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Sensitivity and Specificity of Different Methods for the Isolation of *Salmonella* from Pigs

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Bager, F. and J. Petersen: Sensitivity and specificity of dfferent methods for the isolation of *Salmonella* from pigs. Acta vet. scand. 1991, 32, 473–481. – Three different selective enrichment media, Rappaport-Vassiliadis broth (RV), selenite broth (SB) and Müller-Kauffmann tetrathionate broth (MKTB), in combination with plating on modified brilliant green agar (BGA), were compared for the isolation of *Salmonella* from samples of pig feces. These conventional methods were also compared with a new ELISA kit in conjunction with RV and SB enrichment. Of the conventional methods, enrichment in RV had a higher sensitivity and selectivity than SB and MKTB. Recovery of *S. typhimurium* from MKTB was significantly poorer than recovery of other serotypes. The combination of RV enrichment and ELISA was as good as the conventional method involving RV enrichment, with a similar high sensitivity and specificity.

microbiological methods; ELISA; RV; rapid methods.

Introduction

Epidemiological investigations of disease depend heavily on the availability of accurate means of discriminating between diseased and non-diseased individuals.

In this respect, investigations of *salmonella* in domestic animals are bedeviled by a number of factors, including the variable sensitivity of current microbiological technique (*Harvey et al.* 1977, *Bailey et al.* 1981, *Vassiliadis et al.* 1981). The intermittent fecal shedding of low numbers of *salmonella* bacteria by symptomless carriers constitutes a different, but related problem (*McCall et al.* 1966, *Haddock* 1970). An isolation procedure that is capable of detecting *salmonellae* when these are present in feces in large numbers during clinical salmonellosis may not be sufficiently sensitive to permit detection of the small numbers shed by symptomless carriers.

Tetrathionate broths are widely used as enrichment media in standard methods, although the exact formulation and preparation can vary markedly. The principal selective agent in the medium is the tetrathionate ion, usually in synergism with incubation at elevated temperatres at 42 or 43 °C. However, the presence of membrane-bound reductase in *Citrobacter, Serratia* and *Proteus* genera, in addition to *Salmonella,* provides some resistance to the toxic effects of tetrathionate and results in reduced selectivity (*D'Aoust* 1989). Furthermore, some modifications of tetrathionate broth, notably the Müller-Kauffmann tetrathionate brilliant green broth very widely used in Europe, has been associated with disturbing levels of toxicity to *Salmonella* (*D'Aoust* 1989).

Another very widely accepted group of enrichment media for *Salmonella* is the family of selenite broths. The selectivity of the unmodified selenite medium is based on the differential uptake of toxic selenite and on the presence of selenopolythionates formed

in the liquid medium. The uptake of selenium is generally more rapid by non-salmonellae and incorporation in cell proteins as a sulphur analog hinders proliferation of the competitive microflora. However, as is the case with tetrathionate broth the selectivity of the selenite medium is limited, as has been documented in a number of reports (*D'Aoust* 1989).

In contrast to the 2 preceding groups of enrichment media, Rappaport broth as modified by *Vassiliadis* (1983) is a relatively recent innovation. This medium has achieved eminence because it ensures excellent recovery of salmonellae, combined with a high selectivity. The performance of the Rappaport-Vassiliadis (RV) broth is based on the ability of *Salmonellae* to multiply at a high osmotic pressure and at a low pH-value and on the resistance of *Salmonellae* to malachite green (*Peterz et al.* 1989).

In addition, the broth is usually incubated at elevated temperatures at 42 or 43 °C. It is critical that the inoculum is small, usually at the ratio of 1:100, as heavier inoculation compromises both the selectivity and the sensitivity of the medium (*Vassiliadis et al.* 1981, *D'Aoust* 1989). It should be noted that a recent publication reported that some commercial preparations of RV broth have proved to be more inhibitory to *Salmonellae* than the original preparation by *Vassiliadis,* and therefore lack sensitivity (*Peterz et al.* 1989).

A drawback common to all conventional *Salmonella* isolation procedures is the time elapsed before results of the analyses are available, at least 84–96 h. For this reason, considerable efforts have been invested in the development of techniques that permit rapid and reliable detection of *Salmonella.* Techniques under investigation include impedance and conductance measurements, use of salmonella-specific bacteriophages, DNA-probes and a variety of immunological methods, including ELISA (Enzyme-Linked Immuno Sorbent Assay) techniques. Of these rapid methods, the ELISA technique is probably closest to gaining wide acceptance as routine diagnostic tests. The ELISA technique is based on the detection of *Salmonella* antigens by polyclonal or monoclonal antibodies that are conjugated with an enzyme. After incubation with an enzyme substrate, the resulting colour reaction is measured in a photometer at a wavelength specific to the combination of enzyme and substrate.

The present study was designed to compare the ability of the MKTB, SB and RV media to isolate *Salmonella* from samples of pig feces naturally contaminated with low numbers of these bacteria, and to compare traditional isolation of salmonella on brilliant green agar with detection by a new commercial ELISA kit. The objective of the study was to get an estimate of the sensitivity and specificity of the techniques for use in epidemilogical investigations.

Materials and methods

Samples

A total of 373 fecal samples were obtained from 4 different pig herds, where outbreaks of clinical salmonellosis had previously been diagnosed. The outbreaks involved only a small number of animals in each herd. At the time of sampling no animal was showing sign of clinical disease. The samples were transported chilled to the laboratory and processed the same day.

Microbiological analysis

Media. Fecal samples of 25 g were inoculated in 225 ml of buffered peptone water (BPW) (Merck 7228) for pre-enrichment and incubated at 37 °C for 16–18 h. Following pre-enrichment each sample was inoculated

in the following selective media: Selenite broth 1:9 (SB) (Merck 7717); Müller-Kauffmann tetrathionate broth 1:9 (MKTB), (Merck 10863); Rappaport-Vassiliadis broth (0.1:9.9 (RV), prepared according to *Vassiliadis* (1983). SB was incubated at 37 °C, MKTB at 43 °C and RV at 42 °C.

After selective enrichment for 24 and 48 h, material was inoculated on Modified Brilliant Green agar (BGA; Oxoid CM 329). *Salmonella* suspect colonies were inoculated on Triple-Sugar Iron (TSI; Merck 3915, Lysine-Iron agar (LIA; Merck 11640) and on Prilane Sorbitol agar (PSA).

PSA was prepared as recommended by *Lund* (1990):

Substrate base: 15 g Bacto agar (Difco); 10 g Bacto peptone (Difco); 5 g beef extract (Difco); 1 l deionized water. Phenol red 0.1 %: 1 g phenol red (Merck 7241) is dissolved in 40 ml 0.1 N NaOH by heating in boiling water. Distilled water is added to a total volume of 1000 ml.

The finished substrate is prepared by adding to each 1000 ml of substrate base: 80 ml phenol red 0.1 %; 2 ml brilliant green (Merck 1374); 2 g of freshly boiled and cooled Teepol 610 (Serva Fine Biochemica); 50 ml of a sterile 40 % sorbitol solution. pH is adjusted to 7.0–7.2.

Salmonella produces green or, very rarely, red colonies.

Isolates that produced red/yellow reaction with gas formation and with or without H_2S production in TSI, and which gave a violet/violet reaction with gas formation and with or without H_2S production in LIA, and which formed green colonies on PSA, were sent to the National Veterinary Laboratory for verification and serotyping.

ELISA procedure. The ELISA test used a recently developed kit (FastScreen Elisa Kit[1]). The test is based on detection of *Salmonella* with polyclonal antibodies, conjugated to peroxidase. Sub-samples for the ELISA test were obtained from the SB and RV broths used in the conventional procedure, after 24 and 48 h incubation. The bacteria were destroyed by boiling the sub-samples for 20 min. After cooling, 100 µl of sub-sample were pipetted into microtitre wells coated with the polyclonal salmonella antibodies and the wells incubated for 1 h at 35–37 °C. The wells were washed and 100 µl of a solution containing antibody-peroxidase conjugate were added followed by incubation for 1 h at 35–37 °C. After washing, 100 µl of peroxidase substrate (ABTS: 2,2 AzinoBis (3-ethylbenzThiazolineSulfonic acid) were pipetted into each well, followed by incubation at room temperature for 30 min. The enzymatic reaction was stopped by adding 25 µl of 1.25 % NaF, and the resulting colour reaction was measured photometrically at 405 nm on a TIM 10 photometer[2].

The samples were analyzed in replicate, and each series of tests incorporated positive and negative controls, as well as blinds.

Data evaluation

All data were processed using a statistical software package (*SAS* 1988). Results obtained by the enrichment procedure, followed by conventional biotyping were tabulated against the true *Salmonella* status of the sample. The true status was defined as *Salmonella* positive when *Salmonella* was isolated from a sample by at least 1 of the 3 enrichment media after either 24 or 48 h enrichment and plating on BGA. The sensitivity of each method was calculated as the number of salmonella positive samples detected by that method, divided by the total number of true positive samples. The selec-

[1] Bioteknisk Institut, Holbergsvej 10, DK-6000 Kolding, Denmark.

[2] Cambridge Life Science, Cambridge, UK.

tivity was calculated for each enrichment broth as the number of samples classified as salmonella negative on BGA, divided by the total number of verified true negative samples, i.e. negative in all enrichment media. The selectivity was therefore calculated in the same way as specificity (*Martin et al.* 1987). However, "selectivity" refers to the enrichment step only, while "specificity" refers to the entire isolation procedure.

For the ELISA test the optical density of the blind controls was subtracted from each sample reading, and the mean of each pair of replicates calculated to give an optical density for each sample tested. In order to establish the optimum sensitivity and specificity of the ELISA test in combination with the RV and SB enrichment media, the samples were classified as *Salmonella* positive or negative by increasing cut-off values of optical density. For each cut-off value, the results of the test were tabulated against the true sample status as defined above, and the sensitivity and specificity calculated and plotted graphically against the optical density. A smooth line was fitted to the points using a cubic spline. The statistical tests used were McNemar's test for paired comparisons, and, for unpaired observations, Fisher's exact test for differences between proportions.

Results and discussion

Conventional methods

The optimum method for the isolation of *Salmonella* in an epidemiological investigation should have a high sensitivity (few false negatives) and also be highly specific that is, produce few false positive isolates. However, the combination of sensitivity and specificity deemed adequate for a particular investigation also depends on the prevalence of the disease under investigation. Estimation of the true prevalence of a low prevalence disease requires a method with a higher sensitivity than does a disease with a high prevalence, in which case a high specificity may be more desirable.

Conventional microbiological *Salmonella* tests consist of a number of steps, each of which has the objective of eliminating bacteria that may appear to be *Salmonella,* but which are not. That is, each step produces a number of false positive isolates. At the final step, an isolate that has been verified and serotyped as *Salmonella* is, by definition, a true positive. That is, the specificity of the entire procedure is 100 %, and these pathognomonic methods are characterized by differences in sensitivity alone.

For this reason, the selectivity of the enrichment media was compared on the basis of the presence or absence of *Salmonella* suspect colonies on BGA. The 3 conventional methods differed only in the enrichment step, and the selectivity as calculated becomes a surrogate measure of the specificity of the methods. The selectivity is a utility measure of considerable practical value. A high selectivity implies that little laboratory effort will be expended on isolates that turn out not to be *Salmonella.* In other words, it is a measure of the economy of the particular enrichment methods.

One advantage of using naturally contaminated material for the present evaluation is that it avoids the bias associated with artificial inoculation of sample material with *Salmonella* strains previously isolated by 1 of the methods being compared.

However, bias could still arise if 1 of the methods showed enhanced recovery of particular serotypes of *Salmonella.*

Of the 4 pig herds sampled, 1 herd proved to be *Salmonella* negative in all samples. Six different serotypes were isolated from the remaining 3 herds (Table 1). The routine diagnostic microbiological tests following the

Table 1. *Salmonella* serotypes isolated from 115 fecal samples.

Serotype	No. isolates
S. typhimurium	57
S. 4:12:b	27
S. give	21
S. senftenberg	10
S. derby	1
S. agona	1
Total no. isolates	117*

* 2 samples each contained 2 serotypes.

outbreaks of clinical disease had detected only 3 of them, namely *S. typhimurium, S. give* and *S. 4.12:b.*

Salmonella was isolated from 112 samples out of 373 after 24 h incubation, and from only 105 when the enrichment media were re-examined after 48 h. A total of 115 samples were salmonella positive in at least one of the media after either 24 or 48 h incubation. All other 258 samples were salmonella negative.

Sensitivity

Table 2 shows data on the 115 *Salmonella* positive fecal samples, examined by enrichment followed by traditional plating. The comparison found a marked difference in the sensitivity of the enrichment media. With 24 h incubation at 42 °C, the RV broth was able to detect 88 % of the samples that contained salmonella, compared with 51 % and 30 % for SB and MKTB, respectively ($p < 0.001$). Swarming by *Proteus* spp. was a considerable problem on BGA inoculated from MKTB and, to a lesser extent, from SB, so that salmonella colonies actually present may have been missed, compounding the effect of the low sensitivity of these media. Therefore, the poor performance of these substrates, and of MKTB in particular, may be regarded in part as a reflection of their low selectivity, compared with RV broth.

It is evident from Table 2 that the sensitivity of SB and MKTB was improved with plating on BGA at both 24 and 48 h ($p < 0.001$). This increased the sensitivity from about 0.50 and 0.30 to 0.64 and 0.44, respectively. In contrast, repeated plating from RV produced results that did not differ statistically from plating after 24 h only.

Table 3 shows the recovery of each serotype of *Salmonella* from each of the enrichment media. 'Other' serotypes include *S. senftenberg, S. agona* and *S. derby.* SB isolated all the serotypes equally well, while RV showed a decreased recovery of other *S. give* ($p < 0.01$) compared with the recovery of serotypes in this medium. In contrast, MKTB

Table 2. Recovery by 3 different enrichment broths of *Salmonella* from 115 *Salmonella*-positive fecal samples. After non-selective enrichment, each sample was inoculated in selenite broth (SB), Müller-Kauffmann tetrathionate broth (MKTB) and Rappaport-Vassiliadis broth (RV) and plated on modified brilliant green agar after 24 and 48 h incubation.

Salmonella	24 h			48 h			24 or 48 h		
	SB	MKTB	RV	SB	MKTB	RV	SB	MKTB	RV
+	59	34	101	55	36	96	73	51	103
–	56	81	14	60	79	19	42	64	12
Sensitivity	0.51	0.30	0.88	0.48	0.31	0.84	0.64	0.44	0.90

Table 3. Comparison of the sensitivity of RV, SE and MKTB with respect to different serotypes. The group 'Other' includes *S. senftenberg*, *S. derby* and *S. agona*.

	Serotype			
Medium	4.12:b	Give	Typhimurium	Other
RV	0.89	0.71**	0.95	1.00
SB	0.62	0.57	0.50	0.69
MKTB	0.46	0.91***	0.26***	0.50
n	26	21	58	10

** and *** indicates a statistically significant difference at the $p \leq 0.01$ and $p \leq 0.001$ level, respectively. See Table 2 for abbreviations.

showed enhanced recovery of *S. give* ($p < 0.001$) and a very marked reduction in the recovery of *S. typhimurium* ($p < 0.001$). This low recovery of *S. typhimurium* may be a result of the toxicity of MKTB described by *D'Aoust* (1989), and contributed to the overall low sensitivity of MKTB.

The prejudice of RV against *S. give* is relatively unimportant, as RV still has a relatively high sensitivity, even to this serotype. The prejudice of MKTB against *S. typhimurium* is a matter of greater concern, when the public health importance of this serotype is taken into consideration.

In a similar investigation, *Munch & Larsen* (1990) compared methods for the isolation of salmonella from effluent. These authors found the sensitivity of commercial preparations of RV, SB and MKTB to be 0.63, 0.39 and 0.44, respectively, using an incubation temperature of 43 °C and plating on BGA after 24 and 48 h. The low sensitivity of commercially prepared RV compared with the results of the present investigation, where home-made RV broth was used, appears to confirm the finding of *Peterz et al.* (1989) that some commercial preparations of RV are not as sensitive as the original preparation by *Vassiliadis* (1983). The sensitivity of SB found by *Munch & Larsen* (1990) is also considerably lower than the one found in the present work. However, as different commercial preparations of the broth were used in the 2 investigations, in addition to different incubation temperatures, the reasons for this discrepancy cannot be readily established.

Furthermore, composition of the samples with respect to salmonella serotypes will affect the overall sensitivity of the media. The results of *Munch & Larsen* (1990) confirm the low sensitivity of MKTB to *S. typhimurium* found in the present investigation. On the other hand, we found the sensitivity of RV to *S. 4.12:b* to be as high as the overall sensitivity, while *Munch & Larsen* (1990) found it to be low. Therefore, direct comparisons between different investigations must be made with caution.

Selectivity

The higher the selectivity of a particular enrichment broth, the lower the number of false positives relative to the total number of true negative samples.

The selectivity of the enrichment media, calculated as the proportion of samples that were salmonella negative on BGA to the total number of true negative samples, is shown in Table 4. It is apparent that SB and MKTB broth produced more false positive salmonella colonies on BG agar, than did

Table 4. Selectivity of SB, MKTB and RV enrichment, calculated as the proportion of samples that were *Salmonella* negative on brilliant-green agar to the total number of true negative samples. See Table 2 for abbreviations.

Incubation time	SB	MKTB	RV
24 h	0.87	0.82	0.97
48 h	0.85	0.78	0.99

RV broth ($p < 0.001$). RV broth is, therefore, better than SB and MKTB at suppressing competing bacteria, a conclusion shared by *Vassiliadis* (1983) in a review of culture methods. In other words: in the present material RV broth, incubated at 42 °C was able to detect more salmonella positive samples than either SB or MKTB, incubated at 37 °C and 43 °C, respectively. The RV also had a higher selectivity with the result that a higher percentage of the isolates were true positives.

ELISA test

A total of 150 samples were examined by ELISA after 24 and 48 h incubation; an additional 66 samples were examined after 48 h only. Calculations of sensitivity and specificity are based on the 150 paired samples only.

In contrast to the traditional microbiological techniques, the sensitivity and specificity of the ELISA test depends on a subjective decision on how high an optical density is required for a sample to be regarded as salmonella positive. The results of calculating the sensitivity and specificity for increasing cut-off values of optical density are displayed graphically in Fig. 1.

In the present case, RV/ELISA is superior to the combination of SB/ELISA: For RV there is a relatively wide range of cut-off values

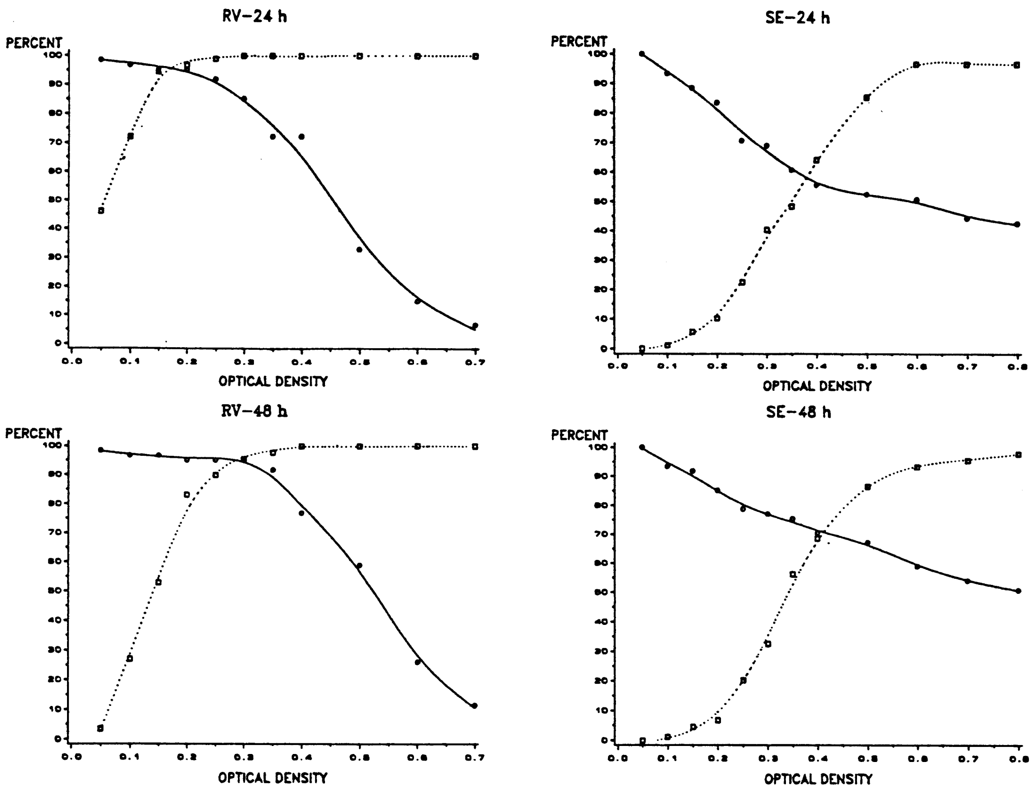

Figure 1. Sensitivity and specificity of the ELISA test after enrichment in RV and SB for 24 and 48 h, plottet against optical density. · · · · · · : Specificity; ——— : Sensitivity.

where *both* sensitivity and specificity are high. No such range is apparent in the curve for SB. The present evaluation of the ELISA test is an evaluation of the kit, in conjunction with the 2 different enrichment media used. It is apparent that the performance of the ELISA kit is very much influenced by the selection of enrichment medium: the RV broth is superior to selenite in terms of both sensitivity and selectivity. This is in agreement with the results from the comparisn of these media in the conventional methods. Appropriate selection of cut-off point when the combination SB/ELISA is used will produce a sensitivity as high as for RV/ELISA, but at the expense of a very low specificity. This is in accordance with the low selectivity of selenite broths mentioned by *D'Aoust* (1989). In practical terms, with a low specificity considerable laboratory effort would be expended, attempting to isolate for serotyping *Salmonella* from samples that were false positives in the ELISA test.

In conclusion it may be stated that of the 3 enrichment media compared in this evaluation, the RV broth, incubated at 42 °C, gave considerably better recovery than did the selenite and tetrathionate broths, incubated at 37 °C and 43 °C, respectively. The RV also exhibited a higher selectivity, with fewer false positive isolates on BGA. If SB or MKTB are to be used, plating on BG after 24 as well as 48 h incubation is recommended to increase recovery, while for RV, 24 h is sufficient. The MKTB broth appears to be somewhat toxic to the strain of *S. typhimurium* found in this investigation, with a sensitivity that is significantly lower than to the other types found.

The combination RV/ELISA has a similar high sensitivity and specificity to that of the traditional combination of RV enrichment with isolation of salmonella on BGA.

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References

Bailey JS, Cox NA, Thomson JE: Efficiency of selenite cystine and TT enrichment broths for the detection of *Salmonella.* J. appl. Bacteriol. 1981, *51,* 409–414.

D'Aoust JY: *Salmonella.* In: Doyle MP (ed): Foodborne Bacterial Pathogens. Marcel Dekker, New York 1989.

Haddock RL: Efficacy of examining rectal swabs to detect swine Salmonella carriers. Amer. J. vet. Res. 1970, *31,* 1509–1512.

Harvey RWS, Price TH, Morgan J: Salmonella surveillance with reference to pigs – Cardiff abattoir, 1968–1975. J. Hyg. (Camb.) 1977, *78,* 439–448.

Lund C: Personal communication, 19 . ..

Martin SW, Meek AH, Willeberg P: Veterinary Epidemiology. Iowa State University Press, Ames, Iowa, USA 1987, 367 pp.

Munch B, Bonde Larsen A: Veterinær forskning, overvågning og rådgivning i forbindelse med etablering og drift af biogasfællesanlæg. (Veterinary research, surveillance and councelling in relation to the establishment and operation of communal biogas digestors). Report to the Danish Veterinary Service, 1990, 188 pp.

Peterz M, Wiberg C, Norberg P: The effect of incubation temperature and magnesium chloride concentration on growth of *Salmonella* in homemade and in commercially available dehydrated Rappaport-Vassiliadis broths. J. Appl. Bacteriol. 1989, *66,* 523–528.

SAS Institute Inc.: SAS/STAT User's guide, Release 6.03 Edition. SAS/Graph User's guide, Release 6.03 Edition.

Vassiliadis P, Trichopoulos D, Papadakis J, Kalapothaki V, Zavitsos X, Serie C: *Salmonella* isolation with Rappaports's enrichment medium of different compositions. Zbl. Bakt., I. Abt. Orig. B. 1981, *173,* 382–389.

Vassiliadis P: The Rappaport-Vassiliadis (RV) enrichment medium for the isolation of *Salmonellas:* An overview. J. Appl Bacteriol. 1983, *54,* 69–76.

Sammendrag

Sensitivitet og specificitet af forskellige metoder til påvisning af salmonellabakterier fra svin.

Tre salmonella opformeringsmetoder, Müller-Kauffmann tetrathionat (MKTB), selenit bouillon (SB) og Rappaport-Vassiliadis bouillon (RV), inkuberet ved henholdsvis 43°C, 37°C og 42°C, blev sammenlignet med hensyn til sensitivitet og selektivitet ved undersøgelse af gødningsprøver fra svin. Efter selektiv opformering blev salmonella påvist, dels traditionelt ved udsæd på modificeret brilliantgrøn agar (BGA), og dels immunologisk ved hjælp af et nyt kommercielt ELISA kit. RV havde en statistisk signifikant højere sensitivitet og selektivitet end både SB og MKTB. Opformering i MKTB gav en statistisk signifikant lavere påvisning af *S. typhimurium* end af andre serotyper. Den anvendte ELISA teknik, kombineret med opformering i RV havde en lige så høj sensitivitet og specificitet som ved traditionel udsæd fra RV-mediet.

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