

THE NEUTRAL-RED REACTION AS A MEANS  
OF DETECTING BACILLUS COLI IN WATER SUPPLIES<sup>1</sup>.

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THE object of the experiments detailed below, which were made at the suggestion of Dr W. Hunter, was to ascertain whether media containing neutral-red afford a rapid means of detecting *Bacillus coli* in water and estimating the number present.

In 1898 Rothberger<sup>2</sup> discovered that *B. coli* reduces solutions of neutral-red, the colour changing to canary-yellow, accompanied by green fluorescence. Since *B. typhosus* does not do so a valuable means of distinguishing between these two organisms was afforded. Scheffler<sup>3</sup> has confirmed Rothberger's work, extending the investigation to a large number of races of *B. coli*, obtained from different sources. With all these the reaction was constantly obtained, and he considers it so characteristic that any organism which fails to give it may be excluded from the *coli* group. Hunter<sup>4</sup> has recently obtained identical results, and has further shown that *B. enteritidis* (Gaertner) also reduces neutral-red—a fact which places this bacillus closer to the *coli* group, and separates it more sharply from *B. typhosus*.

Rothberger<sup>5</sup> found that certain anaerobic bacteria—*B. tetani*, *B. anthracis symptomatici*, and *B. oedematis maligni*—have the same power of reducing neutral-red, and Scheffler mentions that he separated from water and from faeces several species of micro-organisms which produce a green fluorescence, the part of the reaction on which he appears to lay most stress. Unfortunately he gives no hint as to their nature, beyond stating that they were not *B. coli*. On the other hand Rothberger and

<sup>1</sup> MS. received April, 1901.

<sup>2</sup> *Centralbl. f. Bakteriol.*, vol. xxiv. p. 513, 1898.

<sup>3</sup> *Ibid.*, vol. xxviii. p. 199, 1900.

<sup>4</sup> *Lancet*, March 2, 1901, p. 613.

<sup>5</sup> *Centralbl. f. Bakteriol.*, vol. xxv. p. 69, 1899.

Hunter have tested most of the aerobic pathogenic organisms with negative results. Thus the *Staphylococcus*, *Streptococcus*, *Pneumococcus*, *Friedländer's Bacillus*, *B. diphtheriae*, *B. pyocyaneus*, *B. mallei*, *B. anthracis*, *Vibrio cholerae*, and many of the allied *Vibrios* failed to alter neutral-red.

I found that *B. tetani* and *B. oedematis maligni* produced in glucose-agar the same appearances as *B. coli*, even when the surface of the medium was exposed to air. With all three a layer of unreduced red was left at the top of the tube. In bouillon, however, the anaerobic bacilli only produced the reaction when oxygen was excluded. A reaction due to these organisms would thus appear to be easily distinguished, although we must remember that anaerobic bacteria have been observed to develop in cultures exposed to the air when associated with aerobic bacteria.

One organism isolated from tap-water—apparently a variety of *B. mesentericus*—was observed to change the red to a dull orange colour, both in bouillon and glucose-agar. The change began on the second day of incubation. The surface layer was, however, the part first affected, while with *B. coli* the change begins at the bottom of the tube, and in glucose-agar never reaches the surface. In a tube inoculated with both organisms it is possible to distinguish the two reactions in the upper and lower layers, when the bright yellow due to the *coli* contrasts with the dull orange of the *mesentericus*. The latter also acts much more slowly, and does not form gas in glucose-agar.

A number of other water organisms were tested, none of which gave the reaction. My experiments, so far as they have gone, seem to indicate that a water producing a typical canary-yellow colour in neutral-red media, within 48 hours in bouillon, and accompanied in glucose-agar by green fluorescence and gas-formation, may be considered to contain *B. coli*. At any rate in every case in which these appearances were obtained further examination revealed the presence of an organism with all the essential characters of the *coli* group.

A large number of experiments were made to test the delicacy of the reaction as an indication of the presence of *B. coli*. Flasks containing a known amount of sterilised tap-water were inoculated with varying quantities of 24-hour old bouillon cultures (themselves inoculated from old agar cultures) of *B. coli*. From each of these flasks quantities of 1 c.c. were added to tubes of bouillon and glucose-agar containing 1% of a saturated watery solution of neutral-red. The tubes were afterwards incubated at 37°. At the same time agar plates

TABLE I.

	FLASK A. Dil. $\frac{1}{1000}$	FLASK B. Dil. $\frac{1}{10,000}$	FLASK C. Dil. $\frac{1}{100,000}$	FLASK D. Dil. $\frac{1}{1,000,000}$	FLASK E. Dil. $\frac{1}{10,000,000}$	FLASK F. Dil. $\frac{1}{100,000,000}$	FLASK G. Dil. $\frac{1}{1,000,000,000}$	FLASK H. Dil. $\frac{1}{10,000,000,000}$
	1000 c.c. of sterile tap-water+1 c.c. bouillon culture <i>B. coli</i>	1000 c.c. of sterile tap-water+1 c.c. coli bouillon	1000 c.c. of sterile tap-water+0.1 c.c. of coli bouillon	1000 c.c. of sterile tap-water+1 c.c. of flask A	1000 c.c. of sterile tap-water+1 c.c. of flask B	1000 c.c. of sterile tap-water+1 c.c. of flask C	1000 c.c. of sterile tap-water+1 c.c. of flask D	1000 c.c. of sterile tap-water+1 c.c. of flask E
Neutral-red in 24 hours	1 c.c. of this dilution in each tube 1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +
Neutral-red glucose-agar in 24 hours	1 c.c. per tube 1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. + 2. + 3. +	1. A few bubbles in 24 hours 2. Ditto 3. Ditto All three gave yellow fluid in 24 hours
No. of colonies on agar-plates	1. } Innumerable 2. }	1. } Innumerable 2. }	1. } Innumerable 2. }	1. } Numerous 2. }	1. } 30-40 2. } 3. }	1. } 10-20 2. } 3. }	1. } 1-5 2. } 3. }	1. } 1-2 2. } 3. }

containing corresponding amounts of the water were prepared, and the colonies counted after 48 hours' incubation. Uniformity in method was thus obtained, and the results of many observations were fairly even.

It was evident that neutral-red affords a very delicate test. In bouillon a reaction could be constantly obtained within 24 hours even with dilutions corresponding to from 1 to 5 organisms per c.c. It consisted of a diffuse canary-yellow colour which in extreme dilutions did not reach the surface of the fluid in 24 hours, while the lower parts had a more orange tint than when *B. coli* was plentiful. If larger amounts of the culture were added complete change occurred within 12 hours. In glucose-agar the reaction was much less prompt, and with extreme dilutions it took 5 or 6 days for a complete reaction (medium broken up by gas-formation, and coloured yellow along with greenish fluorescence except at the upper  $\frac{1}{2}$  inch) to develop. Gas-bubbles appeared first, usually within 24 hours, then a yellow fluid in the spaces at the bottom of the medium, and finally the full reaction.

It is unnecessary to detail the results of each series of dilutions. The above table, representing one series, will serve as an illustration.

In the table the sign + indicates a positive reaction irrespective of whether there was complete yellow coloration of the glucose-agar, or merely the appearance of yellow fluid at the bottom of the tube.

The experiments are summarised in Table II.

TABLE II.

Dilution	$\frac{1}{1000}$ to $\frac{1}{1,000,000}$	$\frac{1}{100,000,000}$	$\frac{1}{1,000,000,000}$	$\frac{1}{10,000,000,000}$	$\frac{1}{100,000,000,000}$
Neutral-red bouillon	Constant reaction in 24 hrs.	Constant reaction in 24 hrs.	Reaction generally in 24 hrs. Absent in about 10% of observations	Reaction in 24 hrs. in 40%. No reaction in 60%	Reaction in 24 hrs. very rarely
Neutral-red glucose-agar	Reaction in 24 hrs. complete yellowness or yellow fluid only	Reaction in 24 hrs. Medium broken up and yellow fluid in bottom of tube	Reaction in 24 hrs. in 25%; in 2-5 days in 50%. No reaction at all in 25%	Reaction in 48 hrs. in about 50%	No reaction
No. of colonies on agar plates	Very numerous	9-20	1-5	0-2	

It will be noticed in the tables that the colonies which developed on the agar plates are more numerous than corresponds to the increasing dilution. This was doubtless due to the rapid multiplication of the bacteria while the dilutions were being made.

These experiments were made with pure cultures of *B. coli*, and it was of course necessary, before applying the method to the examination of water-supplies, to determine how far the presence of other organisms might retard or alter the reaction. The time at my disposal only permitted a few experiments in this direction.

The whole series of flasks described in Table I. was prepared again, but this time *unsterile* tap-water was used—with the result that in neutral-red broth as far as Flask G (1 to 5 *coli* organisms per c.c.) all the tubes showed the reaction in 24 hours. Flask H, however, gave a reaction in only one out of three tubes, the others remaining unchanged in colour even after 48 hours. In glucose-agar an inhibitory action was more apparent, as Flask F (10 to 20 *coli* organisms per c.c.) gave a reaction in only one tube after 24 hours. The others were delayed five days, while those from Flasks G and H remained unchanged after six days. It should be added that, as the tap-water itself sometimes gave a reaction when 1 c.c. was added to a tube, the dilutions in the flasks were made double the strength given in Table I., so that it was only necessary to use .5 c.c. for inoculating each tube. This amount of pure tap-water rarely gave a reaction.

In another experiment a large carboy of 40 litres capacity was filled with unsterilised tap-water. To this was added .004 c.c. of *coli* bouillon, making a dilution of  $\frac{1}{10,000,000}$ . Tubes of neutral-red media were inoculated with amounts of this mixture varying from .5 to .01 c.c. In bouillon the reaction was present in 24 hours in all the tubes, and in glucose-agar where .5 and .25 c.c. were used. The same amounts of water taken from the carboy before the culture of *B. coli* was added gave no reaction. The results therefore corresponded to those of the previous experiment, an inhibitory action being apparent in the case of the glucose-agar tubes.

In a further experiment to each of the flasks E, F and G, Table I., 1 c.c. of a 24-hour old bouillon culture of *B. mesentericus* was added. Tubes of neutral-red media were then inoculated with 1 c.c. of this mixture and incubated at 37°, with the result shown in Table III.

TABLE III.

	FLASK E.	FLASK F.	FLASK G.
	Dilution $\frac{1}{10,000,000}$ <i>coli</i> bouillon $\frac{1}{1000}$ <i>mesentericus</i> „	Dilution $\frac{1}{100,000,000}$ <i>coli</i> bouillon $\frac{1}{1000}$ <i>mesentericus</i> „	Dilution $\frac{1}{1000,000,000}$ <i>coli</i> bouillon $\frac{1}{1000}$ <i>mesentericus</i> „
1 c.c. in neutral-red bouillon	In 24 hrs. fluorescence but little yellow In 48 hrs. full reaction	In 24 hrs. slight fluorescence below in some tubes. No reaction in one In 48 hrs. full reaction in all	In 24 hrs. no change In 48 hrs. reaction in two tubes In 72 hrs. reaction in all
1 c.c. in neutral-red glucose-agar	Coli reaction in 24 hrs.	Coli reaction after 72 hrs.	

The *Bacillus mesentericus*, therefore, when present in large excess, was able to delay the reaction considerably.

The laboratory tap-water was well adapted for estimating the value of neutral-red media as a test for minute quantities of *B. coli*. This water normally contains very few *coli* bacilli, and by the usual methods it is difficult to detect their presence. Thus of five agar plates, each containing .2 c.c. of water, only one showed a colony of *B. coli*. In neutral-red media the tap-water gave the following results:—

In bouillon :

- 2 c.c. Positive reaction in 24 hours.
- 1 c.c. Positive reaction in 24 hours in 40% of 15 examinations. No result in other cases.
- .5 c.c. Positive reaction in 14% of tubes. Delayed in one case till third day.
- .25 c.c. and below this. No reaction.

In glucose-agar :

- 2 c.c. No change on first day ; a few bubbles on second ; positive reaction on third or fourth day in every case.
- 1 c.c. Positive reaction in 33% of tubes, generally showing on fourth day.
- .5 c.c. One tube out of seven showed some reaction on the sixth day.
- .25 c.c. and below this. No reaction.

Where reactions were obtained with tap-water the presence of *B. coli* was on most occasions verified by making plates from the tubes. The colonies were then examined as to reaction and mode of growth on

various media, and in every case bacilli having all the characteristics of *B. coli* were demonstrated, save that the coagulation of milk was delayed in some cases till the fourth or fifth day, and was sometimes entirely absent.

In making the plates, more especially from glucose-agar tubes, it was remarkable how nearly pure were the cultures of *B. coli*. The yellow fluid found on the third day at the bottom of these tubes when 2 c.c. of tap-water had been used, was found generally to contain few other organisms.

As a qualitative means of detecting *B. coli* neutral-red media evidently offer great advantages owing to the large amount of water which can be examined. By the ordinary plate method minute quantities of *B. coli* are of course crowded out by other organisms.

I am deeply indebted to Dr W. Hunter, who planned the investigation and gave me much valuable assistance, and to Dr Bullock, in whose laboratory the research was made. A preliminary summary of the results was given by Dr Hunter in the *Lancet*, April 14, 1901, p. 1079.

#### CONCLUSIONS.

1. Neutral-red media afford a rapid and very delicate test of the presence of *Bacillus coli* in water.
2. By using varying quantities of water a rough estimate can be obtained of the number present, allowance being made for the influence of inhibiting organisms.
3. A negative result where a fair sample of water is examined may be taken as evidence of the absence of *Bacillus coli*.
4. Further investigation is needed to decide whether or not a positive reaction always indicates the presence of *Bacillus coli*; but as yet no case has been observed by the writer in which this bacillus was absent from a sample of water which gave a typical positive reaction.