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Studies on the Methods for the Isolation of Salmonella from Pigs*

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Introduction

The results of Salmonalla isolation are greatly influenced by the choice of enrichment media, selective media and the duration of enrichment. Different types of specimen may require different methods of examination. The type of specimen to be examined and the presence of other competing organisms in the specimen are important factors affecting the efficiency of *Salmonella* isolation. Thus the composition and the amount of material, particularly in food products, may have an adverse effect on the selectivity and enrichement quality of selective media[1][2] The microflora of the specimen is another important determinant in the method selected for isolation[3]. In general, material highly contaminated with competing bacteria, particularly enteric bacteria and closely related gram-negative organisms as found in faeces, requires more selective media for both enrichment and selective media.

In the present study attempts were made to compare the relative efficiency of enrichment media, selective media and incubation period of enrichment for the isolation of Salmonella from mesenteric lymph nodes and faeces of pigs slaughtered at a Brisbane abattoir.

Table 1. Number of Pigs and Procedures Employed for Comparative Study

Comparison	No. of pigs studied	Subgroups of pigs studied
Enrichment media: Tet. & Sel. Selective media; BG & SS	359	B1, B2, B3
Inc. P. of enrichment; 24, 48 & 72 hours	224	B2, B3
BG McC. broth & Tet. broth BG McC. agar *& Tet. broth	174	B3

* Direct culture of faeces only
Tet.=tetrathionate broth
Sel.=seoenite broth
Inc. P.=incubation period
BG McC.=brilliant green MacConkey

Meterials and Methods

1. Collection of Samples

Samples of mesenteric lymph node and faeces were collected from pigs slaughtered at a Brisbane abattoir during the period October, 1965 to April, 1966. The pigs originated from various parts of south eastern Queensland and were aged approximately 3-6 months. All the pigs examined were apparently healthy and were passed for human consumption. The pigs were divided into three groups and each group was examined by one or more of the comparative methods. The total number of pigs was 359, and 135 were included in Group A, 50 in Group B and 174 in Group C. The examinations undertaken are as outlined in Table 1.

Faeces were collected by removing a portion of caecum containing a suffieient amount of intestinal content. Mesenteric lymph nodes were collected by removing several lymph nodes together with surrounding tissues from the viscera. Samples were brought to the laboratory within two hours after collection and kept at 4°C until examined.

* This work has been carried out at the Department of Preventive Medicine, School of Veterinary Science, University of Queensland, Australia as a part of the author's Ph. D. Thesis.

2. Preparation of Inoculum

Mesenteric lymph nodes were subjected to further procedures before inoculation into enrichment media. The lymph nodes with surrounding tissues were immersed in boiling water for approximately 30 seconds to minimise surface contamination. Tissue surrounding lymph nodes was removed using sterile scissors and the lymph node out into small pieces and homogenized in an electric homogeniser with a small amount of saline.

Table 2. Salmonella Isolations by Various Culture Methods

Salmonella Strain	M.L.N.				Faeces			
	Tet.		Sel.		Tet.		Sel.	
	BG	SS	BG	SS	BG	SS	BG	SS
S. derby					−	−	+	+
S. cholerae-suis	+	+	−	−				
S. chester	+	+	−	−				
S. muenchen	+	+	−	−	+	−	−	−
S. give	−	−	+	+	−	+	−	−
S. anatum	+	−	+	−	+	−	+	−
S. anatum	+	−	+	−				
S. anatum	+	−	+	−				
S. anatum	+	+	+	+	+	−	−	−
S. anatum	+	−	+	−				
S. anatum	+	+	+	−	−	+	−	−
S. anatum	+	−	−	−				
S. new-brunswick					+	−	+	−
S. birkenhead	−	−	+	−				
S. javiana					+	−	−	−
S. javiana	+	+	+	+	+	+	+	+
S. anatum	+	−	−	−				
S. muenchen	+	+	+	−				
S. javiana					−	−	−	+
S. oranienburg	+	−	−	−				
S. javiana					−	−	+	+
S. javiana					−	−	+	+
S. javiana					+	−	−	−
S. javiana					−	+	−	+
S. derby	+	−	−	−	+	+	+	+
S. javiana	+	+	+	+				
S. muenchen	+	+	−	−	+	−	+	−
S. oranienburg					−	−	+	−
S. muenchen					+	+	−	−
S. muenchen					−	−	+	−
S. muenchen	+	−	+	−				
S. muenchen					+	+	+	+
S. anatum	+	+	−	−				
S. anatum					+	+	+	+
Total	19	10	12	5	12	8	11	9

M.L.N.=mesenteric lymph node
Tet.=tetrationate broth
Sel.=selenite broth
BG=brilliant green agar
SS=SS agar

3. Bacteriological Procedures

Each specimen was inoculated into enrichment media at the ratio of 3 g of original sample to 15 m*l* quantity of enrichment media.

The enrichment media used were tetrathionate broth (Difco), selenite broth (Difco) and brilliant green MacConkey broth. Tetrathionate broth contained 0.1 m*l* iodine solution per 10 m*l* of the broth. Brilliant green MacConkey broth was prepared by adding brilliant green to MacConkey broth (Difco) at the ratio of 1 : 5000. The enrichment media were then incubated at 37°C for 24 to 72 hours. Subcultures were made on both of SS agar (Difco) and brilliant green agar (Difco). Direct culture of faeces on brilliant green MacConkey agar was also tried in the examination of 174 pigs of Group C. This media was prepared by adding brilliant green to MacConkey agar (Difco) at the ratio of 1 : 10000.

Three to five suspect lactose-negative colonies were picked from each of the selective media and further examined after the method described by Edwards and Ewing[4]. Strains identified as genus *Salmonella* were sent to the Salmonella Reference Laboratory, Adelaide for the identification of serotype.

Results

The results of salmonella isolation using the various methods are shown in Table 2. Thirty-three strains of *Salmonella* representing ten different serotypes were isolated from 31 pigs.

1. Enrichment and Selective Media

a) Comparison of Tetrathionate and Selenite Broths

Both enrichment media were subcultured on brilliant green (BG) agar and SS agar simultaneously and the total number of positive specimens was compared. A summary of results is shown in Table 3.

Table 3. Comparison of Tetrathionate Broth and Selenite Broth for the Isolation of Salmonellae from 21 positive M.L.N. and 20 positive Faeces

Enrichment Media	M.L.N.		Faces	
	No.	%	No.	%
Tet. and Sel. combined total	21	100	20	100
Tet. total	19	90.5	15	75.0
Sel. total	12	57.1	13	65.0
Tet. and Sel. together	10	47.6	8	40.0
Tet. only, but not Sel.	9	42.9	7	35.0
Sel. only, but not Tet.	2	9.5	5	25.0

M.L.N.=mesenteric lymph node
Tet.=tetrathionate broth
Sel.=selenite broth

Table 4. Comparison of Brilliant Green Agar and SS Agar for the Efficiency of Salmonella Isolation

Sample	Enrichment media	No. of strains isolated on		
		BG	SS	Both
Mesenteric lymph node	Tet.	19	9	19
	Sel.	12	5	12
	Both	21	12	21
Faeces	Tet.	12	8	15
	Sel.	11	9	20
	Both	16	12	20

Tet.=tetrathionate broth
Sel.=selenite broth

Tetrathionate broth was significantly superior to selenite broth($x^2=6.03$, $0.008<P<0.014$) when mesenteric lymph nodes were examined. All the strains except two (90.5%) were recovered from tetrathionate broth, but only 57.1 per cent of the total strains were recovered from selenite broth.

However, no significant difference was found between the two enrichment media when faeces were examined, although slightly more strains were isolated from tetrathionate broth. The simultaneous use of both enrichment media yielded more isolations than the use of any single medium. Thus 25 per cent of the strains would have been missed if tetrathionate broth alone was used and 35 per cent with selenite broth alone.

b) Comparison of Brilliant Green Agar and SS Agar

The number of salmonella strains isolated by subculturing onto BG agar and SS agar were compared. A summary of results is shown in Table 4.

When mesenteric lymph nodes were examined, strains from either tetrathionate broth or selenite broth were all recovered on BG agar. However, SS agar was much less effective. Only 47.4 per cent of those recovered on BG agar from tetrathionate broth were isolated on this medium, and only 41.7 per cent of those isolated on BG agar from selenite broth. SS agar was significantly inferior to BG agar when both tetrathionate broth($x^2=13.57$, $P<0.001$) and selenite broth($x^2=7.26$, $0.005<P<0.008$) were used as enrichment media.

No significant difference was found between the two selective media when faecal samples were examined, although the number of positive findings was slightly higher with BG agar than with SS agar. However, more satisfactory results were obtained by using the two selective media simultaneously in combination with both tetrathionate broth and selenite broth.

c) Comparison of Results Obtained from Various Combinations of Enrichment Media and Selective Media

The number of salmonella strains isolated by each of the four different combinations of enrichment and selective media was compared. The results are shown below.

	Mesenteric lymph node	Faeces
Tetrathionate broth-BG agar	19	12
Selenite broth-BG agar	12	11
Tetrathionate broth-SS agar	10	8
Selenite broth-SS agar	5	9
Total strains isolated	21	20

Tetrathionate broth-BGagar combination was the best of the four methods, 90.5 per cent of the total isolates from mesenteric lymph nodes being isolated by this combination. Neither the use of the two enrichment media and any single selective media could isolate more than 80 per cent of the total strains from faecal samples. Conseduently, the use of the two enrichment media and the two selective media simultaneously was necessary to obtain the best result from faeces.

2. Brilliant Green MacConkey (BM) Media

In an attempt to compare the efficiency for the isolation of *S. cholerae-suis*,BM media were used.

S. cholerae-suis, however, was not isolated by any of

Table 5. Comparison of Brilliant Green MacConkey Media and Tetrathionate Broth for the Efficiency of Salmonella Isolation

Salmonella	M.L.N.		Faeces		
	Tet.	MC broth	Tet.	MC broth	Direct MCagar
S. javiana			−	−	+
S. javiana	+	+	+	+	−
S. anatum	+	−			
S. muenchen	−	+			
S. javiana			+	−	−
S. oranienburg	+	+			
S. javiana			+	−	−
S. javiana			+	−	−
S. javiana			+	−	−
S. derby	+	−			
S. javiana	+	−			
S. muenchen	+	−	+	−	−
S. oranienburg			+	−	−
S. muenchen			+	−	−
S. muenchen			−	−	+
S. muenchen	+	−			
S. muenchen			+	−	+
S. anatum	−	+			
S. anatum			+	−	−
Total	3/9	4/9	10/12	1/12	3/12

the media used and thus the efficiency for the isolation of this serotype could not be compared. The isolation efficiency of BM media for the other serotypes was much inferior to that of tetrathionate beoth (Table 5). Of the twelve strains isolated from faeces, only one strain was isolated by enriching in BM broth, while ten strains were isolated by enriching in tetrathionate broth. Only three strains were isolated by culturing directly on BM agar. Similar results were obtained when mesenteric lymph nodes were examined. Of the nine strains isolated from mesenteric lymph nodes, seven strains were isolated from tetrathionate broth, but only four were isolated from BM broth.

3. Incubation Periods of Enrichment

Salmonella isolation obtained by applying three different incubation periods of enrichment, 24, 48 and 72 hours, were compared by examining both mesenteric lymph nodes and faeces of 224 pigs. Each sample was enriched in tetrathionate broth and selenite broth simultaneously and subcultures on both BG and SS agar were made after 24, 48 and 72 hours incubation respectively. Combined results from BG and SS agar were used and are shown in Table 6.

For the samples of mesenteric lymph nodes, an incubation period of 48 hours in both tetrathionate broth and selenite broth was the most effective. All the 17 strains isolated from tetrathionate broth and 11 out of 12 strains from selenite broth were recovered when subcultures were made after 48 hours' incubation.

For the faecal samples, however, an incubation period of 24 hours was the most effective. Thus, of 16 strains from tetrathionate broth, 15 were isolated after 24 hours, and similarly, 8 of 9 strains from selenite broth were isolated at this time.

At the three incubation periods, the behaviour of each of the two enrichment media was similar. Thus, the yield of salmonella increased to 48 hours, then declined at 72 hours in the examination of mesenteric lymph nodes; for faeces, yield from both media declined after

Table 6. Comparison of Three Incubation Periods of Enrichment in the Isolation of Salmonella

Sample	Enrichment	No. of strains isolated after incubation period			Total strains isolated
		24 hrs.	48 hrs.	72 hrs.	
Mesenteric lymph nodes	Tet.	10	17	9	17
	Sel.	9	11	7	12
	Both	12	19	13	19
Faeces	Tet.	15	13	8	16
	Sel.	8	7	4	9
	Both	17	16	9	19

Each media plated on both BG and SS agar, cotmbined results considered.
Tet.=tetrathionate broth Sel.=selenite broth

24 hours.

In the examination of both mesenteric lymph nodes and faeces, the number of strains isolated by examining twice after incubation for 24 and 48 hours was not incerased by an additional examination after 72 hours.

Discussion

For the examination of mesenteric lymph nodes of pigs, tetrathionate broth as used in this study with a reduced amount of iodine solution was found to be significanly superior to selenite broth. It is interesting to note that this result was in contrast to that of similar works carried out by Guinee and Kampelmacher[5] and Guinee *et al*[6]. They concluded that selenite broth probably provided a better medium for the examination of lymph nodes of pigs, although they used tetrathionate and selenite broths to which brilliant green was added.

Unlike the results obtained from mesenteric lymph nodes, no significant difference was found between the two enrichment media when faecal samples were examined, although the number of positive findings was slightly higher with tetrathionate than with selenite broth. Similar results showing the superority of tetrathionate broth over selenite broth were observed from faeces of pigs by Guinee and Kampelmacher[5] aed Guinee *et al*[6] and from artificially infected human faeces by McCullough and Byrne.[7] Furthermore, in the present study it was found that the use of both enrichment broths was necessary for the maximum number of recoveries of salmonella from both mesenteric lymph nodes and faeces. This conclusion was also emphasized by many workers[5][6][8][9][10].

Smith[11] also compared the two enrichment media by examining faeces of many domestic animals including pigs. Even though he obtained more positive findings using selenite broth from the total of all species of animals, the number of positive samples from pigs(4 out of 600 samples) was too small to be assessed for the comparison of the two media.

Superiority of enrichment media in the isolation of salmonella from faeces is influenced by the animal species. Thus, Smith[9] in his experiment with artificially infected faeces of various animals found that selenite broth was preferable to tetrathionate broth for examining cow and chicken faeces but the reverse was true in the case of dog faeces; slight differences only were noted in other species including pigs.

Of the selective media, brilliant green agar was significantly superior to SS agar in the examination of mesenteric lymph nodes. As all the positive samples were detected on BG agar, there was no need to use SS agar in addition. However, this remarkable superiortity of BG agar was greatly reduced when faecal samples were examined, while the recovery rate on SS agar remained almost the same (Table 4). This variety in recovery rate on BG agar seemed due to its mild inhibitory action for the numerous enteric bacteria present in faeces, particularly for *Proteus* organisms. The swarming of *Proteus* colonies, which makes difficult the isolation of salmonella colonies, was encountered very often on BG agar but seldom on SS agar. It is necessary to suppress on heavily contaminated material. Galton and coworkers[12] added sulphathiazole to tetrathionate broth(0.125 mg/100 ml) to prevent the excessive growth of *Proteus* orgauisms and compared BG agar and SS agar for the recovery of salmonallae from the faeces of dogs. They obtained far better results with BG agar than with SS agar. For this reason, the use of SS agar in addition to BG agar is preferable to obtain satisfactory results from faecal samples, unless sulphathiazole is added to the media, particularly when one was more selective than the other, was indicated by Hobbs[3].

As no *S. cholerae-suis* was isolated by any method applied, the efficiency of BM broth for the isolation of this serotype could not be determined. Whether this serotype was present in the pigs examined in this study or whether the media were not efficient enough to isolate the serotype is unknown. Smith[11] isolated all the eleven strains of *S. cholerae-suis* from mesenteric lymph nodes of pigs from BM broth but none of these was isolated from faeces using any media. He also found that BM broth was as efficient as selenite broth for the isolation of serotypes other than *S. cholerae-suis* from mesenteric lymph nodes but was not so efficient as selenite broth in the examination of faeces. In the present study BM broth was found neither as efficient as tetrathionate nor selenite broths in the examination of both sites for salmonellae.

However, there are some differences in the methods used by Smith[11] and those of the present study. He used 100 ml quantities of BM broth in which lactose was replaced by mannitol, and subcultures were made

onto desoxycholate-citrate agar. The present author used 15 ml quantities of BM broth in which lactose was not replaced by mannitol and BG agar was used as selective media in the present work.

When incubation periods were considered, 24 hours for faeces and 48 hours for mesenteric lymphnodes were found to give maximum yields of salmonellae. While both enrichment media behaved in this way, tetrathionate was still superior to selenite broth. However, different results have been reported by other workers. Thus Smith[9] found 24-30 hours for tetrathionate broth and 30-48 hours for selenite broth were the optimum times for the incubation of faeces from man, dog, horse, pig, cow, seep, chicken, turkey and duck. Taylor and Silliker[12] also found similar results by examining naturally infected egg albumen. They concluded that selenite broth produced more positive isolations at 48 hours than at 24 hours but no change was found in tetrathionate broth. Guinee and Kampelmacher[5] and Guinee *et al*[6] observed two different incubation periods, 24 and 72 hours, in the examination of mesenteric lymph nodes and faeces of pigs. They concluded that plating after 24 and 72 hour incubation periods resulted in an increase in the number of positive findings over a single plating.

Summary

Various methods for the isolation of salmonellae were compared by examining mesenteric lymph nodes and faeces of 395 pigs at slaughter. The conclusions were as follows.

(i) Tetrathionate broth was significantly superior to selenite broth for the examination of mesenteric lymph nodes.

(ii) There was no significant difference between tetrathionate broth and selenite broth for the examination of faeces. The use of both tetrathionate broth and selenite broth as enrichment media for faeces produced far better results than the use of any single enrichment medium.

(iii) Brilliant green agar was significantly superior to SS agar for the examination of mesenteric lymph nodes.

(iv) There was no significant difference between brilliant green agar and SS agar when faeces were examined. The use of both media produced far better results than the use of either singly.

(v) Brilliant green MacConkey broth was much inferior to tetrathionate broth as an enrichment broth. Direct culture of faeces on brilliant green MacConkey agar yielded less isolates than prior enrichment in tetrathionate or selenite broth.

(vi) The optimum incubation period of enrichment was 24 hours for faeces and 48 hours for mesenteric lymph nodes when either tetrathionate broth or selenite broth was used as enrichment media.

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돼지에서 살모넬라菌을 分離하는 方法에 관한 硏究

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健康屠殺豚의 腸間膜淋巴腺과 糞(大腸內容物)으로부터 살모넬라菌의 分離方法을 비교연구하여 다음과 같은 결론을 얻었다.

1. 비교적 다른 세균에 의한 汚染度가 낮은 腸間膜淋巴腺을 검사하였을 경우, 增菌培地로서는 tetrathionate broth(TB)가 selenite broth(SB) 보다 살모넬라菌 分離率이 월등히 높았으며, 選擇培地로서는 brilliant green agar(BG)가 SS agar(SS) 보다 역시 월등히 높았다.
2. 여러가지 腸內細菌에 의한 汚染度가 높은 糞을 검사하였을 경우에는, 살모넬라菌의 分離率에 있어서 增菌培地로 사용한 TB와 SB의 優劣을 인정할만한 차이를 보지 못하였으며, 選擇培地로 사용한 BG와 SS의 성적도 비슷하였다. 增菌培地나 選擇培地를 어느 한가지 培地만 사용한 경우보다는 두가지 培地를 동시에 같이 사용함으로써 살모넬라菌의 分離率이 크게 높았다.
3. Brilliant green McConkey broth를 增菌培地로 사용하였을 경우에는 腸間膜淋巴腺이나 糞의 두가지 재료중 어느것이나 TB 보다 살모넬라菌의 分離率이 훨씬 낮았다. 糞을 brilliant green McConeky agar에 직접 培養하는 방법은, TB나 SB에 增菌培養을 한 뒤에 BG나 SS의 選擇培地를 사용하는 방법보다 또한 훨씬 分離率이 낮았다.
4. TB 및 SB의 增菌培養時間은 37°C에서 24, 48 및 72時間등 세가지를 비교해본 결과 腸間膜淋巴腺은 48時間 培養했을 때에 살모넬라菌의 分離率이 가장 높았으나 糞은 24時間 배양했을 때에 가장 分離率이 높았다.