986; Thiry et al., 1987). A difficult parturition, weaning, social hierarchy, infection with *Dictyocaulus viviparus* (Msolla et al., 1983), but also transport to and introduction into a new herd may be considered stressful enough to reactivate latent BoHV-1. Following the administration of high doses of corticosteroids, latent virus can be reactivated and re-excreted as well (Sheffy and Rodman, 1973; Pastoret et al., 1980; Kaashoek et al., 1996) and transmitted to susceptible animals (Pastoret et al., 1984; Hage et al., 1996). Latent or sub-clinical infections are suggested as the
mechanism of virus persistence within the populations since the 1960s (Davies and Duncan, 1974). In unvaccinated herds, Van Nieuwstadt and Verhoeff (1983) observed an average frequency of BoHV-1 outbreaks of one every 3.5 years, whereas Bosh et al. (1997) found it to be more frequently, on average one every 1.5 years.

Transmission

After a primary infection with BoHV-1, cessation of virus excretion and resolution of clinical disease happens 10 to 14 days PI (Davies and Duncan, 1974; Kaashoek et al., 1996). The virus is perpetuated in populations by direct contact with cattle shedding the virus (Kahrs, 1977), but also aerosols over a short distance have been suggested (Janzen et al., 1980; Wentink G.H. et al., 1993). Indirect infection by other contagious contacts (practitioner, manure, trader) are also possible, e.g. at agricultural shows (Wentink G.H. et al., 1993).

Virus from balanoposthitis lesions in bulls can contaminate semen and constitute a hazard in natural breeding. In the artificial insemination industry, BoHV-1 is of major concern because it may be present at any time in the semen of acutely- or latently-infected bulls (White and Snowdon, 1973; van Oirschot et al., 1993) and may thereby be transmitted to inseminated cows (Philpott, 1993). After artificial insemination with infected semen, endometritis can arise (Kendrick and McEntee, 1967). If semen is frozen, the virus may be preserved. Also embryos constitute a hazard (Philpott, 1993).

Although cattle are the principal reservoir of BoHV-1, other species like sheep and goats may contribute to interspecies transmission (Whetstone and Evermann, 1998). However, this mode of transmission does not seem to be epidemiologically important (Pirak et al., 1983; Hage et al., 1997).

Diagnosis

A diagnosis of infection with BoHV-1 in cattle is established by isolating the virus from living animals’ secretions from affected tissues, e.g. from the nose, eyes, vagina, etc… Virus isolation can also be tempted post-mortem from mucous membranes of the respiratory tract, and portions of the tonsil, lung and bronchial lymph nodes. BoHV-1 genomes can be identified in secretions or organ samples by polymerase chain reaction (PCR). Viral BoHV-1 DNA detection methods may prove to be particularly useful for testing semen samples. These techniques that can identify infected animals are only useful during the acute periods of infection in which viral shedding occurs.

Mass diagnosis of BoHV-1 infection is made indirectly by detecting a serological response in the live animal. Several authors have reported that BoHV-1 infections induce variable or low antibody responses in serum or elicit no antibody response at all (Snowdon, 1965; Parsonson and
Snowdon, 1975; Dennett et al., 1976). However, in most of these studies rather insensitive antibody detection methods were used. Because it is important for eradication programmes to be able to detect cattle latently infected with BoHV-1, serological methods must be very sensitive and highly specific (Ackermann et al., 1990). In the last few years several very sensitive antibody detection techniques have been developed. A Danish blocking and indirect ELISA (Kramps et al., 1993) and a BoHV-1 glycoprotein B (gB) blocking ELISA (Kramps et al., 1994) have been reported to be able to detect low levels of BoHV-1 specific antibodies. (In the format of an indirect or non-competitive ELISA, specific antibodies in the test sample that have been bound to the immobilised antigen are detected by enzyme-conjugated immunoglobulin-specific antibodies. In a blocking ELISA, the test sample is first added to the antigen, and the conjugated antibodies are added subsequently, after a washing step (Schrijver and Kramps, 1998).) These tests do not allow differentiation between BoHV-1 - infected and BoHV-1 - vaccinated cattle. In contrast, the gE-blocking ELISA (van Oirschot et al., 1997) is able to discriminate between antibodies induced by an infection with a BoHV-1 field strain and antibodies induced after vaccination with a BoHV-1 marker vaccine that lacks gE. These latter are currently the only commercially available marker vaccines.

**Control - eradication**

Within the EU, there are differences in the BoHV-1 status of the member states: Denmark, Finland and Sweden are officially free of BoHV-1. Austria has a EU approved national compulsory BoHV-1 eradication programme. In these countries vaccination has never been practised and a test-and-cull programme has eliminated the virus. To prevent re-infection, restrictions have been imposed on the import of cattle. Such restrictions are incompatible with the principle of free trade within the EU, and other member states are therefore being encouraged to eradicate BoHV-1.

Directives of the EU (88/407 and 93/60) oblige artificial insemination centres to admit only seronegative bulls. From 1999 artificial insemination centres are no longer allowed to keep seropositive bulls or to use their semen. This policy puts great pressure on BoHV-1 - infected countries, that export cattle, semen, or embryos. These countries may suffer considerable financial loss from restrictive regulations imposed by importing countries from competition with BoHV-1 - free member states. For these economic and political reasons, a BoHV-1 - control programme in cattle has been implemented in Belgium. Control of BoHV-1 in cattle in Belgium where these infections are highly prevalent, is only economically feasible by first reducing the prevalence, through mass vaccination, eventually followed by test and removal procedures of infected cattle. The exclusive use of marker vaccines against BoHV-1 in cattle was imposed by law in 1997 by the Belgian Veterinary Authorities. In The Netherlands, BoHV-1 marker vaccines have been
demonstrated to be tools for BoHV-1 control (Bosch et al., 1998). Available BoHV-1 vaccines are used to prevent clinical manifestations of a BoHV-1 infection in individual cattle and herds. Vaccination, in general, may increase the virus dose needed to establish an infection, as shown for pseudorabies virus in pigs (Wittmann et al., 1982; Pensaert et al., 1990), and reduce the severity of clinical signs and the level and duration of virus shedding after intranasal challenge to low or even undetectable levels (van Drunen Littel-van den Hurk et al., 1993; Kaashoek et al., 1994). Furthermore, fewer cattle show virus reactivation after dexamethasone treatment, and reactivated BoHV-1 is shed for a shorter period (Kaashoek et al., 1994). Vaccination of seropositive cattle considerably reduces the re-excretion of BoHV-1 after dexamethasone treatment (Bosch et al., 1997).
INFECTION WITH *Mycobacterium avium* subsp. *paratuberculosis*

Para-tuberculosis is a chronic granulomatous gastrointestinal disorder of ruminants. The aetiological agent is *Mycobacterium avium* subsp. *paratuberculosis* (Map), previously named *Mycobacterium paratuberculosis*. Bovine paratuberculosis (PTB) (Johne’s disease) is incurable. Map infects a wide range of domestic and wild animals, especially ruminants like cattle, sheep, goats, deer, and llamas (Fawcett et al., 1995) but also rabbits (Greig et al., 1997). Currently available evidence points out genetic homogeneity within Map subspecies, notably between Map and *Mycobacterium avium* subsp. *avium*, and *Mycobacterium avium* subsp. *silvaticum*. Thus, 100% specific identification seems difficult. However, different strains of Map have been isolated from ruminants but none of them was specific for a single ruminant species (Pavlik et al., 1995).

**Distribution**

Clinical Johne’s disease has been reported similarly from almost all countries in the world. PTB has been recognized as a fairly common condition in both Western Europe and North America for many years. A regional survey in Southern Belgium found 12% of the cattle seropositive to PTB (Vannuffel et al., 1994). The national Map prevalence was not known before the work of this thesis begun.

**Pathogenesis and immunity**

PTB typically starts as an infection in calves. Infection with Map is usually through oral uptake of faecal contaminated feed or raw milk in which the bacteria have been shed. In utero transmission is also a possibility (Seitz et al., 1989). Calves at the age of 0-6 months are considered to be most susceptible to infection with Map (Collins, 1994). This susceptibility decreases until the age of one year when the resistance seems to be equal to that of adult cattle (Larsen et al., 1975).

(Sweeney et al., 1992)Map is an obligate intracellular bacterium; hence cell-mediated immunity is the primary immunological response. How the bacteria enter intestinal tissues is still not fully elucidated. With the phagocytosis of the mycobacteria by sub- and intraepithelial macrophages, the immunological reaction has commenced. Next, one of the two main immunological responses occurs: the cell-mediated dominated reaction or the humoral dominated reaction. The type of immune response depends on the individual animal, but there is usually a cell-mediated immune response and a low humoral response in the beginning, and as the disease progresses, the situation reverses: there is a stronger humoral response initiated by the release of bacteria by dying macrophages (Bakker et al., 2000). Experimental studies indicate that cellular immunity may be detected 1-2 months after infection whereas the humoral immune function
Introduction of infections with PRV, BoHV-1 and Map

potentially can occur 10-17 months after infection (Lepper et al., 1989). It is often assumed that cellular immune response can be detected in calfhood shortly (i.e. a few months) after infection and that the humoral immune response can be detected in adulthood with a fixed likelihood of a diagnosis no matter how old the animal is. This is of great importance because the usability of the diagnostic tests will depend on the type of immune response raised by the individual animal and/or the number of bacteria shed in the faeces by this animal.

During the cell-mediated immunological reactions the humoral response with production of antibodies (IgM, IgA, IgG₂ and small amounts of IgG₁) is depressed, but the infection is still ongoing (Chiodini, 1996). The multiplication of Map in the mucous membrane of the intestine causes chronic enteritis leading to prolonged diarrhoea, poor digestion, excessive weight loss, and decreased milk production (Whitlock and Buergelt, 1996). Map causes a gradually worsening disease condition, and is highly resistant to the infected animal’s immune defences. Therefore, infected animals harbour the organism for years before they develop disease signs. The incubation period of the disease in cattle varies, in general, from 1.5 to more than 5 years. A fixed incubation time for PTB is in many instances self-contradictory. This is due to the chronic nature of the disease, possible latency of infection (Whitlock and Buergelt, 1996) and, though usually assumed to be in calfhood, the unknown time of infection. Thus, the dynamics of the infection in a natural setting covering all potential stages of infection and disease have jet to be described relative to the routinely applied diagnostic tools.

Transmission

The main route of transmission is the faecal-oral route. Cattle in advanced stages of infection can excrete the bacterium in their faeces, but also in colostrum, and milk (Streeter et al., 1995). These bacteria are typically shed in varying numbers into the environment. The bacteria may then be picked up in faecally contaminated feed or water by non-infected animals. Other transmission modes are either via contaminated teats during suckling, or by drinking contaminated colostrum or milk. The newly exposed animals may develop disease and spread it within the herd. As PTB is just beginning to spread in a herd, there may be only one or two animals showing clinical disease signs at a time. These sick animals are culled and the disease may go unrecognised as a whole herd problem for some time.

Map has been cultured from semen (Larsen et al., 1981) but the risk of transmission through artificial breeding techniques is considered to be very low (Eamens, 1996).

Once outside the animal, the bacterium is quite hardy, living for months in water, feed and manure. It persists in soil, water and slurry for periods of several months until more than one year (Jorgensen, 1977).


Chapter 4

Diagnosis

Diagnosis of PTB follows the patterns of pathogenesis. The available diagnostic tests can be divided into two groups. The first group of tests aim at direct detection of Map. These tests have high specificity and, since the number of bacteria present increases, the sensitivity increases as the disease progresses. These tests are microscopic examination of faecal smears, culture of the organism from faecal samples, and detection by polymerase chain reaction (PCR). Isolation of Map by traditional culturing from faeces can take 2 to 4 months (Whipple et al., 1991), since one of the main characteristics of Map is its slow growth. Faster techniques have been developed (Cousins et al., 1995) but these are still labour-intensive and require that excretion of the bacteria takes place at the time of sampling.

The second group consists of the indirect, immunological tests. These are the ELISA, using a variety of different antigens, the complement fixation test (CFT), the agargel-immuno diffusion test (AGID), the delayed type hypersensitivity test or intra-dermal test, and the gamma-interferon test (Stabel, 1996). However, prior to detection of the immune response, the reactions have to have occurred. The convenience of sample collection, large capacity, rapid laboratory turnaround time, and low cost make a serological test in an ELISA format the preferred test for the large-scale testing needed for a certification and/or eradication programme. However, faecal culture and antibody ELISA’s are only suitable for use as tests on older animals because the antibody response increases at a late stage of the disease. Therefore, the ELISA will have a sensitivity rising from zero at early stages of disease to 100% when the animal starts to show clinical symptoms (Bakker et al., 2000).

PTB in cattle can also be diagnosed at post-mortem examination (Whitlock and Buergelt, 1996), by which the terminal jejunum, ileum, mesenterial and ileocaecal lymph nodes are examined macroscopically and tissue samples are taken for histology, Ziehl-Neelsen staining and culture of Map.

Control - eradication

PTB on the other hand is of public health concern as a potential zoonosis and increased public awareness and concern for food safety and quality. Moreover, the detrimental effects on the individual animal, decreasing its welfare (due to diarrhoea, emaciation etc.) and the production loss affecting the farmer’s income, are even more devastating compared to infection with PRV and BoHV-1.

For many years it has been recognised that Crohn’s disease in humans and Johne’s disease have many clinical and pathological features in common. Crohn’s disease is a chronic inflammatory disorder affecting most parts of the intestinal tract, especially the ileocaecal area. Patients with Crohn’s disease present symptoms of chronic weight loss, abdominal pain, nausea,
Introduction of infections with PRV, BoHV-1 and Map

vomiting, diarrhoea and general malaise (Chiodini, 1989). While the mortality from Crohn’s disease is low, patients often live with chronic pain and frequently require surgical resection of diseased intestine. It is a life long disease and has no cure.

Map has been isolated from humans with Crohn’s disease (Chiodini et al., 1984; Kreuzpainter et al., 1992). This led to the hypothesis that CD was caused by Map. As yet, the cause of Crohn’s disease remains unknown, but a multi factorial aetiology including environmental, genetic, immunological and microbial factors have been suggested. Epidemiological data point to the possibility of more than one cause, while genetic susceptibility and the host immune response are likely to play a major role in the pathogenesis of this disease. The current available evidence does not suffice to confirm or refute that Map is a causative agent of at least some cases of CD in man (Van Kruiningen, 1999; Hubbard and Surawicz, 1999; Badiola et al., 2003).

Vaccination is used worldwide since decades to control PTB in cattle, goats and sheep. In Belgium a heat-killed vaccine (VAR, Brussels) was used in cattle herds. Calves in the first month after birth were vaccinated sub-cutaneously in the dewlap. Vaccination reduces both the number of clinical cases in cattle (Wentink et al., 1988) and the number of faecal shedders (Körmédny, 1994). Because vaccination does not prevent calves becoming infected (Wentink et al., 1988), additional preventive management measures in vaccinated herds are necessary to decrease the infection rate. The vaccine interferes with the skin test used for diagnosing tuberculosis (Wentink G.H. et al., 1988), therefore vaccination is restricted to heavily, clinically infected cattle herds in Belgium and these cattle were registered by the authority of the Veterinary Inspection as being vaccinated. Vaccination also influences the diagnosis of PTB. In a study by Spanger et al. (1991) the majority of calves became ELISA positive and remained seropositive until the end of the study period, which was one year after vaccination.

The principles of Johne’s disease control include reducing exposure and infection of replacement cattle within infected farms, and monitoring and identifying the highly infected cattle. Detecting infected cattle by faecal culture and immediate culling of the cows tested positive (Obasanjo et al., 1997), is essential to decrease the PTB prevalence. Within infected herds, the chance of newborn calves to be infected must be limited. Immediately at birth the susceptible calf is at risk for infection by entering an environment, which can be contaminated by Map from the faeces of the own mother or from other adult cattle. Hygienic measures at calving are needed to decrease this infection risk (Johnson-Ifearulundu and Kaneene, 1998). After birth, the risk of transmission of Map must be diminished by removing the calf immediately from the mother (Goodger et al., 1996) and by housing the calves separated from adult cattle (Thoen and Baum, 1988). The calf can also ingest the bacterium by Map contaminated feed. Feeding the colostrum of
the own mother, milk replacer and hay (Cetinkaya et al., 1997) are therefore components of a PTB control strategy.

Also, screening sources of off-farm replacements must prevent introduction of infection. The purchase of cattle is considered to be the most important way to introduce Map into a herd (Sweeney, 1996).

(Roizman, 1996)
(Martin and Wardley, 1987)
(Zuckermann et al., 1990)
(Babiuk et al., 1996)
REFERENCES


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**ABSTRACT**

In Belgium, pseudorabies in swine has been the subject of a mandatory eradication programme since 1993. From December 1995 to February 1996, a survey was conducted in the five provinces of northern Belgium to estimate the provincial pseudorabies virus (PRV) herd seroprevalence. Seven hundred and twenty randomly selected herds were included in this survey. To detect recently infected animals, only young sows were sampled. The results show that 44% of these herds had an important number of PRV-seropositive young sows. The highest herd seroprevalence was observed in West Flanders (68%), followed by Antwerp (60%), East Flanders (43%), Limburg (18%), and Flemish Brabant (8%). Assuming a diagnostic test sensitivity and specificity of 95 % and 99 % respectively, and a true PRV within-herd prevalence of 43 %, the overall true PRV herd prevalence was estimated to be 35 %. A logistic multiple-regression revealed that the presence of finishing pigs was associated with a two-fold increase in odds of a herd being seropositive (odds ratio (OR)=2.07, 95% confidence interval (CI)=1.31-3.26); a breeding herd size ≥ 70 sows was associated with a four-fold increase in odds of a herd being seropositive (OR=4.09, 95% CI=2.18-7.67); a pig density in the municipality of ≥ 455 pigs per km² was associated with a ten-fold increase in odds of a herd being seropositive (OR=9.68, 95% CI=5.17-18.12). No association was detected between the PRV herd seroprevalence and purchase policy of breeding pigs (purchased gilts, or use of homebred gilts only).

**INTRODUCTION**

Pseudorabies is caused by the pseudorabies virus (PRV) (Aujeszky’s disease virus; Suid herpesvirus 1). It is an enzootic disease on the B List of the ‘Office International des Epizooties’ (O.I.E.). Programmes to eradicate PRV have been implemented in several European countries to facilitate the free trade of pigs within the European Community.

Northern Belgium (an area with a very high pig density where 96% of the Belgian pig population is located) is enzootically infected with pseudorabies (Pensaert et al., 1987). In this region, an intensive mass-vaccination programme (vaccination with marker vaccines) has been implemented to eradicate PRV since 1993. Area-wide vaccination has worked in The Netherlands (Stegeman, 1995a). All pigs have to be vaccinated according to the following scheme: sows and boars are vaccinated simultaneously twice a year with an inactivated vaccine or three times a year with a live vaccine. Replacement gilts and boars receive the first vaccination at 10 to 12 weeks (wk) of age, a second at 14 to 16 wk and a third at 26 wk. Two years after the start of the eradication campaign, finishing pigs had to be vaccinated twice (at 10 to 12 and 14 to 16 wk of age) instead of just once as was previously done. Marker vaccines that lack PRV glycoprotein E
(gE) do not induce antibodies against gE, whereas wild-type PRV strains do. Therefore, vaccination with these vaccines does not preclude the serodiagnosis of PRV-infected pigs.

Surveying disease prevalence over time is essential in the initial stage of an eradication campaign. A survey is an examination of an aggregate of units (Kendall and Buckland, 1982). In animal populations, sample surveys for estimating the prevalence of diseases are undertaken on a sample of the population, as opposed to census surveys that are carried out on every unit of the population. Sampling units may be individual animals or aggregates such as herds, and prevalence may be calculated in relation to these different units. However, when dealing with infectious diseases, the group of animals which is of epidemiological importance in terms of the transmission and maintenance of infection -- and therefore of disease control and eradication -- is the herd (Thrusfield, 1995). This is particularly true for PRV because the control and eradication measures implicate the herd -- not the animal -- (International Animal Health Code, 1996). Therefore, in this survey, the sampling units were defined as the swine herds, notwithstanding the fact that individual-animal prevalence is a more-suitable indicator for the spread of PRV on a yearly basis. In a PRV-eradication campaign, the individual-animal prevalence will decrease faster than the herd prevalence, because one of the first notable effects of vaccination is the reduction of the proportion of positive animals within the herd. Individual-animal prevalence is also used to determine the point at which culling of seropositive pigs can start.

To date, few surveys have estimated PRV herd or individual-animal prevalence at the regional or national levels. In Germany, Leontides et al. (1994) found serological evidence of PRV infection in 66.5% herds with breeding sows. Elbers et al. (1995) and Elbers and Stegeman (1996) surveyed the national individual-animal seroprevalence in The Netherlands and noted a marked reduction of the PRV seroprevalence in pig-dense areas. In sows, the seroprevalence decreased significantly from 27% to 17% in the eastern region; from 32% to 17% in the southern region; and from 18% to 12% in the western-central region. A non-significant decrease from 10% to 6% was observed in the Northern region. Some reported PRV herd-seroprevalence surveys used census data. In Hungary, the PRV breeding-herd prevalence was 55.2% between 1976 and 1991 (Medveczky and Lomniczi, 1996).

In Belgium, Maes et al. (1998) organized a regional sample survey in 1994 and estimated the PRV herd seroprevalence in farrow-to-finish herds to be 76%. At the national level, sample surveys monitoring the PRV herd seroprevalence are organized on a yearly basis. A pilot survey involving only herds with breeding sows was conducted from December 1995 to February 1996 in the five provinces of northern Belgium. The primary goal of this first survey was to provide an unbiased estimate of the provincial PRV herd seroprevalence of herds with an important number of PRV-infected young sows, by random selection of herds to sample. A second objective was to
identify some herd and management risk factors associated with the presence of PRV-seropositive young sows.

**MATERIAL AND METHODS**

**Survey design**

The survey was organized using the co-ordinates for the pig herds registered in SANITEL-Pigs, the central computerized database created in 1991 for the identification and registration of the Belgian pig population (Ministry of Small Enterprises, Traders and Agriculture, Belgium). By law, all Belgian pig keepers have to be registered in SANITEL-Pigs and have the duty to report all the necessary data that are needed for making up their herd and pig movements inventories. This information is updated daily in SANITEL-Pigs by the Regional Veterinary Investigation Centres. In SANITEL-Pigs, a herd is defined as a stock of pigs kept in a geographical entity -- containing one or several buildings with adjacent premises -- that makes up a clear and distinct unit on the basis of epidemiological bounds set by the Veterinary Inspection. Therefore, in this survey, the sampling units were defined as these swine herds.

The survey was conducted only on herds with breeding sows. From December 1995 to February 1996, blood samples were collected from young sows that were 8 to 20 months (mo) old.

A two-stage sample design was followed. First, in each province, herds with breeding sows were randomly selected from SANITEL-Pigs. The selection was performed using a software random generator function of Visual Basic 3.0 (Microsoft Corp., 1993). The number of herds to be sampled per province was determined considering the number of herds present, an expected herd seroprevalence of 50% (considering no a priori knowledge of the herd seroprevalence), and a 10% desired accuracy for a 99% level of confidence (Table 1) (Cannon and Roe, 1982). Secondly, the within-herd sample-size was determined to have at least a 99% confidence of detecting infection, providing that a seroprevalence of 20% or higher (Cannon and Roe, 1982) was present. The number of young sows to be sampled was set at 20 for herds with more than 25 young sows; 15 for herds with 15 to 25 young sows. All sows had to be sampled in herds with less than 15 young sows. A herd was defined to be seropositive if at least one PRV-seropositive young sow was present. Young sows were sampled across a spectrum of ages between 8 and 20 mo and were selected from different pens and different units in the herd using systematic sampling. The sampling interval was calculated by dividing the number of young sows present by the number of young sows to sample.

The number of herds figuring on the computer-generated sampling lists exceeded the calculated number, to allow for replacement of herds which did not have sows between 8 and 20
mo old on the sampling date. As soon as a herd was confirmed as not having sows of the required age category, it was replaced by the next herd figuring on the reserve list. In 137 instances, herds were replaced.

**Collection of samples and herd and management characteristics**

The blood samples were taken by the veterinary practitioners and sent to the Regional Veterinary Investigation Centres. The veterinary practitioners also noted the age of the sampled sows and interviewed the farmer concerning the following herd and management characteristics: herd type (breeding herd with or without finishing pigs), breeding-herd size (number of sows on the premises), and origin of replacement gilts in the year before the survey was conducted (use of homebred and/or purchased replacement gilts). In addition, the density of the pig population in the municipalities where the herds were located was determined by dividing the number of pigs (SANITEL-Pigs) by the amount of effective agricultural land. Data on the amount of agricultural land per municipality were obtained from the Belgian Agricultural Statistics Yearbook, 1995 (National Institute of Statistics, 1995).

**Serological testing**

The serum samples were tested for antibodies to gE of PRV wild virus as described by Van Oirschot et al. (1988), using a commercially available competitive ELISA (Chekit-PRV-gI EIA®, Bommeli, Switzerland). This test allows differentiation of vaccinated from infected pigs. All samples were tested using plates from one batch of test kits. Sera with optical density (OD)-values \( \leq 40\% \) and \( \geq 50\% \) of the OD\textsubscript{positive control} were considered negative and positive, respectively. Intermediate OD-values were considered doubtful and classified as negative in the data analysis.

**Data analysis**

The inclusion criteria were as follows: (1) the age of all the sampled sows had to be mentioned; and (2) the samples had to be obtained from sows between 8 and 20 mo old. Data originating from herds with sampled sows outside the required age category or where no age data were provided were excluded from the analysis. All analyses were performed using the statistical software Statistix 1.0 (Analytical Software, Tallahassee FL 32317-2185, USA).

The provincial herd seroprevalence was analyzed with a \( \chi^2 \) test for two-dimensional contingency tables. The significance level for pairwise comparison of individual \( \chi^2 \) tests was adjusted according to the Bonferroni method (Altman, 1991).
The true PRV herd prevalence was estimated according to the methodology described by Martin et al. (1992). These calculations incorporate factors such as test sensitivity and specificity, true within-herd prevalence, sample size, and the cut-off number of reactors required to call a herd truly positive. First, assumptions from literature were made concerning the test sensitivity and specificity of the used ELISA. Secondly, the true within-herd prevalence was estimated based on the results from the PRV seropositive herds, assuming non-reactor herds were non-infected. This true PRV within-herd prevalence was calculated as the median estimated true within-herd prevalence of the seropositive herds. Thirdly, the sample size was calculated as the median number of sampled young sows per herd. Based on the aforementioned parameters, the herd sensitivity, the herd specificity and the true herd prevalence were calculated.

Bivariable analyses were conducted to compare the overall herd seroprevalence to each recorded herd and management characteristic. The significance of categorical variables was tested using Pearson’s $\chi^2$ test for independence. Continuous variables were compared using a Wilcoxon rank-sum test. Factors significant in the bivariable analyses were combined in a logistic multiple-regression analysis with a seropositive herd as the response variable. Collinearity between the herd size and the local pig density was verified by the Pearson correlation coefficient. All interactions and second-order factors were considered in a hierarchical backward stepwise-selection procedure (Kleinbaum, 1994). Finally, continuous variables were dichotomized using the median in the whole sample as a cut-off point and logistic regression coefficients were recomputed, in order to derive an adjusted odds ratio (OR) for each possible combination of z-level factors. All tests were two-tailed and a p-value < 0.05 was considered as significant.

**RESULTS**

**PRV herd seroprevalence**

An overview of the sample composition is given in Table 1. The sample consisted of 720 randomly selected herds. There were 66 nonresponding herds (9.2% of the 720 herds) from which no samples were received and for which no replacement occurred either. The three major reasons for no response were: (1) no sows of the required age category were present (30 herds, 4.2%); (2) no sows were blood sampled in due time, by lack of coordination between different project partners (24 herds, 3.3%); and (3) the farmer had ceased his activities (12 herds, 1.7%). Samples were received from 654 herds (90.8%), including 101 herds (14.0%) for which either all sampled sows were outside the required age category (n=17) or where no age data were provided (n= 84). Thus, 553 herds (76.8%) met the inclusion criteria. In 524 of these herds, all the samples were obtained from sows between 8 and 20 mo old; for 29 of these herds, a minority (average: 17%) of
the sampled sows were outside the required age category. The latter samples obtained from sows outside the required age category were excluded from the analysis. In 179 of the 553 herds, all sows between 8 and 20 mo old were sampled due to the small herd size.

As presented in Table 1, 242 of the 553 herds had at least 1 gE-seropositive young sow. The frequency distribution of the within-herd prevalence of gE-seropositive young sows is shown in Figure 1. Of the positive herds, 31% had 20% or less seropositive samples; 16% of the positive herds had more than 90% gE-positive samples.

The pairwise comparison of the provincial herd seroprevalence showed that the herd seroprevalence of West Flanders did not differ from that of Antwerp (p=1), whereas it was different from that of East Flanders (p<0.005); the herd seroprevalence of Antwerp did not differ from that of East Flanders (p=0.16); the mentioned seroprevalence figures were higher than those for the provinces of Limburg and Flemish Brabant (p<0.001); and there was no difference between the herd seroprevalence figures of Limburg and Flemish Brabant (p=0.36).

![Figure 1. Frequency distribution of the within-herd prevalence of young sows seropositive to pseudorabies, in northern Belgium, 1996](image)

**a** In parentheses: number of positive herds

**b** Percentage of positive herds
Table 1. Provincial prevalence of herds with young sows seropositive to pseudorabies in northern Belgium, 1996

<table>
<thead>
<tr>
<th>Province</th>
<th>Number of herds</th>
<th>PRV herd seroprevalence</th>
<th>PRV herd true prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>To be sampled</td>
<td>Actually sampled</td>
<td>With sampled sows</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>West Flanders</td>
<td>3,953</td>
<td>160</td>
<td>159</td>
</tr>
<tr>
<td>East Flanders</td>
<td>2,157</td>
<td>150</td>
<td>140</td>
</tr>
<tr>
<td>Antwerp</td>
<td>828</td>
<td>140</td>
<td>133</td>
</tr>
<tr>
<td>Limburg</td>
<td>1,001</td>
<td>140</td>
<td>113</td>
</tr>
<tr>
<td>Flemish Brabant</td>
<td>585</td>
<td>130</td>
<td>109</td>
</tr>
<tr>
<td>northern</td>
<td>8,524</td>
<td>720</td>
<td>654</td>
</tr>
</tbody>
</table>

$^a$ SANITEL-Pigs, 1995. Ministry of Small Enterprises, Traders and Agriculture, Belgium

$^b$ confidence interval
PRV herd true prevalence

A test sensitivity of 95% and a test specificity of 99% were assumed. Secondly, the true overall within-herd prevalence was estimated to be 43%. Thirdly, the overall median sample size was 15. Based on the three aforementioned parameters, the overall PRV herd sensitivity, herd specificity and herd true prevalence were respectively 99%, 86% and 35%. The provincial PRV herd true prevalences are in Table 1. The calculation of the provincial herd true prevalence was based upon the overall true PRV within-herd prevalence of 43%, and the provincial median sample size, which was 19 for West Flanders, 20 for Antwerp, 15 for East Flanders, 15 for Limburg, and 8 for Flemish Brabant.

Bivariant analysis of the recorded herd and management characteristics

The distribution of the herd and management risk factors between seronegative and seropositive herds is summarized in Table 2. The herd seroprevalence increased with a larger breeding-herd size, and ranged from 14.5% for herds with a maximum of 24 sows to 67.0% for herds with a minimum of 150 sows (Figure 2). At the municipal level, the herd seroprevalence varied from 17.7% for herds located in municipalities with a maximum 50 pigs/km² to 88.9% for herds located in municipalities with more than 2,000 pigs/km² (Figure 3).

Figure 2. Distribution of the prevalence of herds with young sows seropositive to pseudorabies per herd size, in northern Belgium, 1996

a In parentheses: number of herds
b Herd seroprevalence
Table 2. Bivariable distribution of herd and management factors between herds with and without young sows seropositive to pseudorabies in northern Belgium, 1996

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Serology</th>
<th>positive herds</th>
<th>negative herds</th>
</tr>
</thead>
<tbody>
<tr>
<td>(all P&lt;0.001 comparing positive to negative)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd type: breeding herd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with finishing pigs</td>
<td></td>
<td>195 (51%)</td>
<td>184 (49%)</td>
</tr>
<tr>
<td>without finishing pigs</td>
<td></td>
<td>47 (27.0%)</td>
<td>127 (73%)</td>
</tr>
<tr>
<td>Herd size (number of sows)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 70</td>
<td></td>
<td>164 (58%)</td>
<td>121 (42%)</td>
</tr>
<tr>
<td>&lt; 70</td>
<td></td>
<td>78 (29%)</td>
<td>190 (71%)</td>
</tr>
<tr>
<td>Origin of replacement gilts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>homebred and purchased or only</td>
<td></td>
<td>104 (54%)</td>
<td>88 (46%)</td>
</tr>
<tr>
<td>purchased</td>
<td></td>
<td>138 (38%)</td>
<td>223 (72%)</td>
</tr>
<tr>
<td>only homebred</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig density (pigs per km²/100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 4.55</td>
<td></td>
<td>181 (64%)</td>
<td>100 (36%)</td>
</tr>
<tr>
<td>&lt; 4.55</td>
<td></td>
<td>61 (22%)</td>
<td>211 (78%)</td>
</tr>
</tbody>
</table>

Logistic-regression analysis of the recorded herd and management risk factors associated with PRV herd seroprevalence

Among the four risk factors submitted to the logistic regression, purchase policy was not associated with the frequency of herds having young sows seropositive to PRV (Table 3). The model with dichotomized continuous variables and adjusted odds ratio’s is in Table 4. The latter table shows a multiplicative relationship between the OR for herds being seropositive when exposed to the risk factors pig density and presence of finishing pigs (9.7 x 2.1 = 20.0), and between the OR for herds being seropositive when exposed to the risk factors herd size and presence of finishing pigs (4.1 x 2.1 = 8.5). Due to interaction, this is not the case for the OR for herds being seropositive when exposed to the risk factors pig density and herd size (15.4 < 9.7 x 4.1). These observations are visualized in Figure 4.
Figure 3. Distribution of the prevalence of herds with young sows seropositive to pseudorabies per pig density of the municipality, in northern Belgium, 1996

Table 3. Final logistic-regression model of risk factors for herds with presence of young sows seropositive to pseudorabies, in northern Belgium, 1996

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Coefficient</th>
<th>SE a</th>
<th>p-value</th>
<th>odds ratio</th>
<th>95% CI b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd type c (1=yes, 0=no)</td>
<td>0.75</td>
<td>0.23</td>
<td>&lt; 0.002</td>
<td>2.12</td>
<td>1.34-3.35</td>
</tr>
<tr>
<td>Herd size ≥ 70 sows (1=yes, 0=no)</td>
<td>1.36</td>
<td>0.32</td>
<td>&lt; 0.0001</td>
<td>3.90</td>
<td>2.07-7.35</td>
</tr>
<tr>
<td>Purchase policy (1=yes, 0=no)</td>
<td>-0.31</td>
<td>0.21</td>
<td>0.15</td>
<td>0.74</td>
<td>0.49-1.11</td>
</tr>
<tr>
<td>Pig density ≥ 455 pigs/km²</td>
<td>2.25</td>
<td>0.32</td>
<td>&lt; 0.0001</td>
<td>9.44</td>
<td>5.03-17.7</td>
</tr>
<tr>
<td>Interaction Herd size x Pig density</td>
<td>-0.96</td>
<td>0.41</td>
<td>&lt; 0.02</td>
<td>0.39</td>
<td>0.17-0.86</td>
</tr>
<tr>
<td>(Constant)</td>
<td>-2.25</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a  standard error
b  confidence interval
c  breeding herd with finishing pigs
Table 4. Estimated odds ratio and 95% confidence intervals (CI) within each combined level of the risk factors pig density, herd size, and herd type, for herds with presence of young sows seropositive to pseudorabies, in northern Belgium, 1996

<table>
<thead>
<tr>
<th>Pig density</th>
<th>Herd size</th>
<th>Herd type</th>
<th>odds ratio</th>
<th>95% CI a</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 455 pigs/km²</td>
<td>≥ 70 sows</td>
<td>with finishing pigs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) no</td>
<td>no</td>
<td>no</td>
<td>1.</td>
<td>baseline</td>
</tr>
<tr>
<td>(b) yes</td>
<td>no</td>
<td>no</td>
<td>9.7</td>
<td>5.2-18.1</td>
</tr>
<tr>
<td>(c) no</td>
<td>yes</td>
<td>no</td>
<td>4.1</td>
<td>2.2-7.7</td>
</tr>
<tr>
<td>(d) yes</td>
<td>yes</td>
<td>no</td>
<td>15.4</td>
<td>8.4-28.1</td>
</tr>
<tr>
<td>(e) no</td>
<td>no</td>
<td>yes</td>
<td>2.1</td>
<td>1.3-3.3</td>
</tr>
<tr>
<td>(f) yes</td>
<td>no</td>
<td>yes</td>
<td>20.0</td>
<td>8.9-44.9</td>
</tr>
<tr>
<td>(g) no</td>
<td>yes</td>
<td>yes</td>
<td>8.5</td>
<td>4.1-17.7</td>
</tr>
<tr>
<td>(h) yes</td>
<td>yes</td>
<td>yes</td>
<td>31.9</td>
<td>15.8-64.2</td>
</tr>
</tbody>
</table>

a confidence interval

Figure 4. OR for herds with young sows seropositive to pseudorabies, by increasing herd sizes, per pig density of the municipality, in northern Belgium, 1996

* odds ratio
** fattening pigs
*** number of sows
DISCUSSION

PRV herd seroprevalence and PRV herd true prevalence

To date, few surveys have estimated PRV prevalence at the regional or national levels. Moreover, these surveys are affected by the nature of the study design, the type and size of herds studied, the type of prevalence parameters studied (herd, individual-animal or within-herd prevalence), and the age of the tested animals. Comparison of these survey results is difficult.

The present survey aimed to provide an unbiased estimate of the PRV herd seroprevalence, because these herds were randomly selected. Because the percentage of nonresponding herds was fairly low (9%), this random sample of herds can be considered to be representative for the population of breeding swine herds in northern Belgium (Thrusfield, 1995). However, there is a potential for bias due to nonresponding herds or herds which provided samples which did not meet the inclusion criteria. Particularly, the 101 herds where all sampled sows were outside the selected age category or where no age data were provided can jeopardize the estimated seroprevalence. Additionally, the sampling and testing procedures had inherent probabilities of misclassification.

The within-herd sample-size, which was calculated to detect a minimum prevalence of 20% gE-seropositive young sows, was determined by budget considerations. Due to this cut-off level, the probability of failing to detect seropositive herds with a lower within-herd seroprevalence than the minimum detectable one is increased. Therefore, this study design potentially misclassifies some herds as seronegative when actually they are seropositive. The true herd seroprevalence may thus have been underestimated. The potential existence of low-PRV within-herd seroprevalence herds can be suspected by the fact that, due to the intensive obligatory mass-vaccination programme in northern Belgium, the PRV transmission among pigs and pig herds is diminished, creating these low-PRV within-herd seroprevalence cases. The impact of this misclassification could be evaluated by true-PRV within-herd seroprevalence data, based on testing a census of sows in vaccinating breeding herds. However, no such reports exist. Morrison et al. (1991) reported the PRV within-herd seroprevalence of sows of all ages to be distributed bimodally (modes = 0% and 90%), based on a within-herd sample-size calculated to detect a minimum prevalence of 10%, whereas Leontides et al. (1994) and Stegeman et al. (1994 and 1995b) fixed the sampled size at 20 and 25, respectively. Based on sampling 25 sows per herd, stratified by parity, Stegeman et al. (1994) found the mean within-herd seroprevalence of first-parity sows in breeding herds to be at least 24%. In Belgium, Maes et al. (1996) organized a regional sample survey in 1994, and found a median PRV within-herd seroprevalence of 50% in slaughter pigs from farrow-to-finish herds, based on a fix sampled size of 25 slaughter pigs per herd. In the present survey, a strong variation of the within-herd seroprevalence
(as depicted in Figure 1) for first- or second-parity sows, was observed. This variation might be because replacement gilts (which can be infected with PRV as a gilt or as a breeding sow) are usually added to the herd in small groups. Although the theoretical sample scheme was designed to evaluate herd seroprevalence in a large number of herds with a 20% within-herd seroprevalence and tends to underestimate the true herd seroprevalence, practical considerations contradict this statement. Indeed, more information could be obtained because the number of herds that were examined was small. First, in one-third of the included herds, all the young sows were sampled (due to the small herd size). This suggests that if no sample was positive, the within-herd seroprevalence was 0%. The estimated herd seroprevalence may thus be overestimated for this category of herds. Second, within-herd seroprevalence lower than 20% could be demonstrated even when one or more samples of a herd were positive (but not with a 99% confidence). An example can be cited of a herd with 1 infected young sow out of 20 young sows. If 15 young sow are sampled, the probability of the infected young sow to be sampled was about 15%, according to the hypergeometric distribution (Cameron and Baldoc, 1998). In this case, the herd was declared seropositive with an estimated within-herd seroprevalence of at least 20% -- which was an overestimation because the true within-herd seroprevalence was 5%.

The aforementioned calculations assume a perfect test sensitivity and specificity of 100%. Because no test is perfect, the testing procedure could also have been a source of information bias. Martin et al. (1992) showed that the calculation of the true herd prevalence incorporate factors such as herd sensitivity and specificity, true within-herd prevalence, sample size, and the cut-off number of reactors required to call a herd truly positive. No published data of sensitivity and specificity of the ELISA kit we used in this survey exist. However, the actual generation of ELISA tests that allow differentiation of vaccinated from infected pigs is considered to have a high sensitivity and specificity (Arias et al., 1992). Because a randomized survey design was followed, a test sensitivity of 95% and a test specificity of 99% were assumed. This ELISA lacks some sensitivity, inducing a problem of false-negative tested animals. The specificity is high, implying only a minor problem of false-positive tested animals. The problem of false-positive test results was further diminished by the protocol of the data analysis, whereby doubtful OD-test values were classified negative. The calculated PRV true within-herd prevalence (43%) assumed a census of young sows to be sampled and non-reactor herds to be non-infected. The first assumption could result in an overestimation of the true PRV within-herd prevalence, because -- as explained before -- the sample design of the present study probably fails to detect herds with low PRV within-herd prevalence. An overestimation of the true PRV within-herd prevalence would result in an underestimation of the PRV herd true prevalence, which was calculated to be 35%. The importance of this theoretical underestimation of the PRV herd true prevalence is probably diminished in practice, due to the small number of examined herds and the census sampling in one-third of the selected herds, as for the PRV herd seroprevalence. The bias created by the second
assumption is probably small, because the herd negative predictive value, which estimates the impact of false-negative herds, was 100%. This indicates that all seronegative herds were non infected. The herd positive predictive value, assuming -- as for the calculation of the herd negative predictive value -- the calculated true herd prevalence to be the true prevalence of infected herds was 79% indicating that 21% of the seropositive herds were not infected by PRV.

The results of this pilot survey show that a large proportion of herds with breeding sows harbored recently infected sows. This finding is consistent with results obtained by Maes (1998), and indicates that in northern Belgium, the PRV-eradication campaign (primarily implemented through an intensive obligatory mass-vaccination campaign since 1993) did not stop PRV circulation. There were marked statistical differences in the provincial herd seroprevalence. From an epidemiological viewpoint, a first subregion had a high herd seroprevalence and covered the provinces of West Flanders, Antwerp and East Flanders, whereas a second one had a low herd seroprevalence and covered the provinces of Limburg and Flemish Brabant. Because this study was a pilot survey and no comparison could be made with previous herd-seroprevalence figures, it was impossible to identify trends in the PRV herd seroprevalence at this time.

Herd and management risk factors associated with PRV herd seroprevalence

This cross-sectional study measures the disease status of the members of a population at a particular time. The disease patterns indicated only reflect associations, and do not permit causal interpretations about the results obtained. However, for purposes of causal interpretations, cross-sectional studies are suited to studying permanent factors, because such factors can not be altered by the passage of time or by the presence or absence of disease (Martin et al., 1987). Therefore, in this survey, the recorded herd and management characteristics are of a permanent type with regard to the lifetime of a breeding sow.

The recorded risk factors were examined according to their association with a herd being seropositive. In contrast, most reports identify PRV risk factors that are associated with the PRV within-herd seroprevalence. The former factors are primarily responsible for the introduction or presence of PRV or PRV seropositive pigs into the herd. The latter are, in addition, also related to maintenance and transmission of PRV within an infected herd. However, the distinction between those two types of risk factors may be difficult to make. According to Johnson-Ifeearulundu and Kaneene (1998), studies about risk factors affecting the within-herd transmission -- measured by the within-herd prevalence -- of infectious disease have an inherent bias, if they have a within-herd sample-based design. Herds with low within-herd prevalence have an increased probability of testing
negative. If these are herds whose management practices prevent transmission to the cut-off level of
detection or more, then the impact of the mentioned risk factors is overestimated.

Pig density in the municipality of \( \geq 455 \) pigs per km\(^2\) was associated with an almost ten-fold
increase in odds of a herd being seropositive. The positive relationship between the pig density and
the PRV herd seroprevalence was already seen in the provincial herd seroprevalence. The pig density
is highest in West Flanders, followed by, in decreasing order, Antwerp, East Flanders, Limburg and
Flemish Brabant. The provincial herd seroprevalence decreases in the same order. High pig density in
the municipality is considered to be a risk factor for the circulation and transmission of PRV by many
authors (Morrison, 1991; Marsh et al., 1991; Austin and Weigel, 1992; Weigel et al., 1992; Auvigne
and Hery, 1995; Stegeman et al., 1995b; and Lambers and Crauwels, 1996). In pig-dense areas, the
contacts by area spread increase and as a result, PRV may circulate more easily.

A breeding herd size of \( \geq 70 \) sows was associated with a four-fold increase in odds of a herd
being seropositive. These results corroborate with Medveczky and Lomniczi (1996) who pointed out
that the probability of reintroduction of PRV infection on PRV free herds is positively correlated with
the herd size. Also, Christensen et al. (1990) and Leontides et al. (1994) found a positive correlation
between the breeding herd size and the herd seroprevalence. This may be attributed to the increased
probability of introduction of PRV in large herds because they usually have more-frequent contacts
outside the herd than small herds. Also, the larger area of farms may increase the risk of airborne
introduction of PRV (Willeberg et al., 1994). Furthermore, the sustainability of PRV infection in large
herds may be increased because susceptible pigs are usually present, whereas the infection may fade
out in small herds. Reports concerning the influence of herd size on PRV within-herd seroprevalence
are conflicting. Stegeman (1995a) suggested that these conflicting results indicate the presence of
confounders, such as stocking density and management procedures.

The presence of (infected) finishing pigs was associated with a two-fold increase in odds of a
herd being seropositive. This result is consistent with results obtained by Marsh et al. (1991). Reports
concerning the influence of the presence of (infected) finishing pigs on PRV within-herd
seroprevalence of breeding sows also indicate a positive association (Morrison et al., 1991; Duffy et
al., 1991; Siegel et al., 1993; Leontides et al., 1995). Better management and vaccination protocols in
breeding herds without finishing pigs may also explain these differences in seroprevalence. Stegeman
et al. (1995b, 1996) showed that PRV can be transmitted even by single vaccinated finishing pigs,
causing infections in breeding pigs. Double vaccination of the finishing pigs however, abolishes the
increased risk of new infections of sows by the presence of finishing pigs. From one year before this
survey, finishing pigs in northern Belgium had to be vaccinated twice instead of just once as was
previously done. A possible reason for the observed OR could be that the vaccination scheme was not
carried out as prescribed. The quality of the vaccination and, as a consequence, the herd immunity was probably still to heterogeneous to prevent PRV circulation.

The analysis of the interaction between the three mentioned risk factors leaded to the observation of a synergetic effect between presence of finishing pigs and pig density, and between presence of finishing pigs and herd size. A synergetic effect was also observed between pig density and herd size, as visualized by Figure 4. However, this multiplicative relationship was less-pronounced for herds with a large breeding-herd size that were situated in municipalities with a high pig density.

We found no association between the purchase policy and the herd seroprevalence. This finding is in contrast with several studies. The purchase of infectious pigs is reported as the most important source of PRV introduction (Beran, 1992). Observations of Stegeman et al. (1994) and Leontides et al. (1994) also suggest purchased stock as a major source of virus introduction into vaccinating breeding herds. In a second study however, Stegeman et al. (1995b) found no association between the purchase policy and the within-herd seroprevalence of breeding herds. According to these authors, PRV area spread could have masked the importance of introducing PRV by gilts actively shedding virus. As to the epidemiological situation regarding PRV in northern Belgium described in this paper, this hypothesis is most likely.

These results show that high pig density in the municipality, breeding herd of large size, and presence of finishing pigs are associated with the proportion of PRV seropositive herds. Moreover, a multiplicative relationship exists between the ORs for herds being seropositive when exposed to the risk factors pig density and presence of finishing pigs, and between the ORs for herds being seropositive when exposed to the risk factors herd size and presence of finishing pigs. Due to interaction, this was not the case the ORs for herds being seropositive when exposed to the risk factors pig density and herd size.

We conclude that based on the results of this survey, northern Belgium can be categorized into two subregions: the first subregion with a high herd seroprevalence covers the provinces of West Flanders, Antwerp and East Flanders; and a second one with a low herd seroprevalence covers the provinces of Limburg and Flemish Brabant. The eradication campaign in these two subregions should be adapted with specific measures. Future surveys involving not only herds with sows but also herds with finishing pigs will monitor the expected reduction of the PRV circulation.

The results also show that high pig density in the municipality, breeding herd of large size, and presence of finishing pigs are associated with the PRV herd seroprevalence. These risk factors can not be directly managed by farmers, but should be considered by the government in its PRV eradication programme (stressing the need for a regional approach). A longitudinal risk factor study considering
all herd, management and vaccination characteristics possibly associated with the PRV herd seroprevalence would be extremely beneficial.

ACKNOWLEDGEMENTS

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REFERENCES


ABSTRACT

The national bovine herpesvirus 1 (BoHV-1) seroprevalence (apparent prevalence) in the Belgian cattle population was determined by a serological survey that was conducted from December 1997 to March 1998. In a random sample of herds (N = 556), all cattle (N = 28,478) were tested for the presence of antibodies to glycoprotein B of BoHV-1. No differentiation could be made between vaccinated and infected animals, because the exclusive use of marker vaccines was only imposed by law in 1997 by the Belgian Veterinary Authorities. Twenty-one % of the farmers vaccinated continuously against BoHV-1.

In the unvaccinated group, the overall herd, individual-animal and median within-herd seroprevalence were estimated to be 67% (95% confidence interval (CI)=62-72), 35.9% (95% CI=35.0-36.8) and 33% (quartiles = 14-62), respectively.

Assuming a test sensitivity and specificity of 99% and 99.7% respectively, the true herd, individual-animal and median within-herd prevalence for the unvaccinated group of herds were estimated to be 65%, 36% and 34% respectively. The true herd prevalence for dairy, mixed and beef herds was respectively 84%, 89% and 53%; the true individual-animal prevalence for those herds was respectively 35%, 43% and 31%; whereas the true median within-herd prevalence was 36%, 29% and 38%.

INTRODUCTION

Infectious bovine rhinotracheitis is caused by the bovine herpesvirus 1 (BoHV-1). It is an enzootic disease on the B List of the Office International des Epizooties (O.I.E.). Programs to eradicate BoHV-1 have been implemented in several European countries to facilitate the free trade of cattle, semen and embryos within the European Community. Therefore, Belgium will have an incentive to control and eradicate BoHV-1.

A preparatory step towards the design of an eradication program consisted in investigating the BoHV-1 prevalence. The group of animals which is of epidemiological importance in terms of the transmission and maintenance of infection -- and therefore of disease control and eradication -- is the herd (Thrusfield, 1995). This is particularly true for BoHV-1 because the control and eradication measures implicate the herd - not the animal (O.I.E., 1996). Therefore, in this survey, the sampling units were defined as the cattle herds.

To date, few surveys have estimated BoHV-1 prevalence at the regional or national level. These surveys are affected by the nature of the study design (sample or census survey), the study population (subclinical or clinical, vaccinated or unvaccinated), the type of prevalence parameters
studied (herd prevalence, individual-animal prevalence or within-herd prevalence), the diagnostic test used, and the age of the tested animals. Comparison of these survey results is difficult. Moreover, few studies adjust the seroprevalence for factors such as test sensitivity and specificity to calculate the true prevalence (creating further difficulty in comparison across studies).

In Europe, the BoHV-1 seroprevalence in herds with dairy cows (both dairy and mixed herds), was surveyed several times in the Netherlands. Van Wuyckhuisen et al. (1993) investigated a non-random sample of unvaccinated herds by blood testing in March 1992, and found 93% seropositive herds, 36% seropositive animals, whereas the within-herd seroprevalence varied from 0 to 100% based on a within-herd sample survey. Without considering the vaccination status of herds, the Dutch herds with dairy cattle were investigated by a random-sample survey in the winter of 1992-1993 by van Wuyckhuisen et al. (1993), and by a census survey in November 1994 by van Wuyckhuisen et al. (1998); they found the BoHV-1 bulk-milk herd apparent prevalence to be 75% and 84%, respectively. In a more-recent sample survey that also did not take into account the vaccination status of herds, Hartman et al. (1997) found the herd apparent prevalence (based on individual blood samples) to be 92%, whereas 89% of the herds were positive by bulk-milk sampling.

In Belgium, regional BoHV-1 surveys that did not take into account the vaccination status of herds, estimated the herd seroprevalence in mixed and beef herds to be 62% in 1986 (Van Malderen et al., 1987), whereas the individual-animal and the within-herd seroprevalence in herds of these types were estimated to be 64% and 51%, respectively, in 1995 (Lemaire et al., 1997). The national prevalence of BoHV-1 in Belgian dairy, mixed and beef herds is unknown.

To investigate BoHV-1 prevalences in the Belgian cattle population, a survey was conducted from December 1997 to March 1998 in all the provinces of Belgium. The primary goal of this survey was to provide an unbiased estimate of the true national BoHV-1 herd-level prevalence in dairy, mixed and beef herds, by random selection of herds to sample. A second objective was to estimate the true national BoHV-1 individual-animal and within-herd prevalences in dairy, mixed and beef herds.

MATERIAL AND METHODS

Survey design

The survey was organized using the co-ordinates for the cattle herds registered in SANITEL-Cattle, the central computerized database for the identification and registration of the Belgian cattle population (Ministry of Small Enterprises, Traders and Agriculture, Belgium). By
law, all Belgian cattle keepers have to be registered in SANITEL-Cattle and have the duty to report all the necessary data that are needed for making up their herd and cattle-movement inventories. This information is updated daily in SANITEL-Cattle by the Regional Veterinary Investigation Centers. In SANITEL-Cattle, a herd is defined as a stock of bovids kept in a geographical entity -- containing one or several buildings with adjacent premises -- that makes up a clear and distinct unit on the basis of epidemiological bounds set by the Veterinary Inspection. Therefore, in this survey, the sampling units were defined as these cattle herds.

The survey was conducted on herds of all types from December 1997 to March 1998. A stratified random sample design was followed. The total number of herds to be sampled was set at 1% of the total number of Belgian cattle herds. The sample was stratified by province. The number of herds to be sampled in each province was determined by proportional allocation (Thrusfield, 1995). Herds were randomly selected from SANITEL-Cattle using a software random generator function of Visual Basic 3.0 (Microsoft Corp., 1993). In the selected herds, all animals were blood sampled. A herd was defined to be BoHV-1-seropositive if at least one BoHV-1 seropositive bovid was present.

The number of herds on the computer-generated sampling lists exceeded the calculated number, to allow for replacement of herds which did not have cattle on the sampling date. As soon as a herd was confirmed as not having cattle, it was replaced by the next herd on the reserve list. In 185 instances, herds were replaced.

Collection of samples and herd and management characteristics

The blood samples were taken by the veterinary practitioners and sent to the Veterinary and Agrochemical Research Center. The age of the cattle was known from the SANITEL-Cattle herd inventories. The veterinary practitioners also interviewed the farmer concerning the following herd and management characteristics: herd type (dairy herd, mixed herd or beef herd), herd size (number of cattle on the premises), and whether the farmer vaccinated continuously, intermittently, or not, against BoHV-1 (before 1996, during 1996-1997 and in 1998), or whether he did not know the BoHV-1 vaccination status.

Serological testing and interpretation

The serum samples were tested for antibodies against BoHV-1 by using a commercially available blocking ELISA (HerdChek®, Idexx, France), specific for BoHV-1 glycoprotein B (gB)
(Kramps et al., 1994). All samples were tested according to the manufacturer’s instructions. Doubtful test results were classified negative in the data analysis.

**Statistical methods used to calculate the true prevalence for unvaccinated herds**

The seropositive herds will have one or more infected animals, or have veal with maternal antibodies, or have vaccinated animals. Because of the latter, the vaccination status of tested herds was investigated, and the BoHV-1 true prevalences for unvaccinated herds were calculated as follows. First, assumptions found in the literature concerning the intrinsic properties of the gB blocking ELISA were made: a diagnostic test sensitivity (Se) of 99% (Kramps et al., 1994), and a diagnostic test specificity (Sp) of 99.7% (de Wit et al., 1998). Second, true within-herd prevalence (WHP) for each of the BoHV-1 seropositive herds was estimated according to the standard equation of Marchevsky (1974). Third, for each of the BoHV-1 seropositive herds, the infected herd detectability (IHD) was calculated based on the following probabilities formula:

\[
IHD = 1 - \left[ (1 - Se)^m \cdot WHP \cdot (Sp)^m \cdot (1 - WHP) \right]
\]

whereby \(m\) is the sample or herd size because all cattle present were sampled.

This formula is the equivalent of the herd-level sensitivity formula developed by Martin et al. (1992), adapted for sampling of all animals present in the herds. The overall and herd-type-specific IHD were calculated as the median IHD of the BoHV-1 seropositive herds and of the seropositive dairy, mixed and beef herds respectively. Fourth, the herd-level specificity (HSp) was calculated according to Martin et al. (1992);

\[
HSp = (Sp)^m
\]

whereby \(m\) is the median sample or herd size because - as for the IHD - all cattle present were sampled.

Fifth, based on the calculated IHD and HSp, the true herd-level prevalence was estimated according to the standard equation of Marchevsky (1974).
The true individual-animal prevalence (P) was calculated according as:

\[ P = \frac{\sum_{i=1}^{n} d_i}{N} \]

whereby \( d_i \) (the number of infected animals) was estimated for each seropositive herd by multiplying the sample or herd size by the WHP, and N was total number of animals held in the unvaccinated herds.

The overall and herd-type-specific WHP were estimated based on the survey results from the BoHV-1 seropositive herds, assuming non-reactor herds were non-infected. This calculation consisted in calculating the median of the estimations of the WHP for each of the BoHV-1 seropositive herds.

Lastly, the infected herd detectability, herd specificity, and herd true prevalence were estimated according to a range of test sensitivities and specificities of respectively 70% - 99% and 96% - 99.7%.

**Data analysis**

The prevalences were analyzed per herd type to allow comparison with other published BoHV-1 prevalence figures. Data originating from herds without herd-type specification were excluded from the analysis. The median herd size of different herd types were compared using a Wilcoxon rank-sum test. The proportion of farmers of different herd types that vaccinated continuously against BoHV-1 were compared using a logistic regression model using PROC GENMOD (Statistical Analysis Systems institute Inc., 1996) with herd type as independent variable and vaccination status as the response variable. All tests were two-tailed and a p-value < 0.05 was considered as significant.

**RESULTS**

**General features of the target and study population**

The sample consisted of 594 randomly selected herds (Table 1). There were 38 nonresponding herds (6% of the 594 herds) from which no samples were received and for which
no replacement occurred either. The reasons for non-response were: (1) the farmer had ceased his activities (24 herds, 4.0%); and (2) no cattle were blood sampled in due time, due to lack of coordination between different project partners (14 herds, 2.3%). The study population consisted of 106 (20%) dairy herds, 113 (21%) mixed herds and 309 (59%) beef herds. At the animal-level the total numbers of bovines held in dairy, mixed and beef herds were 8,360 (31%), 10,206 (37%) and 8,892 (32%), respectively. The median and the range of the herd size of herds were: 81, 2-238 for dairy herds; 73, 4-252 for mixed herds; and 11, 1-326 for beef herds.

Table 1. Sample composition and national seroprevalence of bovine herpesvirus-1 (BoHV-1) in Belgium, 1998

<table>
<thead>
<tr>
<th>Number of herds</th>
<th>Number of cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>58,811</td>
</tr>
<tr>
<td><strong>To be sampled</strong></td>
<td>594</td>
</tr>
<tr>
<td><strong>Actually sampled</strong></td>
<td>556</td>
</tr>
<tr>
<td><strong>Actually sampled unvaccinated herds</strong></td>
<td>309</td>
</tr>
<tr>
<td><strong>BoHV-1 seroprevalence for</strong></td>
<td></td>
</tr>
<tr>
<td><strong>unvaccinated herds, [95% CI]</strong></td>
<td>207</td>
</tr>
</tbody>
</table>

**Vaccination against BoHV-1**

The vaccination status of the 534 herds that sent in a questionnaire, are shown in Table 2. The median herd size of vaccinated herds was; 88 overall and 91 for dairy herds; 114 for mixed herds; and 48 for beef herds; whereas the median herd size of unvaccinated herds was; 19 overall and 60 for dairy herds; 59 for mixed herds; and 7 for beef herds. Herds that vaccinated continuously had thus a larger herd size compared to unvaccinated herds, overall and specific for all herd types.

The proportion of farmers that vaccinated continuously against BoHV-1, was 21% (102/(102+309+64)). Compared to the proportion of beef herds that were vaccinated continuously (17%), more mixed herds were vaccinated (28%), whereas this proportion differed not with that for dairy (24%).
Table 2. Vaccination status against bovine herpesvirus-1 (BoHV-1) and herd size of cattle herds, per herd type, Belgium, 1998

<table>
<thead>
<tr>
<th>Type of herd</th>
<th>Variable being cited (number)</th>
<th>Vaccinated</th>
<th>Unvaccinated</th>
<th>Unknown vaccination status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Continuously</td>
<td>Intermittently</td>
<td></td>
</tr>
<tr>
<td>All, combined</td>
<td>Herds</td>
<td>102</td>
<td>64</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>9,313</td>
<td>4,686</td>
<td>11,284</td>
</tr>
<tr>
<td>Dairy</td>
<td>Herds</td>
<td>23</td>
<td>11</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>2,073</td>
<td>1,156</td>
<td>4,112</td>
</tr>
<tr>
<td>Mixed</td>
<td>Herds</td>
<td>29</td>
<td>22</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>3,624</td>
<td>2,305</td>
<td>3,724</td>
</tr>
<tr>
<td>Beef</td>
<td>Herds</td>
<td>47</td>
<td>31</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>3,446</td>
<td>1,225</td>
<td>3,378</td>
</tr>
</tbody>
</table>

a herds that were vaccinated continuously
b herds that were never vaccinated
c 3 herds of unknown herd type
d, e 2 herds of unknown herd type
Table 3. National seroprevalence of bovine herpesvirus-1 (BoHV-1), in Belgium, 1998

<table>
<thead>
<tr>
<th>Type of herd</th>
<th>Herd seroprevalence</th>
<th>Individual-animal seroprevalence</th>
<th>Within-herd seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>% positive [95% CI]</td>
<td>Number tested</td>
</tr>
<tr>
<td>Dairy</td>
<td>59</td>
<td>86 [77.7-95.2]</td>
<td>4,112</td>
</tr>
<tr>
<td>Mixed</td>
<td>54</td>
<td>91 [83.0-98.5]</td>
<td>3,724</td>
</tr>
<tr>
<td>Beef</td>
<td>194</td>
<td>54 [47.1-61.1]</td>
<td>3,378</td>
</tr>
</tbody>
</table>

* confidence intervals

Table 4. Infected herd detectability, herd specificity, and true prevalences at the herd-level, animal-level and within-herd, of bovine herpesvirus-1 (BoHV-1), Belgium, 1998 *

<table>
<thead>
<tr>
<th>Type of herd</th>
<th>Infected herd detectability (%)</th>
<th>Herd specificity (%)</th>
<th>True herd prevalence (%)</th>
<th>True individual-animal prevalence</th>
<th>Within-herd prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Npos(%)</td>
<td>N</td>
<td>median (quartiles)</td>
<td>average</td>
</tr>
<tr>
<td>Dairy</td>
<td>100</td>
<td>84</td>
<td>84</td>
<td>4,112</td>
<td>1,448(35%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>100</td>
<td>84</td>
<td>89</td>
<td>3,724</td>
<td>1,584(43%)</td>
</tr>
<tr>
<td>Beef</td>
<td>100</td>
<td>98</td>
<td>53</td>
<td>3,378</td>
<td>1,059(31%)</td>
</tr>
</tbody>
</table>

* Assuming a true overall within-herd prevalence of 34%; a true within-herd prevalence of 36% for dairy herds, 29% for mixed herds, and 38% for beef herds; a median of 19 animals per herd, a median sample size of 60 for dairy herds, 59 for mixed herds, and 7 for beef herds; a test sensitivity and specificity of 99% and of 99.7%, respectively; and non-reactor herds non-infected.
BoHV-1 seroprevalence in unvaccinated herds

The BoHV-1 overall herd and individual-animal seroprevalence (95% confidence interval) for unvaccinated herds were respectively 67% (62-72) and 35.9% (35.0-36.8) (Table 1). The overall median (quartiles) and average within-herd seroprevalence were respectively 33% (14-62) and 41%. The herd-type-specific seroprevalence parameters are summarized in Table 3.

BoHV-1 true prevalence in unvaccinated herds

The median WHP was estimated to be 34%. The overall frequency distribution of the BoHV-1 within-herd prevalence is shown in Figure 1; of the positive herds, 57% had ≤ 40% seropositive samples, -- but 12% of the positive herds had > 90% gB-positive samples. The overall IHD was calculated to be 100%. Based on a median sample size of 19, the overall HSp was 94%. Based on the aforementioned parameters, the true overall herd prevalence was 65%. The true overall individual-animal prevalence was 36% (4,095/11,284). The herd-type-specific prevalence parameters are summarized in Table 4.

In Figure 2 the infected herd detectability, herd specificity, and herd true prevalence are estimated according to a range of test sensitivities and specificities of respectively 70% - 99% and 96% - 99.7%, a median sample or herd size of 19 animals, and a BoHV-1 overall within-herd prevalence of 34%. Assuming a Se of 99% and a Sp of 99.7%, the herd true prevalence estimation was 65%, whereas with the same Se and a Sp of 96%, the herd true prevalence estimation was 28%.
Figure 1. Frequency distribution of the BoHV-1 true within-herd prevalence in Belgium, 1998

a Assuming a test sensitivity and specificity of 99% and of 99.7%, respectively
b In parentheses: number of positive herds

Figure 2. BoHV-1 infected herd detectability (IHD), herd specificity (HSp) and herd true prevalence (HP) in non-vaccinated herds, calculated for a range of test sensitivities (Se) and specificities (Sp), Belgium 1998

a Assuming a true overall within-herd prevalence of 34%, a median herd size of 19 animals, and a test sensitivity and specificity of 99% and of 99.7%, respectively.
DISCUSSION

The present sample survey aimed to provide an unbiased estimate of the true national BoHV-1 herd prevalence, and so these herds were randomly selected. Because the percentage of nonresponding herds was low (6%), this random sample of herds can be considered to be representative for the Belgian cattle population (Thrusfield, 1995). Because all cattle in the selected herds were tested, the reactor herds provided data without sampling bias for estimation of the apparent and true within-herd prevalence, compared to studies with a within-herd sample-based design.

Vaccination against BoHV-1

The vaccination percentage for dairy herds (24%) was in line with those of Dutch dairy herds (about 20% before the start of a BoHV-1 eradication program based on compulsory vaccination) (Vonk Noordegraaf et al., 1998). The herd-type-specific vaccination percentage was highest for mixed herds. Since vaccination against BoHV-1 is usually practiced with the aim of clinical protection, this could indicate that herd type is a possible risk factor for BoHV-1 infection.

BoHV-1 seroprevalence

In Belgium, only live non-marker vaccines were commercialized before 1997. Since it is not possible to discriminate between antibody response following vaccination and the antibody response following infection, vaccination with these non-marker vaccines has in fact only increased the prevalence of gB-seropositive cattle. In this survey, seropositive unvaccinated herds could thus have purchased animals vaccinated with conventional vaccines or non-infected veal with maternal antibodies, aside one or more infected animals. This could have resulted in an overestimation of the true overall prevalences. However, the unvaccinated herds had a smaller herd size compared to vaccinating herds. Consequently, the seroprevalences for the unvaccinated herds might also underestimate the true overall prevalences, because larger herd size is a possible risk factor for BoHV-1 infection (Van Wuijckhuise et al., 1998).

When comparing the apparent prevalences of the unvaccinated herds to published figures of seroprevalence that did not take into account the herd vaccination status, the following observations can be made. The overall herd seroprevalence for unvaccinated mixed and beef herds, 62% (49+105/54+194) equals the regional seroprevalence figure obtained by Van Malderen et al. (1987). The BoHV-1 herd seroprevalence of Belgian herds with dairy cows, taking into
consideration dairy and mixed herds, was 88.5% (51+49/59+54) with 95% confidence interval (CI) of [82.6-94.8], is comparable to the herd seroprevalences of analogue Dutch herds in 1992, 93%, reported by van Wuyckhuise et al. (1993) and in 1997, 92%, reported by Hartman et al. (1997). The former Dutch study concerned only unvaccinated herds. The individual-animal seroprevalence for herds with dairy cows, 38.3% (1,436+1,567/4,112+3,724) with 95% CI of [37.2-39.4], was comparable to the Dutch analogue figure of 36% in unvaccinated herds, in 1992 (van Wuyckhuise et al., 1993). The individual-animal seroprevalence for mixed and beef herds, 36.9% (1,567+1,051/3,724+3,378) with 95% CI of [35.7-38.0], was lower than the regional figure of 64% obtained by Lemaire et al. (1997). These authors found a regional average within-herd seroprevalence figure for mixed and beef herds of 51%, whereas in the present study the average (quartiles) within-herd seroprevalence was 42% [16-67].

**BoHV-1 true prevalence of unvaccinated herds**

The testing procedure could also have been a source of information bias, because the aforementioned apparent prevalences assume a perfect test sensitivity and specificity of 100%. No published data of sensitivity and specificity of the ELISA kit, used in this survey, exist. Kramps et al. (1994) estimated the diagnostic sensitivity of the gB-blocking ELISA to be 99%. In their study no information was reported on the reference test (gold standard) or the vaccination status of the positive reference sera. The estimated diagnostic specificity of the gB-blocking ELISA in a BoHV-1 free and unvaccinated animal population ranged from 96% (Kramps et al., 1994) to 99.7% (de Wit et al., 1998). For the true prevalence calculations, the most recent published test specificity figure was assumed. The gB ELISA is very sensitive, inducing a negligible problem of false negative animals. The assumed specificity is very high -- implying only a minor problem with false-positive animals. The problem of false-positive test results was further diminished by the protocol of the data analysis, whereby doubtful OD-test values were classified as negative. The estimations of the true within-herd prevalence (34%) and of the infected herd detectability (100%) assumed non-reactor herds to be non-infected. Corrected for testing procedures the true prevalence figures were comparable to the apparent prevalences.

Since these intrinsic test characteristics could have been too optimistic, the herd true prevalence was verified for a set of test characteristics. For these calculations, a fix true within-herd prevalence of 34% was assumed, as this parameter did not vary substantially according to the different test characteristics mentioned hereafter. The calculations show that the herd true prevalence stays the same for a varying test sensitivity ranging from 70% to 99%. The infected herd detectability was 99.99% to 100% for all parameter combination under consideration. However, the lack of test specificity has a dramatic effect on the estimation of the herd true
prevalence; the true herd prevalence estimation decreased from 65% to 28% if the test specificity decreased from 99.7% (de Wit et al., 1998) to 96% (Kramps et al., 1994), respectively, for a varying test sensitivity ranging from 70% to 99%. These calculations showed that the practical limits of the accuracy of the used screening test, a gB ELISA, jeopardize the estimation of the herd true prevalence within reasonable confidence intervals.

**CONCLUSION**

The results of this survey show that the Belgian cattle population is endemically infected with BoHV-1. Eradication of BoHV-1 will be only economically feasible by first lowering the prevalence, possibly followed by test and removal procedures. To anticipate such BoHV-1 eradication program, the Belgian Veterinary Authorities imposed by law the exclusive use of marker vaccines since 1997. Vonk Noordegraaf et al. (1998) simulated the epidemiological and economic consequences of various control strategies of BoHV-1 infection in the Dutch pure dairy herds, and found that compulsory vaccination would be necessary to reach a BoHV-1-free status. The rate of Belgian dairy farmers vaccinated continuously against BoHV-1, 24%, would be too low. However, the model developed by Vonk Noordegraaf et al. (1998) should be run on the basis of parameters characterizing the Belgian dairy cattle population.

**ACKNOWLEDGMENTS**

We thank all the veterinary surgeons and farmers who participated in the study, the Regional Veterinary Investigation Centers and Inspection Services for execution of the survey, Christel De Smedt and Amédée Massart for skilled technical and administrative assistance. The support of the Directors and the staff of V.A.R. is gratefully acknowledged. This study was supported by the Fund for Animal Health and Production, Ministry of Small Enterprises, Traders and Agriculture, Belgium.
REFERENCES

ABSTRACT

The national bovine paratuberculosis (PTB) seroprevalence (apparent prevalence) in the Belgian cattle population was determined by a serological survey that was conducted from December 1997 to March 1998. In a random sample of herds (N = 556, 9.5%), all adult cattle of 24 months of age or older (N = 13,317, 0.4%) were tested for the presence of antibodies using a commercially available absorbed ELISA test kit. The PTB median within-herd seroprevalence (proportion of detected animals within the seropositive herds) and the PTB individual-animal seroprevalence (proportion of detected animals) were, respectively, 2.9% (quartiles=1.6-5.6) and 0.87% (95% CI=0.71-1.03). The PTB herd seroprevalence (proportion of detected herds) was 18% (95% confidence interval (CI)=14-21).

Assuming a test sensitivity and specificity of 45 and 99% (Sweeney et al., 1995 and Sockey et al., 1992) respectively, the median true within-herd prevalence and the true individual-animal were estimated to be 7 and 2% respectively. The true herd prevalence of *M. paratuberculosis* infection was first estimated according to currently accepted methodology. This calculation revealed that the specificity of the used test has a dramatic effect on the estimation; assuming a test sensitivity of 45% and a true within-herd prevalence of 7%, the true herd prevalence estimation decreased from 36 to 0.8% if the test specificity decreased from 99.9 to 99% respectively. This sensitivity analysis showed that the practical limits of the accuracy of the used screening test jeopardize the estimation of the true herd prevalence within reasonable confidence limits, because the within-herd PTB true prevalence was low.

For this reason we augmented the herd specificity for herds with larger adult herd size (> 5). This was done by increasing the cut-off number of positive cattle required (≥ 2) to classify a herd truly positive and including herds with one positive test result if there was historical evidence of PTB (previous diagnosis and/or clinical signs). This approach resulted in an estimated true herd prevalence of *M. paratuberculosis* infection of 6%. The true herd prevalence for dairy, mixed and beef herds was respectively 10, 11 and 3%.

INTRODUCTION

Paratuberculosis (PTB), or Johne’s disease, is a chronic infectious disease of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis*. It is an enzootic disease on the B List of the ‘Office International des Epizooties’ (O.I.E.), and is characterized by chronic, granulomatous degenerative enteritis that causes intermittent but persistent diarrhea, progressive weight loss, and eventually, death. The disease is untreatable and slowly progressive. Paratuberculosis is probably the most widespread infectious disease of domestic animals and
causes important economic losses in ruminants, particularly cattle, worldwide (Buergelt and Duncan, 1978; Chiodini et al., 1984; Chiodini and Van Kruiningen, 1986; Benedictus et al., 1987). Expanded efforts to control this disease, including regulatory programs in some countries, may lead to future market restrictions.

In Belgium PTB is not a notifiable disease, and hence no official control or eradication program is executed. Vaccination has been recommended in heavily, clinically infected herds. However, vaccination precludes the serodiagnosis of PTB-infected cattle, and is administered under the authority of the Veterinary Inspection since it interferes with the diagnosis of bovine tuberculosis.

When dealing with infectious diseases, the group of animals which is of epidemiological importance in terms of the transmission and maintenance of infection - and therefore of disease control and eradication - is the herd (Thrusfield, 1995). This is particularly true for PTB because the control and eradication measures implicate the herd - not the animal - (O.I.E., 1998). Therefore, in this survey, the sampling units were defined as the cattle herds.

To date, few methodological serological surveys have been organized to estimate the PTB prevalence at the regional or national levels. Moreover, these surveys are affected by the nature of the study design (sample or census surveys), the study population (subclinical or clinical), the type of prevalence parameters studied (herd, individual animal or within-herd prevalence), the diagnostic test used, and the age of the tested animals. Comparison of these survey results is virtually impossible. Moreover, only very few studies adjust the seroprevalence for factors such as test sensitivity and specificity to calculate the true prevalence, creating further difficulty in comparison across studies.

Estimations of the herd seroprevalence in the USA range from 50% in Wisconsin (Collins et al., 1994) to 74% in Missouri (Thorne and Hardin, 1997) for dairy herds. In Louisiana beef herds, Turnquist et al. (1991) found a herd seroprevalence of 30%, whereas Thorne and Hardin (1997) estimated it to be 40%. At the individual-animal level, serological surveys results range from 7.3% in Wisconsin (Collins et al., 1994) to 17.1% in Florida (Braun et al., 1990) for dairy cattle, and from 1.2% in Finland (Hintikka, 1998) to 25.2% in Texan beef cattle (Alexander et al., 1993). In Belgium, a regional survey in Southern Belgium found 12% of the cattle seropositive to PTB (Vannuffel et al., 1994).

Published estimations of the true prevalence are only available for dairy herds. The true herd prevalence is estimated to range from 1.3% in England, United Kingdom (Cetinkaya et al., 1998) to 34% in Wisconsin (Collins et al., 1994). The true individual-animal prevalence in dairy cattle is estimated to range from 4.8% in Wisconsin (Collins et al., 1994) to 6.1% in Ontario, Canada (NeNab et al., 1991). Hardly any data exist on the true within-herd prevalence of PTB. Estimations
range from 5% (Obasanjo et al., 1997) based on whole herd examination by fecal culture, to 20% based on sample surveys by absorbed ELISA (Collins et al., 1994).

To investigate PTB prevalences in the Belgian adult cattle population, a pilot survey was conducted from December 1997 to March 1998 in all the provinces of Belgium. The goal of this survey was first to provide an unbiased estimate of the national herd-level seroprevalence of *M. paratuberculosis* infected dairy, mixed and beef herds, by random selection of herds to sample, and second to calculate the true national PTB herd prevalence.

**MATERIAL AND METHODS**

**Survey design**

The survey was organized using the co-ordinates for the cattle herds registered in SANITEL-Cattle, the central computerized database for the identification and registration of the Belgian cattle population (Ministry of Small Enterprises, Traders and Agriculture, Belgium). SANITEL-Cattle constitutes a permanent basis for efficient organized disease control. By law, all Belgian cattle keepers have to be registered in SANITEL-Cattle and have the duty to report all the necessary data that are needed for making up their herd and cattle-movement inventories. This information is updated daily in SANITEL-Cattle by the Regional Veterinary Investigation Centers. In SANITEL-Cattle, a herd is defined as a stock of cattle kept in a geographical entity - containing one or several buildings with adjacent premises - that makes up a clear and distinct unit on the basis of epidemiological bounds set by the Veterinary Inspection. Therefore, in this survey, the sampling units were defined as these cattle herds.

The survey was conducted on herds of all types from December 1997 to March 1998. A stratified random sample design was followed. The total number of herds to be sampled was set at 1% of the total number of Belgian cattle herds. The sample was stratified by province. The number of herds to be sampled in each province was determined by proportional allocation (Thrusfield, 1995). Herds were randomly selected from SANITEL-Cattle using a software random generator function of Visual Basic 3.0 (Microsoft Corp., 1993). In the selected herds, all of the adult herd, i.e. all cattle over 24 months of age were blood sampled. A herd was defined to be PTB-seropositive if at least one PTB-seropositive adult bovine was present.

The number of herds on the computer-generated sampling lists exceeded the calculated number, to allow for replacement of herds which did not have cattle on the sampling date. In 185 instances, herds were replaced.
Collection of samples and herd and management characteristics

The blood samples were taken by the veterinary practitioners and sent to the Veterinary and Agrochemical Research Center. The age of the cattle was known by the SANITEL-Cattle herd inventories. By means of a questionnaire, the veterinary practitioners also interviewed the farmer concerning the following herd and management characteristics: herd type (dairy herd, mixed herd or beef herd), herd size (number of cattle on the premises), whether the farmer vaccinated yearlong against PTB, and whether there was historical evidence of PTB (previous diagnosis and/or clinical signs).

Serological testing

The serum samples were tested for antibodies to *M. paratuberculosis*, using a commercially available Absorbed ELISA (HerdChek®, IDEXX, France). All samples were tested using one batch of test kits, according to the manufacturer’s instructions. Sera with corrected optical density (OD)-values < 0.2 and ≥ 0.3 were considered negative and positive, respectively. Intermediate OD-values were considered doubtful and classified as negative in the data analysis.

Data analysis

The inclusion criteria were as follows: (1) the samples had to be obtained from adult cattle; (2) the samples had to be obtained from herds that never vaccinated against PTB. Data originating from herds with all sampled cattle outside the required age category or that ever vaccinated against PTB were excluded from the analysis. The prevalences were analyzed per herd type to allow comparison with other published PTB prevalence figures. Data originating from herds without herd type specification were excluded from the analysis.

Statistical methods used to calculate the true herd prevalence for unvaccinated herds

The overall and herd type specific true within-herd prevalence (WHP) were estimated based on the survey results from the PTB seropositive herds, assuming that non-reactor herds were non-infected. This consisted in calculating the median of the estimations of the WHP for each of the PTB seropositive herds. The WHP for each of the PTB seropositive herds was estimated according to the standard equation of Marchevsky (1974).
The true individual-animal prevalence (P) was calculated according to:

\[ P = \frac{\sum_{i=1}^{n} d_i}{N} \]

whereby \( d_i \) is the number of infected animals that was estimated for each seropositive herd by multiplying the sample size by the WHP, and whereby \( N \) was total number of adult animals held in the unvaccinated herds.

Estimation of the true herd prevalence of *M. paratuberculosis* infection should incorporate factors, such as test sensitivity and specificity, true within-herd prevalence, sample size and the cut-off number of reactors required to call a herd truly positive, that lead to uncertainty in the observed herd prevalence (Martin et al, 1992). First, the following assumptions found in the literature concerning the intrinsic properties of the absorbed ELISA were made: an overall diagnostic test sensitivity (Se) and specificity (Sp) of respectively 45% and greater than 99% (Collins, 1996). Second, true within-herd prevalence (WHP) for each of the PTB seropositive herds was estimated as described above. Third, the infected herd detectability (IHD) was calculated based on the following exact probabilities formula (Boelaert et al., 2000):

\[
IHD = 1 - [(1-Se)^{m \times \text{WHP}} \times (Sp)^{m \times (1-\text{WHP})}] 
\]

whereby \( m \) is the median sample or adult herd size because all adult cattle present were sampled.

This formula is the equivalent of the Herd Sensitivity formula developed by Martin et al. (1992), adapted for sampling of all adult animals present in the herds. The overall and herd-type-specific IHD were calculated as the median IHD of the PTB seropositive herds and the median of the seropositive dairy, mixed and beef herds respectively. Fourth, the herd-level specificity (HSp) was calculated according to Martin et al. (1992):

\[
\text{HSp} = (Sp)^{m}
\]

whereby \( m \) is the median sample or adult herd size because - as for the IHD - all adult cattle present were sampled.

Fifth, based on the calculated IHD and HSp, the true herd-level prevalence was estimated according to the standard equation of Marchevsky (1974). The IHD, the HSp, and herd true
prevalence were also estimated according to a range of test sensitivities and specificities of respectively 25 - 55% and 99.0 - 99.9%.

Apart from the above method of true herd prevalence calculation, we used another approach to augment the HSp for herds with more than 5 adult cattle since for these herds the HSp drops below 95% if the Sp is 99% (Martin et al. 1992). This approach consisted in increasing the cut-off number of positive cattle required to classify a herd truly positive, as described by Jordan (1996), and adding herds with one positive test result if there was historical evidence of PTB (previous diagnosis and/or clinical signs) on the farm.

**RESULTS**

**General features of the target and study population**

In 1997 there were 3,242,600 cattle and 58,811 cattle herds in Belgium. The average herd size was 55.

The sample consisted of 594 randomly selected herds (Table 1). There were 83 nonresponding herds (14% of the 594 herds) from which no samples were received and for which no replacement occurred either. The major reasons for no response were: (1) the farmer had ceased his activities (53 herds, 8.9%); (2) no adult cattle were present (26 herds, 4.4%); and (3) no cattle were blood sampled in due time, due to lack of coordination between different project partners (4 herds, 0.7%). A total of 14,699 adult cattle from 511 herds (86.0%) were tested for *M. paratuberculosis* during this survey. There were 47 tested herds that did not meet the inclusion criteria for data analysis. The reasons for this were: (1) no complete information was available about the PTB vaccination scheme (29 herds, 4.9%); (2) no questionnaire was send in (14 herds, 2.4%); and (3) the herd was vaccinated against PTB (4 herds, 0.7%). A total of 13,317 adult cattle from 464 (78.1%) herds that did not vaccinate against PTB, met the inclusion criteria. The median, the average and the range of the herd size were 38, 55, and 1-326. The study population was made up of 98 (21%) dairy herds, 101 (22%) mixed herds and 259 (56%) beef herds. At the animal-level the total numbers of animals held in dairy, mixed and beef herds were 7,775 (31%), 9,137 (36%) and 8,303 (33%), respectively. The median and the range of the herd size of herds were: 81, 2-238 for dairy herds; 72, 4-252 for mixed herds; 14, and 1-326 for beef herds.
Table 1. National seroprevalence of paratuberculosis in Belgium, 1998

<table>
<thead>
<tr>
<th>Number of herds</th>
<th>Number of cattle of 24 months of age or older</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Total</td>
<td>58,811</td>
</tr>
<tr>
<td>To be sampled</td>
<td>594</td>
</tr>
<tr>
<td>Actually sampled</td>
<td>511</td>
</tr>
<tr>
<td>Actually sampled non-vaccinated herds</td>
<td>464</td>
</tr>
<tr>
<td>Actually sampled non-vaccinated herds, with herd type specification</td>
<td>458</td>
</tr>
<tr>
<td>PTB seroprevalence, [95% CI ( b )]</td>
<td>82</td>
</tr>
</tbody>
</table>

\( a \) SANITEL-Cattle, 1997. Ministry of Small Enterprises, Traders and Agriculture, Belgium

\( b \) confidence intervals
Table 2. Distribution of test results of non-vaccinated herds with adult cattle seropositive to paratuberculosis in Belgium, 1998

a. Number of adult cattle tested for *M. paratuberculosis* per herd:

<table>
<thead>
<tr>
<th>Number of cattle sampled</th>
<th>Number of herds</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>5 or fewer</td>
<td>129 27.8</td>
</tr>
<tr>
<td>6-25</td>
<td>132 28.4</td>
</tr>
<tr>
<td>26-50</td>
<td>106 22.8</td>
</tr>
<tr>
<td>51-75</td>
<td>57 12.3</td>
</tr>
<tr>
<td>76-100</td>
<td>26 5.6</td>
</tr>
<tr>
<td>101-250</td>
<td>14 3.0</td>
</tr>
<tr>
<td>Total</td>
<td>464 100</td>
</tr>
</tbody>
</table>

Average: 29; minimum:1; first quartile:5; median:19; third quartile:43; maximum:213.

b. Number of *M. paratuberculosis*-seropositive adult cattle per herd:

<table>
<thead>
<tr>
<th>Number of test positive cattle</th>
<th>Number of herds</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>382 82.3</td>
</tr>
<tr>
<td>1</td>
<td>60 12.9</td>
</tr>
<tr>
<td>2</td>
<td>17 3.7</td>
</tr>
<tr>
<td>3</td>
<td>2 0.4</td>
</tr>
<tr>
<td>4</td>
<td>1 0.2</td>
</tr>
<tr>
<td>5</td>
<td>1 0.2</td>
</tr>
<tr>
<td>6</td>
<td>0 0.0</td>
</tr>
<tr>
<td>7</td>
<td>1 0.2</td>
</tr>
<tr>
<td>Total</td>
<td>464 100</td>
</tr>
</tbody>
</table>

Average: 1.4; minimum:1; first quartile:5; median:1; third quartile:2; maximum:7.

**PTB seroprevalence in unvaccinated herds**

The PTB overall herd and individual-animal seroprevalence (95% confidence interval) for unvaccinated herds were respectively 18% (14.2-21.1) and 0.87% (0.71-1.03) (Table 1).

The distribution of the herd test results is depicted in Table 2. Seventy three percent of herds testing positive (60/82) had only one single positive test result. The overall median (quartiles) and average within-herd seroprevalence were respectively 2.9% (1.6-5.6) and 7.1%. The frequency
distribution of the PTB within-herd seroprevalence is shown in Figure 1. Of the positive herds, 90% had a maximum within-herd seroprevalence of 10%. The herd-type-specific seroprevalence parameters are summarized in Table 3.

Figure 1. Frequency distribution of the within-herd seroprevalence and prevalence of adult cattle seropositive to paratuberculosis in Belgium, 1998

* Number of positive herds

a Assuming a test sensitivity of 45%, and a test specificity of 99%
Table 3. National seroprevalence of paratuberculosis in non-vaccinated herds, per herd type, Belgium, 1998

<table>
<thead>
<tr>
<th></th>
<th>Within-herd seroprevalence (%)</th>
<th>Individual-animal seroprevalence</th>
<th>Herd seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (quartiles) average</td>
<td>N</td>
<td>Npos(%)</td>
</tr>
<tr>
<td>Dairy herd</td>
<td>2.2 (1.7-4.9)</td>
<td>3.3</td>
<td>4,497</td>
</tr>
<tr>
<td>Mixed herd</td>
<td>2.9 (1.7-4.7)</td>
<td>4.4</td>
<td>4,643</td>
</tr>
<tr>
<td>Beef herd</td>
<td>4.2 (1.1-17.5)</td>
<td>18.4</td>
<td>4,010</td>
</tr>
</tbody>
</table>

Table 4. Estimates of the national prevalences of paratuberculosis in non-vaccinated herds, per herd type, Belgium, 1998

<table>
<thead>
<tr>
<th></th>
<th>Within-herd prevalence (%) a</th>
<th>True individual-animal prevalence a</th>
<th>True herd prevalence (%) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (quartiles) average</td>
<td>N</td>
<td>Npos(%)</td>
</tr>
<tr>
<td>Dairy herd</td>
<td>5 (4-11)</td>
<td>8</td>
<td>4,497</td>
</tr>
<tr>
<td>Mixed herd</td>
<td>7 (4-10)</td>
<td>10</td>
<td>4,643</td>
</tr>
<tr>
<td>Beef herd</td>
<td>9 (3-36)</td>
<td>27</td>
<td>4,010</td>
</tr>
</tbody>
</table>

a Assuming (1) a median sample size of 46 for dairy herds; 39 for mixed herds; and 6 for beef herds, (2) a test sensitivity of 45%, and a test specificity of 99%, and (3) non-reactor herds non-infected.

b Including herds with ≥ 2 positive animals, herds with one positive test result if there was historical evidence of PTB (previous diagnosis and/or clinical signs) on the farm, and herds with small adult herd size (≤ 5) with one positive test result.
PTB true prevalence

As a randomized survey design was followed, and adult cattle of 24 months of age or older were sampled, a test sensitivity of 45% and a test specificity of 99% were assumed, according to Sweeney et al. (1995) and Sackett et al. (1992).

The overall median (quartiles) and average within-herd prevalence were respectively 7% (4-12) and 13%. The overall frequency distribution of the PTB true within-herd prevalence is shown in Figure 1; of the positive herds, 93% had a maximum true within-herd prevalence of 30%. The true overall individual-animal prevalence was 2%.

Based on the aforementioned parameters, the overall IHD and the overall HSp were both 83%. Consequently, the overall true herd prevalence was 0.8%.

Figure 2 depicts the calculated IHD, HSp, and herd true prevalences according to a range of test sensitivities and specificities of respectively 25 - 55% and 99.0 - 99.9%, a median adult herd sample size of 19 animals, and a PTB overall true within-herd prevalence of 7%. It shows that, for a test sensitivity of 45%, the true herd prevalence estimation decreased from 36 to 0.8% if the test specificity decreased from 99.9 to 99% respectively.

The alternative approach consisting in using the cut-off of two positive test results, led to a true herd prevalence estimation of 4.7% (22/464) (Table 2). In the group of 60 herds with only one positive test result there was one herd with historical evidence of PTB, and five other herds with small adult herd size (≤ 5). Consequently, the estimated true herd prevalence is 6% (28/464). The herd-type-specific true prevalence parameters are summarized in Table 4.
Figure 2. Sensitivity analysis of the paratuberculosis infected herd detectability, herd specificity and herd true prevalence in non-vaccinated herds, Belgium 1998.

Assuming a true within-herd prevalence of 7%.

where: IHD; infected herd detectability, HSp; herd specificity, HP; herd true prevalence, Se; test sensitivity, Sp; test specificity
DISCUSSION

PTB seroprevalence

The present survey aimed to provide an unbiased estimate of the true national PTB herd prevalence by random selection of herds to sample. Because the percentage of nonresponding herds was low (14%), this random sample of herds can be considered to be representative for the Belgian cattle population (Thrusfield, 1995). This was also evidenced by the fact that there was no difference in the average herd size of the target and study population.

When comparing the apparent prevalences to published figures of seroprevalence, the following observations can be made. The average Belgian PTB within-herd seroprevalence of dairy herds, 3.3% was lower than the average figure of 20% found by Collins et al. (1994). At the individual-animal level, the Belgian figures for dairy and beef cattle, 1.16 and 0.52% respectively, are lower than analogue figures for other countries published so far, ranging from 7.3% in Wisconsin (Collins et al., 1994) to 17.1% in Florida (Braun et al., 1990) for dairy cattle and from 1.2% in Finland (Hintikka, 1998) to 25.2% in beef cattle in Texas (Alexander et al., 1993). Also the Belgian dairy and beef herd seroprevalences, 32 and 7% respectively, are lower than analogue figures for other countries published so far, ranging from 50% in Wisconsin, USA (Collins et al., 1994) to 74% in Missouri (Thorne and Hardin, 1997) for dairy herds, and from 30% in Louisiana (Turnquist et al., 1991) to 40% in Missouri (Thorne and Hardin, 1997) for beef herds.

PTB true prevalence

The aforementioned calculations assume a perfect test sensitivity and specificity of 100%. Because no test is perfect, the testing procedure could also have been a source of information bias.

Considering that in the selected herds all adult animals were tested, the reactor herds provided data without sampling bias for estimation of the true within-herd prevalence, compared to studies with a within-herd sample-based design. The median true within-herd prevalence of PTB seropositive herds was 7%. This estimation assumed non-reactor herds to be non-infected, which is a potential bias, because the use of tests of poor sensitivity to attempt to substantiate freedom from diseases of low within-herd prevalence is extremely difficult (Cameron and Baldoc, 1998). Consequently, the present estimated PTB within-herd prevalence, based on seropositive herds, could be an overestimation.

Corrected for testing procedures the overall true within-herd prevalence and the overall true individual-animal prevalences increased to 7 and 2% respectively. The Belgian true PTB within-herd prevalence of dairy herds, 5% was comparable with the figure of 5% found by Obasanjo et al.
(1997) based on whole herd examination by fecal culture. The Belgian true individual-animal prevalence in dairy cattle, 5%, was in line with the estimation of 4.8% in Wisconsin (Collins et al., 1994) and of 6.1% in Ontario (NeNab et al., 1991).

When true herd prevalence calculations were applied to PTB, problems arose because of the poor sensitivity of the available diagnostic tests, the low within-herd prevalence of infection, and clustering of false positives within a herd (Jordan, 1996). In the case of PTB, animals usually become infected as calves and develop clinical disease as adults several years later (Chiodini et al., 1984). Antibodies to *M. paratuberculosis* appear to occur late in the course of the infection, albeit before the onset of clinical signs. Thus, the pathobiology of PTB somewhat limits the ability of tests for serum antibodies to detect animals in the early stages of a *M. paratuberculosis* infection (Collins, 1996). The absorbed ELISA is, at present, the most sensitive and specific test for serum antibodies to *M. paratuberculosis* (O.I.E., 1996). Ridge et al. (1991) found an absorbed ELISA to have a sensitivity of 88.3% in clinical cases, and 48.8% in subclinical cases; whereas the specificity was 99.8%. Sweeney et al. (1995) showed that the sensitivity in low-level fecal shedders could be as low as 15%. Although no published data of sensitivity and specificity of the absorbed ELISA kit, used in this survey, exist, the overall sensitivity and specificity of absorbed ELISA’s are considered to be respectively 45% and greater than 99% (Collins, 1996). The probability of false positives created problems in classifying seropositive herds as being infected herds, especially those with only one single positive test result. This classification problem was particularly important in this study because 73% of herds testing positive had only one single positive test result. The lack of test specificity has a dramatic effect on the estimation of the true herd prevalence; some decimal changes in test specificity result in a true herd prevalence being 2, 3 or more times higher or lower, for constant test sensitivity, true within-herd prevalence and sample size. For instance, assuming a test sensitivity of 45% and a true within-herd prevalence of 7%, the true herd prevalence estimation decreases from 36 to 0.8% if the test specificity of the absorbed ELISA decreases from 99.9 to 99% respectively. Lack of test sensitivity leads to higher estimations of the true herd prevalence, with a greater impact at higher specificity levels. The true herd prevalence calculations revealed the implications of the aforementioned parameters, as depicted in Figure 2. This sensitivity analysis showed that the practical limits of the accuracy of the used screening test jeopardize the estimation of the true herd prevalence within reasonable confidence intervals.

For this reason we used an approach that increased the herd specificity. If the herd specificity was less then 95%, we raised the cut-off number of positive cattle required, as described by Jordan (1996), and we included herds with one positive test result if there was historical evidence of PTB (previous diagnosis and/or clinical signs) on the farm. The latter was an attempt to use available information from the herds to correctly identify herds that have only one
positive test as truly positive herds. If the herd specificity was at least 95%, i.e. if the adult herd size was at maximum 5, we assumed that herds were truly infected even if they had only one positive test result. Consequently, our best estimate of the true herd prevalence of *M. paratuberculosis* infection is 6%. The Belgian true dairy herd prevalence, 10%, is higher than in England, 1.3% (Cetinkaya et al., 1998) and lower than in Wisconsin, 34% (Collins et al., 1994).

This pilot study provides estimates regarding the PTB prevalence in the Belgian dairy, mixed and beef cattle population. A risk factor study considering all herd and management characteristics possibly associated with the PTB herd prevalence would be extremely beneficial.

**ACKNOWLEDGMENTS**

We thank Christel De Smedt and Amédée Massart for skilled technical and administrative assistance. The support of the farmers, the veterinary practitioners and the regional investigation centers is gratefully acknowledged. This study was supported by the Fund for Animal Health and Production, Ministry of Small Enterprises, Traders and Agriculture, Belgium.
REFERENCES


Descriptive surveys

CHAPTER 5:

DESCRIPTIVE SURVEY OF PSEUDORABIES VIRUS, OF BOVINE HERPESVIRUS 1,
AND OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS

5.1 Prevalence of herds with young sows seropositive to pseudorabies (Aujeszky’s Disease) in northern Belgium

5.2 Prevalence of Bovine Herpesvirus 1 in the Belgian Cattle Population

5.3 Prevalence of Paratuberculosis (Johne’s Disease) in the Belgian Cattle Population
Chapter 5.1:

based on:

Prevalence of herds with young sows seropositive to pseudorabies
(Aujeszky’s Disease) in northern Belgium


Chapter 5.2:

based on:

**Prevalence of Bovine Herpesvirus 1 in the Belgian Cattle Population**


Chapter 5.3:

based on:

Prevalence of Paratuberculosis (Johne’s Disease) in the Belgian Cattle Population

F. Boelaert, K. Walravens, P. Biront, J.P. Vermeersch, D. Berkvens, and J. Godfroid

SUMMARY

This paper critically assesses the design implications on the analysis of surveys of infections. It indicates the danger of not accounting for the study design in the statistical investigation of risk factors. A stratified design often implies an increased precision while clustering of infection results in a decreased precision. Through pseudo-likelihood estimation and linearisation of the variance estimator, the design effects can be taken into account in the analysis. The intra-cluster-correlation can be investigated through a logistic random effect model and a generalised estimating equation (GEE), allowing the investigation of the extent of spread of infections in a herd (cluster). The advantage of using adaptive Gaussian quadrature in a logistic random effect model is exemplified. Applicable software is briefly reviewed. The aforementioned methods were illustrated with data of a Bovine Herpesvirus 1 (BoHV-1) serosurvey of Belgian cattle.

INTRODUCTION

One of the aspects of veterinary epidemiology involves quantitative investigations of disease occurrence (Thrusfield, 1997). These include surveys, monitoring, and surveillance that often deal with binary data. Three important characteristics of survey data are stratification, clustering and sampling weights.

Stratification is a sampling method aimed at reducing variance, if a known factor causes significant variation in the outcome variable, but is not the target of analysis. For example, in the case of beef production in a population of two different breeds, sampling variation of estimates will be substantial, largely due to genetic differences affecting beef production between the two breeds. Stratification on breed will allow reducing the overall variation of the beef production estimate. The technique also allows easy access to information about the sub-populations represented by the strata. For stratified sampling to be effective at reducing variation, the elements within the strata should be homogeneous and variance between the strata should be large. As a disadvantage, the status of the sampling units with respect to the stratification factor must be known and more complex methods are required to obtain correct variance estimates (Pfeiffer, 1999).

Clustering of data may be due to repeated measurements of subjects over time or due to sub-sampling of the primary sampling units. Livestock disease clustering is a consequence of unequal distribution of the disease agents throughout the animal population (Rothman, 1990). Interest primary concerns individual-level characteristics, such as the disease status of the animal, but the sampling unit becomes a grouping of individual animals such as the herd to which they belong. The groups or clusters can represent natural groupings such as litters or herds, or they can be based on artificial groupings such as geographic areas or administrative units. The random selection of the
clusters as the sampling units can be performed using simple random, systematic or stratified random sampling.

Clustering can help to reduce the sampling and data collection costs. However, since independence among sample observations is a key assumption underlying standard statistical procedures, the presence of clustering in the data may raise important statistical issues, which should be addressed in the analysis. With data collected on the basis of clusters, the variance is largely influenced by the number of clusters, not the number of animals in the sample. Cluster sampling can lead to an increased sampling variance following the saying that “birds of a feather flock together”. In this situation, a larger sample size would be required to reduce variance to acceptable levels (Pfeiffer, 1999). Unfortunately, scientific reports often present, for example, confidence intervals that assume simple random sampling where the design involved clustered units.

In sample surveys observations are selected through a random process, but different observations may have differing selection probabilities. These probabilities should be accounted for in the analysis of the survey; otherwise, biased estimates may be obtained (Stata Corporation, 2001).

The objective of this paper is to present methods for analysing survey data where clustering, stratification and differing sampling probabilities may be present. The emphasis is directed towards the estimation of the effect of risk factors on the presence of infectious diseases. As the main tool, logistic regression models, taking into account the effects of clustering and stratification, are considered. Furthermore, ways of calculating the intra-cluster correlation are presented. Various software packages, which can be used to apply the aforementioned methods of analysis, are considered. Effects of ignoring sampling design characteristics are demonstrated and discussed. The techniques are illustrated using data of a Bovine Herpesvirus 1 (BoHV-1) serosurvey of Belgian cattle, reported by Boelaert et al. (2000). BoHV-1 causes infectious bovine rhinotracheitis, an enzootic disease. Programs to eradicate BoHV-1 have been implemented in several European countries to facilitate the free trade of cattle within the European Union.

**METHODS**

**The data**

Figures 1a-1d present the design of the BoHV-1 serosurvey of the Belgian cattle population. The survey was conducted on cattle herds of all types from December 1997 to March 1998 in Belgium. The sample was stratified by province (ten provinces in Belgium). Within each province, 1% of the total number of herds was sampled. In the selected herds, all animals were blood sampled. The sera were tested for antibodies against BoHV-1 by using a commercially available
blocking ELISA (HerdChek®, Idexx, France), specific for BoHV-1 glycoprotein B (Kramps et al., 1994). The age and sex of 11284 animals originating from 309 BoHV-1 unvaccinated herds were collected. Also, the type (dairy, mixed or beef) and size of the herds were registered. Due to computational problems, the variable “herd size” was dichotomised as 0 (respectively 1) for farms smaller (respectively larger) than the average herd size (=36.5) in the final models presented in Section 3.

Figures 1a-d. Design of the BoHV-1 serosurvey of the Belgian cattle population, 1998.
a: In every province the farms are listed.
b: A same proportion of the farms is selected in each province.
c: In each selected farm all the animals are sampled.
d: A total overview of the Survey: a same proportion of the farms is selected in each province and in each selected farm all the animals are sampled.
The survey is an example of an one-stage cluster sampling design. The individual subjects (animals) still remain the target units so that animal-level disease can be studied, but the (primary) sampling unit becomes a group of individuals (herd). All elements within a randomly selected group are included in the sample. This technique does only require a sampling frame for the groups, but not for the members within the groups. In the present example, the random selection of clusters (herds) as the sampling units was performed using stratified random sampling, but in general it can also be performed using systematic or simple random sampling (Pfeiffer, 1999).

**Important Features of Survey Data**

In this section, the following features of survey data are shortly discussed: stratification, clustering and sampling probabilities.

**Stratification**

The argument in favour of stratification can be illustrated using a simple example. Consider a population of 3 male and 3 female animals being infected (Y=1) or not (Y=0). Clearly, the prevalence of the disease equals 50%. Now, assume that from the above population independent samples of size 2 are to be taken using simple random sampling without replacement. Within the above population, the probability of estimating prevalences as 0% is 0.2, as 50% is 0.6 and as 100% is 0.2. As a consequence there is a probability of 0.4 that the estimated prevalence differs markedly from the true value. However, if we consider sampling stratified by sex, with one animal sampled from males and one from females, the prevalence will be estimated as 50% for every sample, the variability of the estimate thus being greatly reduced.

In real settings, stratification is an effective method for reducing variability of an estimator if a known factor, which is not the target of analysis, causes substantial variation in the outcome variable such that the elements within the strata are homogeneous and variability between the strata is large. Stratified sampling also leads to a straightforward computation of estimates for the sub-populations represented by the strata. Obviously, the technique requires that the status of the sampling units with respect to stratification factors be known. Also, more complex methods are required to obtain precision estimates referring to the global population (Pfeiffer, 1999).

**Clustering**

Clustering can be seen as a form of stratified design, where instead of selecting some individuals from each (large) stratum, we aim at selecting some (from a large number of relatively
small) strata with, possibly, each individual within a selected group included in the sample (Barnett, 1991). To reflect this difference, the strata are called clusters. If all individuals in a sampled cluster are included in the sample, as in the BoHV-1 survey, this is referred to as one-stage cluster sampling.

If a disease is contagious, the clustering (or grouping) of animals within herds may result in a higher chance for an animal becoming infected once the infection is introduced into the herd. Consequently, individual responses, i.e., whether the animals are infected or not, are more homogeneously distributed within herds than in the whole population. If the response is distributed in a homogeneous way within a cluster, considering the sample as a simple random sample can lead to erroneous conclusions. Assume for example the extreme case of herds in which either all or none of the animals are infected. In this situation, the calculation of the standard errors of the parameter estimates should be based on the number of farms rather than on the number of animals since the information provided by a single animal would amount to the total information provided by the whole herd to which the animal belongs.

Consequently, in the presence of clustering, the calculation of the variance of the prevalence using formulae for simple random sampling may yield overly optimistic estimates. Unfortunately, it appears that in surveillance studies investigating prevalence of diseases the precision of the prevalence is often overestimated (McDermott et al., 1994).

**Sampling probabilities**

As a result of the choice of a sampling design, each individual member of a population is assigned a probability with which it can be included in the sample. If these probabilities differ between members, they should be accounted for in the analysis of the survey. Consider the BoHV-1 survey. Since in the sampled herds, all animals were included in the sample, the probability of being sampled was the same for each animal in the population, as the herds were sampled with equal probabilities. Suppose, however, that from each of the sampled herds only one animal would have been sampled. (In such case the sampling would have been an example of two-stage sampling, with herds considered as primary sampling units and the animals as secondary sampling units.) Then the probability of being selected would have been higher for animals from small herds than from large herds. As a result, the animals from small herds would have been over-represented in the sample. It follows that the estimates obtained from the analysis ignoring the sampling probabilities would have been biased towards the characteristics of the sub-population of small-herd animals. This was not the case for the BoHV-1 survey.

The analysis of a survey can be adjusted for unequal sampling probabilities by applying appropriate weights to the observed results (Stata Corporation, 2001). In general, different sampling probabilities arise most naturally in multistage sampling designs. Such designs, in
Analytical BoHV-1 survey: statistical design-based analysis

combination with stratification, are recommended by the Office International des Epizooties as part of the official pathway to declaration of freedom from infection with the rinderpest virus.

In short, adjusting for unequal sampling probabilities allows obtaining unbiased point estimates from survey data. Taking into account clustering and stratification results in appropriate precision measures for the point estimates. Adjusting for sampling probabilities can also influence the precision.

Statistical Methodology

We will consider the situation of a binary response variable $Y$. In our example, $Y$ will indicate whether an animal is infected ($Y=1$) or not ($Y=0$). To investigate the effect of explanatory variables (age or sex of an animal, for example) on the probability of infection, we will consider a logistic regression model. Denoting by $\pi$(covariates) the probability of infection as a function of covariates, we can write the model symbolically as follows:

$$ \text{logit} \{ \pi(\text{covariates}) \} = \log\left( \frac{\pi(\text{covariates})}{1 - \pi(\text{covariates})} \right) = \alpha + \beta^* \text{covariates} \quad (1) $$

The parameters $\alpha$ and $\beta$ (which is a vector) have to be defined.

For the BoHV-1 survey, age and sex of the animals, as well as type (dairy, mixed, beef) and size of the herd, will be used as covariates.

Adjustment for sampling probabilities and stratification

In general, this is done by appropriate weighting of the data. The weights are taken as the inverse of the sampling probabilities (Skinner et al., 1989; Sarndal et al., 1992). Here, we will not discuss the techniques any further; the interested reader can find them in any elementary textbook on survey sampling (Kish, 1965; Cochran, 1977; Levy and Lemeshow, 1999).

Using a 'pseudo' likelihood (Skinner et al., 1989) and deriving the variance estimator through 'linearisation' is one way to account for the effects of sampling probabilities and stratification as well as clustering (see next section) in the analysis. A pseudo-likelihood is needed since the standard ML estimator does not give a true 'likelihood' under a complex design and therefore estimates of model parameters are obtained by solving weighted analogues of likelihood equations based on the probability sampled data. The full MLE would require an expression for the exact likelihood, which may be very complicated and require many assumptions since it involves modelling the relation between the response and the design variables (Skinner et al., 1989). With probability sampling, each unit in the survey population has a known, positive probability of
selection. This property of probability sampling avoids selection bias and enables one to use statistical theory to make valid inferences from the sample to the survey population. As a consequence of these issues, the likelihood-ratio test is invalid with weighted data of this kind.

**Adjustment for clustering**

There are a lot of methods available for the analysis of clustered binary data. In general, one can distinguish between marginal, conditional and random-effects approaches, which can be applied using different inferential methods (likelihood, quasi- or pseudo-likelihood, generalised estimating equations). Unlike the Gaussian setting, they tend to give dissimilar results. Reviews can be found in Diggle et al. (1994), Fahrmeir and Tutz (1994) or Pendergast et al. (1996). We will discuss only the approaches used most frequently in the survey context.

**The analysis at herd level**

In an attempt to account for clustering of the animals, one may consider analysing the data at herd level. In such an analysis, a herd with at least one seropositive animal is called positive, otherwise it is called negative. A logistic regression with binary response Z, indicating whether a herd is positive (Z=1) or not (Z=0), can be carried out. Only herd-level covariates can be used in such a model, for example, the type and size of the herd, the average age of the animals and the proportion of males by herd. A herd-prevalence could be defined as the proportion of herds with at least one positive animal.

An advantage of the herd-level analysis is that it focuses on the probability of infection in a herd, which is economically important information. However, there are several disadvantages. As already mentioned, only herd-level covariates can be considered in the analysis. Moreover, the associations detected at the herd level do not necessarily correspond to those existing at the animal level. Thus, there might be some confusion between aggregate and individual effects, an issue that is often referred to as the ecological fallacy (Robinson, 1950).

**Marginal model fitted using Generalised Estimating Equations**

One way to address the disadvantages associated with the herd-level analysis is to fit the logistic regression model (1), while correcting estimated standard errors of parameters $\beta$ for clustering. The approach can be applied using the generalised estimating equations (GEE) technique developed by Zeger and Liang (1986).
Logistic random-effects model

The use of a random-effects model approach (see Agresti et al. (2000) for a recent review in the broader context of categorical response data) can be motivated by arguing that animals belonging to a herd share the same environment (physical location), as well as characteristics such as the type of farm (milk- or meat-oriented). These shared factors, whose effects can change from herd to herd, create dependencies between responses observed for the individual animals.

In its simplest form, the model can be symbolically written as follows (with $b_i$ a random variable representing the effect of factors shared by the animals belonging to herd $i$):

$$\text{logit} \{ \pi(\text{covariates}, b_i) \} = \alpha + \beta^*\text{covariates} + b_i, \quad (2)$$

where $i$ is an index for herds, $\pi(\text{covariates}, b_i)$ denotes the conditional probability of infection (conditionally on the covariates and the random effect $b_i$). Usually, these random variables are assumed to be normally distributed.

Likelihood inference in this type of model can proceed by integrating over the random effects $b_i$ to derive the marginal likelihood, which can practically be done by numerical integration (Gaussian quadrature for instance). Note that the interpretation of the $\beta$ coefficients in this model is conditional on the (unobserved) value of the random variable $b_i$ and is, therefore, called “individual-specific”. In model (1), on the other hand, $\beta$ can be interpreted as describing marginal (so-called “population-averaged”) effects of the covariates. This model does not take into account clustering and other design effects.

It should also be noted that model (2) assumes that, conditionally on the value of $b_i$, the response has a binomial error distribution. A different kind of “random-effects” model would be to assume that conditional on each herd, the response is binomial and that the response probabilities follow, for instance, a beta distribution (thus yielding the so-called beta-binomial model). We will not illustrate this approach in the present paper. A description of the approach can be found in Kleinman (1973).

Comparison with Pseudo-Likelihood Methodology

Unlike pseudo-likelihood methodology (Stata Corporation, 2001), standard marginal and random-effects models cannot directly account for survey-related issues but simply afford a more flexible way to account for clustering in the analysis of the data. It can be noticed, however, that for
the data at hand a way to take for example the stratification into consideration is to incorporate strata indicators as covariates.

**Quantifying the influence of sampling design on the precision: design effect**

The influence of sampling design on the precision of estimates can be quantified using the measure of design effect (Kish, 1965). It is defined as

\[
deff = \frac{\hat{V}}{\hat{V}_{srworr}}
\]

where \( \hat{V} \) is the design-based estimate of the parameter variance and \( \hat{V}_{srworr} \) is an estimate of the variance for a hypothetical simple-random-sampling design in place of the complex design that was actually used. \( \hat{V} \) can be computed by adapting the formulae for variance estimation by using techniques such as (balanced) replicated sampling, jackknife repeated replication and the Taylor series method (Lee et al., 1989).

In case where the quantity of interest is a proportion (such as the prevalence discussed so far) the design effect is directly proportional to the size of the intra-cluster-correlation and the cluster size (Kish and Frankel, 1974):

\[
deff = 1 + \rho(m-1),
\]

where \( m \) denotes the cluster size (assumed to be constant) and \( \rho \) is the intra-cluster correlation. For a variable cluster size, a reasonable approximation for \( m \) is the average cluster size.

Marginal logistic regression models fitted using GEE and random-effect models allow \( \rho \) to be estimated. Apart from indicating the amount of association between responses within a cluster, the intra-cluster correlation can be interpreted as measuring the part of the total variance explained by the clusters (Kish, 1965; Lehtonen and Pahkinen, 1965).

In the following section, software, which can be used to carry out the aforementioned models, is presented.
Software

There are several commercially available statistical software packages where methods for analysing survey data are implemented: STATA, SAS and SUDAAN, amongst others. Since the latter was not available to us, we will only consider STATA and SAS.

Stata

STATA (Stata Corporation, Texas, USA) is a multi-purpose interactive statistical package. It includes a set of so-called svy commands implementing methods for analysing surveys.

A pseudo-likelihood method is used to calculate estimates of model parameters by solving weighted analogues of likelihood equations based on the probability sampled data. For all models, STATA can adapt standard errors and confidence intervals for estimates taking into account clustering, stratification and sampling probabilities. All svy commands can compute design effects for their estimates. STATA currently uses the Taylor series linearisation estimator as the variance estimation method. This method approximates the complex formulas for the adapted variance, by writing it as a series of functions. Lee et al. (1989) give a detailed account of this method. Multistage designs are handled by the “ultimate” cluster sample selection paradigm (Stata Corporation, 2001). Kish (1965) defines the term ultimate clusters. The ultimate cluster is a grouping of sampled cases for variance estimation purposes. In general, the use of ultimate clusters for sampling error estimation reflects the gains in precision from stratification and the loss in precision from the clustering of cases within primary sampling units. Under the ultimate cluster-sampling model, elements within primary sampling units are divided into ultimate clusters and a sample of these clusters is drawn without replacement across the primary sampling units. Variance estimates are computed using only between first stage unit totals without having to compute variance components at each stage of selection.

Svyplogit fits logistic regression models for survey data and is able to incorporate probability sampling weights, stratification and clustering (one level only) or any combination of these three. Associated variance estimates and design effects (deff) are computed. Clustering is taken into account by pseudo-likelihood estimation and precision estimates are calculated in a way similar to the sandwich estimator in GEE.

The xtplogit command can fit marginal (using GEE) as well as random-effects logistic regression models. In the latter case, numerical integration is based on simple Gaussian quadrature, although a limitation of the command is that it cannot handle more than 30 quadrature points.
SAS

SAS (SAS Institute Inc., North Carolina, USA) is also a multi-purpose statistical package. From version 7 of the SAS system onwards, a few procedures has been available for the analysis of data from complex sample surveys. Precisely, the SURVEYSELECT procedure selects probability samples using various sampling designs; the SURVEYMEANS procedure computes descriptive statistics for sample survey data; and the SURVEYREG procedure fits linear regression models for such data.

Two other procedures are of interest in the present context. These are the GENMOD procedure, which can fit models for correlated data using the GEE method, and the NLMIXED procedure, which fits nonlinear mixed models, that is, models in which both fixed and random effects enter nonlinearly. NLMIXED can, in particular, handle logistic random-effects regression models and use adaptive Gaussian quadrature to approximate the likelihood, which should be preferred to simple Gaussian quadrature in general (Lesaffre and Spiessens, 2001).

RESULTS

In this section, we illustrate the issues previously discussed using the data from the BoHV-1 survey. At first, the data are analysed at herd level and this is compared with a naïve analysis at animal level. Thereafter, the data are analysed at animal level, using the more sophisticated methods described in Section 2.

Analysis at animal level vs. analysis at herd level

The herd seroprevalence was estimated as 67% (207 of 309 farms had at least one animal infected). The estimated proportion of seropositive animals was 36%, which confirms the figure of Boelaert et al. (2000).

Table 1 shows the average seroprevalence for animals and herds by type of farm. Interestingly, beef farms show a much lower herd seroprevalence. The lower seroprevalence in beef farms can be explained by the fact that these farms are typically small as shown in Table 2, but carry the same weight in the calculation of the herd-seroprevalence. As the prevalence tends to increase with size, the large number of small beef farms results in a lower herd-prevalence for this type of farm. This illustrates how size of the farm acts as a confounding factor in the analysis at herd level. This was confirmed by the logistic regression at the herd level, which indicated that beef farms had a significantly (p<0.001) lower prevalence than the other types of farms. However, including size of the farm in the model showed that a larger size was significantly (p<0.001) related
to a higher risk of herd seroprevalence and that beef farms had no longer a significantly lower prevalence than the other types of farms (p=0.331).

At the animal level, the difference in seroprevalence between the different types of farms was not so pronounced. This can be explained by the stronger weight of big farms in the final seroprevalence calculations. The seroprevalence at animal level in large farms is close to the figures obtained for animal level in Table 1.

Table 1: Average seroprevalence of animals and herds, by type of farm.

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>Animal level (n=11284)</th>
<th>Herd level (n=309)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy</td>
<td>0.345</td>
<td>0.864</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.429</td>
<td>0.907</td>
</tr>
<tr>
<td>Beef</td>
<td>0.320</td>
<td>0.541</td>
</tr>
</tbody>
</table>

Table 2: Frequency distribution of farm size per type of farm.

<table>
<thead>
<tr>
<th>Size (number of animals)</th>
<th>Dairy Farms</th>
<th>Mixed Farms</th>
<th>Beef Farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 20</td>
<td>6</td>
<td>5</td>
<td>152</td>
</tr>
<tr>
<td>≤ 40</td>
<td>8</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>≤ 60</td>
<td>16</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>≤ 80</td>
<td>5</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>≤ 100</td>
<td>9</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>≤ 120</td>
<td>8</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>≤ 140</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>≤ 160</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>≤ 180</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>&gt;180</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>54</td>
<td>194</td>
</tr>
</tbody>
</table>

**Effects of the sampling design on confidence intervals**

Figure 2 presents, by type of farm, the estimated seroprevalence at animal level together with 95% confidence intervals (in brackets) computed with and without the effects of clustering and stratification taken into account. The results were obtained using the command `svy:mean` in
STATA. Clearly, if the sampling design is not accounted for, the precision of the estimates is considerably overestimated.

For the data at hand, it is interesting to investigate which of the sampling design features, (stratification or clustering) is mainly responsible for the adjustment of the confidence intervals. The estimates of BoHV-1 seroprevalence by province (proportion of animals infected by province) ranged from 2 to 42.2%. A strong difference between provinces indicate that including this factor into the analysis could improve the estimation in terms of efficiency. However, with the command `svymean` of STATA the design effect for stratification was only 0.96, while the design effect for clustering was 45.6, which is very similar to the design effect for clustering and stratification together (45.5).

A similar effect of adjustment for sampling design on the precision estimates can be seen when considering a logistic regression model with type and size (binary variable) of the farm, and age and sex of the animal, included as co-variates. Table 3 presents the coefficients of the model as well as estimates of their standard errors obtained using the STATA command `svylofit` with and without adjustment for clustering and stratification. Without adjusting for sampling design, Table 3 (misleadingly) indicates that the seroprevalence among animals in mixed farms is significantly higher than in dairy and beef farms. Also, the naïve model suggests significant effects of size of the farm, age and sex of animals.

When sampling design is accounted for, the results change substantially, owing to the increase in standard errors of the coefficients. Conclusions for the herd-level covariates are mostly affected: the effect of mixed farms becomes non-significant, while the effect of size of the farm becomes borderline significant at the 5% level.

As in the case of the overall BoHV-1 seroprevalence, design effect associated with adjusting the logistic regression model for stratification was small (0.96). It seems that the homogeneity in the strata was not high enough compared to the variability between the strata.

**Figure 2.** Estimates and 95% confidence intervals for the overall BoHV-1 seroprevalence by type of farm, respectively taking into account (confidence intervals as top-bar) and not taking into account (confidence intervals as bottom-bar) the effects of the sampling design.
Table 3: Effect of type and size of the farm, and age and sex of the animal, on the BoHV-1 seroprevalence in Belgian cattle, with and without adjustment for clustering and stratification.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Without design effect</th>
<th>With design effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>Standard Error</td>
</tr>
<tr>
<td>Herd size (binary)</td>
<td>0.691</td>
<td>0.066</td>
</tr>
<tr>
<td>Age</td>
<td>0.215</td>
<td>0.011</td>
</tr>
<tr>
<td>Sex</td>
<td>0.324</td>
<td>0.067</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.348</td>
<td>0.048</td>
</tr>
<tr>
<td>Beef</td>
<td>0.072</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Sex: 0=female, 1= male; Herd size (binary) =0 if herd size ≤ 36.5, and =1 otherwise

Marginal (GEE) Model

Using the same covariates as in the previous model, a marginal model (GEE with exchangeable working correlation structure) was fitted with the `xtlogit` command in STATA and the results are shown in Table 4.

The value of the intra-herd correlation coefficient was estimated as 0.526.

To further account for omitted aspects of the sampling design, we could, in principle, incorporate indicator variables for provinces in the above model but this led to convergence problems.

Table 4: Estimates with standard errors and significance level for a marginal model.

| Variable          | Coefficient | Standard Error | p>|t|  |
|-------------------|-------------|----------------|------|
| Herd size (binary)| 0.838       | 0.245          | 0.001|
| Age               | 0.214       | 0.007          | 0.000|
| Sex               | 0.137       | 0.050          | 0.006|
| Mixed             | -0.365      | 0.276          | 0.187|
| Beef              | -0.285      | 0.275          | 0.300|
Random-effects Logistic Regression

With simple Gaussian quadrature, the estimated coefficients and their standard errors were strongly dependent on the number of quadrature points specified in the algorithm. This is a typical signal that Gaussian quadrature works poorly and that adaptive Gaussian quadrature should be utilised instead. In fact, the latter should be preferred as it has, in general, better numerical properties than simple Gaussian quadrature. For this reason, we illustrate the fitting of the random-effects model solely with the SAS procedure NLMIXED. The results are shown in Table 5.

The value of $\sigma^2$, the between-herd variance, was estimated as 5.964 (SE=0.732). From this model, the derived value of the intra-herd correlation coefficient can be calculated as:

$$\rho = \frac{\sigma^2}{\pi^2/3 + \sigma^2},$$

which gives 0.645 (SE=0.028).

Table 5: Estimates with standard errors and significance level for a random-effects logistic model.

| Variable      | Coefficient | Standard Error | p>|t| |
|---------------|-------------|----------------|-----|
| Herd size (binary) | 1.024 | 0.416 | 0.014 |
| Age           | 0.424       | 0.016         | 0.000 |
| Sex           | 0.301       | 0.100         | 0.003 |
| Mixed         | 0.284       | 0.478         | 0.553 |
| Beef          | -0.069      | 0.472         | 0.884 |

Model Comparison

The conclusions reached by the pseudo likelihood model, the marginal model and the random effect model are similar: Herd size, age and sex are significantly related with the seroprevalence of Bovine Herpesvirus 1 at the 5% level. The signs of the coefficients differ only for non-significant parameters: the type of the farm. The intra cluster correlation is above 50% with a marginal model as well as with a random effect model.
DISCUSSION

In general, while adjusting for the sampling design effects in a complex survey, two approaches might be considered to analyse data from a survey with (clustered) binary responses indicating, e.g., whether animals are seropositive or not. First, one might consider clusters (herds) as the units of analysis. Information at the herd-level may be sufficient when one aims to eradicate disease from the herd. This is particularly true for BoHV-1, because control and eradication measures implicate the herd - not the animal (O.I.E., 1999). In the analysis at herd level of the BoHV-1 survey, the smaller size of the majority of the beef farms resulted in a lower herd seroprevalence for beef farms. This was due to the confounding effect of the size of the farm. Hence, demonstration is made of the importance of correcting for an important covariate. Secondly, one might treat animals as the units of analysis and adjust the results for the effects of clustering, stratification and weighting. Such an analysis at animal level offers an additional and vital insight into the understanding of the epidemiology, since a sole analysis at herd level does not allow an analysis of the factors measured at animal level. The adjustment for the effects of clustering, stratification and weighting can be done using pseudo-likelihood methods with a linearisation estimator for the variance.

Although a BoHV-1 risk factor study was outside of the scope of this paper, the influence of four different parameters on the serological results was investigated with the sole aim of illustrating the impact of the design on the analysis; type and size (binary variable) of the farm, and age and sex of the animal. Size of the farm and age of the animals seemed to be statistically important predictors for BoHV-1 seropositivity of an animal. The older an animal is, or the larger the farm it belongs to, the higher is the chance to show a serological positive result. Both risk factors can be explained from a biological viewpoint. The risk of BoHV-1 transmission among cattle within herds is higher at larger herd size. This may be explained by the within-herd contact structure. In smaller herds the number of susceptible animals is smaller throughout the year, so infection may not be sustained. Larger herds usually have loose-housing barns, creating more contact between infected and susceptible animals. These herds possibly also have more visits by animal handlers (farmers, inseminators, veterinarians, traders) (van Wuijckhuise et al., 1998). Also the double-track survival strategy of herpesviruses in nature sustains the biological explanation of herd size being a risk factor. During primary infection, herpesviruses are disseminated within susceptible populations, which raise strong immune responses and overcome in most cases the diseases associated with the infections. The latent viruses represent a long-term reservoir that becomes meaningful upon reactivation. Then, seemingly healthy animals are able to re-excrete and transmit the virus to non-immune as well as to immune hosts (Thiry et al., 1985; Thiry et al., 1987; Engels and Ackermann, 1996).
The advantage of the pseudo-likelihood methods is that these methods account for all the design effects simultaneously. However, they do not provide an estimated intra-cluster correlation and do not allow for more than one level of clustering in STATA. Variance estimation for multistage sample data is carried out through the customary between-primary sampling units-differences calculation. The generalised estimating equations and the random-effects logistic regression model that can be carried out with respectively the commands GENMOD and NLMIXED in SAS cannot directly take into account stratification. However, these commands are not specially designed to analyse survey data and by using the stratum as a fixed effect in the model, the stratification variable is accounted for in a somewhat less efficient way.

The sampling design must be taken into account when analysing surveys. Otherwise, wrong conclusions may be drawn. In general, stratification may lead to an increase of the precision of the estimates, while clustering may decrease the precision. This was illustrated using the pseudo-likelihood methods with a Taylor series linearisation estimator as the variance estimation method. The type of a farm can erroneously be considered as significantly related to the seroprevalence by not including an adjustment for the design. Adjusting for unequal sampling probabilities results in unbiased point estimates. Accounting for stratification and clustering allows correct standard errors to be made. Thrusfield (1997) stated that the group of animals which is of epidemiological importance in terms of the transmission and maintenance of infection -- and therefore of disease control and eradication -- is the herd. In the mentioned methods for analysis at animal level, the herd was still considered as the primary sampling unit.

It can be mentioned that SUDAAN is also a program suitable to analyse data from complex sample surveys. In particular, beside the Taylor series linearisation as a robust variance estimation method, it allows also for the use of replication methods.

The precision of the estimates for the data of BoHV-1 was slightly increased by including the effect of stratification at the province level. However, this effect was only minor (0.96, with 1 meaning no effect) for the parameter estimates, which might be explained by a variability within provinces not being smaller as compared to the overall variability. The effect of clustering was much stronger, as could be seen from the widened confidence intervals (pseudo-likelihood approach) or by the relatively high values obtained for the intra-herd correlation coefficient in the marginal and random-effects model. Also, the sampling design effects influenced mostly the herd-level (e.g. type of farm) rather than the animal-level covariates (e.g. age of the animal). Cattle in Belgian farms are kept together in lots. This constitutes conditions for the infection to spread, which results in more homogeneous clusters with respect to the presence or absence of the infection. This was supported, in the analysis, by the value of the intra-cluster correlation, which was higher than 50%. As an alternative for using pseudo-likelihood methods, logistic random effect models were used. The importance of using adaptive Gaussian quadrature for the random effect
models was illustrated. By not using it different conclusions with respect to significance and signs of the coefficients are reached depending on the number of quadratures used.

Additionally, the testing procedure had inherent probabilities of misclassification, due to diagnostic test inaccuracy. If one would know the diagnostic sensitivity and specificity of the test, the true prevalence could be estimated. Unfortunately, the test characteristics are, in general, not known. Moreover, these test characteristics vary among subpopulations (Greiner and Gardner, 2000). The impact of this misclassification on the BoHV-1 risk factor analysis is currently being investigated. In conclusion, cross-sectional surveys based on diagnostic test results will always be concealed by the inaccuracy of the diagnostic test and this cannot be solved by a complex analysis.

ACKNOWLEDGEMENTS

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SUMMARY

This study reports the epidemiological investigation of risk factors for bovine herpesvirus 1 seropositivity, based on a cluster sample survey of the Belgian cattle population.

An increasing age and male sex were risk factors for seropositivity. The effects of the risk factors origin (homebred or purchased) and farm size interacted such that the overall effect of purchase status was different for smaller and larger farms. For smaller farms purchase status and increasing farm size were risk factors, whereas these effects were not observed for larger farms.

However, seroprevalence figures, and consequently the statistical modelling outcome, are a post-epidemic snapshot and non-informative regarding the within-farm index case and the within-farm infection dynamic. In larger farms, the contagion of homebred herd mates could have masked the importance of purchased index cases as direct sources of virus introduction. Biologically, purchase status would be a direct risk factor for farms of any size.

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) infection is a worldwide distributed transmissible disease of economic importance and significance to international trade. Hence, it is a disease on the B List of the Office International des Epizooties (O.I.E., 1999). Programs to eradicate BoHV-1 have been implemented in several European countries to facilitate the free trade of cattle, semen and embryos within the European Community. The BoHV-1 seroprevalence (apparent prevalence) in the Belgian cattle population was determined in 1998 by a serological survey (Boelaert et al., 2000). In 309 unvaccinated farms, the overall farm seroprevalence was estimated to be 67% (95% confidence interval (CI)=62-72). A design-based analysis, taking into account the effects of clustering and stratification estimated the animal-level seroprevalence to be 36% (95% CI=30-42) (Speybroeck et al., 2003). As these authors reported, clustering is a key feature of the BoHV-1 1998-serosurvey data, with an intra-cluster (intra-farm) correlation coefficient of 0.64. Indeed, in the context of intensive animal husbandry the animal population is clustered, since animals are kept in farms or herds (clusters), rather than on an individual basis. In clustered populations, disease has a strong tendency to focus (Rothman, 1990). Animals within such clusters share common characteristics such as nutrition, housing, and exposure (e.g. contacts) to infectious agents, and can be considered as a homogeneously mixed population. Exposure to infectious agents probably results in a more homogeneous serological status of animals within clusters as measured by the intra-cluster correlation coefficient (Donald and Donner, 1987), since the interrelatedness of incidence and prevalence is the very characteristic that defines infectious diseases (Koopman et al., 1991; Susser, 1994; De Jong, 1995). Consequently it cannot be assumed that the animals are independent units (Schukken et al., 1991; Elbers and
Schukken, 1995; Cameron and Baldock, 1998), because compared to other population members animals within clusters have a higher chance to become infected once the infection is introduced in the farm.

In literature, only farm-level BoHV-1 seroprevalence (proportion of seropositive farms) risk factors of Dutch farms with dairy cows are reported. Van Wuijckhuise et al. (1998) found that larger farm size, dairy farms with beef/veal animals, and higher density of farms in the municipality were associated with the farm BoHV-1 seroprevalence. Also, significant interactions between farm type and purchase of stock were observed. Also in the Netherlands, van Schaik et al. (1998), reported that BoHV-1 seropositive farms purchased cattle and participated in cattle shows more often compared to BoHV-1 negative farms. BHV1-seropositive farms also had more (professional) visitors who used farm clothing less often. BoHV-1 positive farms were found to be situated closer to other cattle farms compared to BoHV-1 negative farms. Van Schaik et al. (2001) further documented the following risk factors for introduction of BoHV-1 in BoHV-1 free dairy farms: failure of professional visitors (e.g. veterinarians, insemination technicians) to wear protective farm clothing when handling cattle, and cattle escaping and mingling with other cattle.

The objective of the present study was to investigate the 1998-serosurvey results of the Belgian cattle population for animal-level and farm-level risk factors associated with BoHV-1 seropositivity.

**METHODS**

**The BoHV-1 serosurvey data**

A detailed description of the BoHV-1 serosurvey can be found elsewhere (Boelaert et al., 2000). Short, within each of the ten provinces in Belgium, 1% of the total number of farms was randomly selected via SANITEL, the central computerized database for the identification and registration of the Belgian cattle population (Federal Public Service Health, Food Chain Safety and Environment, Belgium). Since in the selected farms, all animals were blood sampled, this was a one-stage cluster sample survey. Blood samples were tested for the presence of antibodies to glycoprotein B (Kramps et al., 1994) of BoHV-1, using a commercially available blocking ELISA (HerdChek®, Idexx, France). The BoHV-1 survey database comprised 11,284 cattle living in 309 unvaccinated farms.

Different covariates, obtained via SANITEL, exist. First, there are the animal-level factors: age, sex and purchase (purchased or homebred). Second, there are the farm-level covariates: farm type (dairy, mixed or beef) and farm size (number of cattle on the premises). Last, the densities of the cattle and of the farms in the municipalities were determined by dividing the number of cattle and farms, respectively, by the effective agricultural land (Anonymous, 1997).
Risk factors associated with BoHV-1 seropositivity

Effects of risk factors can be assessed using a multiple variable analysis. Given the dichotomous outcome of infection, the most likely choice for such an analysis is a logistic regression analysis. Logistic regression parameters can be translated into odds ratios. All animal records with one or more missing covariate or response values were deleted (complete case analysis). Analyses were performed using the statistical software Stata®, Version 8.0/SE (Stata Corporation, Texas, USA, 2003).

Cluster-specific random-effects logistic regression models were implemented. In these models, the intra-cluster (intra-farm) correlation, which induces extra variation between clusters (heterogeneity, over-dispersion), is assumed to arise from natural heterogeneity (in the success probability) across clusters. This extra variation (so-called extra-binomial variation) is statistically taken into account by the use of a random effect.

The random-effects model approach can be motivated by arguing that animals belonging to a farm share observed factors such as the same environment (physical location), as well as characteristics such as the type of farm (milk- or meat-oriented), and also unobserved factors. These farm-specific factors create dependencies between responses observed for the individual animals within farms. Likelihood inference in this type of model proceeds by integrating over the distribution of random effects to derive the marginal likelihood, which can practically be done by numerical integration (Gaussian quadrature for instance). Thus the cluster effects are removed by assuming that they are realizations of a random variable.

The Stata® procedure `gllamm` was used for implementing these cluster-specific random-effects logistic regression models. Gllamm, “generalized linear and latent mixed models”, is a Stata command that fits a class of multilevel latent variable models including multilevel generalized linear mixed models. Recently, adaptive quadrature has been proposed for multilevel models (Rabe-Hesketh et al., 2002). This allows using adaptive Gaussian quadrature to approximate the likelihood, which should be preferred to simple Gaussian quadrature in general (Lesaffre and Spiessens, 2001), as also demonstrated in the case of the BoHV-1 serosurvey (Speybroeck et al., 2003).

For the random-effects models, all two-by-two interactions with a biologically meaningful interpretation and second-order (quadratic) factors were considered in a forward stepwise-selection procedure. The polynomial predictor variables were centred to avoid problems of multicollinearity (Neter et al., 1996). Selection of the (relative) most important models was based on Akaike’s Information Criterion (Akaike, 1974).
RESULTS

Data exploration

At the animal level there were missing values for the exposures age (26), sex (14) and purchase (2,091), and for the BoHV-1 test result (36). At the farm level, the farm size variable was right-skewed (plots not shown). The median farm size was 19 overall, 60 for dairy farms, 59 for mixed farms and seven for beef farms. The density variables were less skewed. The average age (years) of the animals was lowest in beef farms (2.8), higher for mixed farms (2.9) and highest for dairy farms (3.1).

The proportion of missing covariate observations for the variable purchase was considerable with an average of 27% per farm. A design-based logistic regression taking account of the clustering at farm-level indicated that the age of the animal and the BoHV-1 test result were positively associated with missingness of the variable purchase (Table 1).

Exploration of the missingness of the variable purchase, by farm size, revealed that the largest farm, a beef farm with size 264, was most affected: 98% (259/264) of its animal records lacked the purchase information (Figure 1). Omission of this farm lead to different logistic regression results with age of animals and farm size being respectively positively and negatively associated with purchase missingness (Table 1). The effect of BoHV-1 test result became non-significant at the 5% level. The within-farm seroprevalence for this farm was 80% (212/264).

Table 1: Design-based logistic regression model of factors associated with purchase missingness.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Model with outlying farm</th>
<th>Model without outlying farm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (SE)</td>
<td>P-value</td>
</tr>
<tr>
<td>BoHV-1 test result (0=negative)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.766 (0.231)</td>
<td>&lt; 0.001</td>
<td>[1.365;2.282]</td>
</tr>
<tr>
<td>Farm size</td>
<td>1.001 (0.006)</td>
<td>0.879</td>
</tr>
<tr>
<td>Age (months)</td>
<td>1.016 (0.001)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

OR: odds ratio, SE: standard error, CI: confidence interval, and farm size: number of animals on the premises
No collinearity was discerned between the continuous covariates. The variable purchase was associated with sex (Pearson chi-square (1) = 41.0549, p < 0.001): one in four cows are purchased compared to one in three bulls. But purchase was also associated with farm type (Pearson chi-square (2) = 418.3857, p < 0.001): in dairy farms 17.19% of the cattle was purchased, compared to 23.65% and 40.29% for mixed and beef farms, respectively.

![Scatterplot of the farm-specific proportion of missing observations, by farm size.](image)

**Figure 1.** Scatterplot of the farm-specific proportion of missing observations, by farm size.

**Seroprevalence risk factor analysis**

**Bivariant analysis**

Table 2 shows that there were no obvious differences between the seroprevalence of cows and bulls, and between homebred or purchased animals. Seroprevalence increased with age and with farm size, with a hint of a non-linear effect for both effects, prompting the inclusion of quadratic terms (results not shown).

**Table 2: Seroprevalence of bovine herpesvirus 1, by sex and purchase category, in Belgium, 1998.**

<table>
<thead>
<tr>
<th>Animal-level seroprevalence</th>
<th>Number of observations</th>
<th>Seroprevalence (%) [95% Confidence interval ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>9,950</td>
<td>0.36 [0.30 – 0.42]</td>
</tr>
<tr>
<td>Males</td>
<td>1,320</td>
<td>0.34 [0.25 – 0.43]</td>
</tr>
<tr>
<td>Homebred</td>
<td>6,833</td>
<td>0.327 [0.259 – 0.393]</td>
</tr>
</tbody>
</table>
The final model included the animal-level factors age, sex and origin (purchased or homebred), and the farm-level covariate farm size. An increasing (centred) age was a risk factor for seropositivity, but this effect levelled off at higher age (significant negative quadratic term). Bulls were more at risk, compared to cows. The effects of the risk factors origin and farm size interacted. A significant negative interaction was observed between purchase status and (centred) farm size and a positive one between the same variable and the square of (centred) farm size. The final model is in Table 3. The log likelihood and the Akaike’s Information Criterion for this model estimating 9 parameters were -3289.0833 and 3307.0833, respectively.

The final model defined the following equation:

\[
\text{Logit}(\pi) = -2.349621 + b_i + 0.513734 \times \text{purchase} + 0.3169284 \times \text{sex} + 0.0395207 \times \text{farm size} - 0.0002016 \times \text{farm size}^2 + 0.0419751 \times \text{age} - 0.0000898 \times \text{age}^2 - 0.013023 \times \text{farm size} \times \text{purchase} + 0.0000634 \times \text{farm size}^2 \times \text{purchase}
\]

With: \(b_i\); the farm-level, random intercept

Table 3: Final logistic random-effects model.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>OR (SE)</th>
<th>P-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (months)</td>
<td>1.042 (0.002)</td>
<td>&lt; 0.001</td>
<td>[1.038 1.046]</td>
</tr>
<tr>
<td>Age^2 (months)</td>
<td>0.99991 (0.00003)</td>
<td>0.005</td>
<td>[0.99980 0.9999]</td>
</tr>
<tr>
<td>Sex (cow=0)</td>
<td>1.372 (0.165)</td>
<td>0.009</td>
<td>[1.084 1.738]</td>
</tr>
<tr>
<td>Purchase (no=0)</td>
<td>1.671 (0.203)</td>
<td>&lt; 0.001</td>
<td>[1.316 2.122]</td>
</tr>
<tr>
<td>Centred farm size</td>
<td>1.040 (0.006)</td>
<td>&lt; 0.001</td>
<td>[1.026 1.054]</td>
</tr>
<tr>
<td>Centred farm size^2</td>
<td>0.9997 (0.00005)</td>
<td>&lt; 0.001</td>
<td>[0.99960 0.9999]</td>
</tr>
<tr>
<td>interaction Centred farm size by Purchase</td>
<td>0.987 (0.003)</td>
<td>0.001</td>
<td>[0.979 0.994]</td>
</tr>
<tr>
<td>interaction Centred farm size^2 by Purchase</td>
<td>1.00006 (0.00002)</td>
<td>0.014</td>
<td>[1.00001 1.00001]</td>
</tr>
</tbody>
</table>

OR: odds ratio, SE: standard error, CI: confidence interval, farm size: number of animals on the premises
Figures 2 and 3 plot this equation respectively for bulls and cows, by farm size. A grid of observed farm size values was chosen. These figures first show that age is a risk factor for BoHV-1 seropositivity. Second, bulls have a persistently higher probability to be BoHV-1 seropositive, compared to cows. Third, these figures show that the effects of the risk factors origin and farm size interact. The significant negative interaction between purchase status and farm size plus the positive interaction between the same variable and the square of farm size means that its overall effect was different for smaller and larger farms. For small sized farms (up to 50 animals on the premises), purchased cattle were more at risk, compared to homebred ones (curve of purchased origin persistently higher than those of homebred origin), and increasing farm size was also a risk factor (positive slope). For larger farms, these overall effects were not observed.

Relation between centred farm size and the probability for bulls (Figure 2, up) or cows (Figure 3, down) to be seropositive to BoHV-1, for different ages and for different origins, by farm size.
DISCUSSION

Purchase missingness

The age of cattle and the BoHV-1 test result were positively associated with missingness of the variable purchase. But, the results of one highly seropositive farm, the largest one in the database, were outlying and most influential: omission of this beef farm lead to the odds for purchase missingness to be significantly increased respectively decreased with the age of cattle and the size of farms, whereas the BoHV-1 test results were not significantly associated any more, at the 5% significance level. These results are plausible, since almost all purchase information was lacking for this farm, the fact that naturally led to the farm’s omission in further analysis. As demonstrated graphically, there were hardly any other farms of this size in the sample. Consequently, relevant information is lacking to conclude anything with confidence regarding these large beef farms.

More importantly, omission of this outlying farm lead to a subsample for which purchase missingness did not depend any more on the BoHV-1 test result. Equally cardinal knowledge is that purchase missingness was caused by a technical problem while conducting the survey. Notably, for animal-level identification, the animals’ working ear tag numbers were noted, not their official ones. The advantage of the former ones is higher readability and consequently less subject to errors. Unfortunately, trying to fill in the purchase missingness values through SANITEL, it turned out that these working ear tag numbers were not indexed in SANITEL. Consequently, for every missing purchase value, all cattle registered in SANITEL, some 11 billion at the time of the survey, were to be verified. The computer runtime needed to retrieve this information was too high, in practice. Since the cause of missingness was very likely completely random, we hypothesise that the complete case analysis, as implemented, can be considered to be unbiased with respect to purchase being a BoHV-1 seroprevalence risk factor or not (Zhao et al., 1996). The missingness thus only constitutes a sample size reduction, while the resulting subsample can still be considered to be representative for the total sample.

Risk factors for BoHV-1 seropositivity

The objective of the present study was to investigate a basic network of risk factors for BoHV-1 seropositivity. The bivariant analysis clearly shows the dangers of presenting results based on collapsed (marginal) tables, which ignore the effects of other covariates. Marginally, the seroprevalence does not at all differ between bulls and cows, and between homebred and purchased
animals. But these comparisons are unfair, as demonstrated by the results of the design-based multiple logistic regression analysis.

The rationale for the animal-level analysis was to unravel the phenomena emerging when animals, the individual units of the usual analytic currency of epidemiology (Susser, 1994), are assembled into larger units (clusters, farms). To this end, cluster-specific (farm-specific) random-effects models are suitable, since they model the response rates as a function of covariates and parameters that are specific to a cluster (cluster varying covariates, animal-level covariates) (Neuhaus, 1992; Neuhaus and Kalbfleisch, 1998).

To the knowledge of the authors, no cluster sample surveys are reported in the literature investigating risk factors for BoHV-1 seropositivity at the animal-level, incorporating an unvaccinated cattle population and farms of all types. Speybroeck et al. (Speybroeck et al., 2003) analysed a subset of the present BoHV-1 survey data to exemplify population-averaged and cluster-specific models.

The most important finding in this study was the interaction between the effects of the risk factors origin and farm size. For farms up to 50 animals, purchased status and increasing farm size were risk factors, whereas these effects were not observed for larger farms. The interpretation of this marked difference in risk assessment for cattle in smaller farms, compared to larger ones, considers the biology of bovine herpesvirus 1 infection. A key epidemiological feature of BoHV-1 is its contagion. Contagion is a group effect of the dependent variable. It is a fundamental dynamic of communicable disease (Susser, 1994; De Jong, 1995), and explains why infections follow the clustering of animal populations. This is also the case in the BoHV-1 survey, since the intra-cluster correlation coefficient, which investigates the extent of spread of infections in a farm, was about 0.64 (Speybroeck et al., 2003), which is high. Once the infection is introduced in a farm it quickly spreads within-farm, to purchased and to homebred herd mates. This within-farm infection dynamic is cardinal, since the seroprevalence figures, and consequently the statistical model outcome, are a post-epidemic snapshot. Indeed, the serological status of the animals only marks past infection exposure, since the sampling of animals was performed - on average - at a time after the BoHV-1 ‘mini-epidemic’ had occurred in the farms. The probability of an animal to be sampled in the serological silent period - about 7 to 10 days (Davies and Carmichael, 1973; Kramps et al., 1994) - is low. Also, once infected, most cattle have antibodies, lifelong (Chow, 1972; Kahrs, 1977). Consequently, seroprevalence figures are non-informative with regard to the within-farm index case and the within-farm infection dynamic. We hypothesise that in larger farms, the contagion of homebred herd mates, which are more numerous compared to smaller farms, could have masked the importance of purchased index cases as direct sources of virus introduction (Figure 4). From a biological viewpoint, purchase status would be a direct risk factor for farms of any size. The finding that purchase was a major direct risk factor is consistent with other studies (Wentink G.H. et al., 1993; van Schaik et al.,
The BoHV-1 seems mainly introduced to cattle farms by cattle in the incubation period or in the acute phase of the infection, and by latently infected cattle.

Also the farm size was a risk factor for BoHV-1 seropositivity. In smaller farms the number of susceptible animals is smaller throughout the year, so infection may not be maintained. Also, the risk of BoHV-1 transmission among cattle within infected farms is higher at larger farm size. This may be explained by the within-farm contact structure. Larger farms usually have loose-housing barns, creating more contact between infected and susceptible animals. These farms possibly also have more visits by animal handlers (farmers, inseminators, veterinarians, traders) (van Wuijckhuise et al., 1998). Also the double-track survival strategy of herpesviruses in nature sustains the biological explanation of farm size being a risk factor. During primary infection, herpesviruses are disseminated within susceptible populations, which raise strong immune responses and overcome in most cases the diseases associated with the infections. The latent viruses represent a long-term reservoir that becomes meaningful upon reactivation. Then, seemingly healthy animals are able to re-excrete and transmit the virus to non-immune as well as to immune hosts (Thiry et al., 1985; Thiry et al., 1987; Engels and Ackermann, 1996). Indeed, the average frequency of outbreak is estimated to be once every 3.5 years (van Nieuwstadt and Verhoeff, 1983) to once every 1.5 years (Bosch et al., 1998).

Furthermore, bulls were more at risk of being seropositive, compared to cows. This finding is new. It could be that bulls have more ‘risky’ contacts compared to cows. This hypothesis matches the observation that the animal’s sex was significantly associated with its origin: one in three bulls were purchased compared to one in four cows. Sex could have been a marker for purchase. Another example of risky contacts that bulls could have more often are participation in cattle shows. Also, bulls possibly display a more risky behaviour than cows. Cattle that escape and mingle with other cattle are a risk for virus introduction into farms (van Schaik et al., 2001). If bulls escape more often than cows, this would explain our findings.

Last, an increasing age was a risk factor for seropositivity, but this effect levelled off at higher age (significant negative quadratic term). This result was expected since age is a surrogate measure of amount of exposure-time. Antibodies are markers for present or past infection, and persist mostly lifelong in the case of infection with BoHV-1 (Chow, 1972; Kahrs, 1977). This result is consistent with other studies (van Wuijckhuise et al., 1993; Kadohira et al., 1996).

In conclusion, prevention of BoHV-1 seropositivity should primarily focus on purchase that directly introduces virus into the farms. The farmer should prevent direct and lengthy animal contacts in order to reduce the risk of introduction of BoHV-1 considerably. Only certified BoHV-1 uninfected cattle should be purchased. After purchase of cattle, quarantine measures should be rigorously applied. Uninfected cattle should further be prevented to get in contact – directly and indirectly - with bulls or older cattle with unknown BoHV-1 infection status, or cattle originating from larger farms.
that are not certified BoHV-1 free. Due to sample limitations, these conclusions cannot be extrapolated to large beef farms.

Figures 4 a, b. Post-epidemic prevalence masking the importance of purchased index cases as direct sources of virus introduction in large farms.

a: Post-epidemic prevalence in small farms. In this scenario 100 farms consist each of size 2 (only 8 farms are drawn). Eight farms purchase 1 animal. In total there are 8 purchased cattle: 2 infected index cases (black square icons), and 6 non-infected ones (white square icons). The post-epidemic prevalence would consist of 4 infected cattle: 2 purchased and 2 homebred (grey circle icons), and 196 non-infected cattle: 6 purchased and 190 homebred (white circle icons animals). Statistically this results in purchase being a risk factor for seropositivity (Fisher’s exact P-value = 0.008).

b: Post-epidemic prevalence in large farms. In this scenario 4 farms consist each of size 50. Each farm purchases 2 animals. In total there are 8 purchased cattle including 2 infected index cases (black square icons). The post-epidemic prevalence would consist of 100 infected cattle: 4 purchased (including 2 non-index cases - grey square icons) and 96 homebred (grey circle icons), and 100 non-infected cattle: 4 purchased (white square icons) and 96 homebred (white circle icons animals). Statistically this results in purchase not being a risk factor for seropositivity (Fisher’s exact P-value = 1).
ACKNOWLEDGEMENTS

The BoHV-1 survey was supported by the Fund for Animal Health and Production, Federal Public Service Health, Food Chain Safety and Environment, Belgium.

(van Wuijkhuise et al., 1998)
(van Schaik et al., 1998)
(van Schaik et al., 2001)
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SUMMARY

Population-averaged modeling of the serological results of a bovine herpesvirus 1 survey indicated that the animal-level factors age, sex and origin (purchased or homebred), and the herd-level covariate herd size were associated with seropositivity to the bovine herpesvirus 1. The final regression model included significant quadratic terms, as well as interactions between all main factors.

The present study explored the impact of test misclassification bias on the interpretation of the results of this seroprevalence risk factor study. This exploration was implemented via a sensitivity analysis using age-related test misclassification probabilities, which were obtained by expert opinion. The range of expert values for the diagnostic sensitivity [0.960; 0.995] was comparable for different age groups of cattle, except for bovids younger than 1 year having lost their maternal immunity. For this young stock the diagnostic sensitivity was [0.800; 0.995]. The range of expert values for the diagnostic specificity [0.930; 0.995] was surprisingly wide with a low lower limit. It was the same for different age groups of cattle. The range of expert values for the duration of BoHV-1 maternal immunity was wide, with a minimum of 2.5 and a maximum of 11 months.

The population-averaged based sensitivity analysis indicated that the final model based on calculated true prevalence data, giving the - rather extreme - expert opinion, was more parsimonious compared to that based on seroprevalence data. Age, herd size and purchase were stable risk factors. Moreover, the sensitivity analysis reduced the uncertainty region of the seroprevalence odds ratios [0; + ∞] to [1.016; 1.028] and [1.016; 1.023] for an increase in age of 1 month and an increase in herd size of 1 animal, respectively. The uncertainty region of the seroprevalence odds ratios [0; + ∞] for purchase status was reduced to [1.360; 1.724]. We conclude that, taking account of expert values regarding diagnostic sensitivity and specificity, the age and purchase status of cattle, and the size of herds are potential risk factors for BoHV-1 infection.

INTRODUCTION

The BoHV-1 serosurvey data

In 1998, a large one-stage cluster sample survey of the cattle population estimated the bovine herpesvirus 1 (BoHV-1) herd seroprevalence to be 67%. An extensive description of the BoHV-1 serosurvey data can be found elsewhere (Boelaert et al., 2000). Seroprevalence estimation
and assessment of risk factors for BoHV-1 seropositivity has also been reported (Speybroeck et al., 2003; Boelaert et al., 2003).

**Test misclassification bias**

The globalization of trade in animals and animal products has resulted in efforts to improve and control the analytical and diagnostic quality of tests. However, little attention has been given to the post-analytical phase of the diagnostic process (which includes data management, analysis and interpretation) (Greiner and Gardner, 2000a). This is also the case for observational or epidemiological studies of infectious and other diseases that assess the association between disease and its determinants or between disease and economically important traits such as milk production or growth rate. The objective typically is to estimate the ‘effect’ of an exposure on a response variable. For infectious diseases, diagnostic tests are used frequently to determine the disease state. Therefore, statements will often refer to the serological rather than to the unknown true state of animals. One of the major challenges in the interpretation of these serodiagnostic data is the need to adjust test results for misclassification. In the veterinary literature, test misclassification bias has received only scant coverage, to date. Indeed, in the evaluation of diagnostic tests for infectious diseases in the biomedical literature, primarily laboratory aspects of test validation get the focus, as exemplified in an issue of the O.I.E. Scientific and Technical Review (Rev. Sci. Tech. Off. Int. Epiz. 17 (1998) 2).

**Classical definition of diagnostic sensitivity and specificity**

The medical value of a laboratory test is commonly determined by its medical (or diagnostic or epidemiological) characteristics in relation to a disease. These characteristics (e.g. diagnostic sensitivity, diagnostic specificity, positive and negative predictive value) deal with the uncertainties and errors that can occur when a test result is applied to make a medical decision. In addition, the biological variation that occurs between subjects and within the same subject over time contributes to a pre-analytic source of uncertainty. In determining the diagnostic test characteristics, the stochastic variability usually is of minor importance compared with bias in the parameter estimates (Greiner and Gardner, 2000a). Uncertainty arises when the true value of the parameter is unknown. This shift from the certainty we associate with logical truth when reasoning in exact or ‘hard’ sciences to the notions of correctness or satisfactoriness in inexact or ‘soft’ sciences, like medicine, results from the increased degrees of freedom characterizing living beings and processes. These always have unstated (and hence unexamined) properties compared to objects or processes in exact sciences (Blois, 1988).
The diagnostic sensitivity and specificity are classically defined as intrinsic characteristics of the test (Thrusfield, 1986; Jacobson, 1991; Martin et al., 1992). The diagnostic sensitivity and specificity are usually determined for a specific test and a specific disease or infection. They can be determined from data that describe the results of the test on one population, all of whom are known to be disease free and a second population, all of whom are known to have the disease of interest. The existence or nonexistence of the particular disease in the individuals of the two populations is based on a ‘definitive’ or ‘gold standard’ method, which is error free. If you know that an individual has a specific disease, diagnostic sensitivity (Se) indicates the probability that the patient will be positive for the particular test. If you know that an individual is free of a specific disease, diagnostic specificity (Sp) indicates the probability that the subject will be negative for a certain test.

Because of limitations in current diagnostic test technology, it has been impossible to devise the perfect test, which would predict the infection status of subjects with total accuracy. Consequently, all tests have non-perfect Se and Sp. The false negative rate for a test equals 1 minus the Se, whereas the false positive rate for a test is 1 minus the Sp. There are numerous reasons for lack of Se leading to false negative test results: natural or induced tolerance (animals that do not produce antibodies in contact with a specific antigen), improper timing (sampling during the serological ‘silent’ period after infection, or a period close to parturition), non-specific inhibitors, blocking antibodies, immunosuppression, laboratory errors, etc.) However, many more biological variables may contribute to lack of Sp leading to false positive results (cross-reactive antibodies to many other agents, non-specific reactions, true exposures unrelated to the present disease status – maternal antibodies, vaccination, previous exposure -, laboratory errors, etc.) than to false negative results, since animals generally develop antibody responses towards for most but not all pathogens, and thus are not falsely negative (Schrijver and Kramps, 1998).

**Diagnostic sensitivity and specificity are operational population parameters**

Assumptions that the values for Se and Sp are known and fixed fail to reflect reality. More specifically, Se and Sp are (sub-) population operational parameters that describe the test performance for a given reference population, i.e. conditional on a given distribution of influential covariate factors, under defined conditions (laboratory, gold standard, cut-off, etc…) (Greiner and Gardner, 2000b). They are strata (covariate) specific parameters, meaning that wherever possible, results of validation studies should be categorised into stratum-specific estimates. A few examples of these influential variables are breed, age, sex, nutritional status, pregnancy, stage of infection, differing responses of individuals to infectious agents and differing host responses in chronic versus peracute infections. In practice, the factors, their impacts and their interrelationships are
mostly unknown and Se and Sp estimates are considered to be average values calculated in non-homogenous populations. Thus, Se and Sp are only estimable using an epidemiological approach.

Two examples of reported influential variables for the Se are the prevalence of the disease, and the age of animals. Goddard (1977) challenged the idea that Se does not vary with changes in exposure or disease prevalence, and allowed the Se of a test for schistosomiasis to depend on prevalence. He felt that higher disease prevalence would correspond to a higher intensity of infection, hence decreasing the chance of a false negative result. A negative exponential relationship was assumed between the false negative rate and the disease prevalence. Sockett et al. (1994) reported that the Se of a non-serological test for bovine paratuberculosis changed depending on the prevalence of the disease in the herd. This phenomenon was also reported for serological tests for bovine paratuberculosis (Sockett et al., 1992). Although no formal relationship is reported to exist between Se and prevalence, a reasonable assumption for serologic tests is that the Se varies with the stage of infection or with the immune status of the host. Therefore, the case mix in the diseased reference sample will influence the Se estimate. In the case of the BoHV-1, there are indications that the Se of serological tests is age-related. Hage et al. (1998) and Lemaire et al. (2001) demonstrated the generation of seronegative latent carrier calves. These are calves that are infected with BoHV-1 in the presence of maternal antibodies, which is possible since the maternal immunity to herpes viruses, as BoHV-1, does not offer a complete protection against infection. Once these passive antibodies naturally decay, these calves become seronegative although they are still infected with the BoHV-1 virus. Conversion to seropositivity due to actively produced antibodies only happens after some months at a time when the virus is reactivated from its latent state. Aside from disease prevalence and age, it is worth mentioning also the maternal immunity as influencing Se and Sp, since it is naturally involved in a dynamic interplay with the disease prevalence.

**Diagnostic sensitivity and specificity of the used BoHV-1 survey test**

The BoHV-1 survey sera were tested for antibodies against BoHV-1 by using one assay, notably the assay with the highest Se (Kramps et al., 1994) (with a commensurate reduction in Sp). No published data of Se and Sp of the used assay, exist. Kramps et al. (1994) estimated the Se of an analogous non-commercial test to be 0.990. In their study, no information was reported on the gold standard or the vaccination status of the positive reference sera. The estimated Sp of this non-commercial test in a BoHV-1 free and unvaccinated animal population ranged from 0.960 (Kramps et al., 1994) to 0.997 (de Wit et al., 1998).
Impact and adjustment of test misclassification bias

Using multiple tests it is possible, under certain conditions, to estimate error parameters such as Se, Sp, relative risk, or predictive value, even though no gold standard is available. The parameter estimates are obtained by modelling the data, using maximum likelihood, with or without some constraints. The models recognize that the true classification of an individual is unknown, and so are sometimes referred to as ‘latent class’ models. The latent class approach provides a unified framework for various methods, characterising each by the number of (sub-)populations that are sampled, and the number of observations (tests) made on each individual. Both numbers together determine the number of cross-classifications into which the data are grouped, and hence the number of degrees of freedom that are available for parameter estimation. Thus, the number of degrees of freedom is implied by the sampling design. Data sets with three or more observations (tests) per individual lead directly to estimates of the misclassification rates, subject to some simple assumptions (Walter and Irwig, 1988). If there are too many parameters to be estimated from the available degrees of freedom (parameter redundancy, overparametrized model, overspecified model), constraints must be applied to render the model identifiable. For instance one may regard some of the parameters as known constants, which is often done in single-test surveys. The sampling design of single-test surveys implies an overparametrized model if estimation of the true prevalence and Se and Sp is intended. Indeed, animals from a single source population are then classified by a single test as positive or negative. This is the seroprevalence (apparent or measured prevalence), which depends on the Se and the Sp of the used test, and on the true prevalence in the animal population. Regarding the total number of individuals observed as fixed, the number of test positives determines the number of test negatives, and vice versa; thus only 1 degree of freedom is available, whereas there are 3 parameters to estimate (the prevalence, and the Se and Sp). Thus, two constraints must be applied if parameter estimation is intended. A common option in standard statistical practice is to impose the two constraints by regarding the Se and the Sp as known, and then to estimate the prevalence.

Few findings are reported concerning the exploration of the impact of test misclassification bias on the results of seroprevalence risk factor studies. Greiner and Gardner (2000a) considered the impact of adjustment for test misclassification in a hypothetical single-test cross-sectional risk factor study. They stated that the unadjusted estimator of the OR systematically underestimates the true OR if misclassification is non-differential. However, most epidemiological studies consider multiple covariates (risk factors, confounders and effect modifiers); hence, adjustment approaches are often more complicated than those described by the authors. Meta-analytic methods are described to combine estimates from different primary studies and to investigate covariate effects (Irwig et al., 1995).
Objective

The objective was to explore the impact of the test misclassification bias, in a frequentist framework, on the interpretation of the single-test BoHV-1 survey results.

MATERIALS AND METHODS

The exploration of the impact of test misclassification bias was implemented in a frequentist framework, as follows. First, the effect of risk factors related to BoHV-1 seroprevalence (apparent or measured prevalence) was estimated. As the main tool, logistic regression models, taking into account the effects of clustering, were considered. Second, expert opinion was elicited regarding the age-related Se and Sp of the used survey test. Third, the post-diagnostic (medical or epidemiological) uncertainty was explored. Via sensitivity analysis an uncertainty region for a set of risk factor parameters was constructed with a calculated true infection status as the response variable.

Statistical design-based investigation of risk factors associated with BoHV-1 seropositivity

The analysis should match the design of the study that was a one-stage cluster sample survey. Marginal (population-averaged) models were opted for, using pseudo-likelihood methodology. Essentially, small computer run-times motivated this choice (Geys et al., 1997; Geys et al., 1998). A pseudo-likelihood was used since the standard maximum likelihood estimator does not give a true 'likelihood' under a complex design and therefore estimates of model parameters are obtained by solving weighted analogues of likelihood equations based on the probability sampled data. The full maximum likelihood estimator would require an expression for the exact likelihood, which may be very complicated and require many assumptions since it involves modelling the relation between the response and the design variables (Skinner et al., 1989).

All analyses were performed using the statistical software Stata®, (StataCorp. Stata Statistical Software: Release 8.0, 2003). The STATA procedure svylogit was implemented. Svyllogit fits logistic regression models for survey data and is able to take account of clustering (one level only), by adapting standard errors and confidence intervals for estimates. STATA currently uses the Taylor series linearization estimator as the variance estimation method. This method approximates the complex formulas for the adapted variance, by writing it as a series of functions.

Using this pseudo likelihood method, two-by-two interactions and second-order (quadratic) factors were considered in a hierarchical backward stepwise-selection procedure. Selection of the
(relative) most important models was based on the significance level. All tests were two-tailed and a p-value < 0.05 was considered as significant.

**Quantification of expert opinion**

Eight experts: from Denmark (1), Germany (1) Sweden (1), Belgium (2), The Netherlands (2) and The United States of America (1) filled in a questionnaire (Annex A). They expressed their current belief and uncertainty about the diagnostic characteristics of the used BoHV-1 survey test. Experts were selected from the O.I.E. reference laboratoria for BoHV-1 together with veterinary epidemiologists with a doctoral thesis on BoHV-1 epidemiology. They were contacted by electronic mail only. The main objective was the investigation of the age-related Se and Sp of the used BoHV-1 test in an unvaccinated cattle population. For the purpose of quantifying beliefs, a trial roulette form was circulated as developed by Gore (Errington et al., 1987; Gore, 1987a; Gore, 1987b; Gore, 1987c). The trial roulette are diagrams showing betting ‘streets’ (as in a game of gambling). Each of the diagrams addresses the following main question: *Suppose you were involved in a game of gambling, where would you place your 20 gaming tokens to represent your current belief and uncertainty (i.e. bets) about the given key parameter in an unvaccinated cattle population?* Please, place your 20 gaming tokens (X) in some or all of the ‘streets’.

**Sensitivity analysis  exploring the impact of test misclassification bias**

*The concept of sensitivity analysis*

It is useful to distinguish between two types of statistical uncertainty. The first one, statistical imprecision, is due to finite sampling. A second source of uncertainty, due to incompleteness, can be called statistical ignorance. Both combine into the statistical uncertainty. These definitions were recently formalized (Kenward et al., 2001) (Molenberghs et al., 2001). The concept of fitting a single model is then replaced by that of sensitivity analysis, where several plausible non-ignorable models are contrasted. Thus, a natural way to proceed is to acknowledge the inherent ambiguity and explore the range of inferences that are consistent with the gap in our knowledge.

The sampling design of the BoHV-1 1998 survey implies that we have an overparametrized model if estimation of the true prevalence and Se and Sp is intended. The number of parameters to estimate and the available number of degrees of freedom for this single-test survey, are 3 and 1, respectively. Therefore expert knowledge regarding the Se and Sp of the survey test was included to render the model identifiable. All expert values were considered in a frequentist framework.
Chapter 6.3

The Stata ado-file

The test misclassification probabilities were factored in with the seroprevalence risk factor analysis using a Stata® ado-file. Via this program the dataset was transformed a number of times (grid) according to the range of values obtained by the expert opinion for the age-related Se and Sp. For each unique combination of expert values a unique ‘true infection state’ binary response variable was calculated. Different true prevalences, 0.2, 0.3, 0.4 and 0.5, were assumed to be able to create this latter variable, via the predictive values and the random generator function ‘runiform’. The final model selected with seroprevalence as response was re-run with the ‘true infection state’ as new response, for each of these 160 datasets. In total, 3 runs were implemented. This resulted in a gridanalysis comprising 480 transformations of the database, through all possible combinations of the range of values obtained by expert opinion. The STATA ado-command file is in Annex B.

Gridanalysis

The gridanalysis was considered as an experiment. The results of the 3 runs were combined in a multiple-regression analysis with the seroprevalence risk factors and their interactions as the response variables. The assumed true prevalences and the range of expert opinion values were the independent variables. All interactions and second-order factors were considered in a hierarchical backward stepwise-selection procedure. All tests were two-tailed and a p-value < 0.05 was considered as significant. Finally, uncertainty regions were described for the response variables. These were the ranges of the observed response values.
RESULTS

The BoHV-1 seroprevalence risk factor analysis

The final model is in Table 1. The animal-level factors age, sex and origin (purchased or homebred), and the herd-level covariate herd size were associated with seropositivity to the bovine herpesvirus 1. Significant quadratic terms were included in the final model, as well as interactions between all main factors.

Table 1. Final logistic regression model

<table>
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<tr>
<th>Risk Factor</th>
<th>Odds ratio</th>
<th>Standard error</th>
<th>p</th>
<th>95% Confidence interval</th>
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<tr>
<td>Centred age (months)</td>
<td>1.023</td>
<td>0.003</td>
<td>&lt; 0.001</td>
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<td>Centred age^2 (months)</td>
<td>0.99997</td>
<td>0.00004</td>
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<td>[0.999891 0.00006]</td>
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<td>[0.736 1.696]</td>
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<td>0.006</td>
<td>[1.158 2.367]</td>
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<td>0.024</td>
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<td>interaction Age * Centred herd size^2</td>
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With: sex; 1=male 0=female, purchase; 1=yes 0=no, herd size; number of animals on the premises

Expert opinion

Table 2 reports the expert-specific distributions of values on the duration of BoHV-1 maternal immunity, as detected by the BoHV-1 test. The bar chart in Figure 1 plots the overall distribution of the expert values, in order to clarify overall patterns. The range of the duration of BoHV-1 maternal immunity was wide, with a minimum of 2.5 and a maximum of 11 months.

Table 3 reports the expert-specific distributions of values on BoHV-1 test Se, when testing a representative subpopulation boids younger than 1 year having lost their maternal immunity. The bar chart in Figure 2 plots the overall distribution of the expert values, in order to clarify overall patterns. The range of BoHV-1 test Se for this subpopulation of cattle was wide, with a minimum of 0.800 and a maximum of 0.995.
Table 2. Distribution of expert opinion values on the duration of BoHV-1 maternal immunity

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Figure 1. Overall distribution of the expert opinion values on the duration of BoHV-1 maternal immunity

Table 3. Distribution of expert opinion values on BoHV-1 test diagnostic sensitivity, when testing a representative subpopulation of unvaccinated bovids younger than 1 year having lost their maternal immunity.
Positioning of 20 gaming tokens to represent current belief and uncertainty (i.e. bets) about the BoHV-1 test diagnostic sensitivity, when testing a representative subpopulation of unvaccinated bovids younger than 1 year having lost their maternal immunity

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Figure 2. Overall distribution of the expert opinion values on BoHV-1 test diagnostic sensitivity, when testing a representative subpopulation of unvaccinated bovids younger than 1 year having lost their maternal immunity.

Table 4 reports some summary statistics of the overall distribution of expert values regarding the duration of the serologically silent period after a primary infection and the age-related diagnostic test characteristics. The range of expert values for the Se [0.960; 0.995] was comparable for different age groups of cattle, except for bovids younger than 1 year having lost their maternal immunity [0.80; 99.5]. The range of expert values for the Sp [0.930; 0.995] was surprisingly wide with a low lower limit. It was the same for different age groups of cattle.
Table 4 Distribution of expert opinion values on BoHV-1 test, when testing a representative subpopulation of unvaccinated cattle.

<table>
<thead>
<tr>
<th>Questions</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (days) of the serologically silent period after a primary infection</td>
<td>9</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>Duration (days) of BoHV-1 maternal immunity</td>
<td>6.5</td>
<td>2.5</td>
<td>11</td>
</tr>
<tr>
<td>Se , when testing unvaccinated cattle never having had maternal antibodies, and younger than 1 year</td>
<td>0.990</td>
<td>0.960</td>
<td>0.995</td>
</tr>
<tr>
<td>Sp , when testing unvaccinated cattle never having had maternal antibodies, and younger than 1 year</td>
<td>0.990</td>
<td>0.930</td>
<td>0.995</td>
</tr>
<tr>
<td>Se , when testing unvaccinated cattle, having had maternal antibodies, and infected with BoHV-1 under maternal immunity, and younger than 1 year, but having lost their maternal immunity</td>
<td>0.980</td>
<td>0.800</td>
<td>0.995</td>
</tr>
<tr>
<td>Sp, when testing unvaccinated cattle, having had maternal antibodies, younger than 1 year, but having lost their maternal immunity.</td>
<td>0.985</td>
<td>0.930</td>
<td>0.995</td>
</tr>
<tr>
<td>Se , when testing unvaccinated cattle, aged 1 to 3 years</td>
<td>0.990</td>
<td>0.965</td>
<td>0.995</td>
</tr>
<tr>
<td>Sp, when testing unvaccinated cattle, aged 1 to 3 years</td>
<td>0.990</td>
<td>0.930</td>
<td>0.995</td>
</tr>
<tr>
<td>Se , when testing of unvaccinated cattle, 3 years or older</td>
<td>0.990</td>
<td>0.960</td>
<td>0.995</td>
</tr>
<tr>
<td>Sp, when testing of unvaccinated cattle, 3 years or older</td>
<td>0.990</td>
<td>0.930</td>
<td>0.995</td>
</tr>
</tbody>
</table>

The expert opinion results define a scheme of parameters as represented in Table 5. These parameters are the backbone of the gridanalysis.

Table 5. Scheme of grid parameters

<table>
<thead>
<tr>
<th>Age of cattle (years)</th>
<th>0 – a (^1)</th>
<th>a – 1</th>
<th>&gt; 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle with</td>
<td>Se (^2)</td>
<td></td>
<td></td>
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<tr>
<td>maternal immunity</td>
<td>l</td>
<td>b</td>
<td>d</td>
</tr>
<tr>
<td>Cattle without</td>
<td>Sp (^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>maternal immunity</td>
<td>0</td>
<td>c</td>
<td>c</td>
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<tr>
<td></td>
<td>d</td>
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<td>d</td>
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</tbody>
</table>

\(^1\) Duration of maternal immunity  
\(^2\) Diagnostic sensitivity  
\(^3\) Diagnostic specificity

By definition, the Se of the BoHV-1 test for a subpopulation of calves having maternal antibodies, is 1. On the contrary, its Sp is, by definition, 0.
Sensitivity analysis exploring the impact of test misclassification bias

Results of the grid

Age, herd size and purchase were stable risk factors. All their coefficients were significant, except for the variable purchase (1/160 coefficients not significant). Moreover, it reduced the uncertainty region of the seroprevalence ORs $[0; + \infty]$ to $[1.016; 1.028]$ and $[1.016; 1.023]$ for an increase in age of 1 month and an increase in herd size of 1 animal, respectively. The uncertainty region of the seroprevalence ORs $[0; + \infty]$ for purchase status was reduced to $[1.360; 1.724]$.

Sensitivity analysis

The independent variables significantly related with the age, herds size and purchase coefficient response variable are reported in Table 6, 7 and 8. The regression indicates that the coefficients for age, herds size and purchase are significantly related to the same grid analysis parameters (independant variables), with the exception of purchase that does not depend on the parameter $a$ (duration of maternal immunity). But, the effect of the grid analysis parameters do not have the same direction (sign).

Table 6. Multiple-regression analysis with age as the response variables.

| Coefficients | Standard error | t   | $p>|t|$ | 95% CI     |
|--------------|----------------|-----|--------|------------|
| $p$          | -0.01217       | 0.021678 | -0.56 | 0.575      | -0.05477 | 0.030429 |
| $a$          | -0.00171       | 0.000753 | -2.27 | 0.024      | -0.00319 | -0.00023 |
| $b$          | -0.04375       | 0.003722 | -11.76 | < 0.000    | -0.05106 | -0.03644 |
| $c$          | 0.052982       | 0.003797 | 13.95 | < 0.000    | 0.04552  | 0.060444 |
| $d$          | 0.081663       | 0.020734 | 3.94  | < 0.000    | 0.04092  | 0.122406 |
| $pa$         | 0.001047       | 0.000119 | 8.81  | < 0.000    | 0.000813 | 0.00128  |
| $pb$         | 0.0637         | 0.003445 | 18.49 | < 0.000    | 0.05693  | 0.070469 |
| $pc$         | -0.06835       | 0.010335 | -6.61 | < 0.000    | -0.08866 | -0.04804 |
| $pd$         | 0.047933       | 0.019193 | 2.5   | 0.013      | 0.010218 | 0.085648 |
| $ab$         | -0.00126       | 0.000136 | -9.29 | < 0.000    | -0.00153 | -0.001   |
| $ad$         | 0.002924       | 0.000759 | 3.85  | < 0.000    | 0.001434 | 0.004415 |
| _cons_       | -0.0677        | 0.020894 | -3.24 | 0.001      | -0.10876 | -0.02664 |

With: $p$=prevalence, $a$= duration of maternal immunity, $b$ and $d$ are Se parameters, and $c$ is the Sp.
Table 7. Multiple-regression analysis with herd size as the response variables.

| Coefficients | Standard error | t    | p>|t| | 95% CI    |
|--------------|----------------|------|-------|--------|
| p            | -0.05643       | 0.01788 | -3.16 | 0.002  | -0.09156 | -0.02129 |
| a            | 0.000385       | 0.000102 | 3.76  | < 0.000 | 0.000184 | 0.000587 |
| b            | 0.008526       | 0.009996 | 0.85  | 0.394  | -0.01112 | 0.028168 |
| c            | 0.067646       | 0.009409 | 7.19  | < 0.000 | 0.049157 | 0.086134 |
| d            | -0.00157       | 0.005875 | -0.27 | 0.79   | -0.01311 | 0.009976 |
| pb           | 0.042953       | 0.00287 | 14.97 | < 0.000 | 0.037313 | 0.048592 |
| pc           | -0.06614       | 0.00861 | -7.68 | < 0.000 | -0.08306 | -0.04922 |
| pd           | 0.078974       | 0.01599 | 4.94  | < 0.000 | 0.047554 | 0.110395 |
| ab           | -0.0004        | 0.000113 | -3.54 | < 0.000 | -0.00063 | -0.00018 |
| bc           | -0.02571       | 0.009873 | -2.6  | 0.009  | -0.04511 | -0.00631 |
| _cons        | -0.02647       | 0.011083 | -2.39 | 0.017  | -0.04824 | -0.00469 |

With; p=prevalence, a= duration of maternal immunity, b and d are Se parameters, and c is the Sp.

Table 8. Multiple-regression analysis with purchase as the response variables.

| Coefficients | Standard error | t    | p>|t| | 95% CI    |
|--------------|----------------|------|-------|--------|
| p            | 1.259981       | 0.513137 | 2.46  | 0.014  | 0.251672 | 2.268291 |
| b            | -0.00506       | 0.06232 | -0.08 | 0.935  | -0.12752 | 0.1174   |
| c            | 1.080075       | 0.186959 | 5.78  | < 0.000 | 0.712701 | 1.447448 |
| d            | 0.873096       | 0.105653 | 8.26  | < 0.000 | 0.665489 | 1.080702 |
| pb           | 0.471758       | 0.169613 | 2.78  | 0.006  | 0.138469 | 0.805046 |
| pc           | -1.81716       | 0.508839 | -3.57 | < 0.000 | -2.81703 | -0.8173  |
| _cons        | -1.41143       | 0.214971 | -6.57 | < 0.000 | -1.83385 | -0.98902 |

With; p=prevalence, a= duration of maternal immunity, b and d are Se parameters, and c is the Sp.

DISCUSSION

BoHV-1 seroprevalence risk factor analysis

A through discussion of the risk factors associated with animal-level BoHV-1 seropositivity can be find elsewhere (Boelaert et al., 2003). The present population-averaged analysis confirms that the animal-level factors age, sex and origin (purchased or homebred), and the herd-level covariate herd size were associated with seropositivity to the bovine herpesvirus 1. The final regression model included significant quadratic terms, as well as interactions between all main factors.
Expert opinion

In the light of the uncertainty regarding the Se and Sp, there are two basic ways of deriving the distributions needed for modelling this uncertainty. The first is directly from observed or experimental data, and the second is using expert opinion when data are scarce or missing (Vose, 1996). The quantification of expert opinions in the veterinary context has been recently discussed by Stærk et al. (2002). The expert opinion results of the present study were rather extreme. The range for the Sp values, for instance, was surprisingly wide with a low lower limit.

The expert opinion results rely on a relatively small number of experts and may thus be criticized for a perceived lack of objectivity, as opinions may not only reflect scientific knowledge but also preferences and cultural values. However, in this study, the experts had different backgrounds (countries). It must be emphasised that the sole aim of the expert opinion questionnaire was to gather data about the uncertainty regarding the age-related Se and Sp. As to experts, overall, the Sp of the used BoHV-1 test is not age-related, whereas the Se is. We believe a range of expert values as in Appendix C to be highly useful for exploring the impact of misclassification bias.

Sensitivity analysis towards test misclassification bias

Proper adjustment requires both uncertainty-reducing strategies and methods that adequately account for the remaining uncertainty. The single-test BoHV-1 survey implied that we had an overparametrized model. Also, the error probabilities of the used survey test were not well established, globally nor locally in Belgium. Therefore it was opted for eliciting expert knowledge regarding the Se and Sp of the survey test, to render the model identifiable. The population-averaged based sensitivity analysis provided us with an uncertainty region for risk factors associated with a calculated BoHV-1 true infection status, conditional on the expert opinion. Age, herd size and purchase were stable risk factors for BoHV-1 infection. Moreover, it reduced the uncertainty region of the seroprevalence ORs \([0; + \infty]\) to \([1.016; 1.028]\) and \([1.016; 1.023]\) for an increase in age of 1 month and an increase in herd size of 1 animal, respectively. The uncertainty region of the seroprevalence ORs \([0; + \infty]\) for purchase status was reduced to \([1.360; 1.724]\). The stability of the final model based on seroprevalence data and on a calculated true prevalence conditional on expert Se and Sp values can probably partly be explained by the high sample survey size. Also, the BoHV-1 infection turned out to be endemically in the Belgian cattle population.

Specificity errors are reported to be a more important source of bias in the prevalence estimate than are errors of Se (Quade et al., 1980). This is especially true when a disease has a low
prevalence since in this situation the positive predictive value will largely depend on the Sp. This becomes the case in due course of a BoHV-1 eradication program.

**Concluding Remarks**

The population-averaged based sensitivity analysis provided us with a fairly stable model and uncertainty region for risk factors associated with a calculated BoHV-1 true infection status, taking account expert opinion regarding the Se and Sp of a BoHV-1 screening test. The age of cattle, their purchase status and the size of herds are potential risk factors for BoHV-1 infection. Consequently, one should prevent uninfected cattle to get in contact – directly and indirectly - with older cattle, or cattle originating from larger herds. Also, purchased cattle should be quarantined, until their sanitary status is cleared out.
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EXPERT OPINION

Diagnostic test characteristic of the BHV-1 gB-blocking ELISA,
in an unvaccinated cattle population

Trial Roulette Form (Gore, 1987)

Prepared by Frank Boelaert and Dirk Berkvens

Objective

In this study we call for your expert opinion to investigate the diagnostic sensitivity and specificity of a commercially available test, a blocking ELISA, which detects antibodies specific for bovine herpesvirus type 1 (BHV-1) glycoprotein B (gB). The gB-blocking ELISA is considered as the most sensitive test to detect serum antibodies to BHV-1.

Definition of diagnostic sensitivity and specificity

Determining the meaning of a laboratory test result is often a difficult task because of the many sources of uncertainty in the various steps involved in generating the result. The analytic (or measurement) characteristics of a test deal with the uncertainties and errors that can occur in the analytic aspects of a test. There are two kinds of analytic errors: systematic errors affecting the test accuracy or validity, and random errors affecting the test precision or variability. A systematic error is the difference between the measured amount and the true amount, whereas a random error is observed when one property of the same sample is repeatedly measured. The medical value of a laboratory test is commonly determined by its medical (or diagnostic or epidemiological) characteristics in relation to a disease. These characteristics (e.g. diagnostic sensitivity, diagnostic specificity, positive and negative predictive value) deal with the uncertainties and errors that can occur when a test result is applied to make a medical decision. Again these errors can be systematic or random. In addition, the biological variation that occurs between subjects and within the same subject over time contributes a pre-analytic source of uncertainty. The diagnostic sensitivity and specificity are usually determined for a specific test and a specific disease or infection. They can be determined from data that describe the results of the test on two populations, one population known to be free of the disease and one population known to have the disease. The existence or nonexistence of the particular disease in the individuals of the two populations is based on an explicit set of criteria (a “gold standard”). If you know that an individual
has a specific disease, **diagnostic sensitivity** indicates the likelihood that the patient will be positive for the particular test. The false negative rate for a test equals 100% minus the diagnostic sensitivity. If you know that an individual is free of a specific disease, **diagnostic specificity** indicates the likelihood that the subject will be negative for a certain test. The false positive rate for a test is 100% minus the diagnostic specificity. A perfect test tests all diseased patients (or animals in our case) as positive (100% diagnostic sensitivity) and all non-infected subjects as negative (100% diagnostic specificity).

Because of limitations in current diagnostic test technology, it has been impossible to devise the perfect test, which would predict the infection status of subjects with total accuracy. This is also the case for the gB-blocking ELISA. Kramps et al. (1994) estimated the diagnostic sensitivity of the gB-blocking ELISA to be 99%. The estimated diagnostic specificity of this test in a BHV-1 free and unvaccinated animal population ranges from 96% (Kramps et al., 1994) to 99.7% (de Wit et al., 1998).

**Your expert opinion**

On the next pages, we ask you to express your current belief and uncertainty about the **diagnostic characteristics of the BHV-1 gB-blocking ELISA (generation of 1998, Idexx® ELISA test)**. Our main objective is the investigation of the age-related diagnostic sensitivity and specificity of this test in an **unvaccinated cattle population** (part III of the questionnaire).

However, two minor topics, the duration of the serologically silent period after a primary infection, and the duration of the maternal immunity, are discussed first (part I and II).

For the purpose of quantifying your beliefs, a trial roulette form is proposed as developed by Sheila Gore (1987a-b). Sheila Gore is senior biostatistician and member of the Medical Research Council, Biostatistics Unit, Cambridge, and uses the trial roulette in the design of trials (Gore, 1987c; Errington et al., 1991). The trial roulette are diagrams showing betting ‘streets’ (as in a game of gambling). Each of the diagrams addresses the following main question: **Suppose you were involved in a game of gambling, where would you place your 20 gaming tokens to represent your current belief and uncertainty (i.e. bets) about the given key parameter in an unvaccinated cattle population?** Please, place your 20 gaming tokens (X) in some or all of the ‘streets’. An example on the next page illustrates the point (Table 1).
Example

The following diagram shows betting streets for the diagnostic sensitivity of a BHV-1 gB-blocking ELISA (generation of 1998, Idexx® ELISA test) in an unvaccinated cattle population. Each ‘street’ in the diagram represents a range of diagnostic sensitivity values, e.g. in the table below the first ‘street’ (left side of diagram) goes from 79.5% to 80%, the second from 80% to 80.5%, … Suppose that I believe that the diagnostic sensitivity of the gB-blocking ELISA, in an unvaccinated cattle population, tends to be higher than 99%. Then I would place more gaming tokens (X) near values larger than 99%: thus in the last street (right side of diagram). Table 1 shows my 20 gaming tokens. The large spread on my tokens indicates that I am not very certain and leave a possibility for the diagnostic sensitivity to be smaller than 99%.

Table 1. Betting streets for the diagnostic sensitivity of the gB-blocking ELISA (generation of 1998, Idexx® ELISA test) in an unvaccinated cattle population.

| 80  | 80.5 | 81   | 81.5 | 82   | 82.5 | 83   | 83.5 | 84   | 84.5 | 85   | 85.5 | 86   | 86.5 | 87   | 87.5 | 88   | 88.5 | 89   | 89.5 | 90   | 91   | 91.5 | 92   | 92.5 | 93   | 93.5 | 94   | 94.5 | 95   | 95.5 | 96   | 96.5 | 97   | 97.5 | 98   | 98.5 | 99   | 99.5 |
|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| X   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| X   | X    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| X   | X    | X    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| X   | X    | X    | X    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| X   | X    | X    | X    | X    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

For reference: Kramps et al. (1994) estimated the diagnostic sensitivity of a gB-blocking ELISA to be 99% (generation of 1994).
As mentioned, our main objective is the investigation of the age-related diagnostic sensitivity and specificity of the BHV-1 gB-blocking ELISA in an unvaccinated cattle population. Therefore, we propose you to fill in the following eight age-specific tables (part III of our questionnaire):

**Tables 4 and 5**: concern the following representative subpopulation of unvaccinated cattle;
- not having and never having had maternal antibodies, and
- younger than 1 year.

As guidance, the following graph (Tizard, 1992) is provided:

![Graph](image)

**Tables 6 and 7**: concern the following representative subpopulation of unvaccinated cattle;
- having had maternal antibodies, and
- infected with BHV-1 under maternal immunity, and
- younger than 1 year, but having lost their maternal immunity.

Tables 6 and 7 partly relate to the existence or nonexistence of seronegative carrier calves.

**Tables 8 and 9**: concern a representative subpopulation of unvaccinated cattle, aged 1 to 3 years.

**Tables 10 and 11**: concern a representative subpopulation of unvaccinated cattle of 3 years or older.

Tables 10 and 11 partly relate to the question of the existence or nonexistence of seronegative adult carrier cattle. BHV-1 reactivations cause re-exposure to antigen, which results in increase of antibody titre and partly explains the persistence of humoral antibody for the life of some animals. But can adult cattle become seronegative?

Please, keep in mind that we investigate **diagnostic characteristics of the BHV-1 gB-blocking ELISA** (generation of 1998, Idexx® ELISA test) in an unvaccinated cattle population.
Part I. Duration of the serologically silent period after a primary infection

Your opinion about the duration of the serologically silent period (gap between the time of a primary infection until the time of seroconversion) as detected by the BHV-1 gB-blocking ELISA (generation of 1998, Idexx® ELISA test), in cattle that have NO ANTIBODIES at the time of infection with BHV-1 (no passively acquired maternal antibodies, nor actively induced antibodies via vaccination, nor actively synthesized antibodies after infection)?

a) What is your opinion the largest value for the mean serologically silent period (in days) that you still consider plausible? Please write absolute numbers, e.g. 10 (days).

b) Please, place your 20 gaming tokens (X) on some or all of the ‘streets’ to represent your current belief and uncertainty about the duration (in days) of the serologically silent period.

Table 2. Betting streets for the duration of the serologically silent period.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |

For reference: The BHV-1 gB-blocking ELISA is able to detect specific antibodies in serum as early as 7 days postinfection (Kramps et al., 1994).
Part II. Duration of maternal immunity

Your opinion about the duration of BHV-1 maternal immunity, as detected by the gB-blocking ELISA (generation of 1998, Idexx® ELISA test)?

a) What is in your opinion the largest value for the mean duration (in days) of maternal immunity that you still consider plausible? Please write absolute numbers, e.g. 180 (days).

b) Please, place your 20 gaming tokens (X) on some or all of the ‘streets’ to represent your current belief and uncertainty about the mean duration (in days) of maternal immunity?

Table 3. Betting streets for the mean duration of maternal immunity.

| 0  | 15 | 30 | 45 | 60 | 75 | 90 | 105 | 120 | 135 | 150 | 165 | 180 | 195 | 210 | 225 | 240 | 255 | 270 | 285 | 300 | 315 | 330 |

For reference: The maternal antibodies titre, detected by a serum-neutralisation test, decays to undetectable levels between 95 and 231 days, depending upon the initial titre of the calves (Brar et al., 1978).
Part III. Age-specific diagnostic test characteristics

Your opinion about the BHV-1 gB-blocking ELISA diagnostic SENSITIVITY (generation of 1998, Idexx® ELISA test) when testing the following representative subpopulation of unvaccinated cattle;

- not having and never having had maternal antibodies, and
- younger than 1 year.

Table 4. Betting streets for the diagnostic SENSITIVITY of the gB-blocking ELISA when testing unvaccinated cattle younger than 1 year, not having and never having had maternal antibodies.

<table>
<thead>
<tr>
<th>80</th>
<th>80.5</th>
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<th>98</th>
<th>98.5</th>
<th>99</th>
<th>99.5</th>
</tr>
</thead>
</table>

For reference: Kramps et al. (1994) estimated the diagnostic sensitivity of a gB-blocking ELISA to be 99% (age of study population not specified).
Your opinion about the BHV-1 gB-blocking ELISA diagnostic SPECIFICITY (generation of 1998, Idexx® ELISA test) when testing the following representative subpopulation of unvaccinated cattle:

- not having and never having had maternal antibodies, and
- younger than 1 year.

Table 5. Betting streets for the diagnostic SPECIFICITY of the gB-blocking ELISA when testing unvaccinated cattle younger than 1 year, not having and never having had maternal antibodies.

For reference: The estimated diagnostic specificity of a gB-blocking ELISA in a BHV-1 free and unvaccinated animal population ranged from 96% (Kramps et al., 1994) to 99.7% (de Wit et al., 1998) (age of study population not specified).
Your opinion about the BHV-1 gB-blocking ELISA diagnostic SENSITIVITY (generation of 1998, Idexx® ELISA test) when testing the following representative subpopulation of unvaccinated cattle;

- having had maternal antibodies, and
- infected with BHV-1 under maternal immunity, and
- younger than 1 year, but having lost their maternal immunity.

Table 6. Betting streets for the diagnostic SENSITIVITY of the gB-blocking ELISA when testing an unvaccinated subpopulation of cattle as specified above.

| 80 | 80.5 | 81 | 81.5 | 82 | 82.5 | 83 | 83.5 | 84 | 84.5 | 85 | 85.5 | 86 | 86.5 | 87 | 87.5 | 88 | 88.5 | 89 | 89.5 | 90 | 90.5 | 91 | 91.5 | 92 | 92.5 | 93 | 93.5 | 94 | 94.5 | 95 | 95.5 | 96 | 96.5 | 97 | 97.5 | 98 | 98.5 | 99 | 99.5 |
|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|----|------|

For reference: None
Your opinion about the BHV-1 gB-blocking ELISA diagnostic SPECIFICITY (generation of 1998, Idexx® ELISA test) when testing the following representative subpopulation of unvaccinated cattle;

- having had maternal antibodies, and
- younger than 1 year, but having lost their maternal immunity.

Table 7. Betting streets for the diagnostic SPECIFICITY of the gB-blocking ELISA when testing an unvaccinated subpopulation of cattle as specified above.

| 80  | 80.5 | 81  | 81.5 | 82  | 82.5 | 83  | 83.5 | 84  | 84.5 | 85  | 85.5 | 86  | 86.5 | 87  | 87.5 | 88  | 88.5 | 89  | 89.5 | 90  | 90.5 | 91  | 91.5 | 92  | 92.5 | 93  | 93.5 | 94  | 94.5 | 95  | 95.5 | 96  | 96.5 | 97  | 97.5 | 98  | 98.5 | 99  | 99.5 |
|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|

For reference: The estimated diagnostic specificity of a gB-blocking ELISA in a BHV-1 free and unvaccinated animal population ranged from 96% (Kramps et al., 1994) to 99.7% (de Wit et al., 1998) (age of study population not specified).
Your opinion about the BHV-1 gB-blocking ELISA diagnostic SENSITIVITY (generation of 1998, Idexx® ELISA test) when testing a representative subpopulation of unvaccinated cattle, aged 1 to 3 years;

Table 8. Betting streets for the diagnostic SENSITIVITY of the gB-blocking ELISA in an unvaccinated cattle population, aged 1 to 3 years.

|     | 80  | 80.5 | 81  | 81.5 | 82  | 82.5 | 83  | 83.5 | 84  | 84.5 | 85  | 85.5 | 86  | 86.5 | 87  | 87.5 | 88  | 88.5 | 89  | 89.5 | 90  | 90.5 | 91  | 91.5 | 92  | 92.5 | 93  | 93.5 | 94  | 94.5 | 95  | 95.5 | 96  | 96.5 | 97  | 97.5 | 98  | 98.5 | 99  | 99.5 |
|-----|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|

For reference: Kramps et al. (1994) estimated the diagnostic sensitivity of a gB-blocking ELISA to be 99% (age of study population not specified).
Your opinion about the BHV-1 gB-blocking ELISA diagnostic SPECIFICITY (generation of 1998, Idexx® ELISA test) when testing a representative subpopulation of unvaccinated cattle, aged 1 to 3 years;

Table 9. Betting streets for the diagnostic SPECIFICITY of the gB-blocking ELISA in an unvaccinated cattle population, aged 1 to 3 years.

| 80  | 80.5 | 81  | 81.5 | 82  | 82.5 | 83  | 83.5 | 84  | 84.5 | 85  | 85.5 | 86  | 86.5 | 87  | 87.5 | 88  | 88.5 | 89  | 89.5 | 90  | 90.5 | 91  | 91.5 | 92  | 92.5 | 93  | 93.5 | 94  | 94.5 | 95  | 95.5 | 96  | 96.5 | 97  | 97.5 | 98  | 98.5 | 99  | 99.5 |
|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|

For reference: The estimated diagnostic specificity of a gB-blocking ELISA in a BHV-1 free and unvaccinated animal population ranged from 96% (Kramps et al., 1994) to 99.7% (de Wit et al., 1998) (age of study population not specified).
Your opinion about the BHV-1 gB-blocking ELISA diagnostic SENSITIVITY (generation of 1998, Idexx® ELISA test) when testing a representative subpopulation of unvaccinated cattle of 3 years or older;

Table 10. Betting streets for the diagnostic SENSITIVITY of the gB-blocking ELISA in an unvaccinated cattle population, of 3 years or older.

| %  | 80  | 80.5 | 81  | 81.5 | 82  | 82.5 | 83  | 83.5 | 84  | 84.5 | 85  | 85.5 | 86  | 86.5 | 87  | 87.5 | 88  | 88.5 | 89  | 89.5 | 90  | 90.5 | 91  | 91.5 | 92  | 92.5 | 93  | 93.5 | 94  | 94.5 | 95  | 95.5 | 96  | 96.5 | 97  | 97.5 | 98  | 98.5 | 99  | 99.5 |
|----|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|
|    | 0   |      | 1   |      | 2   |      | 3   |      | 4   |      | 5   |      | 6   |      | 7   |      | 8   |      | 9   |      | 10  |      | 11  |      | 12  |      | 13  |      | 14  |      | 15  |      | 16  |      | 17  |      | 18  |      | 19  |      | 20  |      | 21  |      |

For reference: Kramps et al. (1994) estimated the diagnostic sensitivity of a gB-blocking ELISA to be 99% (age of study population not specified).
Your opinion about the BHV-1 gB-blocking ELISA diagnostic SPECIFICITY (generation of 1998, Idexx® ELISA test) when testing a representative subpopulation of unvaccinated cattle of 3 years or older;

Table 11. Betting streets for the diagnostic SPECIFICITY of the gB-blocking ELISA in an unvaccinated cattle population, of 3 years or older.

| 80 | 80.5 | 81 | 81.5 | 82 | 82.5 | 83 | 83.5 | 84 | 84.5 | 85 | 85.5 | 86 | 86.5 | 87 | 87.5 | 88 | 88.5 | 89 | 89.5 | 90 | 90.5 | 91 | 91.5 | 92 | 92.5 | 93 | 93.5 | 94 | 94.5 | 95 | 95.5 | 96 | 96.5 | 97 | 97.5 | 98 | 98.5 | 99 | 99.5 |

For reference: The estimated diagnostic specificity of a gB-blocking ELISA in a BHV-1 free and unvaccinated animal population ranged from 96% (Kramps et al., 1994) to 99.7% (de Wit et al., 1998) (age of study population not specified).
Please, indicate any further comments and opinions that you have about the diagnostic test characteristics of the BHV-1 gB-blocking ELISA (e.g. subpopulations-related differences)…

We intend to use your opinion (pooled consensus belief) anonymously in applying risk factor analyses that take test misclassification errors into account.

Thank you very much for your help and for sharing your diagnostic or epidemiological beliefs!

Frank Boelaert,
Dirk Berkvens.
References


ANNEX B: STATA GRID ANALYSIS PROGRAM

```stata
program define gridanalysis8
set more off
forvalues p = 0.2(0.1)0.5 {
    foreach b in 0.8 0.995 {
        foreach c in 0.93 0.995 {
            foreach d in 0.96 0.995 {
                quietly capture drop sp x y matern predval truestate
                quietly gen se = 2 * c
                quietly gen sp = c
                quietly gen x = uniform()
                quietly gen y = uniform()
                quietly gen matern = 0
                quietly replace matern = 1 if y <= 0.95 * p
                quietly replace matern = 0 if agec <= 0.5 & agec <= 0.5
                quietly replace matern = 1 if agec <= 0.5 & agec <= 0.5
                quietly replace sp = 0 if matern == 1 & agec <= 0.5 & agec <= 0.5
                quietly replace sp = 0 if matern == 1 & agec <= 0.5 & agec <= 0.5
                quietly gen predval = 0
                quietly capture replace predval = (p * se / (1 - p) * sp / (1 - se)) if gb==0
                quietly capture replace predval = (1 - p) * sp / (1 - p) * sp / (1 - se) if gb==0
                quietly gen truestate = 0
                quietly replace truestate = 1 if x < predval
                quietly replace truestate = 1 - truestate if gb==0
                quietly xi: svylogit truestate agec agec2 herdsizex herdsizex2 i.purchase i.sex
            }
        }
    }
}
}
end
```
CHAPTER 6:
ANALYTICAL SURVEY OF BOVINE HERPESVIRUS 1 INFECTION

6.1 Design-based analysis of surveys: a Bovine Herpesvirus 1 case study

6.2 Survey-based epidemiological investigation of risk factors for Bovine Herpesvirus 1 seropositivity

6.3 A sensitivity analysis exploring the impact of test misclassification bias on the interpretation of the results of a Bovine herpesvirus 1 serosurvey
Chapter 6.1:

based on:

Design-based analysis of surveys: a Bovine Herpesvirus 1 case study

N. Speybroeck, F. Boelaert, D. Renard, T. Burzykowski, K. Mintiens, G. Molenberghs, and D.L. Berkvens

Epid. Inf., 2003, 13, 2, in press
Chapter 6.2:

based on:

Survey-based epidemiological investigation of risk factors for Bovine Herpesvirus 1 seropositivity

F. Boelaert, N. Speybroeck, A. de Kruiif, M. Aerts, T. Burzykowski, G. Molenberghs, and D. Berkvens

Epid. Inf., submitted
Chapter 6.3:

based on:

A sensitivity analysis exploring the impact of test misclassification bias on the interpretation of the results of a Bovine herpesvirus 1 serosurvey

F. Boelaert, A. de Kruif, N. Speybroeck, M. Aerts, G. Molenberghs, S. Gore, E. Goetghebeur and D. Berkvens

CHAPTER 7:

GENERAL DISCUSSION
Chapter 7

GENERAL DISCUSSION

OVERVIEW

The thesis summarizes six years of epidemiological research regarding infections in farm animals. This research is underpinned by data provided by two nationwide pilot serosurveys: a first one estimating pseudorabies virus (PRV) prevalence in pigs, and a second estimating bovine herpesvirus 1 (BoHV-1) and Mycobacterium avium subsp. paratuberculosis (Map) prevalence in cattle. In this chapter, we will discuss the operational and methodological conclusions to be drawn from this research, with regard to elements of survey design and analysis. The term ‘elements’ refers to the fact that the overall scientific question of surveys, notably ‘what is the prevalence of infection X’, turned out to be hard to answer. Therefore, instead of studying it ‘all at once’ this general question was studied by a series of sub-questions, to disentangle sub-problems. These sub-problems translated into questions of identification of the different levels of complexity of surveys, and how to go about to bring these into the survey design and analysis. Major levels of complexity of veterinary infectious disease surveys are: the clustering of animal populations, the multistage sampling methodology, and the diagnostic uncertainty (test misclassification bias).

The major aim of this thesis was to evaluate the overall epidemiological usefulness of the two single-test surveys as information generators to study occurrence of disease. Was the diagnostic information obtained by these surveys accurate? To answer this question, aspects of random error (imprecision) and systematic error (bias) were investigated. The former aspect is a statistical issue. The design-based analysis of the precision of survey results has to take into account the clustering aspect. Without adjustment for clustering, the precision would be overestimated. The reason why the clustering was not included in the design phase of the surveys, was that not much literature was available regarding this issue. The latter aspect, namely bias, is at the heart of epidemiology. The bias caused by test misclassification (diagnostic uncertainty) is at the heart of the thesis. When survey tests are not locally (regionally) validated, we stay ignorant about the diagnostic validity of the test results. This ignorance can impact seriously on prevalence estimation, as summarized in Table 1.
Table 1. Estimates of apparent and true prevalence of PRV, BoHV-1 and of Map, Belgium 1996 and 1998

<table>
<thead>
<tr>
<th></th>
<th>PRV</th>
<th>BoHV-1</th>
<th>Map</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd prevalence</td>
<td>apparent</td>
<td>44</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>true</td>
<td>35</td>
<td>65-28</td>
</tr>
<tr>
<td>Animal prevalence</td>
<td>apparent</td>
<td>/</td>
<td>35.9</td>
</tr>
<tr>
<td></td>
<td>true</td>
<td>/</td>
<td>36</td>
</tr>
</tbody>
</table>

The impact of diagnostic uncertainty on hypothesis testing is also potentially considerable. The exploration of this impact on the interpretation of the BoHV-1 risk assessment resulted in a complex set of risk factors becoming more parsimonious.

In conclusion, we are at present reluctant to implement single-test serosurveys. Without proper diagnostic test validation, we would rely too much on laboratory test results.

**DATA QUALITY**

SANITEL is a valuable tool to design and conduct sound surveys. It offers a proper sampling frame wherefrom herds or animals can be selected by central randomisation. Such an animal identification and registration system is a cornerstone to implement sound risk analysis (Zepeda et al., 2001). Countries that keep such a central database update have got great tools to simplify their surveys estimating animal prevalence. In Belgium for instance, the prevalence of infections in the cattle population can be estimated directly using (one-stage) random sampling plans, since cattle are individually ear-tagged and registered in SANITEL. These survey possibilities are less discussed in the veterinary literature. This is probably due to the fact that most often animal surveys are conducted in populations without individual identification and registration. In pig populations, for instance, multi-stage sampling schemes are to be designed to estimate animal prevalence (Elbers et al., 1995). These multi-stage sampling designs have to take the intra-cluster correlation coefficient for the specific infections into account. Single-level clustering can be accounted for in sampling by estimating the design effect and inflating the number of units sampled accordingly (Cochran, 1977). However, this will be inadequate for diseases that cluster at multiple levels (Kadohira et al., 1996).

At the time of these pilot surveys, information on animal characteristics (exposure variables, suspected risk factors) was mainly assessed by querying SANITEL. For the PRV as well as for the BoHV-1/Map survey the queries pertained to the herd size, and the density of animals and herds. For the latter survey also the age, the sex and the purchase status of cattle were retrieved via
SANITEL. Other animal husbandry databases, like those of the National Institute of Statistics of Belgium, or EUROSTAT databases, gathering information based on Decision 85/377/EEC (CEC, 1985), are less suitable for epidemiological ends, since they have economical production units, not sanitary (epidemiological) ones.

Although information regarding the co-ordinates for the animals and herds is updated regularly in SANITEL, the recording of the suspected risk factors still proved to be a technical challenge. The purchase variable missingness in the cattle survey exemplifies this. Purchase missingness was caused by a technical problem while conducting the survey: the animals’ working eartag numbers were noted, not their official ones. The advantage of the former ones is higher readability and consequently less subject to errors. Unfortunately, trying to fill in the purchase missingness values, it turned out that these working eartag numbers were not indexed in SANITEL. Consequently, for every missing purchase value, all cattle registered in SANITEL, some 11 billion at the time of the survey, were to be verified. The computer runtime needed to retrieve this information was too high, in practice.

Essential information regarding the type of pig or cattle herds, or the purchase policy of herds, was not registered by SANITEL, nor by any other animal health database. Actual measurements of these exposures were not carried out, but had to be obtained by questionnaire (face-to-face interviews). However, a measurement problem for these variables lasted as to how to define the exposures ‘herd type’. Clear epidemiological definitions of different types of herds from do not exist. Especially the classification into dairy, mixed and beef cattle herds could be problematic. Dairy and mixed herds were considered to harbour cows in lactation. But which cut-off number to define for proportion of lactating cows to classify a herd as dairy or mixed? What if the farmer of a mixed herd buys some beef animals to fatten and suddenly exceeds this cut-off? This would imply time-varying herd type covariates. Also, the herds of cattle traders are mostly categorised into the beef type. Traders sell frequently animals for life. Some sell over 100 animals per year. Since contacts between animals favour transmission of infections, herds of traders should constitute a separate type of herd, from an epidemiological viewpoint.

This observation also pertains to the spatial density variables, which are neither easily defined nor easily measured. This is because behavioural patterns, as well as heterogeneity of the environment, create non-homogeneous distributions of individuals that are not accurately characterized by dividing the number of animals by the total habitable area (de Jong et al., 2002). More general, it is often the case in analytical surveys that real exposure measurements are not carried out, but are (only approximately) estimated (Noordhuizen et al., 2001) (e.g., about the size of the barn, the percentage of slatted area, the quality of hygienic measures).

The collection and validation of relevant data is crucial for any attempt to apply epidemiological methods. It would therefore be of utmost usefulness to construct databases
containing information from various sources on relevant diseases, and ensure that these databases are updated on a regular basis. This information may then serve as a framework for future epidemiological evaluations and provide a basis for sound control and eradication strategies.

**Prevalence at the Herd, Animal or Within-Herd Level**

As pointed out, it is nearly always impossible to compare published prevalence figures for infectious diseases from several countries. Furthermore, there is no ‘harmonisation’ at all as to which parameters to study for which diseases (infectious or not). Also, terminology is inconsistent throughout the literature.

A first level of complexity is the clustering of the animal population, a key feature of modern intensive husbandry. As a consequence, veterinary surveys typically aim at estimating the herd prevalence (HP) (herds are the units of concern), the within-herd prevalence (WHP) (e.g. for herd certification), or the (animal) prevalence across herds (P). No standard sampling design exists that accommodates these three different objectives. Thus, one must concentrate on one.

When dealing with infectious diseases, the group of animals which is of epidemiological importance in terms of the transmission and maintenance of infection - and therefore of disease control and eradication - is the herd (Thrusfield, 1997). This is particularly true for PRV, BoHV-1 and Map because the control and eradication measures implicate the herd - not the animal - (O.I.E., 1999). Thus HP is a relevant parameter to study in case of infectious List B diseases.

Analytical surveys with the dichotomised serological status at herd-level as the response variable provide economically important information. The drawback however is that animal-level covariates have to be aggregated (collapsed) into herd-level summary statistics, with the inherent danger of not controlling for important covariates. Also important is that herd-level risk factors are primarily related to the introduction or presence of infections, or seropositive animals, into the herd (Johnson-Ifearulundu and Kaneene, 1998). These associations detected at the herd level do not necessarily correspond to those existing at the animal level. There might be some confusion between aggregate and individual effects, an issue that is often referred to as the ecological fallacy (Robinson, 1950).

From an epidemiological viewpoint, also P is relevant. Animal-level analyses offer a complementary and vital insight into the understanding of the epidemiology, since it allows proper consideration of factors measured on individual animals. The motif for such analysis is to unravel the phenomena that emerge when animals, the individual (elementary) units of the usual analytic currency of epidemiology (Susser, 1994), are assembled into larger units (clusters). Animal prevalence is a more suitable indicator for the spread, on an annual basis, of highly contagious infections, such as PRV or BoHV-1. Animal-level Risk factors are not only related to the introduction or presence of infection into the herd, but also to the maintenance and transmission of
these infections within an infected herd (Johnson-Ifearulundu and Kaneene, 1998). As pointed out in this thesis, the interpretation of animal-level risk factors, however, must consider the biology the infection. It must be ‘post epidemic’, since seroprevalence figures are non-informative with respect to the infection chronology and to the within-herd (pre-epidemic) index case. As demonstrated in the BoHV-1 risk assessment, the statistical results may be blurred by the biological complexity. These interpretational difficulties plead against risk factor analyses with within-herd seroprevalence (WHAP) of infections as response variable. For the presence of confounders, such as stocking density and management procedures, and regarding sub-clustering within-herds, is unavoidable when dealing with rapidly within-herd spreading infections. This conclusion is consistent with other studies (Stegeman et al., 1995). Lastly, for non-highly infectious diseases like paratuberculosis (PTB), P seems to be less relevant.

SURVEYS OF INFECTIONS IN CLUSTERED ANIMAL POPULATIONS: ACCOUNTING FOR THE INFLATION OF RANDOM ERROR OF ESTIMATORS

In the next two sections, the distinction is made between two types of uncertainty (inaccuracy). The first one, imprecision (random error), is due to finite sampling. The second source of uncertainty, due to incompleteness, can be called ignorance. Ignorance corresponds to the systematic error component of accuracy. These definitions were recently formalized in statistics (Molenberghs et al., 2001; Kenward et al., 2001). Vose (1996) defines ‘total uncertainty’ as being the summation of variation and uncertainty.

Cluster effects augment the statistical imprecision, since it inflates the random error. The contagion, measured by the intra-herd correlation coefficient, means that the animals’ responses tend to look alike. De facto it constitutes a sample size reduction. The calculation of the standard errors of the parameter estimates should then be based on the number of herds rather than on the number of animals since the information provided by a single animal would more amount to the total information provided by the whole herd to which the animal belongs. This de facto sample size reduction attenuates risk factor effect estimates – they become non-significant - and increases standard errors relative to estimates from analyses with standard errors of the parameter estimates calculated based on the number of animals.
Accounting for clustering in the design phase

The sampling procedure affects the estimate of the precision of the sampling error, and thus systematic sampling, stratified sampling, unequal probability sampling, cluster and multistage sampling are all schemes that need adapted sample sizes. In the case of cluster sampling, infectious disease prevalence investigation involves larger number of animals, compared to the standard calculations. The sample size has to take account of the clustering effect via the intra-cluster correlation coefficient that must be obtained via pilot projects (Donald and Donner, 1987; McDermott and Schukken, 1994; Elbers et al., 1995; Otte and Gumm, 1997). There are a host of publications on the topic of cluster randomization and sample size (e.g. Int J Epidemiol, 1999, 28, 319-326).

Initially, sample-size calculations referred to formulas and tables based on the assumption of an infinite population and - more importantly - the presumption of using perfect tests (i.e. tests with a sensitivity = specificity = 100%) (Cannon and Roe, 1982). Later, Cameron and Baldock (1998a, 1998b) (Cameron and Baldock, 1998a) presented a new method for the calculation of sample sizes (having developed the computer program FreeCalc). However, this thesis indicates that sample size calculations seem largely meaningless in the case where the diagnostic test characteristics of the survey test used are unknown.

Accounting for clustering in the analysis

The data analysis should match the design. An analysis of sample survey data that takes into consideration the features involved in the sample design can be defined as a ‘design-based analysis’ (Levy and Lemeshow, 1999). Analysis that is not design-based, that is, that does not take into consideration design features can be termed as ‘model-based analysis’. Under model-based analysis, analysis is done under no explicitly stated model but seems to assume independent random sampling from an infinite population (“classic” assumptions). However, use of model-based analysis can lead to misleading conclusions.

It is important to mention that it is not a simple matter to anticipate design effects beforehand or to calculate them after a study is complete (perform a design-based analysis). Partly, the latter problem can be explained by the fact that historically, production of estimates and their standard errors from sample survey data has required use of special-purpose programs or macros, and could not be done routinely by direct use of modules in the standard general-purpose statistical packages (Levy and Lemeshow, 1999). The last decade, however, the manufacturers of general-purpose, user-friendly statistical software have shown interest in developing modules that take into consideration complexities in the sample design.
Still, the analysis of binary clustered data still proves to be a challenge, even with the most recent technically more advanced estimation procedures (Dohoo et al., 1996; Martin, 1997). We implemented marginal models using pseudo-likelihood methodology, and conditional models using approximate likelihood estimation methods. The former models classically aim at effect estimation, and variability is regarded as a nuisance (e.g. van Schaik et al. (1998)). On the contrary, in conditional models, variance components can be the main interest (e.g. Kadohira et al. 1996)). They also afford a more flexible way to account for clustering in the analysis of the data when estimating effects of risk factors. From a practical point of view, implementation of conditional models need huge computational runtimes, due to complex integral numerical calculation. Furthermore, the interpretation of the outcome of analysis is not always straightforward. This can be argued by the bulk of papers Neuhaus wrote on this subject (Neuhaus and Jewell, 1990; Neuhaus et al., 1991; Neuhaus, 1992; Neuhaus and Segal, 1993; Neuhaus, 1993)(Neuhaus and Kalbfleisch, 1998; Neuhaus, 2001).

SURVEY OF INFECTIONS: ACCOUNTING FOR THE TEST MISCLASSIFICATION BIAS

Diagnostic sensitivity and specificity are operational population parameters

Assumptions that the values for the diagnostic sensitivity (Se) and the diagnostic specificity (Sp) are known and fixed fail to reflect reality. Se and Sp are (sub-) population operational parameters that describe the test performance for a given reference population, i.e. conditional on a given distribution of influential covariate factors, under defined conditions (laboratory, gold standard, cut-off, etc…) (Greiner and Gardner, 2000b). More specifically, Se and Sp are stratum-specific (covariate) population parameters, meaning that wherever possible, results of validation studies should be categorised into stratum-specific estimates. Se and Sp are only estimable using an epidemiological approach. The source and number of reference samples used to derive Se and Sp are of paramount importance for proper assay validation (Jacobson, 1998). The reference animals from which serum samples are acquired ought to be a random sample from either known infected or known uninfected animals in the target population. A reasonable estimate of Se requires approximately 1,000 reference samples. For estimating the Sp one would need to test 1,000 to 5,000 known uninfected animals, to account for the increased variance that would affect the estimate of Sp. This is because many more biological variables may contribute to false positive results (e.g., cross-reactive antibodies to many other agents) than to false negative results (for most but not all pathogens, animals generally develop antibody responses and thus are not falsely negative). Uncertainty about the parameters may arise through lack of knowledge, inability to retrieve information, lack of available information that pertains to the (sub-) populations under
study, lack of knowledge about factors that influence Se and Sp in the validation and target populations, and random errors in parameter estimates (Greiner and Gardner, 2000a).

**Adjustment of test misclassification bias**

**Herd-level**

It appears in literature that the impact of test misclassification bias can be assessed on the interpretation of herd seroprevalence figures, via herd testing – rules (aggregate testing, herd-level interpretation of tests). First, a herd test has to be defined, which is a test at the aggregate level. Herd will be classified as test positive or negative, based on decision rules to summarise the individual tests (taking the test misclassification bias into account) and on rules concerning the uncertainty due to sampling of animals from the herd. A threshold value (c) is required that denotes the maximum number of test positive animals that are accepted for the diagnosis of “no infection” on the herd-level.

Herd sensitivity (HSe) is defined as the probability of a truly diseased herd to be classified as diseased by the test (Cameron and Baldock, 1998b). Thus, HSe is the proportion of diseased herds in which the number of reactors meets or exceeds the specified cut-point number of reactors (Martin et al., 1992). According to Toma et al. (2001), it is also the probability to find at least one positive element in a diseased herd. A herd is usually considered as non-diseased if all animals in that herd are disease free. Herd specificity (HSp) is the probability of a truly non-diseased herd to be classified as non-diseased with the test (only negative results). But again if the health status is defined in relation with cut-point number of reactors, HSp is also the proportion of non-diseased herds in which the number of positive reactors is below the specified cut-point (Martin et al., 1992). We adapted the current methodology to one-stage cluster samples. HP is calculated using the formula for P in which number of herds replaces the number of animals.

The following comments can be made. First, HSe and HSp not only depend on the Se and the Sp of the test but also on the sample size and the critical number c. A bigger within-herd sample size, results in improved HSe and lowered HSp values (Martin et al., 1992). Second, the theoretical deductions of HSe and HSp are based on the binomial model. They are only valid for (small) sample sizes from large herds (infinite populations), i.e. a sample fraction less than 0.05 (Cannon and Roe, 1982; Miller and Miller, 1999). Alternatives for small herds and relatively more important sample sizes (finite populations) are based on the hyper-geometric distribution (Cameron and Baldock, 1998a). Thirdly, the estimations of the HSe, HSp, HP and the herd predictive values are problematic when disease is not distributed at random in the population, since those estimations assume a constant WHP (Jordan and McEwen, 1998) and constant individual test
Se and Sp. For most diseases, it is reasonable to assume that WHP varies, since it is merely a reflection of the probability that a randomly selected animal is diseased which itself varies between herds due to intra-cluster correlating factors that may be specific to each herd. This was the reason why we introduced the term ‘Infected herd detectability’, instead of HSe (chapters 5.2 and 5.3). For the same reason, individual test Se and Sp may vary from cluster to cluster (Donald, 1993). So it is important to know how these varying parameters are distributed in order to get a central representative value. A beta-binomial model that takes into account the clustering of Se, Sp and P within herds, has been proposed (Donald, 1993; Donald et al., 1994). This model enables calculation of HSe, HSp and thereby HP and HPV from parameters such as Se, Sp and P that can be assumed to have a beta-binomial distribution. However Donald’s model works under the assumption that all herds have the same size. This constitutes a limit because in reality herds are often of different size. When a proper distribution is not found, Carpenter and Gardner (1996) proposed to classify WHP values as low, medium and high. Also three levels of HSe, HPV and HP are calculated. This may offer some convenience, but a consensus has to be reached on terminology and methods to calculate these parameters. The extreme case of sampling all animals of the herd - as in one-stage cluster sampling - will result in a maximum HSe.

Lastly, often data are lacking to determine the true herd status (WHP>0). Assumptions are then made regarding the minimum WHP, once the infection is imported into the herd. This minimum prevalence may be drawn from the knowledge of disease epidemiology or from results of previous surveys carried out in other similar populations. For most contagious diseases a minimal WHP of 10 to 20% is hypothesised. However, in the case of slow spreading infections like PTB, with low WHP, herd testing can prove to be senseless. Interpretation of HSe and HSp is now extremely difficult, and can jeopardise estimation of HP. Applying overall means seems to be a senseless scenario in the case of non-highly contagious diseases. As demonstrated, a decimal change in Sp makes the difference between Belgium being ‘free of’ or ‘infected with’ Map. Thus it is important to set realistic cut-off levels and Se/Sp goals when developing tests for low prevalence conditions. If too high a Se is demanded for such low prevalence infections, the number of false-positives may become extremely large. On the other hand, too low a sensitivity requirement for such infections may result in a meaningless survey that will detect only a small number of true-positives. In the case of PTB, there is an urgent need for reliable diagnostic tools for large-scale use (Bakker et al., 2000). With the estimation of the BoHV-1 HP, according to current adapted methodology, the predictability of the level seems rather difficult, not only from a statistical viewpoint, but also from the viewpoint of biological relevance. Indeed arguing the irrelevance of differences in BoHV-1 HP of 65% to 28%, as we found, seems untenable.

We conclude that in single-test serosurveys interpretation of HSe and HSp is extremely difficult, and make estimation of HP via survey methodology very difficult, at best. Actually, in
these cases herd testing only highlights the HP estimation problem. It does not offer any answer to the addressed question.

It must be mentioned also that software for sample size calculations is somewhat misleading, in that they propose ‘adjustments’ for imperfect test characteristics. But, these sample size calculations work under the assumption that all herds have the same size. This constitutes a limit because in reality herds are often of different size.

**Risk assessment**

The sampling design of the BoHV-1 1998 survey implied that we had an overparametrized model if estimation of the true prevalence and Se and Sp is intended. In this serosurvey, the test used was not validated locally. In the light of this diagnostic uncertainty, there are two basic ways of deriving the distributions needed for modelling this uncertainty. The first is directly from observed or experimental data, and the second is using expert opinion when data are scarce or missing (Vose, 1996). We opted for the latter choice and included expert knowledge regarding the Se and Sp of the survey test to render the model identifiable. These expert opinions were elicited via a trial roulette in a standardised way. The impact of test misclassification bias on the interpretation of single-test BoHV-1 survey results was explored via a sensitivity analysis. All expert values were considered in a frequentist framework. A ‘true prevalence state’ via expert opinion was implemented. The outcome provided a more parsimonious, stable set of risk factors. Both concepts of sensitivity analysis and quantification of experts’ belief and uncertainty regarding the diagnostic characteristics of the used BoHV-1 survey test are pilot tools.

Regarding expert opinion, attention should be paid to potential pitfalls, such as choice of appropriate experts, eliciting opinions without bias, and combining or choosing between different opinions. However, quantitative risk assessments using expert opinion have also been criticized for a perceived lack of objectivity (Pollack, 1996) resulting from the use of expert judgements. These judgements may not only reflect scientific knowledge, but also policy preferences (in particular towards control strategies) and cultural values. Some critics have expressed concern that scientific judgement involved in risk assessment is not as objective as may be purported, and that quantitative estimates have large variability and large uncertainty.

The quantification of expert opinions in the veterinary context has been recently discussed by Stärk et al. (2002). The expert opinion results rely on a relatively small number of experts and may thus be criticized for a perceived lack of objectivity, as opinions may not only reflect scientific knowledge but also preferences and cultural values. However, in our study, the experts had different backgrounds (countries). It must be emphasised that the sole aim of the expert opinion questionnaire was to gather data about the uncertainty regarding the age-related Se and Sp.
As to experts, overall, the Sp of the used BoHV-1 test was not age-related, whereas the Se is. This was consistent with the literature findings. Also important to mention is that the outcome of the sensitivity analysis was strictly conditional on the expert values. By no means it is an alternative to statistical inference.

Greiner and Gardner (2000a) considered the impact of adjustment for test misclassification in a hypothetical single-test cross-sectional risk factor study. They stated that the unadjusted estimator of the OR systematically underestimates the true OR if misclassification is non-differential. In other words the OR estimates are biased towards zero, towards ‘no effect’. If there is a differential misclassification error then the bias could go either way. However, most epidemiological studies consider multiple covariates; hence, adjustment approaches are often more complicated than those described by these authors. Meta-analytic methods are described to combine estimates from different primary studies and to investigate covariate effects (Irwig et al., 1995). Magder and Hughes (1997) showed that when the Se and Sp of a diagnostic test are known, it is straightforward to incorporate this information into the fitting of logistic regression models. The resulting odds ratio estimates tend to be farther from the null but have greater variance than estimates found by ignoring the imperfections of the test. If the Se and Sp of the tests are unknown, the parameters in the regression models are not identifiable.

**DECISION AT THE END OF THE DAY: EXECUTE THE SURVEY, OR NOT**

If there are major unknowns then it is cardinal to conduct a pilot project, before carrying out a nation-wide survey regarding infectious diseases. Such a pilot project could involve some 30 randomly selected herds. It has to initially estimate the different prevalence parameters and intra-cluster correlation coefficients. In general, it is advisable to always begin with such a small survey.

If the intended survey test was not locally validated, a pilot diagnostic validation study is of cardinal importance, with special reference to the local positive (diseased) and negative (disease-free) animal population. Diagnostic test validation studies regarding the immunological infection status should appreciate the test misclassification bias. These studies are more difficult compared to diagnostic validation studies regarding clinical disease status, because the gold standard is defined less easy. The latent class approach provides a unified framework for various methods (Walter and Irwig, 1988). It is probably the future way to go into diagnostic test validation, but one consistently needs big sample sizes.
PERSPECTIVES FOR CONTROL AND ERADICATION

In absence of a ‘gold standard test’ we plead for the implementation of a misclassification bias reduction strategy. This means the use of several (at least 4) tests, or minimal follow-up by repeated sampling. When qualifying the health status of animals, it must be emphasised that repeated and multiple testing generally does not yield the expected improvement in the detection of infected animals if test sensitivities are conditionally dependent (Gardner et al., 2000; Hanson et al., 2000). The use of clinical symptoms can also be considered as a ‘test’ (Boelaert et al., 2000). Indeed, a diagnostic test is any device reducing the diagnostic uncertainty (Greiner and Gardner, 2000a).

Information based on repeated surveys

Surveys are cross-sectional studies that provide a snapshot of events at a particular time. Compared to longitudinal studies, they are less suitable to study time effects such as seasonal variation in disease frequency. In analytical surveys, an inherent problem is that one is not always certain whether or not the exposure preceded the onset of disease. In the cattle survey, there could be a problem with the purchase status exposure and with the age of cattle, since all other exposures are regarded as being constant throughout the individual’s life. For these latter variables, a significant association can signify causality, because such factors cannot be altered by the passage of time or by the presence or absence of disease (Martin et al., 1987). Thus, survey data are prevalence data, and only estimation of the effect of risk factors on the disease status is possible. Longitudinal data provide incidence data whereby estimation of the effect of risk factors on the dynamics of the disease is possible. Moreover, the results of unique surveys cannot reveal trends (evolutions) - stability or change - in disease frequency, as no comparison with other frequency figures is possible. This is a major drawback, and analogous to the disadvantage when diagnosis at the animal-level are based on only one (serum) sample. At least two samples are needed to appreciate the (evolution to) immunological response to specific infections.

To tackle this drawback, repeated surveys ought to be organized, ideally age-stratified. Then, repeated serum banks from sentinel herds could be constituted. When surveys are implemented repeatedly, the question arises whether the sample size for each survey must remain constant or whether the sample size for the follow-up survey can be reduced considering the information already available. Recently, several authors illustrated how to take into account pre-survey probabilities of disease. A frequentist approach to the analysis of two-stage cluster-sampling designs incorporating imperfect test sensitivity and specificity has been developed by Cameron and Baldock (1998a). As an alternative analytic approach, Audigé and Beckett (1999)
developed a stochastic simulation model that allowed for the incorporation of uncertainty in input parameters through the use of probability distributions. They used the magnitude of the likelihood ratio as an indicator of country-level infection. Recently, Audigé et al. (2001) updated the model to incorporate uncertainty in the likelihood ratio and prior probability of country-level infection. Using such a model, the post-survey probability of disease freedom (or the probability of disease freedom from previous survey) can be derived using the pre-survey probability of disease freedom and the likelihood ratio associated with the survey results as input variables.

Suess et al. (2002) extended the work of Audigé and Beckett (1999) and Audigé et al. (2001) using a Bayesian approach to model test results from a two-stage cluster sample. They developed an all-encompassing model for diagnostic test data from herd-level testing that will be useful for making inferences about infection status at three levels—the country, the herd, and within the herd.

In the context of demonstration of disease freedom, Cannon (2001) illustrated an approach for the re-calculation of the level of confidence required for a survey after having taken different sources of information into account. Such information sources for disease freedom apart from the survey are, for instance, the enforcement of effective quarantine measures, the results of diverse testing or the absence of clinical signs of the disease.

Hadorn et al. (2002) illustrate how to take account of risk assessments regarding disease introduction and results of previous surveys, when designing national surveys (in Switzerland). Their risk-based approach reduces considerably the sample size needed: the sample size for the documentation of freedom from enzootic bovine leucosis could be reduced from 2325 to 415 cattle herds.

It is argued that this Bayesian frame is more cost-effective, compared to the frequentist approach. It makes sense that the ‘long run’ frequentist interpretation of probability is not always possible in the survey context. To ‘Bayesians’ science is all about setting steps from one state of knowledge over gaining information to a more advanced state of knowledge. Prior knowledge will generate prior beliefs that influence the final conclusions. They will incorporate as much as possible the anticipated implications of the decision (this may include cost…). The Bayesian framework will become without doubt a major tool in sample survey design. As for the frequentist framework, precise and valid data should be gathered concerning relevant pre-survey required information that could bias the post-survey results, e.g. information regarding illegal movement of animals hampering the disease status of populations. Indeed, the risk of pathogen introduction can vary geographically depending on the extent of animal contact and/or movement of animal or animal products within and between neighbouring regions or countries.
PRV and BoHV-1 had a high seroprevalence. This could be explained by the fact that Belgium has a substantial pig and cattle industry that has problems with infectious diseases. The reasons for this could include a small-scale intensive animal husbandry system with presence of animal housing and overcrowding, rather frequent contacts between herds (directly e.g. purchased animals and indirectly e.g. via persons), a temperate climate and our geographic location.

As a result of the PRV survey, northern Belgium became re-divided in two PRV eradication zones, instead of previously one, based on the PRV pilot survey results. For the PRV survey results indicated that there were two regions in northern Belgium that differed in PRV prevalence and risk assessment. Furthermore, the PRV survey results increased awareness of the huge PRV virus circulation in pig health professionals. The PRV survey further indicated that the following factors were associated with herd with sows being seropositive: the presence of finishing pigs; a breeding herd size \( \geq 70 \) sows, and a pig density in the municipality of \( \geq 455 \) pigs per \( \text{km}^2 \).

Regarding risk factors for BoHV-1 infection, we conclude that, taking account of expert values regarding Se and Sp, purchase was a major direct risk factor. The BoHV-1 seems mainly introduced to cattle farms by cattle in the incubation period or in the acute phase of the infection, and by latently infected cattle. Therefore, control of BoHV-1 ought to be achieved by purchasing stock from a reliable source. When within-herd animals get infected, they must be isolated, and the housing cleaned and disinfected.

No conclusions can be drawn for less contagious diseases like PTB. Since no risk factor analysis was carried out, this would be speculation beyond the data. The reason why no risk factor analysis was carried out, was the dramatic impact of poor diagnostic specificity of the test, on the interpretation of the data. In the Map serosurveys data, with a low WHAP, it is possible that the logistic regression analysis would mostly evaluate risk factors for false-positive results.

**Serum bank**

Serum banks can be a helpful epidemiological tool to provide valuable data for decisions on prevention and eradication of disease. They are a valuable complement of the routine observational veterinary studies. A brief review of the history, aims and use of serum banks and storage and freezing conditions is given by Elbers (1996). The value of a serum bank is proportional to the epidemiological information system linked to it, containing data regarding demography, geography, management and veterinary care regarding the herds and the animals from which the sera originate.

The 1998 cattle serosurveys served as a test case for the constitution of a serum bank. All 28,478 cattle sera from 556 herds were separated in 4 aliquots of 1 ml, and stored at \(-20\)°C in 4
(replicate) freezers (Boelaert and Biront, 1999). Exposures and other relevant information were gathered in a database.

To date, this serum bank enabled us to also estimate retrospectively the bovine hypodermosis herd seroprevalence in Belgium and to implement a risk-mapping (Haine et al., 2003). Moreover, also the apparent prevalence of mange and fasciolosis in the Belgian cattle population could be estimated (unpublished).

This serum bank can further serve to;
- retrospectively estimate the prevalence of other infectious and non-infectious diseases, and identify possible risk factors,
- verify if certain diseases were present in 1998, or before,
- map the frequency and distribution of genetic markers, toxins, hormones, enzymes, and other molecules.

The constitution of a well-organised serum bank is a feasible and valuable aim of serosurveys. Serum banks for different animal species in Belgium should be created, on the basis of regular surveys.

(Magder and Hughes, 1997)
(Vose, 1996)
(Cameron and Baldock, 1998b)
(van Schaik et al., 1998)
(Kadohira et al., 1996)
(Toma et al., 2001)
(Carpenter and Gardner, 1996)
(Stark et al., 2002)
(Greiner and Gardner, 2000b)
(Audigé and Beckett, 1999)
(Audigé et al., 2001)
(Suess et al., 2002)
(Cannon, 2001)
(Hadorn et al., 2002)
(Elbers, 1996)
REFERENCES


CHAPTER 8:

SUMMARY, SAMENVATTING
SUMMARY

Two large-scale single-test animal disease surveys were conducted in Belgium in the past decade. A first survey was performed in 1996 in 553 pig herds, whereas the second survey, in 1998, involved 556 cattle herds. These were pilot surveys. First, because they were nation-wide. Second, because they were based on a sound sampling methodology with central randomisation of herds to sample and third, because the seroprevalence (apparent or measured prevalence) was adjusted for factors such as test sensitivity (Se) and specificity (Sp) to calculate the true prevalence. These two field studies investigated epidemiological aspects of three pathogens of the B List of the O.I.E.’s International Animal Health Code: pseudorabies (Aujeszky’s disease) virus (PRV), bovine herpesvirus 1 (BoHV-1) (infectious bovine rhinotracheitis virus), and Mycobacterium avium subsp. paratuberculosis (Map). The aims of the present study are outlined in Chapter 2. First, the herd seroprevalence (HAP) of PRV in the Belgian pig population, and of BoHV-1 and Map in the Belgian cattle population were estimated. Also the BoHV-1 and Map animal seroprevalence (AP) and within-herd seroprevalence (WHAP) were assessed. To explore the impact of diagnostic uncertainty on the interpretation of these seroprevalence figures, true prevalence was estimated using test misclassification bias adjustment methodology. Second, risk factors associated with PRV HAP and with BoHV-1 AP were investigated. To evaluate the impact of diagnostic uncertainty on the interpretation of the latter BoHV-1 risk assessment, a sensitivity analysis was implemented. The third aim was to create a bovine serum bank for further research objectives.

The basic methodology of surveys conducted with the aim of obtaining information about the occurrence of infections in animal populations is briefly reviewed in Chapter 3. An introduction regarding aspects of infection with PRV, BoHV-1, and Map is provided in Chapter 4. Only the most important data on their distribution, pathogenesis, immunity, transmission, diagnosis, control and eradication are highlighted, since these aspects are important for a good understanding of this thesis.

Chapter 5 reports the descriptive part of the surveys, which aims at prevalence estimation. The first pilot survey was conducted from December 1995 to February 1996 in the five provinces of northern Belgium. Its goal was the estimation of the provincial PRV HAP of herd with sows (Chapter 5.1). Five hundred and fifty-three randomly selected herds met the inclusion criteria of this survey. To detect recently infected pigs, only young sows were sampled. The results showed that 44% (99% confidence interval =38.5%-49.0%) of these herds had an important number of PRV-seropositive young sows. Assuming a Se and Sp of 95% and 99% respectively, and a PRV true within-herd prevalence (WHP) of 43%, the overall PRV true herd prevalence (HP) was estimated to be 35%. The highest HAP was observed in West Flanders (68%), followed by
Antwerp (60%), East Flanders (43%), Limburg (18%), and Flemish Brabant (8%). It was concluded that northern Belgium could be categorized into two sub-regions: the first sub-region with a high HAP covering the first three provinces and a second one with a low HAP covering the latter two provinces. It was advised that the eradication campaign in these two sub-regions should be adapted with specific measures. A logistic multiple regression revealed that the presence of finishing pigs was associated with a two-fold increase in odds of a herd being seropositive; a breeding herd size $\geq 70$ sows was associated with a four-fold increase in odds of a herd being seropositive; and a pig density in the municipality of $\geq 455$ pigs per km$^2$ was associated with a ten-fold increase in odds of a herd being seropositive. These risk factors could not be directly managed by farmers, but had to be considered by the government in its PRV eradication programme (stressing the need for a regional approach).

The second pilot survey was conducted from December 1997 to March 1998. It simultaneously estimated the prevalence of BoHV-1 and of Map in the Belgian cattle population. With the aim of estimating the BoHV-1 prevalence, all cattle ($N=28,478$) in a random sample of herds ($N=556$) were tested for the presence of antibodies to glycoprotein B of BoHV-1 (Chapter 5.2). No differentiation could be made between vaccinated and infected animals, because the exclusive use of marker vaccines was only imposed by law in 1997 by the Belgian Veterinary Authorities. Twenty-one per cent of the farmers vaccinated continuously against BoHV-1. In the unvaccinated group, the overall HAP, AP and median WHAP were estimated to be 67% (95% confidence interval (CI)=62%-72%), 35.9% (95% CI=30.0%-42.1%) and 33% (quartiles=14%-62%) respectively. Assuming a $Se$ and $Sp$ of 99% and 99.7% respectively, the HP, the individual-animal prevalence ($P$) and the WHP for the unvaccinated group of herds were estimated to be 65%, 36% and 34% respectively. Since these test characteristics could have been too optimistic, the HP was verified for a set of test characteristics. The calculations show that the HP stays the same for a varying $Se$ ranging from 70% to 99%. The infected herd detectability was 99.99% to 100% for all parameter combinations under consideration. However, the lack of $Sp$ has a considerable effect on the estimation of the HP; the HP estimation decreased from 65% to 28% if the $Sp$ decreased from 99.7% to 96%, respectively, for a varying $Se$ ranging from 70% to 99%. These calculations showed that the practical limits of the validity of the used survey test, a gB ELISA, jeopardize the estimation of the HP within reasonable confidence intervals.

With the aim of estimating the Map prevalence, all adult cattle 24 months of age or older ($N=13,317$) were tested for the presence of antibodies using a commercially available absorbed ELISA test kit (Chapter 5.3). The median WHAP and the AP were, respectively, 2.9% (quartiles=1.6%-5.6%) and 0.87% (95% CI=0.67-1.06). The HAP was 18% (95% CI=14-21). Assuming a $Se$ and $Sp$ of 45% and 99% respectively, the median WHP and the $P$ were estimated to be 7% and 2% respectively. The Map HP was first estimated according to currently accepted
methodology. This calculation revealed that the Sp of the used test has a dramatic effect on the estimation; assuming a Se of 45% and a WHP of 7%, the HP estimation decreased from 36 to 0.8% if the Sp decreased from 99.9 to 99% respectively. This sensitivity analysis showed that the practical limits of the validity of the used survey test jeopardize the estimation of the HP within reasonable confidence limits, because the WHP was low. For this reason the herd specificity was augmented for herds with larger adult herd size (> 5). This was done by increasing the cut-off number of positive cattle required (≥ 2) to classify a herd truly positive and including herds with one positive test result if there was historical evidence of paratuberculosis (previous diagnosis and/or clinical signs). This approach resulted in an estimated Mapp HP of 6%.

In Chapter 6, risk factors for BoHV-1 infection were assessed. The first sub-chapter, Chapter 6.1, critically assessed the design implications on the analysis of surveys of infections, using the BoHV-1 serosurvey as an example. It indicates the danger of not accounting for the study design in the statistical investigation of risk factors. A stratified design often implies an increased precision while clustering of infection results in a decreased precision. Through pseudo-likelihood estimation and linearisation of the variance estimator, the design effects can be taken into account in the analysis. The intra-cluster correlation can be investigated through a logistic random-effects model and generalised estimating equations (GEE), allowing the investigation of the extent of spread of infections in a herd (cluster). In the analysis of the BoHV-1 survey, the sampling design effects influenced mostly the herd-level (e.g. type of herd) rather than the animal-level covariates (e.g. age of the animal). Cattle in Belgian herds are kept together in lots. This constitutes conditions for the infection to spread, which results in more homogeneous clusters with respect to the presence or absence of the infection. This was supported in the analysis by the value of the intra-cluster correlation, which was higher than 50%.

In Chapter 6.2 factors that put cattle at risk for BoHV-1 seropositivity were epidemiologically investigated. Cluster-specific random-effects logistic regression models were implemented. Increasing age and being male were risk factors for seropositivity. The effects of the risk factors origin (homebred or purchased) and herd size interacted such that the overall effect of purchase status was different for smaller and larger herds. For smaller herds purchase status and increasing herd size were risk factors, whereas these effects were not observed for larger herds. However, seroprevalence figures, and consequently the statistical modelling outcome, are a post-epidemic snapshot and non-informative regarding the within-herd index case and the within-herd infection dynamic. In larger herds, the contagion of homebred herd mates could have masked the importance of purchased index cases as direct sources of virus introduction. Biologically, purchase status would be a direct risk factor for herds of any size.

Chapter 6.3 explores the impact of test misclassification bias on the interpretation of the single-test BoHV-1 seroprevalence risk assessment. Population-averaged modelling of the
serological results indicated that age, sex, origin (purchased or homebred), and herd size were associated with seropositivity. The final regression model included significant quadratic terms, as well as interactions between all main factors. The exploration of the impact of the bias was implemented via a sensitivity analysis using age-related test misclassification probabilities, which were obtained by expert opinion. The range of expert values for the Se [0.960; 0.995] was comparable for different age groups of cattle, except for cattle younger than one year having lost their maternal immunity. For this young stock the Se was [0.800; 0.995]. The range of expert values for the Sp [0.930; 0.995] was surprisingly wide with a low lower limit. It was the same for different age groups of cattle. The sensitivity analysis indicated that the final model based on calculated true prevalence data, giving the - rather extreme - expert opinion, was more parsimonious compared to that based on seroprevalence data. Age and purchase status of cattle, and size of herds were stable risk factors for BoHV-1 infection. Moreover, the sensitivity analysis reduced the uncertainty region of the seroprevalence odds ratios [0; +∞] to [1.016; 1.028] and [1.016; 1.023] for an increase in age of one month and an increase in herd size of one animal, respectively. The uncertainty region of the seroprevalence odds ratios [0; +∞] for purchase status was reduced to [1.360; 1.724].

**General conclusion:**

The major aim of this thesis was to evaluate the overall epidemiological usefulness of the two single-test surveys as information generators to study occurrence of disease. Was the diagnostic information obtained by these surveys accurate? To answer this question, aspects of random error (imprecision) and systematic error (bias) were investigated. The former aspect is a statistical issue. The design-based analysis of the precision of survey results has to take into account the clustering aspect. Without adjustment for clustering, the precision would be overestimated, impacting considerably on the risk assessment. The latter aspect, namely bias, is at the heart of epidemiology. The bias caused by test misclassification (diagnostic uncertainty) is at the heart of the thesis. When survey tests are not locally (regionally) validated, we stay ignorant about the diagnostic validity of the test results. Moreover, the impact of diagnostic uncertainty on hypothesis testing is also potentially considerable. Consequently, at present we are reluctant to recommend single-test sero-surveys.

As mentioned, the globalisation of trade of animals and animal products exerts a strong pressure on animal disease management. Risk analysis depends on data generated by a comprehensive surveillance system with a sound epidemiological design (Zepeda et al., 2001). However, a sound integral statistical-epidemiological analysis is also important.
SAMENVATTING

De basis van dit onderzoek werd gevormd door twee grootschalige dierenziektensurveys die in het voorbije decennium in België uitgevoerd werden. Een eerste survey werd uitgevoerd in 1996 en onderzocht 553 varkensbedrijven. Een tweede, in 1998, betrok 556 rundveebedrijven. Het waren piloot surveys, ten eerste omdat ze werden uitgevoerd op nationaal vlak, ten tweede omdat ze gebaseerd waren op steekproef methodologie met centrale toevalligheid (ad random) loting van te bemonsteren bedrijven, en ten derde omdat de seroprevalentie (schijnbare of gemeten prevalentie) aangepast werd voor factoren zoals test gevoeligheid (Se) en specificiteit (Sp) om de ware prevalentie te berekenen. Deze twee dwars-doorsnede studies onderzochten epidemiologische aspecten van 3 pathogenen van de B Lijst van de O.I.E.’s International Animal Health Code: het pseudorabies (ziekte van Aujeszky) virus (PRV), het boviene herpesvirus 1 (BoHV-1) (virus van infectieuze boviene rhinotracheïtis), en de Mycobacterium avium subsp. paratuberculosis (Map). De doelstellingen van deze onderzoeken zijn beschreven in Hoofdstuk 2. Een eerste doel was de schatting van de bedrijfsseroprevalentie (BSP) van PRV in de Belgische varkenspopulatie, en van BoHV-1 en Map in de Belgisch rundvee. Ook de BoHV-1 en Map dierseroprevalentie (SP) en binnenbedrijfseroprevalentie (BBSP) werden geschat. Om de impact van de diagnostische onzekerheid op de interpretatie van deze seroprevalentie parameters te evalueren, werd de ware prevalentie berekend op basis van test misclassificatie bias methodologie. Ten tweede werden risicofactoren opgespoord die geassocieerd waren met PRV BSP en met BoHV-1 SP. Om de impact van de diagnostische onzekerheid op de interpretatie van de BoHV-1 risico evaluatie te onderzoeken, werd een gevoeligheidsanalyse uitgevoerd. Het derde doel was de toestand brenging van een rundserotheek voor verdere onderzoeksdoeleinden.

In Hoofdstuk 3 wordt een kort overzicht gegeven van de basis methodologie van surveys uitgevoerd met het doel informatie te vergaren betreffende het voorkomen van infecties in dierpopulaties. Een inleiding tot aspecten van infecties met PRV, BoHV-1, en Map, is verschaf in Hoofdstuk 4. Enkel de belangrijkste gegevens betreffende de distributie, pathogenese, immuniteit, overdracht, diagnose, beheersing en uitroeiing werden aangehaald, omdat deze aspecten belangrijk zijn voor een goed begrip van deze thesis.

Hoofdstuk 5 omvat drie delen. In dit hoofdstuk werd het beschrijvende deel van de surveys besproken, dat prevalentie schatting als doel had. De eerste piloot survey werd uitgevoerd in de periode december 1995 tot februari 1996 in de vijf provincies van het Noorden van België. Het doel was de schatting van de provinciale PRV BSP van bedrijven met zeugen (Hoofdstuk 5.1). Vijfhonderd en driënvijftig bedrijven voldeden aan de inclusie criteria van de survey. Om recentelijk besmette varkens op te sporen, werden enkel jonge zeugen bemonsterd. De resultaten toonden dat 44% (99% betrouwbaarheidsinterval = 38,5%-49,0%) van de bedrijven een belangrijk
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aantal PRV-seropositieve jonge zeugen had. Onder aannamen van een Se en Sp van respectievelijk 95% en 99%, en een PRV ware binnenbedrijfprevalentie (BBP) van 43%, werd de globale PRV ware bedrijfprevalentie (BP) op 35% geschat. De hoogste BSP werd geobserveerd in West-Vlaanderen (68%), gevolgd door Antwerpen (60%), Oost-Vlaanderen (43%), Limburg (18%), en Vlaams-Brabant (8%). Het besluit was dat het Noorden van België in twee subregios kon ingedeeld worden: een eerste subregio met een hoge BSP die de eerste drie provincies omvat en een tweede met lage BSP gevormd door de laatste twee provincies. Het werd aanbevolen de uitroeingsprogramma’s in deze twee subregios aan te passen met specifieke maatregelen.

Onderzoek van risicofactoren op bedrijfsniveau door logistieke regressie had als resultaat dat de aanwezigheid van vleesvarkens geassocieerd was met een twee- en viervoudige meerkans van bedrijven om seropositief te worden; een aantal fokdieren ≥ 70 zeugen was geassocieerd met een viervoudige meerkans van bedrijven om seropositief te worden; en varkensdichtheid ≥ 455 varkens per km² was geassocieerd met een viervoudige meerkans van bedrijven om seropositief te worden. Deze risicofactoren konden moeilijk veranderd worden door de varkenshouders. Ze vereisten meer structurele maatregelen in het PRV uitroeingsprogramma opgelegd door de diergeneeskundige autoriteiten. Dit benadrukte nogmaals de nood aan een regionale aanpak van de uitroeijing.

De tweede piloot survey werd uitgevoerd van december 1997 tot maart 1998. Ze schatte tezelfdertijd de prevalentie van BoHV-1 en van Map in Belgische rundvee. Voor de eerste prevalentiestudie werden alle runderen (N = 28.478) in een toevalsgewijze steekproef van bedrijven (N = 556) getest voor de aanwezigheid van antistoffen tegenover glycoproteïne B van BoHV-1 (Hoofdstuk 5.2). Onderscheid tussen gevaccineerde en besmette runderen was diagnostisch niet mogelijk aangezien het exclusieve gebruik van marker vaccins slechts bij wet opgelegd werd in 1997. Eenentwintigentig percent van de rundveehouders vacineerden hun runderen op continue basis voor BoHV-1. In de niet-gevacineerde groep, waren de globale BSP, SP en de mediane BBSP respectievelijk geschat op 67% (95% betrouwbaarheidsinterval (BI)=62%-72%), 35,9% (95% BI=30,0%-42,1%) en 33% (quartiele=14%-62%). Onder aannamen van een Se en Sp van respectievelijk 99% en 99,7% werden de BP, de ware dierprevalentie (P) en de BBP in de niet-gevacineerde groep van bedrijven respectievelijk geschat op 65%, 36% and 34%. Aangezien deze test eigenschappen te optimistisch zouden kunnen geweest zijn, werd de BP berekend voor een set van test karakteristieken. Deze berekeningen tonen dat de BP dezelfde blijft voor een veranderende Se van 70% tot 99%. De opspoorbaarheid van besmette bedrijven was 99,99% tot 100% voor alle parameter combinaties onder beschouwing. Echter, het gebrek aan Sp heeft een aanzienlijk effect op de schatting van de BP; de schatting van de BP daalde van 65% tot 28% wanneer de Sp respectievelijk daalde van 99,7% tot 96%, voor een veranderende Se van 70% tot 99%. Deze
berekeningen tonen aan dat de praktische limieten van validiteit van de gebruikte survey test, een gB ELISA, het niet toelaten de BoHV-1 BP te schatten binnen aanvaardbare accuraatheidsnormen.

Voor de Map prevalentiestudie werden alle volwassen runderen van 24 maanden of ouder (N = 13.317) getest voor de aanwezigheid van antistoffen met een commercieel beschikbare ELISA met preabsorptie (Hoofdstuk 5.3). De mediane BBSP en de SP waren respectievelijk 2,9% (quartiel=1,6%-5,6%) en 0,87% (95% BI=0,67-1,06). De BSP was 18% (95% BI=14-21). Onder aannemen van een SE en Sp van respectievelijk 45% en 99%, werden de mediane BBP en de P geschat op 7% en 2% respectievelijk. De Map BP werd eerst geschat volgens de gebruikelijke aanvaarde methodologie. Deze berekening toonde dat de Sp van de gebruikte test een dramatisch effect heeft op de schatting; onder aannames van een SE van 45% en een BBP van 7%, daalde de geschatte BP van 36 tot 0,8% wanneer de Sp daalde van 99,9 tot 99% respectievelijk. Deze gevoeligheidsanalyse toonde aan dat de praktische limieten van validiteit van de gebruikte survey test, het niet toelaten de Map BP te schatten binnen aanvaardbare accuraatheidsnormen, omdat de BBP laag was. Om deze reden werd de bedrijfsspecificiteit verhoogd voor bedrijven met grotere volwassen kuddes (>5). Dit werd bekomen door de afkapwaarde van benodigde aantallen positieve runderen (≥2) om een bedrijf als waar positief te verklaren te verhogen, en toch rekening te houden met bedrijven met slechts één positief test resultaat indien er op dat bedrijf historisch bewijs was van paratuberculose (eerdere diagnose en/of klinische symptomen). Deze aanpak resulteerde in een geschatte Map BP van 6%.

In Hoofdstuk 6 werden risicofactoren voor besmetting met BoHV-1 opgespoord. Ook dit hoofdstuk omvat drie delen. Het eerste deel, Hoofdstuk 6.1, evaluateert kritisch de impact van het ontwerp (design) op de analyse van surveys van besmettingen, met de BoHV-1 serosurvey als een voorbeeld. Het duidt de problemen wanneer men het ontwerp niet in aanmerking neemt bij de statistische risicofactoren analyse. Een geleed (gestratificeerd) ontwerp verhoogt vaak de nauwkeurigheid, terwijl clusterings van besmetting deze verlaagt. Het ontwerp kan bij de analyse in aanmerking worden genomen door marginale modellen gebaseerd op pseudo-likelihood schattingsmethodologie en een linearisatie van de schatter van de variatie. De intra-cluster correlatie kan onderzocht worden door cluster-specifieke modellen (o.a. random effect modellen) alsook door marginale modellen gebaseerd op veralgemeende schattingsovergelijkingen (generalised estimating equations, GEE). Op deze wijze kan de uitgebreidheid van de verspreiding van de besmetting in een bedrijf (cluster) worden onderzocht. Het analyseren van dergelijke gecorreleerde binaire gegevens is methodologisch een grote uitdaging. In de analyse van de BoHV-1 survey was de impact van het survey ontwerp het grootst op de bedrijfsdeskarakteristieken (e.g. type van bedrijf), minder op de dierdeskarakteristieken (e.g. leeftijd van het dier). Runderen in Belgische bedrijven werden vaak in groep gehuisvest. Dit bevordert de verspreiding van infecties, wat resulteert in meer homogene clusters met betrekking tot de aanwezigheid, of afwezigheid, van de
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besmetting. Runderen van eenzelfde bedrijf hebben een gelijkwaardiger serologische status dan runderen die afkomstig zijn van verschillende bedrijven. Dit is het zogenaamde “nest-effect” of “cluster-effect”. Dit wordt door de analyse bevestigd door de waarde van de intra-cluster correlatie, die hoger was dan 50%.

In Hoofdstuk 6.2 worden factoren epidemiologisch onderzocht die voor runderen een risico betekenen om seropositief te worden voor BoHV-1. Cluster-specifieke random effect logistieke regressie modellen werden geïmplementeerd. Hogere leeftijd en mannelijk geslacht waren geassocieerd met seropositiviteit. De effecten van de oorsprong van de runderen (geboren in het bedrijf of aangekocht) en bedrijfsgrootte interageerden en waren dus niet louter additief: het globale effect van de status ‘aangekocht’ was anders voor kleinere en grotere bedrijven. Voor kleinere bedrijven waren aankoop en grotere bedrijfsgrootte risicofactoren, terwijl deze effecten niet geobserveerd werden voor grotere bedrijven. Echter, seroprevalentiecijfers, en dus de statistische modeller resultaten zijn feitelijk slechts een post-epidemische momentopname (foto).

Ze geven geen informatie betreffende index case in het bedrijf, noch omtrent de besmettingsdynamiek binnen het bedrijf. In grotere bedrijven kan de besmetting van in het bedrijf geboren runderen het belang verdoozend hebben van aangekochte index cases als mogelijke directe bronnen van virusinsleuip. Biologisch gezien zou aankoop een directe risicofactor zijn voor bedrijven van om het even welke grootte.

Hoofdstuk 6.3 onderzoekt de impact van test misclassificatie bias op de interpretatie van de BoHV-1 seroprevalentie risico evaluatie. Marginale modellering van serologische resultaten toonde aan dat hogere leeftijd, en mannelijk geslacht, oorsprong (geboren in het bedrijf of aangekocht), en bedrijfsgrootte geassocieerd waren met seropositiviteit. Het uiteindelijk regressiemodel bevatte significante kwadratische termen, alsook interacties tussen alle hoofdeffecten. De evaluatie van de impact van de bias werd uitgevoerd via een gevoeligheidsanalyse die gebruikt maakte van leeftijdsgebonden test misclassificatie kansen. Deze probabiliteitscijfers werden verkregen via expert bevraging. De range van expert waarden voor de Se [0,960; 0,995] was vergelijkbaar voor verschillende leeftijdsklassen van runderen, uitgezonderd voor runderen jonger dan een jaar die hun maternale immuniteit verloren hebben. Voor dit jongvee was de Se [0,800; 0,995]. De range van expert waarden voor de Sp [0,930; 0,995] was verrassend breed met een lage ondergrens. Het was dezelfde voor verschillende leeftijdsklassen van runderen. De gevoeligheidsanalyse toonde aan dat het uiteindelijke model gebaseerd op berekende ware prevalentie data, gegeven de – nogal extreme – expert opinie, eenvoudiger was vergeleken met dat gebaseerd op seroprevalentie data. Leeftijd, aankoop, en bedrijfsgrootte waren stabiele risicofactoren voor BoHV-1 besmetting. Verder verklaarde de gevoeligheidsanalyse de onzekerheidsregio voor de seroprevalentie odds ratios [0; +∞] tot [1,016; 1,028] en [1,016; 1,023] voor een stijging in leeftijd van een maand en een stijging in bedrijfsgrootte van een dier,
respectievelijk. De onzekerheidsregio voor de seroprevalentie odds ratio \([0; +\infty]\) voor aankoop werd vernauwd tot \([1,360; 1,724]\).

Als algemeen besluit kan gesteld worden deze thesis tot doel had het globaal epidemiologische nut te onderzoeken van twee surveys waarbij diergezondheidsinformatie gebaseerd was op één test. Was de diagnostische informatie verkregen door deze surveys accuraat? Om deze vraag te beantwoorden werden aspecten van random fout (onnauwkeurigheid) en van systematische fout (bias) onderzocht. Het eerste aspect is een zaak van statistiek. Een ontwerpgebaseerde analyse van de nauwkeurigheid van de survey resultaten moet het cluster-effect in acht nemen. Zonder aanpassing voor clustering wordt de nauwkeurigheid overschat, wat een groot impact heeft op de risico evaluatie. Het tweede aspect, namelijk vertekening of bias, is het hoofdonderwerp van de epidemiologie. De bias veroorzaakt door test misclassificatie (diagnostische onzekerheid) is het hoofdonderwerp van deze thesis. Wanneer survey testen niet lokaal (regionaal) gevalideerd zijn, blijven we in het ongewisse betreffende de diagnostische validiteit van de test resultaten. Verder is de impact van diagnostische onzekerheid op hypothese testen is ook potentieel groot. Dus is het uitvoeren van surveys gebaseerd op één test, niet aan te raden.

Zoals vermeld oefent de globalisering van de handel in dieren en dierlijke producten een sterke druk uit op dierenziekten management. Risico analyse hangt af van data die gegenereerd zijn door een verstaanbaar bewakingssysteem met een juist epidemiologisch ontwerp (Zepeda et al., 2001). Maar, een optimale integraal statistisch-epidemiologische analyse is ook belangrijk.
REFERENTIES