Evaluation of diagnostic methods used in zoonosis-surveillance programs

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Summary: The purpose of the present work is to set focus on the importance of built-in validation of diagnostic methods in zoonoses-surveillance programs. Appropriate and ongoing evaluation schemes are preconditions for estimating true prevalences of zoonotic infections or infestations. Estimates of true prevalences are crucial for optimizing surveillance programs so they are both cost effective and provide the best possible tool for assessing consumer safety. Using 3 examples we illustrate some of the diagnostic challenges in zoonosis surveillance that potentially could be met by appropriate validation schemes and knowledge about the performance of the diagnostic methods. We recommend that estimates of within and between laboratory variation, analytical and diagnostic sensitivity and specificity are made accessible in the public domain as part of a quality-assurance system for diagnostic methods in surveillance programs. Furthermore, we recommend that diagnostic methods be subject to an ongoing validation in any surveillance program.

Keywords: Consumer safety, Diagnostic performance, True prevalence, Built-in validation, Latent-class methodology

Introduction: Worldwide there has been an increased awareness and public concern over food-borne pathogens over the past two decades. To a large extent this has been due to a number of serious human outbreaks caused by e.g. *Salmonella*, *Campylobacter* and Eschericia coli O157:H7 (Nielsen, 2002). In 2001, salmonellosis and *campylobacter*iosis were by far the most frequently reported zoonoses in the European Union with approximately 159,000 human cases each (Anon., 2003a). In addition, the emergence of new zoonoses such as the BSE related variant Creutzfeld-Jakobs disease has enforced the focus on food safety issues. Veterinary surveillance activities in many countries have improved in the same period. This was due partly to a need for better information on the disease status of the national herd in order to support livestock industries and partly due to improved epidemiological methods and implementation of computerized databases. This development has been stimulated by legislation, which governs international trade of animals and animal products and by the concern over food-borne pathogens and antimicrobial drug resistance (Gibbens and Wilesmith, 2000). Although the majority of food-borne diseases most likely are not attributable to pork (Nielsen, 2002), food safety has been a driving incentive for the pork industry in Denmark, giving a high priority to monitoring and surveillance of zoonotic infections in swine. A well-known example is the nation-wide *Salmonella enterica* surveillance and control program in Danish finishing herds (Mousing et al., 1997; Nielsen et al., 2001). In 2002, the expenses on the surveillance of *Salmonella* amounted to Euro 6.9 million (Nielsen and Korsgaard, 2003).

Within the European Union each member state has to collect epidemiological data on zoonoses to comply with the community strategy. All member states are at present contributing to an annual report (Anon., 2003a) on trends and sources of zoonotic agents according to article 5 of Directive 92/117/EEC, which contains information on the situation regarding zoonoses in animals, feedstuffs, food and man. However, the quality of the data suffers from un-harmonized surveillance systems, which makes it very difficult to draw inferences on the trends of the prevalences of zoonotic agents within the community. To overcome the need for harmonized and valid zoonosis-surveillance programs in the European Union and worldwide, it is of utmost importance to document the diagnostic processes involved. Knowledge of diagnostic test characteristics such as diagnostic sensitivity and specificity

is a precondition to obtain reliable estimates of true prevalence. In turn, true prevalence estimates are crucial for optimizing surveillance programs so they are both cost effective and provide the best possible tool for ensuring a high consumer safety. Therefore, an ongoing validation process is needed for the diagnostic methods used in surveillance programs, in order to detect possible changes with time in disease prevalence and diagnostic performance.
We have illustrated a few of the diagnostic challenges in zoonosis surveillance using data from screening, surveillance and monitoring programs of *Salmonella*, *Campylobacter* and verotoxin producing Escherichia coli in Denmark.

Example 1, Salmonella surveillance: In the Danish *Salmonella* surveillance and control program, finisher herds are monitored for the presence of *Salmonella*- specific antibodies in meat juice. Based on the serological results, all finisher herds are assigned to 3 different herds levels on a monthly basis. At the same time, a national average of seropositive samples is calculated. This average is used as a measure for the general level of seropositive finishers in Denmark. Similarly, fresh pork is monitored for S. enterica at the abattoirs every month. From 1993 to 2000, the surveillance was conducted by bacteriological examination of different pork cuts. From 2001 and onwards, *Salmonella* testing on pig meat has been based on swabbing of carcasses. This latter method is twice as sensitive as the one previously used (S_rensen et al. 2001). The apparent *Salmonella* prevalence in pork was adjusted accordingly to allow for comparisons (Figure 1).

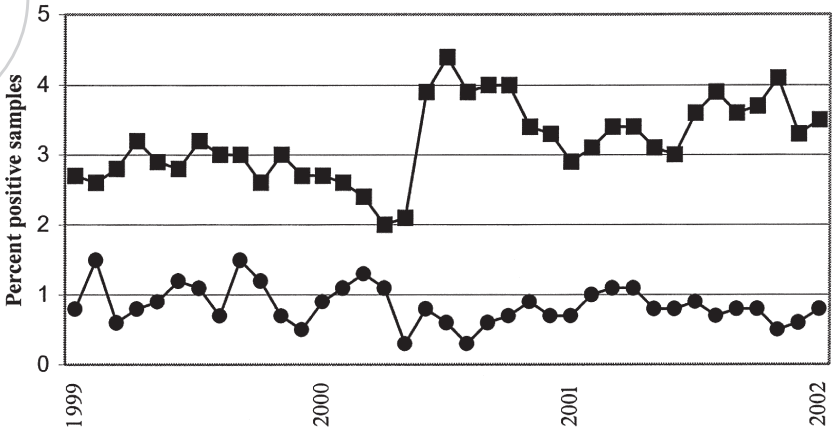

Figure 1. *The prevalence of Salmonella-positive samples assessed by meat-juice ELISA (- -) and bacteriological examination of pork cuts (- -).*

The prevalence of culture-positive samples has declined from 3-4 % in 1993 to 0.7 % in 2000, and has remained at a similar low level since. In contrast, in June 2000, an unusual and unexpected increase in the number of seropositive samples was observed in the course of just 2 weeks. The prevalence of seropositive samples increased from 2 % to 4 %, and remained at higher level for more than a year. However, at the same time the prevalence of Salmonella in pork was lower than usual. These observations were unexpected, as several earlier Danish investigations had demonstrated a clear correlation between the prevalence of seropositive and bacteriological positive samples on herd level. A large investigation was initiated in order to clarify, whether the observed increase in seropositive samples was due to a true increase in Salmonella load in the finishers, or whether the increase was due to a drift in the mix-ELISA (Nielsen et al., 1995). The mix-ELISA is routinely calibrated by use of 8 control

sera on all plates. Additionally, the mix-ELISA is tested on a monthly basis against a panel of 40 other field sera from swine in order to ensure that the results of the ordinary control sera do not drift over time. No deviations in the daily control sera or monthly calibration sera were observed. For comparison, results from the national *Salmonella* surveillance of all breeder and multiplier herds were studied too. In these herds, 10 gilts are blood sampled every month and tested for *Salmonella* antibodies. Half of the herds also produce finishers for slaughter, which are tested for *Salmonella* antibodies as mentioned earlier. In the breeder and multiplier herds, the seroprevalence based on serum samples from gilts remained at a steady level throughout 2000, while the seroprevalence based on meat-juice samples from their finishers increased as was observed in ordinary finisher herds. No satisfactory explanations could be offered for these observations. A similar scenario has not been observed since.

Example 2, ***Campylobacter*** **monitoring and screening:** Samples of intestinal contents are collected routinely from cattle, pigs and poultry at different abattoirs throughout the country, as part of the Danish Antimicrobial Resistance Monitoring Programme (DANMAP). According to the DANMAP procedure, a single caecal sample per herd is collected from 200 to 250 randomly chosen pig herds each year. Samples are analyzed for *Campylobacter* species according to standard bacteriological procedures, followed by determination of antimicrobial resistance profiles. The result – the prevalence of samples positive for *Campylobacter* - thus is an estimate at both pig and herd level. The DANMAP results for *Campylobacter* from 1997 to 2002 are shown in Table 1.

Study	Year	Number of herds tested	*Campylobacter* positive caecal samples (%)		
			Campylobacter spp.	C. coli	C. jejuni
DANMAP	1997	245	56	53	2.4
	1998	194	60	52	6.2
	1999	244	46	41	4.5
	2000	277	60	56	4.0
	2001	238	77	69	2.9
	2002	240	80	79	1.6
DBMC	2002	247	92	90	2.3

Table 1. *Percent Campylobacter positive caecal samples from swine.*

Two observations can be made: (1) the apparent prevalence of *Campylobacter* species (including non-typed isolates) using the DANMAP method ranges from 46-80 % and has been increasing over the past few years; (2) only the prevalence of C. coli has increased (from 41 % to 79 %), while *C. jejuni* in pigs has remained at a relatively constant, low level (1.6-6.2 %). In 2002, the Danish Bacon & Meat Council (DBMC) carried out a screening for *C. jejuni* in pigs at several abattoirs. Caecal samples from 247 herds were examined, and per herd 5 pigs were sampled. Samples were analyzed according to the same bacteriological procedures as in the DANMAP surveys. The results of the screening are summarized in Table 1 (see also Boes et al., 2003, in this issue). All 247 herds were positive for *Campylobacter* species (herd prevalence = 100 %), and 92 % of pigs were *Campylobacter* positive. Pig prevalences of C. coli and *C. jejuni* positive caecal samples were 90.1 % and 2.3 %, respectively. In the DANMAP study, taking 1 sample per pig herd resulted in quite low *Campylobacter* prevalences (especially C. coli), whereas in the DBMC study, herd sensitivity was improved by taking 5 samples per herd, resulting in higher prevalences of positive findings. It should be added that in each of 247

herds in the DBMC screening *Campylobacter* was detected in at least 2 pigs, and usually all 5 pigs sampled per herd were positive. Samples collected in the DBMC screening were analyzed in the laboratory within 24 hours after collection. In contrast, DANMAP samples typically were accumulated over a one-week period, and then analyzed. It might be speculated that this procedure was detrimental to at least a proportion of samples containing *Campylobacter*. Recently, the DANMAP procedure was changed so that samples collected in the DANMAP survey are analyzed more frequently, which could explain the increase in *Campylobacter* prevalence in recent years. Interestingly, the negative effect of storing samples for up to 1 week is more pronounced for C. coli than for *C. jejuni*, as the low apparent *C. jejuni* prevalence is comparable between DANMAP and the DBMC study.

Example 3, Detection of verotoxin producing Escherichia coli: Verotoxin producing *E. coli* (VTEC), like O157:H7 known from ruminants, traditionally has not been related to swine and pork. However, verotoxin producing *E. coli* O157:H7 have recently been detected in swine in several countries (Table 2).

Country	Prevalence of culture-positive faecal samples (%)
Chile	69
Japan	1.4
Netherlands	0.7
Norway	0.1
UK	0.16
USA	3.6

Table 2. *Prevalence of verotoxigenic E. coli O157:H7 in swine faeces.*

Additionally, an experimental inoculation study (Cornick and Helgerson, 2003), demonstrated that swine readily become colonized by *E. coli* O157:H7, and that colonized pigs transmit the infection to naive pen mates. The veterinary and public health focus has so far mainly been on O157:H7, but this strategy might need to be reconsidered. Increasing evidence shows that the virulence cassette of the eae gene in combination with the vtx2 gene – rather than the serotype – is associated with haemolytic uraemic syndrome (HUS) in children and haemorrhagic colitis in adults. Most veterinary and human diagnostic laboratories traditionally only examine for O157:H7 or few other of the most common VTEC serotypes, as detection of all VTEC types demands colony hybridization with probes for verotoxin and eae genes. As a consequence, the non-O157 VTEC are most likely underreported. In a US study (Keen at al., 2003), 1,102 healthy swine were examined for VTEC O157, O111 and O26 while the animals where on display on fairs during 2002. Pigs were found to harbor all 3 VTEC serotypes. At present very little is known about the general occurrence of VTEC in swine and pork worldwide. Future prevalence studies should employ colony hybridization with probes for verotoxin- and eae genes in contrast to slide agglutination for just O157:H7. Until a broad VTEC screening for different VTEC types in swine and pork is conducted, it remains difficult to estimate the human risk for pork related VTEC infections.
The difference between slide agglutination for O157 and colony hybridization can be illustrated by an example from Denmark, where human cases of gastroenteritis are routinely examined for a number of zoonotic agents, including VTEC. Of the 14 counties in Denmark, 7 counties perform slide agglutination for O157:H7 and a few other common VTEC serotypes. The remaining counties submit stool samples to the central Danish diagnostic laboratory, where examination includes colony hybridization using probes for verotoxin- and eae genes prior to slide agglutination. In counties using just slide agglutination the incidence is < 1-2 cases per 100,000 inhabitants per year whereas in counties using the central laboratory VTEC incidence is 3-6 cases per 100,000 inhabitants per year (Anon, 2003b).

Discussion: In the surveillance of *Salmonella* ongoing double-classification of samples from different entities of herds followed by statistical estimation of diagnostic sensitivity and specificity and true

prevalences could potentially have resolved the question whether the increase in seroprevalence in 2000 was real or merely a result of drifts in the diagnostic methods. This could have been accomplished applying latent-class methods (En_e et al., 2000). This approach is possible when 2 or more diagnostic tests are available and applicable. Even if they are far from being perfect they can still be used in the surveillance and will yield valid estimates of the true prevalence. Latent-class methods have been used for validation of the mix-ELISA for detection of *Salmonella*- specific antibodies in meat juice (En_e et al., 2001). Crude estimates of apparent prevalences can be highly biased when compared to estimated true prevalences, potentially leading to serious underreporting (En_e et al., 2003). In the monitoring of *Campylobacter* in DANMAP the importance of common criteria for data collection becomes evident when prevalences of culture positive samples are compared with the results from the DBMC study. Thus a sampling scheme based on 5 caecal samples per herd instead of 1 improves the herd-level sensitivity significantly. A direct comparison of the prevalences from these 2 studies without taking sampling strategy into account will lead to mistakes in inference. It is important to realize that the diagnostic performance of aggregate testing to a large extent depends on the number of samples taken. Thus, uniform sampling strategies including appropriate handling and storing of samples prior to testing and between-laboratory testing could have increased the diagnostic precision and made inference easier. The importance of standardization of diagnostic processes is further illustrated by the differences in incidence of human VTEC infections due to different diagnostic strategies in counties in Denmark.
In the European Union surveillance systems are primarily based on existing programs in member states. However, the community strategy implies common criteria for data collection. Moreover, member states shall ensure that diagnostic laboratories apply quality-assurance systems, which conform to the requirements of Standard EN/ISO 17025 (Table 3).

Management	Technical
Organization	General requirements
Quality system	Personnel
Document control	Accommodation and environmental conditions
Reviews of requests, tenders and contracts	Test methods and test validation
Subcontracting	Equipment
Purchasing	Measurement traceability
Service to clients	Sampling
Complaints	Handling of test samples
Control of non-conforming tests	Assuring quality of results
Corrective actions	Reporting results
Preventive actions	
Control of records	
Internal audits	
Management reviews	

Table 3. *Elements of the standard EN/ISO17025 standard for diagnostic laboratories (from Gajadhar and Forbes, 2002).*

The importance of implementing such systems is illustrated in an excellent way by Gajadhar and Forbes, (2002), who describe an internationally recognized quality-assurance system for diagnostic parasitology, with example data on trichinellosis. Equivalent quality-assurance systems should be set up, if not already in place, for the other important zoonoses in pork and preferably published in peer-reviewed international journals. As part of the quality assurance, diagnostic laboratories should regularly participate in collaborative testing organized or coordinated by the national reference laboratory such as the first international ring trial of ELISAs for *Salmonella*-antibody detection in swine (van der

Heijden, 2001). Basic information gathered in compliance with the ISO/IEC Standard 17025 should be made public e.g. on the Internet or other public domains because it is an absolute precondition for optimizing surveillance or monitoring programs and drawing the right inference from the results. Thus, estimates of diagnostic sensitivity and specificity are needed in order to estimate true prevalences of disease; measures of precision and accuracy are needed for appropriately optimizing diagnostic methods e.g. reduce the number of repeated testing on the same specimen (Ekeroth et al., 2003); ongoing validation (estimates of diagnostic sensitivity and specificity) of diagnostic methods is necessary to assess the possible change of true prevalence with time; innate test characteristics such as analytical sensitivity and specificity are needed to make proper selection of the most appropriate diagnostic measure.

Recommendations and conclusions: To summarize, we recommend that an effort should be made to apply common criteria for data collection. Furthermore, quality-assurance systems that conform to the requirements of ISO/IEC Standard 17025 should be implemented. As a part of the quality-assurance system, basic information should be made available on the Internet or other public domains covering as a minimum requirement:

1. Precision - measured as the variation of repeated analyses on a specimen.
2. Accuracy - measured through ongoing validation and estimates of changes in performance with time and through ring trials.
3. Estimates of analytical sensitivity and specificity.
4. Estimates of sensitivity and specificity or any other relevant measure of validity such as likelihood ratio, assessed in the population or comparable to the population in which the diagnostic method is going to be used.

All this information should be the result of an ongoing validation scheme, as it is evident that the validity of diagnostic methods very likely changes with time. An ongoing validation of disease measures is costly and laborious. However, the latent-class approach (Enøe et al., 2000) may be a valuable contribution to make ongoing validation economically feasible. Latent-class methods should be incorporated in surveillance programs and disease monitoring systems whenever possible in order to provide an ongoing and dynamic validation of diagnostic processes.

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Ensuring the safety of animal feed

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Summary: A general outline is presented of measures for the production of safe animal feed. This is based on the setting of so-called 'feed safety objectives' which make use of principles that relate to animal health, animal welfare, legal aspects of farm practices and human food safety objectives for products of animal origin. Particular emphasis will be put on the types of feed used in relation to feedborne animal diseases caused by infectious and chemical agents and on the relationship between animal feed and zoonotic foodborne diseases. In addition the influence of feed on animal welfare will be discussed.
To produce safe animal feed, a pro-active control system is advocated. This approach has been very successful in relation to human food and involves the use of 'good manufacturing practices' (GMP) and the 'hazard analysis critical control point' (HACCP) concept as the main tools. However, it has been shown that the HACCP-system has certain shortcomings. To counteract these shortcomings, product traceability and hazard early-warning systems have been developed and will also be presented.

Keywords: Feed, health, welfare, environment, legislation, food safety

Introduction: Feedstuffs play an important role in maintaining the health of production animals and therefore of humans. In relation to food safety, the slogan 'healthy animals, healthy humans' is often used to demonstrate the clear relationship that exists between the health status of animals and that of human beings. Experience has shown that the transmission of diseases from domestic animals to man can only be prevented effectively by improving the health care of the animals themselves. It is even more of a challenge to prevent the transmission of zoonotic agents because, as the human population has increased , there has been a concomitant increase in the number of production animals. Factors involved in disease control include the availability of safe feedstuffs, husbandry practices, immunisation and the use of antimicrobials and other veterinary drugs. Strategies that have been explored to control foodborne human pathogens include the administration of selected microbial cultures to piglets and day-old chicks in order to establish a balanced gut microflora and increase colonisation resistance. In the case of ruminants, attempts have been made to reduce carriage of Escherichia coli O157 by using special dietary formulations. However, neither of these approaches to gut flora manipulation has been entirely successful.