Medicine. — *The isolation of typhoid bacilli from water.* By A. CHARLOTTE RUYS. (Communicated by Prof. W. A. P. SCHÜFFNER).

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In several publications WILSON and BLAIR [1]) recommended a new medium containing glucose, bismuth, sulphite, phosphate, iron, brilliant-green, and nutrient agar for the isolation of typhoid bacilli from various materials. They succeeded in isolating typhoid bacilli from sewage and raw river-water, which up till that time was extremely difficult or altogether impossible. HOUSTON [2]) (Metropolitan Water Board London), using WILSON and BLAIR's medium, on several occasions obtained typhoid bacilli in pure culture from raw Thames-water and from London sewage. After an outbreak of paratyphoid-B in Epping he regularly cultivated paratyphoid-B-bacilli from the sewage during nearly a year. So this medium opens a new field for the study of the epidemiology of enteric fevers.

Medium. We have employed WILSON and BLAIR's old standard medium, the formula of which is as follows:
100 c.c. nutrient 3 per cent. agar, 20 c.c. glucose-sulphite-phosphate mixture (a solution of 6 grm. bismuth-ammonium-citrate in 50 c.c. aqua destillata is neutralized and added to 100 c.c. of a 20 per cent. exsiccated sodium sulphite solution, then 10 grm. sodium phosphate and after cooling 10 grm. glucose in 50 c.c. distilled water are added), 1 c.c. 8 per cent. ferrous sulphate solution and 0.5 c.c. 1 per cent. solution of brilliantgreen.

On this medium typhoid and paratyphoid bacilli grow in black colonies with a metallic halo. The growth of most strains of B. coli is inhibited, but there are some strains of B. coli which grow just like typhoid colonies.

When trying out the medium, we first had some difficulties in obtaining constant results, although we carefully observed POT's [3]) prescription never to use anything but a freshly prepared ferrous sulphate solution. We finally found that this precaution is not sufficient because the crystals contained too much ferric sulphate which spoiled the medium. After using ferrous sulphate which had heen precipitated in alcohol and which gave a perfectly clear solution, these difficulties were overcome.

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1) Jl. of Hygiene, Vol. **26**, p. 374 (1927) and Vol. **31**, p. 138 (1931).
2) Reports Metropolitan Water Board, London (1929—1931).
3) Geneesk. Gids, p. 985 (1935).

Atypical strains. All publications on this subject agree that true typhoid colonies should be black with a metallic halo. However, we found a strain isolated from the blood and the urine of a typhoid patient, which could not reduce the iron- and bismuth compounds to the black sulfide. The strain grew quite well on the medium but its colonies were green even if they grew wide apart from each other (fig. 1). This strain, now 8 months in culture, always remained the same. It is possible, however, to make it grow in black colonies (without an halo), if it is cultivated under anaerobic conditions. A return to aerobic conditions causes the colonies to turn from black to green after the lapse of a few days. As the bismuth sulfide, when once it is formed, remains quite constant, this change of colour must be due to the black iron sulfide being oxidated to iron sulphate.

Among 98 strains in the collection of Prof. VAN LOGHEM, Prof. SNIJDERS and our own we found a second strain which grew in green colonies only. This strain was isolated from the blood of a typhoid patient. Neither of the two green growing strains showed any other pecularities.

Consequently, true typhoid colonies may be occasionally overlooked, if subcultures are made from the black colonies with an halo only, and the use of another medium besides that of WILSON and BLAIR is essential for the examination of stools and urine; for the examination of infected water WILSON and BLAIR's will have to suffice, as the other ones are no good at all.

Examination of water.

A. During a milkborn outbreak of typhoid fever occurring in a little village, the milk was asserted to have been contaminated by water from a certain ditch. This ditch communicated with another one to which for some time the stools and urine of a non-recognized typhoid patient had found their way (see PEETERS, RUYS and EPHRAIM) [4]).

On February 13th three samples of water were collected from the polluted ditch, just below the sheat of ice which covered the water since a few days. Typhoid bacilli were recovered from 2 out of the 3 samples by inoculating the surface of a WILSON and BLAIR plate with one drop of water.

Four days later the same method revealed the presence of typhoid bacilli in 3 out of 7 samples collected in various parts of both ditches. The modified method of spreading the centrifugate of 20 c.c. water over two WILSON-BLAIR plates yielded no better results: two negative samples remained negative, two positive ones positive. The other media (Endo-plates, MULLER tetrathionate broth, brilliantgreen-Esbach-broth) all yielded negative results. No other experiment could be tried because the ditches were desinfected after the sampling.

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[4]) Ned. Tijdschr. v. Geneesk. p. 2353 (1936).

B. More complete experiments were carried out in a little town in the neighbourhood of Amsterdam, where every year several cases of typhoid fever continue to occur, without any traceable source of infection. Through the town passes a narrow and shallow canal for shipping. There is no sewerage but a tub-system. Notwithstanding prohibitory bye-laws the inhabitants persist in their objectionable custom of emptying into the canal the tubs containing nightsoil. For years it has been a widespread notion that a person who falls into this canal is liable to acquire typhoid fever. Once every fortnight the canal is flushed with clean water, though quite insufficiently to remove the dirt.

1. On March 26th 2 samples of water from different parts (K and Mg) were examined. Twenty five c.c. of water were mixed with equal parts of the medium and 10 c.c. of water with 25 c.c. of the medium and poured into plates. In order to obtain a better concentration of the bacteria the centrifugate of 60 c.c. of water was spread over 6 WILSON-BLAIR plates. As we had underestimated the pollution of the water, after two days the plates were overgrown with black colonies. The EIJKMAN-test also showed a heavy pollution of the water; in both samples 0.0001 c.c. yielded a positive reaction. From the cultures of the water at K 54 black colonies were picked off and one could be identified as a typhoid colony (biochemically and serologically). Of the water of Mg 50 black colonies were subcultured, but none contained typhoid bacilli. The positive result in K only showed that typhoid bacilli could be isolated from the water; it did not allow of an evaluation of the degree of contamination. In the following experiments we were careful to dilute the water in order to raise better isolated colonies.

2. On April 1st we took 3 samples of water from the main canal at K, KI (at a distance of 30 M from K) and Mz. This time 5 c.c. of water were mixed with 45 c.c. of the medium and 2 c.c. with 48 c.c. of the medium and both poured into dishes. Furthermore the sediment of 80 c.c. of water was spread over 8 WILSON-BLAIR plates. The results are shown in table I. In many plates black colonies were so numerous that only a portion of them could be examined. These experiments showed, that at K and KI the water was heavily contaminated with typhoid bacilli. When working with this strongly polluted water we found that the dilution method gave better results than the plating out of sediment. The latter method, therefore, was discarded in the following experiments.

3. On April 14th 3 samples were examined, one taken at K, another at Kg some 200 M distant and a third from another part of the town not corresponding directly with the main canal (E). At Kg the water from the surface and also from a depth of nearly 60 cM was examined. The table shows that this time the water was again found infected at K and surroundings, but not so heavily as two weeks earlier. There was no difference between the water at the surface and below it. At E no typhoid bacilli could be demonstrated.

TABLE I.

Date	Place	EIJKMAN titer	Quantity of water examined	Number of black colonies	Number of typhoid colonies
26—3—36	K	0.0001	25 c.c.		
			10 c.c.	> 54	1
			60 c.c. *)		
	M g	0.0001	25 c.c.		
			10 c.c.	> 50	0
			60 c.c. *)		
1—4—36	K	0.001	5 c.c.	21	> 4
			2 c.c.	19	> 8
			80 c.c. *)	> 100	> 13
	K I	0.001	5 c.c.	16	11
			2 c.c.	8	7
			80 c.c. *)	> 100	> 15
	M z	0.0001	5 c.c.	6	0
			2 c.c.	6	2
			80 c.c. *)	> 100	1
14—4—36	K	0.0001	5 c.c.	29	3
			0.5 c.c.	3	3
	K g	0.01	5 c.c.	30	7
			0.5 c.c.	0	0
	K g deep	0.0001	5 c.c.	27	6
			0.5 c.c.	2	2
			0.05 c.c.	1	1
	E	0.001	5 c.c.	12	0
			0.5 c.c.	6	0
6—5—36	K	0.001	5 c.c.	12	6
			0.5 c.c.	6	4
	M g	0.001	5 c.c.	17	0
			0.5 c.c.	5	0
	T	0.0001	5 c.c.	12	0
			0.5 c.c.	44	0
9—5—36	K w	0.0001	5 c.c.	92	7

*) Centrifugated.

4. On May 6th a new series of experiments was made, the weather having changed from cold in the previous month to warm and sunny during the first week of May. The temperature of the water was 14½° C. Samples were taken again at K, for the second time at Mz and at T in a canal only indirectly in contact with the main canal. The pollution, as shown by the EIJKMAN-test, was as heavy as in the previous months.

The figures in the table show that at K the contamination of the water with typhoid bacilli was of the same degree as three weeks earlier. Neither at T nor at Mz typhoid bacilli could be isolated this time.

Three days later typhoid bacilli were isolated from a sample of water collected at Kw at a distance of 250 M from K.

These experiments show that the water in the neighbourhood of K must have been continuously contaminated. Up till the present the source of this infection has not been detected. It is difficult to ascertain how long the typhoid bacilli can survive in the water. In the outbreak described under A (see PEETERS, RUYS and EPHRAIM) typhoid bacilli were isolated from the ditch 24 days after the last day the stools had found their way into the ditch. The weather in this period had been rather cold with some days of heavy frost.

In order to get an impression of the longevity of typhoid bacilli under different temperature conditions, some experiments were made with naturally contaminated water. Various samples of this infected water were divided into two parts. One group of them were put in the refrigerator (6° C.), the others in the incubator (24° C.). After 6 days exposure to the temperature of the incubator the typhoid bacilli invariably had disappeared in all of the 6 samples. All of the 6 samples kept in the refrigerator for 5 or 6 days always yielded some typhoid colonies, though less than the recently collected water. Once only we could isolate typhoid colonies from water which had been in the refrigerator for 10 days. From 5 c.c. of the sample of water collected at K on April 1st (which yielded a very large number of typhoid colonies; see pag. 783) fifteen colonies were cultivated on April 6th and seven on April 11th. Five days later no more typhoid bacilli could be recovered from this sample. So the temperature has a considerable influence on the longevity of typhoid bacilli in naturally infected water under laboratory conditions. Further experiments will have to be carried out in order to ascertain which are the other important factors playing a part in this process (protozoa, overgrowing by other bacteria).

SUMMARY.

The medium of WILSON and BLAIR enables us without difficulty to isolate the typhoid bacilli from infected water.

In preparing the medium it is necessary to take care that the ferrous sulphate solution contains no ferric salts.

There are a few strains of typhoid bacilli which in every respect conform to type but which nevertheless grow in green colonies on WILSON and BLAIR's medium and not in the black ones which are supposed to be characteristic of this species.

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(From the laboratories of the City Health Department, Amsterdam.)

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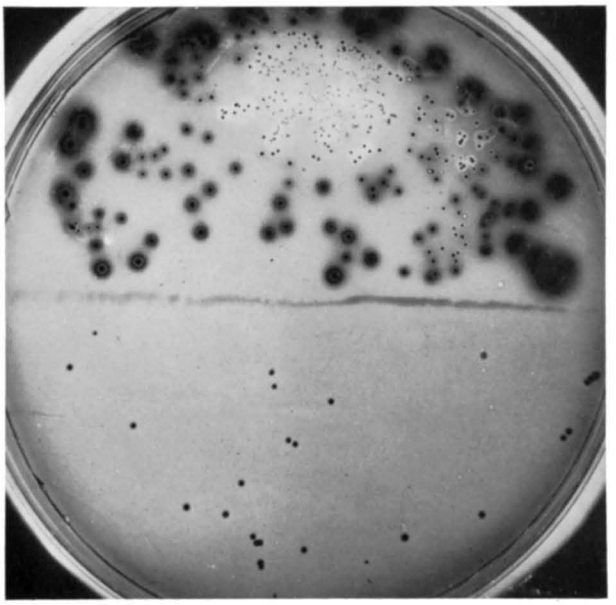

II

Fig. 1.
WILSON and BLAIR's medium incubated at 37° C during 48 hours.
I. typical typhoid colonies.
II. green growing strain.