Update on laboratory diagnosis of subclinical salmonella infections in pigs

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Detection of Salmonella is a key point in veterinary salmonella research and surveillance programmes. The chosen method of detection, direct or indirect, highly affects our conclusions in any examination or investigation. The perfect test is characterized by having a very high specificity, sensitivity and predictive value, being easy to perform, cheap, rapid and having the possibility of large scale examination by automation. So far no single test has been able to fulfil all these requirements.

Especially the lack of sensitivity may be of concern since false negative laboratory results not only give the farmer or the veterinarian a false feeling of being “salmonella negative” but may also seriously affect epidemiological investigations based on laboratory data, giving an underestimated picture of the situation. In epidemiological investigations, it is to some degree possible to adjust the final results if the sensitivity and specificity of the test are known. A major problem is that the diagnostic sensitivity and specificity are dependent on the material examined, whichever detection method is chosen. Consequently, the diagnosis “Salmonella negative” should be changed to “Salmonella not demonstrated”.

The present paper gives an update on laboratory diagnosis of salmonella infections by presenting a number of laboratory methods currently used in diagnostic laboratories as well as different typing methods used for detailed characterization of Salmonella bacteria.

Conventional isolation methods for salmonella

A large number of different culture methods have been used and published during the last century. Many of the original methods were developed for diagnosis of clinical salmonellosis in man or animals. However, clinical salmonellosis in swine is, except for S. Choleraesuis infections, very rare in most swine producing countries.

Isolation of Salmonella depends on the kind of material e.g., faeces, post mortem tissues, feed ingredients or environmental samples which all may harbour different contamination flora competing with the Salmonella during the isolation process. The phase of infection in the animal may also affect the sensitivity of the culture methods. In the acute phase of a gastro intestinal salmonella infection in pigs, massive amounts of Salmonella are excreted in the faeces, giving a nearly pure growth of Salmonella, whereas a chronically infected pig or carrier has an intermittent excretion with a low level of Salmonella in the faeces. As a consequence, the volume of a sample will affect the result. A rectal swab is suitable in the case of acute Salmonella enteritis, but will most likely give many false negative samples from subclinical infections. In subclinical cases, 5-25 grammes of faeces would give a much better estimate of the true infection status compared to rectal swabs. Consequently, the choice of an isolation method must reflect the material for examination. In general it is easier to isolate from post mortem tissues and faeces than to isolate from environmental samples and feed, where salmonella typically may be more or less injured due to the low humidity and possible exposure to disinfectants. During the last decades a fairly

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standardized isolation principle has been developed and is currently used in most routine veterinary laboratories. Basically the principle consists of 5 steps as shown in Table 1.

First step is to inoculate the culture into a non-selective pre-enrichment liquid typically Buffered Peptone Water (BPW) with incubation overnight at 37°C. The first step is beneficial to isolation of salmonella from most types of samples, especially in dry or disinfected samples where the organisms are likely to be in a dormant phase. The pre-enrichment will enhance the growth of salmonella to levels which will be able to survive the second step, selective enrichment.

In the second step, the culture is inoculated into a selective media containing different toxic components. Ideally, the toxic components inhibit competing organisms letting the salmonella survive and proliferate further. The concentration of chemical inhibition is very critical as there may be a narrow safety margin, therefore, errors due to sedimentation during mixing or increased toxicity after storage in daylight can be important. Several toxic components have been used (Table 1) but there appears to be a consensus of opinion, that selenite broth and especially media based on the selective principle of Rappaport-Vassiliadis (RV, RVS, MSRV) are superior to other selective enrichment media (Vassiliadis 1983, Schlundt and Munch 1993, De Smedt et al. 1986, Goosens et al. 1989, Davies and Wray 1994, Dusch and Altwegg 1995, Busse 1995). In general, the selective enrichment is performed overnight in order to speed up the examination, but addition of delayed secondary enrichment (DSE) of 3-5 days has proven to increase the sensitivity for environmental samples (Waltman et al., 1993)

In step 3, the culture is inoculated on selective plates. There are even more types of plating media than enrichment broths in current use for salmonella culture. Commonly used plating media are shown in Table 1. Brilliant green agar is a frequently used plating media in veterinary diagnosis. Lactose positive salmonellae may be missed on BGA, however the occurrence of these is probably low (Maghur 1979). Salmonella which do not produce hydrogen sulphide or are late positives are relatively common so plating media such as XLD and XLT4 may miss these (Miller and Tate 1991, Miller et al., 1995).

Step 4 and 5 are biochemical verification and serological characterization in order to reach a final diagnosis.

Comparison of methods

It is difficult to compare the sensitivity of methods as many recorded studies have used artificially contaminated samples from which it may be unrealistic easy to isolate Salmonella, compared with naturally contaminated samples. Another major problem for comparison is, that many studies have only included very small numbers of positive samples from a narrow range of sources including a limited number of strains and serotypes. Naturally contaminated samples may show a very uneven distribution of contamination which cannot be completely equalised by homogenization. This is reflected in a number of studies where some researchers find very good results with a method while other studies indicate substantially poorer results (Schlundt and Munch 1993, Quinn et al. 1995). There seems to be a general need for a comparison of different media using large numbers of naturally contaminated samples before valid comparisons can be made. Ring trials and quality assurance systems are of great importance for the documentation of methods and the performance of different laboratories. Such systems do exist (In’t Veld and Notermans 1992) in the EU and have proven to be very valuable for the EU National Salmonella Reference Laboratories.
The detection of Salmonella by traditional culture methods takes at least 3-5 days. In view of the increasing number of national and international regulations with respect to the occurrence of Salmonella in production animals and food products there is a need for rapid methods for the detection of this organism. The methods should be applicable for automatization and large scale investigations. The rapid methods will especially be useful for screening of samples and release of animals or products with negative test result as the positive test result in most cases has to be verified by traditional culture methods. In the following an overview of alternative methods for salmonella detection will be given.

**Measurements of metabolism: Conductance**

The conductance method consists of pre-enrichment of samples followed by an assay in selective media. Changes in the electrical conductance of these media, due to the growth and metabolism of the microbes in the sample, are measured through a pair of electrodes in the growth medium. When conductance values reach a certain magnitude, the test is regarded as positive. Several media have been described (Easter and Gibson 1985, Pettipher and Watts 1989, Ogden 1990, Davda and Pugh 1991), with a varying degree of false positive samples mainly due to *Cit. freundii* and *E. coli*.

The conductance method is highly suitable for automation and a number of commercially conductance systems are available e.g. the Bactometer (bioMerieux UK Ltd, Basingstoke), the Malthus (Radiometer, Crawley), the RABIT (Don Whitley Scientific Ltd., Shipley) and the BacTrac (Sy-Lab, Purkersdorf, Austria). In a collaborative study (Gibson et al., 1992), the Malthus was compared with conventional culture methods for the detection of Salmonella in a range of artificially contaminated foods. There was no significant difference between the methods. Subsequently, the conductance method was adopted by AOAC as a first action method (Andrews 1993).

**DNA-based methods for detection of Salmonella**

During the last decade, DNA-based detection methods (hybridization and polymerase chain reaction (PCR)) have become available methods for direct detection of Salmonella in clinical, food, and environmental samples, and for rapid confirmation of traditionally cultured bacteria. A review covering DNA-based methods for detection of food borne bacteria, including Salmonella, has been published by Olsen et al. (1995). Generally, the methods need a preceding enrichment by culture methods to ensure a sufficient high number of bacteria.

**Poly- and oligonucleotide probes**

Several poly- and oligonucleotide probes for detection of Salmonella by hybridization have been published since 1983 (Olsen et al., 1995). Comparative studies on hybridization probe assays and culture methods have almost exclusively been performed with commercially released probe assays for detection of Salmonella. The company Gene-Trak has released two hybridization assays. The first was based on a radio labelled probe developed by Fitts et al. (1983). In general, the performance of the assay was equal to standard culture procedures for detection of salmonella in food and naturally infected raw ground turkey (Flowers et al., 1987). The assay was approved for salmonella detection in food in 1988 by AOAC. Due to the disadvantages with a radio labelled probe, a new colorimetric hybridization assay (colorDNAH) was released. This assay is based on
rRNA probes published by Wilson et al. (1990). In a comparative study on artificially inoculated and naturally contaminated food samples, the colorDNAH assay gave 1.5% false positive reactions and 0.8% false negative reactions on inoculated samples.

Probes may also be used for in situ hybridization. Nordentoft at al. (1997) successfully used a fluorescent labelled specific oligonucleotide probe targeting 23S rRNA for detection of Salmonella in formalin fixed, paraffin embedded tissue sections from mice.

**PCR based assays**

An increasing number of polymerase chain reaction (PCR) based assays for detection of Salmonella has been published during the last years. PCR methods have the potential of reducing the analysis time considerably (24 h), compared to culture methods and with the possible use of magnetic beads to concentrate bacteria from different sample materials, PCR is suitable for automation. PCR assays may be very sensitive to inhibitory compounds such as bilirubin, bile salts in faeces, chemical compounds in selective media and substances inherited by different natural food samples. Problems with inhibitors of the polymerase enzyme may be solved by several adjustments of the methods. One method is to combine immunomagnetic separation with PCR (Widjojoatmodjo et al. 1991, 1992, Olsvik et al. 1991, Fluit et al. 1993, 1995).

PCR assays have been developed in order to detect the entire Salmonella genus (Aabo et al. 1993, 1995, Cohen et al. 1993, 1996, Mahon et al. 1994, Stone et al. 1994, Olsen et al. 1995), as well as for the detection of specific serotypes using specific primers, e.g. for Typhimurium (Joys 1985, Soumet 1997) and Enteritidis (Wood et al. 1994). In general, the sensitivity of PCR methods is considerably higher than traditional culture methods, typically 90-98% for PCR against 50-80% for culture methods. The PCR methods will, without any doubt, become even more used in the future for detection of salmonella.

**Immunomagnetic methods**

Immunomagnetic separation (IMS), i.e., using small super-paramagnetic particles or beads coated with antibodies against surface antigens of cells, has been shown to be efficient for isolation of bacteria from different kinds of sample material (Olsvik et al. 1994). The advantage of this principle has been used in several methods, i.e. as earlier mentioned for concentration of salmonella for PCR. Bacteria, immunologically bound to magnetic beads usually remain viable and can continue to multiply if nutritional requirements are provided. The immunologically bound bacteria can then be washed to remove unspecifically attached organisms before it is placed on suitable growth media (Skjerve and Olsvik 1991). In general, immuno assays require pre-enrichment before the required sensitivity of the assay can be reached.

Paramagnetic beads are very successful used in a semi-automated method (Eia-Foss) as described by Krussel and Skovgaard (1993). The method is based on a two step enrichment procedure (19 and 3h) using two different pre-enrichment broths for raw and processed foods, followed by an automated enzyme immuno assay (EIA) carried out in the "EiaFoss analyser" (A/S Foss Electric, Denmark). In a performance test with naturally salmonella contaminated raw and processed foods, the EiaFoss was compared with a traditional culture, the Rappaport-Vassiliadis procedure. The EiaFoss found 13% more positive samples than the traditional culture method. During a working day one laboratory technician can undertake the analysis of 54-81 samples, depending on the nature of the samples. Salmonella-negative products can be released 24-26 h after starting the analysis. This method is currently, frequently used in Danish industry laboratories.
ELISA’s for detection of Salmonella bacteria

A considerable number of different ELISA’s have been developed for the detection of Salmonella bacteria in several sample materials. The majority of these expressed too low specificity and sensitivity for practical use. The low specificity is mainly due to cross reaction with *Citrobacter* and *E. coli*. All ELISA’s need pre-enrichment to reach a level of detection. A number of commercial ELISA with high specificity and sensitivity is available e.g. the TECRA™ Salmonella Visual Immunoassay (Bioenterprises Pty Ltd. Roseville, Australia), the Assurance Salmonella Enzyme Immunoassay (Biocontrol Systems Inc., Bothwell, Washington, USA) as well as the fully-automated VIDAS (bioMérieux UK Ltd, Basingstoke, UK).

Typing of Salmonella

During the last decade it has become more important to have a rather detailed characterization of Salmonella. Results obtained by traditional culture methods such as determination of biochemical properties and serotyping usually do not provide sufficient data for the exact identification of single strains in order to monitor their spread within or between humans, animal populations and environment. Typing of Salmonella may be done by several pheno- and genotypic methods. There are three general requirements for typing methods:

1) The applicability of the method to all isolates to be tested
2) The reproducibility and comparability of the results obtained within and among different laboratories
3) A high discriminatory power and stability of types during an epidemiological event

The various typing methods (Table 2) are not equally efficient for typing all Salmonella strains.

*Serotyping*, as described by Kaufmann and White and latest updated by Popoff and Le Minor (1992), discriminates Salmonella into more than 2300 serotypes based on O and H antigens. The typeability is generally high but damage of the LPS layer e.g. following environmental stress causes rough and non-typable isolates. Additionally, damages of the flagella can result in mono- or a-phasic isolates where only incomplete serotypes can be detected.

*Phage typing* is commonly used for differentiation among isolates of common serotypes i.e. Typhimurium and Enteritidis. The phage typing system for *S. Typhimurium* described by Anderson et al. (1977) and for *S. Enteritidis* described by Ward et al. (1987) are the most commonly used systems and are additionally approved as the official WHO and EU phage typing systems.

*Antibiogram pattern* has proven to be very useful for monitoring the spread of different antibiotic resistant Salmonella clones of e.g. Typhimurium, Hadar and Virchow. The resistance genes are often situated on mobile DNA elements which may result in instability of the antibiogram pattern. Consequently, the antiogram pattern has its optimal value when compared with other typing methods. The monitoring of the rapid spread of the multiresistant Typhimurium DT 104 in the United Kingdom, Germany and USA is an example of the combined use of phage typing and antibiotic resistance profiling.
Plasmid analysis has been evaluated on many Salmonella serotypes, and has proven useful especially for Typhimurium, Dublin and Choleraesuis (Schwarz et al. 1997). Plasmids are, however, mobile DNA elements which may influence the stability of the profile, and it is therefore recommendable to support the results of plasmid profiling with results of other typing methods. Purification of plasmids can be obtained by several rapid methods, with Kado and Liu (1981) as one of the commonly used methods.

Restriction Fragment Lenght Polymorphism (RFLP) of the chromosomal DNA from Salmonella covers a group of typing methods where the position of different DNA fragments in the genome are detected. The detected DNA fragments used as probes can either represent specific sequences as in ribotyping (rRNA-gene) or IS200-typing (specific IS-sequence) or random DNA sequences of unknown significance. The discriminatory power of either of these methods strongly depends on the rRNA gene probe, the enzyme used for restriction of the whole DNA and, the population of salmonella strains investigated (Schwarz and Liebisch 1994a, Millemann et al. 1995). A general problem of these and other electrophoretic methods is, however, the difficulties in the interpretation of gels between different laboratories.

Ribotyping has frequently been used for typing of Salmonella, however in most cases the discriminatory power has been relatively low (Schwarz et al. 1997).

IS200 typing is basically a detection of restriction fragment length polymorphisms of IS 200 (insertion sequence 200) carrying psrl-fragments of whole DNA. The number of IS 200 elements vary between serovars and between strains of the same serovar. IS200 typing has proven to be highly discriminatory for Typhimurium (Schwarz and Liebisch 1994a, Stanley et al., 1993), whereas the method is of limited value for typing of Enteritidis. S. Dublin only harbours one IS 200 element (Olsen and Skov, 1994) while Choleraesuis has none (Weide-Bootjes et al., 1996), IS 200 typing is consequently of no value for those serotypes.

Macrorestriction analysis is a method for separation of large restriction fragments of whole DNA in pulsed-field gel electrophoresis (Lai et al., 1989) with subsequent comparison of the fragment patterns. High discriminatory power has been obtained for Typhimurium, Choleraesuis, Enteritidis and Dublin (Schwarz et al. 1997). The comparative use of more than one suitable enzyme has shown to increase the discriminatory power of the method. The enzymes which gave the best result in macrorestriction analysis differed from serotype to serotype.

Macrorestriction analysis fulfil the majority of the earlier mentioned requirements for typing methods (Liebisch and Schwarz 1996, Schwarz and Liebisch 1994b, Weide-Bootjes et al. 1996). But even though a large amount of work has been carried out in order to standardize the method, it is not yet possible to compare results between laboratories which is a precondition for the use as a definitive typing system.

In the future new typing systems e.i. highly discriminatory PCR based methods will be developed. These methods allow automatical reading of results and storage of data directly into large databases giving the possibility of comparison to previously analyzed isolates.
Serological detection of salmonella infection

Salmonella infection in animals and humans generally elicit an antibody response towards a variety of Salmonella antigens. This antibody response can be detected by use of ELISA's. Several ELISA's have been developed for detection of Salmonella in poultry and cattle (Hassan et al. 1990, Nicholas and Andrews 1991, Van Zijderveld et al. 1992, Smith et al. 1989, Hoorfar and Wedderkopp 1995) since these species have been recognized as sources of salmonella infection for a longer period. Only a few ELISAs for detection of salmonella infection in swine have been published so far (Nielsen et al. 1995, Srinand et al. 1995, Gray et al. 1996). The studies by Srinand et al. (1995) and Gray et al. (1996) describe ELISAs for detection of S. Choleraesuis infection in experimentally infected pigs in USA. Srinand et al. (1995) used LPS and OMP as antigens and found that both antigens were suitable for detection of the serological response. Gray et al. (1996) similarly used purified LPS and mixed soluble antigen. Antibody response was elicited against both antigens, however the highest response was observed against the soluble antigen. None of the mentioned Choleraesuis ELISAs have, to our knowledge, been evaluated on field material for evaluation of cross reaction.

The ELISA published by Nielsen et al. (1995), designated the mix-ELISA, contains the O-antigens 1,4,5,6,7, and 12. The mix-ELISA makes it possible to detect infection with 95% of the Salmonella serotypes found in Danish pigs (Nielsen et al. 1996). The mix-ELISA has been used for routine screening of breeding, multiplying and slaughter pigs herds in Denmark since 1993 and 1994, respectively. The screening of breeding and multiplying herds is done by ordinary blood sampling using serum in the mix-ELISA, whereas the slaughter pig herds screening is done by examination of meat juice in the mix-ELISA. At the slaughterline, 10 grammes of muscle tissue are taken from the carcass and placed into a container. The container is frozen at -20°C overnight, and subsequently allowed to thaw at 4°C for 24 hours, thereby passively releasing the antibody containing meat juice. The meat juice is diluted 1:30 and examined in the mix-ELISA (Nielsen et al. 1997). The use of meat juice as an antibody source has also been demonstrated for serological detection of Toxoplasma gondii infection in swine (Wingstrand et al. 1997).
Table 1. Principles of conventional culture for Salmonella

<table>
<thead>
<tr>
<th>Culture step</th>
<th>Commonly used components</th>
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<tr>
<td>1. Non-selective pre-enrichment</td>
<td>Buffered Peptone Water (BPW)</td>
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<td>2. Selective enrichment</td>
<td>Rappaport Vassiliadis broth (RV)</td>
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<td>Rappaport Vassiliadis Soya broth (RVS)</td>
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<td>Modified Semi-solid Rappaport Vassiliadis (MSRV)</td>
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<td>Selenite broth</td>
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<td>Selinite brilliant green broth</td>
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<td>Tetrathionate broth</td>
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<td>Tetrathionate brilliant green broth</td>
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<td>Hajna Tetrathionate</td>
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<td>3. Plating agars</td>
<td>Brilliant green agar (BGA)</td>
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<td>Desoxycholate Citrate agar (DCA)</td>
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<td>Rambach agar</td>
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<td>Xylose lysine deoxycholate (XLD)</td>
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<td>Xylose lysine tergitol 4 (XLT4)</td>
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<td>4. Verification</td>
<td>Biochemistry</td>
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<td>Triple Sugar Iron agar</td>
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<td>Rapid test systems (API, Enterotube etc.)</td>
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<td>5. Identification</td>
<td>Biochemistry and serotyping</td>
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Table 2. Typing methods used on Salmonella

- Serotyping
- Phage typing
- Antibiotic resistance
- Plasmid analysis
- RFLP
- Ribotyping
- IS200
- Macrorestriction analysis (PFGE)
References


