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Strontium chloride B and E.E. enrichment broth media for the isolation of *Edwardsiella*, *Salmonella* and *Arizona* species from tiger snakes

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SUMMARY

Strontium chloride B medium and E.E. broth have been found effective in the recovery of *Edwardsiella*, *Salmonella* and *Arizona* species from the cloacal contents of tiger snakes (*Notechis scutatus*). Strontium chloride B medium was superior to E.E. broth.

At least one bacterial species was detected in each of the 60 reptiles examined, and all three organisms were recovered from each of 29 snakes on a single examination.

Strontium chloride M, strontium selenite and Rappaport enrichment media and bismuth sulphite agar, although satisfactory for the isolation of *Salmonella* and *Arizona* species, were found unsuitable for *Edwardsiella tarda*.

INTRODUCTION

A new genus of Enterobacteriaceae, embracing a single species, was described as *Edwardsiella tarda* by Ewing, McWhorter, Escobar & Lubin (1965). The species has been isolated from man (Sonnenwirth & Kallus, 1968; Okubadejo & Alausa, 1968; Jordan & Hadley, 1969) and an increasing range of animals, particularly reptiles. In contrast to the four *Salmonella* subgenera, few details of culture procedures have been published. However, in India, Bhat, Myers & Carpenter (1967) reported four *Edwardsiella* isolations from children and commented that colonies on MacConkey and deoxycholate agar resembled *Salmonella* species. The organisms grew on bismuth sulphite agar and survived culture in selenite F broth. It was further reported by Sakazaki (1967) that strains isolated from Japanese reptiles grew on brilliant green agar. Satisfactory growth of laboratory strains on xylose lysine agar was also noted by Taylor (1965).

In Western Australia the first isolation of *Edwardsiella tarda* was made from the faeces of an infant with acute gastro-enteritis. Subsequently, the organisms were isolated from the cloacal contents of three tortoises, three crocodiles, a sewage effluent swab and the litter from eight tiger snakes. It was observed that *E. tarda* occasionally survived, but did not multiply, in selenite F, Rappaport or strontium selenite enrichment media. It was also noted that the organisms usually failed to produce colonies on bismuth sulphite (B.S. agar).

Further investigations showed that *Edwardsiella* colonies, although somewhat smaller than those of *Salmonella* species, developed satisfactorily on deoxycholate-citrate-agar and S.S. agar. The species also multiplied in the Enterobacteriaceae enrichment broth (E.E. broth) of Mossell, Visser & Cornelissen (1963), and in the strontium chloride medium of Iveson & Mackay-Scollay (1969) when the malachite green was omitted (strontium chloride B medium).

Following favourable preliminary tests comparative trials were undertaken to evaluate the efficiency of E.E. broth (Oxoid) and strontium chloride B medium, for the isolation of *Edwardsiella*, *Salmonella* and *Arizona* species from reptilian materials. The technique involved and the results obtained are presented in this report.

MATERIALS AND METHODS

Specimens

A total of 60 tiger snakes (*Notechis scutatus*), together with 15 contaminated sawdust-litter samples from 39 of the snakes, were examined. The reptiles were active, healthy adults, captured in a remote swampy area near the southern coast of Western Australia. Cloacal swabs were taken on five separate occasions within a few days of capture.

Swabs were placed in 1–2 ml. volumes of Sachs (1939) buffered glycerol saline at the time of sampling, and approximately 2 g. of sawdust litter were sown in 10 ml. volumes of the transport medium. All samples were well mixed before processing the same day.

Plating media

S.S. agar (Oxoid) and modified bismuth sulphite agar (B.S.A.), were used for direct plating of reptile samples and subculturing from enrichment media. The B.S.A. medium was prepared by reconstituting dehydrated bismuth sulphite agar (Oxoid), as recommended by the manufacturer, but immediately before pouring, 10 ml. of 1 % ferrous sulphate and 3·0 ml. 10 % ferric citrate were added to 1 l. of the molten medium. Plates were dried for approximately 30 min. with the lids removed, and were stored at 4° C. overnight, or up to 4 days, before use. The additive solutions were sterilized by heating at 60° C. for 1 hr. and were stored for use at 4° C.

Enrichment media (strontium chloride B enrichment broth)

Bacto tryptone (Difco)	0·5 g.
Sodium chloride	0·8 g.
Potassium dihydrogen phosphate	0·1 g.
60 % strontium chloride	6·0 ml.
Distilled water	100 ml.

The medium was distributed in 10 ml. volumes and sterilized by steaming for 30 min. The final concentration of strontium chloride was 3·4 % and the pH was 5·0–5·5.

Oxoid E.E. broth

The dehydrated medium was reconstituted as recommended by the manufacturers, distributed in 10 ml. volumes and sterilized by steaming for 30 min.

Other enrichment broth media

The strontium selenite, strontium chloride M, Rappaport, Selenite F. and tetrathionate enrichment media used, were prepared as reported by Iveson & Mackay-Scollay* (1969).

Cloacal swabs and litter samples were first inoculated on the solid plating media, and the swabs, together with approximately 0·5 ml. of each sample, were added to 10 ml. volumes of the particular enrichment medium used, in a series of five separate studies.

Subcultures were made from enrichment media after 24 hr. incubation at 37° C. Non-lactose-fermenting colonies on S.S. agar, and colonies resembling *Salmonella* and *Arizona* species on the modified B.S.A. medium, were examined biochemically and serologically.

Approximately 20 colonies were examined from each specimen. During the investigation up to 50 colonies were identified from selected specimens as a control check on routine recoveries.

RESULTS

A total of 241 isolations comprising 109 *Arizona*, 82 *Salmonella* and 50 *Edwardsiella* species were made from 100 cloacal swabs and 15 snake-litter samples. While 117 (49 %) isolations were made by direct culture, 235 (98 %) isolations were made by enrichment culture.

Table 1. *Distribution frequency of* Salmonella, Arizona *and* Edwardsiella *species in* 100 *cloacal samples from* 60 *tiger snakes and* 15 *snake-litter samples*

Species isolations			Specimens		
Salmonella	*Arizona*	*Edwardsiella*	Cloacal swabs	Litter	Totals
+	+	+	34	0	34
+	+	−	32	13	45
+	−	+	1	0	1
−	+	+	12	1	13
+	−	−	2	0	2
−	+	−	16	1	17
−	−	+	2	0	2
	Total positive		99	15	114

Direct plating yielded 83 (76 %) *Arizona*, 24 (29 %) *Salmonella*, and 10 (20 %) *Edwardsiella* isolations, whereas by enrichment methods 105 (96 %) *Arizona*, 82 (100 %) *Salmonella*, and 48 (96 %) *Edwardsiella* isolations were obtained.

* In this paper (*Journal of Hygiene*, 1969, **67**, 457–64) there are three errors on page 459. Line 10: *for* Distilled water 100 ml. *read* Distilled water to 100 ml. Line 14: *for* 10 ml. solution B *read* 6 ml. solution B. Line 36: *for* pH 7·8 *read* pH 6·8.

Table 2. *Relative efficiency of direct and enrichment culture methods in five experiments in isolating* Salmonella, Arizona *and* Edwardsiella *species from* 100 *cloacal swabs of* 60 *tiger snakes and* 15 *snake-litter samples*

			Salmonella		*Arizona*		*Edwardsiella*	
Expt.	Samples	Culture method	Positive	efficiency (%)	Positive	efficiency (%)	Positive	efficiency (%)
I	23	Direct	6	35·3	18	78·3	1	100
		Strontium selenite	11 (17)	64·7	21 (23)	91·3	0 (1)	0·0
		Strontium chloride M	16	94·1	17	73·9	0	0·0
		Rappaport	12	70·6	15	65·2	0	0·0
II	32	Direct	5	21·7	26	81·3	3	25·0
		Strontium selenite	17 (23)	73·9	28 (32)	87·5	0 (12)	0·0
		E.E	15	65·2	24	75·0	11	91·7
III	19	Direct	4	26·7	12	66·7	1	11·1
		Strontium chloride M	13 (15)	86·7	17 (18)	94·5	0 (9)	0·0
		E.E	9	60·0	9	50·0	9	100
IV	20	Direct	1 (11)	9·1	9 (16)	56·3	0 (9)	0·0
		E.E	11	100	14	87·5	9	100
V	21	Direct	8	50·0	18	90·0	5	26·3
		Strontium chloride B	13 (16)	81·3	19 (20)	95·0	16 (19)	84·2
		E.E	11	68·8	11	55·0	10	52·6
Totals	115		(82)		(109)		(50)	

Figures in parentheses indicate samples yielding positive isolations.

* The samples in Experiment I comprised 8 cloacal swabs and 15 snake-litter samples.

At least one bacterial species was recovered from 114 (99%) of the test samples. Of these (see Table 1), 34 (30%) samples yielded all three species together, 59 (52%) two species, and 21 (18%) a single species isolation.

Table 2 (Expt. I) shows the relative efficiency of culture methods before the introduction of E.E. broth and strontium chloride B medium. The Rappaport, strontium chloride M and strontium selenite media combination produced 23 *Arizona* and 17 *Salmonella* isolations from the 23 test samples. The single *Edwardsiella* isolation was recovered by direct plating on S.S. agar, and not by enrichment methods.

The relative efficiency of direct and enrichment culture methods in the recovery of *Salmonella*, *Arizona* and *Edwardsiella* species in the four subsequent experiments are also illustrated in Table 2. In Expt II strontium selenite was slightly superior to E.E. broth in the isolation of *Salmonella* and *Arizona* spp. In Expt. III strontium chloride M was considerably better than E.E. broth for the same two organisms, and in Expt. V a similar difference was seen between strontium chloride B and E.E. broth. In all these experiments and in Expt. IV, where only direct plating and E.E. broth were compared, direct plating was always inferior to the enrichment methods used.

Enrichment in E.E. broth was always superior to direct culture for the isolation of *Edwardsiella* spp. However, in Expt. V strontium chloride B medium was superior to both these procedures in the recovery of the same organism. Edwardsiella organisms were not recovered by strontium selenite or strontium chloride M enrichment media.

The *Salmonella* and *Arizona* serotypes recovered are as follows (the figures in parenthesis indicate the frequency of serotype isolations): *S. adelaide* (3), *S. fremantle* (49), *S. houten* (2), *S. onderstepoort* (7), *S. potsdam* (1), *S. ramat gan* (43), *S. slatograd* (1), *S. wandsbek* (5), *S. species* (1), *Arizona* 5: 29–30 (3), *Arizona* 16: 23–25 (29), *Arizona* 20: 29–25 (46), *Arizona* species (37).

A single *Salmonella* serotype was recovered from 54 samples, 25 samples yielded 2 serotypes, and 3 samples yielded 3 *Salmonella* serotypes. The serotyping of *Arizona* strains has yet to be completed.

DISCUSSION

Using strontium chloride B enrichment medium and E.E. broth it has been demonstrated that tiger snakes in a remote habitat are naturally infected simultaneously with *Salmonella*, *Arizona* and *Edwardsiella* species. The reluctance of the latter species to grow in the widely used isolation media, together with unusual biochemical characteristics, may account in part for its infrequent isolation and tardy recognition by bacteriologists.

The occurrence of *E. tarda* in animals and its pathogenic role in man requires further investigation. The species referred to as 'Serotype 1483–59' was first investigated by Ewing in America, and later designated *Edwardsiella tarda* by Ewing *et al.* (1965). It is the same type organism as the Bartholomew group of King & Adler (1964), the Asakusa group of Sakazaki & Murata (1962) and Sakazaki (1965, 1967).

Human isolations reported in America by Ewing *et al.* (1965), Jordan & Hadley (1969) and from India by Bhat *et al.* (1967) indicate that the organism may be present both with and without symptoms in juveniles and adults. The species has also been recovered in America from a fatal adult meningitis case by Sonnenwirth & Kallus (1968) and from a neonatal meningitis, also fatal, reported from Nigeria by Okubadejo & Alausa (1968). Further isolations, predominantly from snakes but including five from cases of human enteritis and two from seals, were reported in Japan by Sakazaki (1965). The organisms have also been recovered from a sea-lion and two alligators in a Florida zoo by Wallace, White & Gore (1966), and from three samples of pig bile in a Phillipine abattoir by Arambulo, Westerlund & Sarmiento (1968).

The widespread distribution of *Edwardsiella* species is further illustrated in a report by d'Empaire (1969), who investigated the growth requirements of strains isolated in France, Tahiti, Tchad and Vietnam. The study included cultures recovered from humans, cattle, reptiles, a pig and a panther.

In an ecological survey of reptiles in Western Australia conducted before the introduction of E.E. broth and strontium chloride B medium, Iveson, Mackay-Scollay & Bamford (1969) were unable to recover *E. tarda* in 116 reptiles examined, although *Salmonella* and *Arizona* serotypes were detected in 83·6%. However, *E. tarda* was later isolated, using direct culture on S.S. agar, from human faeces, and the cloacal contents of three reptiles, all originating in geographically remote areas. The later recovery of *E. tarda*, again by direct culture from the samples illustrated in Table 1, indicated that routine enrichment methods might be too inhibitory, a factor influencing the selection and trial of the less inhibitory E.E. broth and strontium chloride B enrichment.

E.E. broth was recommended as an enrichment medium for Enterobacteriaceae in the bacteriological examination of foods by Mossell *et al.* (1963), and animal feedingstuffs by Schothorst, Mossell, Kampelmacher & Drion (1966). Additionally, it was suggested that it might be of value as a pre-enrichment medium for labile salmonellas before exposing cells to the more toxic selenite or tetrathionate enrichment broths. The medium was, however, more inhibitory to non-Enterobacteriaceae than the mannite broth of Taylor (1961) and the lactose broth used by North (1961).

The ability of *Escherichia coli*, as well as other non-pathogenic Enterobacteriaceae, to multiply in E.E. broth, severely limits the performance of the medium in the isolation of *Salmonella* and *Edwardsiella* species from human and domestic animal faeces. However, by contrast, the more selective strontium chloride B medium, has shown a marked ability to inhibit other Enterobacteriaceae and to grow *Salmonella*, *Arizona* and *Edwardsiella* species from human and animal excreta when tested at 37° and 43° C., in parallel with our routine isolation procedures.

Salmonella, *Arizona* and *Edwardsiella* species from single cloacal specimens were only recovered together from strontium chloride B or E.E. broth enrichment, subcultured to S.S. agar. The modified B.S. agar in routine use in the laboratory, while it provided ready differentiation of *Salmonella* and *Arizona* colonies, was unsatisfactory for the recognition or recovery of *Edwardsiella*, and these organisms were

identified exclusively on S.S. agar. It was found essential to use the S.S. and B.S. media with their different inhibitory and colony indicator systems, in order to recover the three species together from single samples.

Few workers have reported on the colony morphology of *Arizona* and *Edwardsiella*, or on the suitability of routine enrichment procedures for their simultaneous growth. However, in a report on the occurrence of the *Arizona* group in animals and man, Edwards, McWhorter & Fife (1956) drew attention to the frequent occurrence of lactose-fermenting *Arizona* serotypes, and, as a consequence, isolation difficulties, when only S.S. or D.C. agar were used. Harvey, Price & Dixon (1966) reported that Wilson and Blair medium was more suitable for the easy recognition of *Arizona* spp. In the present study it was noted that, in addition to the familiar black colonies on the modified B.S. agar, *Arizona* species also developed as dark-green to black centred colonies with a pale-green periphery and without a metallic sheen. On S.S. agar *Arizona* colonies often resembled typical lactose-fermenting *Esch. coli*.

The modified B.S. agar was found too inhibitory for *Edwardsiella*. The few laboratory strains which grew on the surface of the medium provided minute, pale-green colonies which failed to develop on continued incubation. On S.S. agar, after incubation for 18–24 hr., *E. tarda* yielded small non-lactose fermenting colonies, frequently resembling *S. pullorum* colonies. With further incubation they became larger and occasionally developed dark centres somewhat resembling *Proteus* colonies.

In separate experiments, using ten pure cultures of *E. tarda* previously isolated in the laboratory, it was found that the species failed to multiply at 37° C. in selenite F, tetrathionate, Rappaport, strontium chloride M or strontium selenite enrichment broths. The sensitivity of *E. tarda* to selenite salt was also observed by Lapage & Bascomb (1968) during selenite reduction tests. All ten strains multiplied rapidly in 1 % peptone water, meat infusion and E.E. broth. The organisms grew readily on D.C. agar, and usually formed characteristic dark-centred colonies within 24 hr.

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