J. Hyg., Camb. (1985), **95**, 69–75
Printed in Great Britain

Salmonellas in Danish pigs: a comparison of three isolation methods

By NIELS SKOVGAARD, SVEN GADE CHRISTENSEN
Institute of Hygiene and Microbiology, Royal Veterinary and Agricultural University, Copenhagen, Denmark

and A. W. GULISTANI
Faculty of Veterinary Science, University of Kabul, Kabul, Afghanistan

(*Received* 20 *December* 1984; *accepted* 31 *January* 1985)

SUMMARY

Caecal samples from 350 Danish bacon pigs were investigated for salmonella using three methods of isolation. (1) Direct inoculation of 1 g of faeces into 10 ml of Muller–Kaufmann medium (MK medium) with addition of 0·3 % Teepol 610 and subculture on Brilliant Green lactose sucrose phenol-red agar (BLSF agar) with 0·3 % Teepol 610. (2) Pre-enrichment of 5 g of faeces into buffered peptone water with addition of 1 % Teepol 610 followed by enrichment of 1 ml in 10 ml MK medium with 1 % Teepol 610 and subculture on BLSF agar with 0·3 % Teepol. (3) Incubation of 0·1 ml of the pre-enrichment (2) into 10 ml Rappaport–Vassiliadis medium (RV 10 medium) incubated at 43 °C, subculture on BLSF agar.

The MK media with and without pre-enrichment yielded higher findings than the RV 10 media. In total, 28 (8 %) of the pigs were found positive, representing 11 (7·4 %) of a total of 142 herds investigated. Lymph glands were collected at a later date from six of the positive herds. Five of the herds were found positive.

The number of salmonellas in the glands was low, probably less than ten per gram.

INTRODUCTION

In 1971 a joint survey on the incidence of salmonella in pigs and feeding stuffs in England and Wales and in Denmark was made (Skovgaard & Nielsen, 1972). Some 3 % of caecal samples and 4 % of lymph node samples from Danish pigs were found positive.

Since these investigations were carried out, many proposals for improvement in the isolation technique for salmonella from highly contaminated material have appeared in the literature. Modifications of the original medium of Rappaport, Konforth & Navon (1956) have been of particular interest, using high incubation temperature and small inoculum doses to reduce the number of competitive bacteria and increase the possibility of isolating salmonellas (Vassiliadis *et al.* 1976; Harvey, Price & Xirouchaki, 1979; Vassiliadis *et al.* 1981; van Schothorst & Renaud, 1983; Harvey & Price, 1983).

In Denmark there has recently been an increase in salmonellosis in cattle herds caused by *Salmonella typhimurium* and *S. dublin* (Anon. 1981*a*; Anon. 1983).

The present study was undertaken to investigate whether the application of new isolation techniques has resulted in an increase of the incidence of salmonella in pigs, and whether the same tendency observed for the incidence of salmonella in the Danish cattle population was also the case in the pig population.

MATERIALS AND METHODS

On the slaughter line of a large co-operative bacon slaughterhouse on Scaland, the pig intestines were marked at the point of the meat inspection, corresponding to the herd numbers of the pigs as well as the slaughtering numbers. Every sample investigated could thus be traced back to the farm of origin. Faecal samples were collected by means of sterile knives and wooden spatulas from the caecum immediately after the evisceration, at a table situated next to the point of the meat inspection.

The media chosen for the investigation were inoculated on the spot and immediately transferred to the incubator. Samples were taken during seven visits to the slaughterhouse in the period December 1983 to April 1984. At each visit, 50 samples were taken, making a total of 350 samples. Samples were taken from not more than 5–6 pigs from the same farms. In total, pigs representing 142 herds were investigated.

From 5 of the 11 herds found to be salmonella-positive, an intact caecal gland was removed from pigs at one of the subsequent deliveries from the farms, within 1–2 weeks after finding the faeces sample positive.

Three methods of enrichment were compared.

(1) Inoculation of 1 g faeces in 10 ml of Muller–Kaufmann tetrathionate broth (MK broth) (Anon. 1981*b*) to which 1 % Teepol 610 (BDH) had been added, and incubated for 24 h at 37 °C. Subcultures were made on Brilliant Green lactose sucrose phenol-red agar (BLSF agar) (Anon. 1981*b*) with the addition of 0·3 % Teepol 610 to prevent swarming. The medium was prepared at the Institute. Brilliant Green agar for microbiology (Merck art. 1319) was used. The ingredients were added to the base according to the ISO formula.

(2) Inoculation of 5 g faeces into 50 ml of buffered peptone water (Anon. 1981*b*) with the addition of 1 % Teepol 610 incubated for 16–20 h at 37 °C. One millilitre of the pre-enrichment broth was transferred to 10 ml of MK medium with 0·3 % Teepol 610 and incubated at 37 °C for 24 h. Subculture was made on BLSF agar with the addition of Teepol 610 as above.

(3) From the buffered peptone enrichment broth, mentioned under (2), 1 ml was transferred into the RV 10 medium, and incubated at 43 °C for 24 h (Vassiliadis, 1983). The medium was prepared at the Institute. Malachite green oxalate GR (Merck art. 1398) was used. Subcultures were made on BLSF agar without Teepol 610. Typical colonies were inoculated on to slanted triple sugar iron agar (Anon. 1981*b*) and at the same time stabbed into iron-sulphite agar tubes (Oxoid, no. CM 79). Salmonella-suspected cultures, that is, cultures which showed blackening of the iron-sulphite agar and red surface, yellow bottom, and gas formation with and without blackening in the triple sugar iron agar, were more fully identified by slide agglutination tests.

The surfaces of the caecal glands were sterilized by immersion in boiling water

Table 1. *Survey of positive findings using three different methods for isolation of salmonella*

	Method 1, MK broth, 37 °C	Method 2, buffered peptone water +MK broth 37 °C	Method 3, buffered peptone water +RV 10, 43 °C	Overall total (All three methods combined)
Number of positive animals	21 (6 %)	22 (6·3 %)	14 (4 %)	28 (8 %)
Total number investigated	350	350	350	350
Number of herds investigated	—	142	—	—
Number of positive herds	—	11 (7·7 %)	—	—

Table 2. *Investigation of caecal glands from pigs originating from salmonella-positive herds*

Herd no.	Number of glands investigated	Number of salmonella-positive glands
1	5	1
2	5	0
3	8	2
4	9	6
5	7	1
6	8	1

for approximately 5 s, and 1 g of an aseptically drawn sample was inoculated into 10 ml MK medium and incubated at 37 °C for 24 h. From a smaller number of glands, 0·1 ml of lymph fluid was inoculated directly on the surface of BLSF agar without addition of Teepol 610 for enumeration of salmonella eventually found.

RESULTS

Table 1 summarizes the salmonella findings from the three different methods of isolation. Approximately the same number of salmonella was detected by using direct inoculation of approx. 1 g faeces into MK broth, incubated at 37 °C (method 1) and pre-enrichment of 5 g faeces in buffered peptone water followed by enrichment of 1 ml in MK broth (method 2) with the addition of 1 % Teepol 610 to suppress the swarmers. By the two methods, 21 (6 %) and 22 (6·3 %) animals respectively, of a total number of 350 pigs, were found salmonella-positive.

Fewer positive samples (14 (4 %)) were detected when inoculating 0·1 ml of the buffered peptone water enrichment into the Rappaport–Vassiliadis medium, incubated at 43 °C (method 3).

In total, 28 positive animals were detected, or 8 % of the investigated animals, by using either of the three methods. Three of the 28 positive animals were demonstrated in the Rappaport–Vassiliadis medium only.

The 28 positive animals represented 11 herds of a total of 142 investigated, corresponding to 7·7 % positive herds.

https://doi.org/10.1017/S002217240006229X Published online by Cambridge University Press

Table 3. *Salmonella serotypes isolated*

Serotypes	Number of positive animals	Number of positive herds
S. typhimurium (*1*, 4, (5), 12:i:1.2)	13	6
S. abony v. haifa (*1*, 4, (5), *27*:b:÷)	9	4
S. derby (*1*, 4, (5), 12:f, g:1.2)	3	1

When two or more animals were examined, half or more of the animals from the positive farms were found positive. One herd was found positive at random at two different sampling dates.

Table 2 shows the findings of salmonella in caecal lymph nodes. Four of five herds were found to harbour salmonellas in the lymph nodes. We did not look for salmonellas in caecal contents when the lymph nodes were examined.

DISCUSSION

The major problem in isolation of salmonella from contaminated material is the inhibition of the competing organisms. When subculturing on solid media, swarming caused by proteus may especially cause trouble. The RV medium is claimed to be superior to most other media in suppressing competing organisms (Vassiliadis, 1983; Papadakis & Efstratiou, 1980; Tongpim *et al.* 1984). Although incubated at 43 °C, growth of swarming bacteria, (*Proteus* species), was very common on BLSF agar using the RV 10 medium. When salmonellas were found on these plates they were, however, usually present either in pure culture or nearly pure culture.

We have for many years made use of anionic active compounds in either the enrichment media and/or the solid media used for isolation of salmonella from contaminated material, adjusting the content of anionic compound according to the load of bacteria in the material to be investigated. In the present investigation 1 % Teepol 610 was added to the buffered peptone water and 0·3 % to the BLSL agar used for subculturing from the MK broth by methods 1 and 2, and better results were obtained using these methods than with the RV medium (method 3).

The addition of the anionic detergent Teepol 610, which is a sodium alkyl sulphate compound, prevents proteus from swarming. The prevention of swarming by using anionic surface active agents as well as neutral surface active agents has been well described by several workers (Andersen, 1957; Jameson & Emberley, 1956).

Andersen considered the effect of anionic active compounds against swarming to be caused by protein denaturation acting on the proteins of the flagella. Doll (1956) showed the effect to be caused by a partly damaging effect on the ciliary apparatus of the bacteria. It has no effect against salmonella. On the contrary, it seems to enhance the growth on solid media by promoting transportation of energy compounds into the cells. Members of the salmonella group produce appreciable amounts of acyl-sulphatase which enables them to utilize the detergent as a source of energy (Köhler, 1961). Colonies of salmonella on BLSF agar with the addition of Teepol 610 often appear irregular and large compared with growth on the medium without the addition of Teepol 610.

The RV medium has a rather low pH, approximately 5·2 (Vassiliadis, 1984, personal communication), which is close to the minimum pH observed to allow growth of many salmonella strains (Chung & Gopfert, 1970; Corlet & Brown, 1980) and must be considered less favourable for propagation of salmonella strains, especially sublethally impaired ones. The low inoculation dose of 0·1 ml recommended for the RV medium is unfavourable for isolation of salmonella present in low number only in the buffered peptone enrichment broth. In the present investigation the use of 10-fold larger inocula improved the isolation rate, and MK broth was better than RV medium.

Tongpim *et al.* (1984), using both natural and contaminated foods, compared the MK medium and the RV medium, using different inoculum ratios (0·1:10, 1:10, 0·1:100 and 10:100 ml), and found after analysis of part of the samples the RV ratio 0·1:100 and MK 10:100 to be optimal. In the present investigation the RV ratio 0·1:10 was used. A ratio of 0·1:100 might have increased the number of positive samples. The general ratios recommended by these authors showed a significant difference in favour of RV 0·1:100 except for the naturally contaminated material. They conclude that at least in some cases the use of RV broth yields better results than that of the MK broth. They also recommend that the use of both of these media is advisable when meat products are examined for salmonella.

Kremastinou *et al.* (1982), when investigating meat products, found the RV 10 medium more sensitive in the isolation of salmonella compared with the MK medium; however, they did not use inhibitors in the pre-enrichment medium.

The number of salmonellas in the faeces samples was not investigated. The rather low percentage of positive lymph glands indicates that in general the number of salmonellas was low.

Attempts were made to enumerate the number of salmonellas in the lymph glands by inoculation of 0·01–0·1 ml tissue fluid on the surface of BLSF agar. Salmonellas were not isolated, indicating either that a very low number of salmonellas were present or that growth might have been prevented by inhibitory substances in the glands.

At the earlier investigation performed in 1969–70 (Skovgaard & Nielsen, 1972), 3% of the caecal faeces samples were found salmonella-positive. In the present investigation 8% of 350 samples were positive.

In the earlier series a slightly different methodology was applied: 10 g of faecal sample was transferred to Muller–Kaufmann tetrathionate broth to which 40 mg/l of the sodium salt of novobiocin was added, and subcultures were also made on BLSF agar without the addition of 0·3% Teepol 610. This methodology is close to method 2 in the present investigation, by which 6·3% positive animals were detected. These results indicate that the incidence of salmonellas in Danish pigs has doubled over the past 14 years. The present prevalence in the pig population is probably about 8%, and some 7% of the herds are positive. The herdwise distribution was not investigated in the earlier investigation. It has to be pointed out that the number of caecal salmonella carriers does not necessarily correspond with the number of faecal excretors (Haddock, 1970).

Three different serotypes were detected. *S. aboney varietas haifa* has not earlier been detected in pigs in Denmark, but has recently been isolated from feeding stuffs supplied to the farms in question (Nielsen, 1984, personal communication).

We thank Sven Skibsted, Chief Veterinary Officer, Forenede Sjaellandske Andelsslagterier, Ringsted, for kind support and continuous interest in the work, and Dr B. Brest Nielsen, State Veterinary Serum Laboratory, for having done some of the salmonella serotyping.

REFERENCES

ANDERSEN, F. M. (1957). Experiments with anion-active or non-ionised, surface-active synthetic compounds for the inhibition of the swarming of proteus bacteria. *Nordisk Veterinärmedicin* **9**, 543–559.

ANON. (1981*a*). SVS-Information (State Veterinary Serum Laboratory), July 1981.

ANON. (1981*b*). International Standard ISO 6579-1981. Microbiology – general guidance on methods for the detection of salmonella. International Organisation for Standardisation, Geneva.

ANON. (1983, 1984). VD sygdomsregistrering (Directorate for Veterinary Services: registration of diseases), juli–september 1983, september–december 1983, january–marts 1984, april–juni 1984.

CHUNG, K. C. & GOEPFERT, J. M. (1970). Growth of salmonella at low pH. *Journal of Food Science* **35**, 326–328.

CORLET, A. & BROWN, M. H. (1980). pH and acidity. In *Microbial Ecology of Foods*, vol. 1 (ed. in chief J. H. Silliker), pp. 92–111. London: Academic Press.

DØLL, W. (1956). Hemmung des Schwärmens von *Proteus*-Bakterien durch oberflächenaktive Substanzen (Prill und Rei). *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. I. Abteilung: Originale* **166**, 43–47.

HADDOCK, R. L. (1970). Efficacy of examining rectal swabs to detect swine salmonella carriers. *American Journal of Veterinary Research* **31**, 1509–1512.

HARVEY, R. W. S., PRICE, T. H. & XIROUCHAKI, E. (1979). Comparison of selenite F, Muller–Kaufmann tetrathionate and Rappaport's medium for the isolation of salmonellas from sewage-polluted natural water using a pre-enrichment technique. *Journal of Hygiene* **83**, 451–460.

HARVEY, R. W. & PRICE, T. H. (1983). A comparison of two modifications of Rappaport's enrichment medium (R25 and RV) for the isolation of salmonellas from sewage polluted natural water. *Journal of Hygiene* **91**, 451–458.

JAMESON, J. E. & EMBERLEY, N. W. (1956). A substitute for bile salts in culture media. *Journal of General Microbiology* **15**, 198.

KOHLER, W., GHATEK, S. N., RISCHE, H. & ZIESCHE, K. (1961). Arylsulfatasebildung durch Mikroorganismen, besonders durch *Salmonella paratyphi* B. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. I. Abteilung: Originale* **201**, 482–488.

KREMASTINOU, J., THERMOYANNI, ST., KALAPOTHAKI, V., MAVROMATTI, CH., VASSILIADIS, P. & SÉRÉ, CH. (1982). Improved isolation of salmonella from meat products with the use of Rappaport–Vassiliadis enrichment medium. *Archives de l'Institut Pasteur Hellénique* **XXVIII**, 27–36.

PAPADAKIS, J. A. & EFSTRATIOU, M. A. (1980). Isolation of salmonellae from fresh vegetables with the use of Rappaport–Vessiliadis magnesium chloride–malachite green enrichment medium. *Hippocrates* **5**, 1–5.

RAPPAPORT, F., KONFORTH, N. & NAVON, B. (1956). A new enrichment medium for certain salmonellas. *Journal of Clinical Pathology* **9**, 261–266.

VAN SCHOTHORST, M. & RENAUD, A. M. (1983). Dynamics of salmonella isolation with modified Rappaport's medium (R10). *Journal of Applied Bacteriology* **54**, 209–215.

SKOVGAARD, N. & NIELSEN, B. B. (1972). Salmonella in pigs and animal feeding stuffs in England and Wales and in Denmark. *Journal of Hygiene* **70**, 127–140.

TONGPIM, S., BEUMER, R. R., TAMMINGA, S. K. & KAMPELMACHER, E. H. (1984). Comparison of modified Rappaport's medium (RV) and Muller–Kaufmann medium (MK-ISO) for the isolation of salmonella from meat products. *International Journal of Food Microbiology* **1**, 33–42.

VASSILIADIS, P., KALANDIDI, A., XEROUCHAKI, A., PALANDIOU, E. & PAPADAKIS, J. (1976). Au suject du pH du milieu de Rappaport. *Achives de l'Institute Pasteur Hellénique* **XXII**, 43–49.

VASSILIADIS, P., TRICHOPOULOS, D., KALAPOTHAKI, V. & SÉRÉ, CH. (1981). Isolation of Salmonella with the use of 100 ml of the R10 modification of Rappaport's enrichment medium. *Journal of Hygiene* **87**, 35–41.

VASSILIADIS, P. (1983). The Rappaport–Vassiliadis (RV) enrichment medium for the isolation of salmonellas: an overview. *Journal of Applied Bacteriology* **54**, 69–76.