THE VALUE OF METHODS FOR THE DIFFERENTIATION OF BACILLI OF THE COLI-AEROGENES GROUP, WHEN APPLIED IN SHANGHAI.

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In the examination of water supplies it is a difficult problem to discriminate between the faecal and non-faecal origin of the lactose-fermenters isolated. At present greatest reliance is placed on the indol test and the combination of the methyl red and Voges-Proskauer reactions. Though these have a great deal of work behind them, it cannot be claimed that they cut a clear division between the members of the coli-aerogenes group. In attempting to do so, Koser (1923, 1924) has devised, among other methods, a citrate test. He makes use of a solution in which sodium citrate is the only source of carbon; and has shown that faecal organisms are not likely to grow in it, whereas the majority of non-faecal organisms do so. The present paper records the results of an investigation into the value in Shanghai of these three tests, the indol, the combined methyl red and Voges-Proskauer and Koser's citrate test.

The collection of organisms from the faeces of man and animals was easy enough; but the collection of non-faecal strains, which were sought in the soil, was much more difficult. Shanghai is surrounded by flat cultivated land heavily manured with human nightsoil. The obvious place to find uncontaminated soil was on the tops of hills; but these are all at some distance, while there are no roads and few railway facilities. On a holiday up-country I secured several samples from hill-sides, but these yielded only two strains of lactose-fermenters. Since then the surrounding country has been too disturbed to allow me to travel far from the Settlement, and I have had recourse to such small elevations as railway embankments, the butts on the rifle range and the grave mounds which are so numerous in China.

The results are set out in Table I.

All the organisms dealt with are small Gram-negative non-sporing bacilli, capable of growing in the presence of bile salt and, in pure culture, forming acid and gas in lactose in 48 hours.

The methyl red is referred to as the MR test. MR + signifies the production of a red colour, MR − of a yellow colour, on the addition of methyl red solution to the glucose peptone water. In doubtful cases, where an orange colour appeared, this is shown as MR ±. The Voges-Proskauer is referred to as the VP test. VP− signifies no change, VP + the production of an eosin-like colour on the addition of caustic potash to the glucose peptone water. The combination of the two tests is referred to as the MRVP test.

Growth in citrate solution is shown as citrate +; no growth as citrate −.

Table I. *Examination of* 200 *organisms of the coli-aerogenes group.*

Incubation for five days at 37° C. The figures are percentages.

Source of strains	Citrate +	Citrate −	Indol +	Indol −	MR + VP −	MR − VP +	MR ± VP −	MR − VP −	Combined Citrate + and Indol −
100 human	7	93	92	8	95	1	3	1	7
50 animal	8	92	90	10	96	0	0	4	6
150 human+animal	7·3	92·7	91·3	8·7	95·3	0·7	2	3·3	6·7
50 soil	80	20	32	68	76	20	0	4	66

The sources of the animal strains were:

Guinea-pig	3 strains	Dog	8 strains
Calf	4 ,,	Rat	7 ,,
Rabbit	10 ,,	Pony	9 ,,
Sheep	5 ,,	Fowl	2 ,,
Mouse	1 strain	Monkey	1 strain

There is no difficulty in finding the reactions typical of faecal organisms. They are citrate negative, indol positive and MR + VP−. Of 150 strains 89·4 per cent. gave the combination of these three reactions.

The problem of the soil organism is not so simple. Of these 32 per cent. were indol positive, while the MRVP test gave little help, for 76 per cent. of the soil organisms were MR + VP −. In fact the failure of the latter is so striking, as compared with the reports of other workers, as to cast suspicion on the results. Two sources of error suggest themselves; either the glucose peptone water was at fault, or a number of faecal organisms have crept into the soil series through the difficulties of collection. That the glucose peptone water is capable of giving a MR − VP + reaction is shown by its having done so in eleven cases. However, it was thought possible that a longer period of incubation might have produced a larger number of such reactions. Therefore ten "soil" strains, of which all were citrate positive and the majority indol negative, were incubated for seven and fourteen days each; in all cases they remained MR + VP − as they were after five days' incubation.

In addition, six MR − VP + strains were examined to discover how quickly this reaction was produced. After 24 hours four gave definite MR − VP + results, while two were indefinite. After 48 hours all six were quite definitely MR − VP +.

With regard to the question of faecal contamination of this series, it is of course impossible to rule it out entirely; in fact it is very probable that some of the strains are of faecal origin, either from man or animals. An example is furnished by one case in which it was found, after the sample of soil had been placed in the enrichment broth, that it contained a small dead beetle. The specimen was set aside from the series on account of the obvious contamination, but the culture was carried on, and finally a bacillus was isolated of the *B. coli communior* type, which gave no growth in citrate.

But in spite of the probability of contamination, the results show that there is some kind of difference between the human and animal strains and those of the "soil" series. There is no question of the origin of the former,

among which 89·4 per cent. are citrate negative, indol positive and MR + VP −. Consequently of any faecal organisms which may have been included in the "soil" series, about nine out of ten will have given the same reactions. As a matter of fact in this series there are ten citrate negative bacilli; nine of these were also indol positive and MR + VP −. Therefore it may be taken as certain that about forty of the "soil" organisms are not derived from faecal contamination. Among the fifty in the "soil" series 80 per cent. were citrate positive and 68 per cent. indol negative. If the contaminating faecal organisms could be recognised with certainty and removed, it is probable that each test would show an improved percentage.

Do these results hold good for organisms isolated from water, or will their sojourn in the water alter their character? Koser (1924 *b*) investigated this point. He inoculated *B. coli* and *B. aerogenes* into sterile water, *B. coli* into sterile soil, and *B. aerogenes* into sterile stool suspensions, and examined them after several months. He also examined at intervals organisms which had been subcultured on artificial media. His conclusion was that "the ability to utilise citrate is apparently a fairly stable character, and evidently is not readily acquired or lost."

Koser has published several papers on the citrate and other distinguishing tests. For example, on one occasion (1924 *a*) he applied the MRVP, the citrate and uric acid tests "to two series of cultures, one from polluted water and the other from water of high sanitary quality as shown by sanitary survey." The MRVP test showed no significant distinction, whereas "the test of citrate utilisation, on the other hand, showed some degree of correlation with the sanitary survey of the water supplies."

Pawan (1925) isolated 240 strains from water which was considered pure. Of these 42·5 per cent. were MR +, while 81·3 per cent. were able to grow in citrate. Consequently the citrate test agreed more closely with the sanitary survey than did the methyl red.

The same author (1926) made a bacteriological survey of the River Lopinot, Trinidad. Among other reactions he applied the indol and Koser's citrate tests. His results supported the value of the citrate test.

Raghavachari (1926) used the citrate and MRVP reactions in examining organisms derived from the soil and from various classes of water. He recognises the possible value of the citrate test and says that while it is worthy of the most careful study, further work appears to be necessary, and that at present he is unwilling to discard the system based on Clemesha's scheme of classification.

It would be out of place in such a paper as this to discuss at length the position of such well-recognised tests as the indol, methyl-red and Voges-Proskauer. It is agreed that nearly all faecal organisms are indol positive and MR + VP −, and that most, but by no means all, non-faecal members of the group are indol negative and MR − VP +. The failure of the latter type to give uniform results with these reactions leaves an opening for a method which will give a clearer distinction. The citrate test may fill this opening,

but a conservative attitude is indicated towards a test which is designed to supplant such well-recognised methods as the indol, methyl red and Voges-Proskauer reactions. But I think it is justifiable to conclude that under local conditions both the indol and citrate tests are valuable as criteria of the origin of the organisms examined, with an advantage in favour of the citrate test. If only bacilli which are citrate positive and indol negative, in combination, are regarded as non-faecal, the error will be on the safe side.

As a matter of interest the citrate test was applied to a number of non-lactose fermenters. After five days' incubation at 37° C. the following results were obtained:

B. typhosus	No growth	*B. enteritidis* (Gaertner)	Growth in 2 days
B. paratyphosus A	,,	*B. paratyphosus* B	,,
B. dysenteriae Flexner V	,,	*B. suipestifer*	,,
B. dysenteriae Flexner Y	,,	*B. faecalis alkaligenes*	,,
V. cholerae	,,	*B. paratyphosus* C	Growth in 4 days

The inoculations were repeated, the only differences being:

B. faecalis alkaligenes	Growth in 1 day
B. paratyphosus C	,, 5 days

On the second occasion those showing no growth were incubated for ten days without the appearance of any turbidity in the solution.

The difference between *B. paratyphosus* A and B is interesting, and if confirmed for other strains, may be important.

Technique.

The majority of the human stools were of the normal formed type; a few were diarrhoeic or dysenteric. They were plated on MacConkey's neutral red bile salt lactose agar, from which suitable colonies were subcultured on to agar slopes.

Most of the animal faeces were plated on to MacConkey's agar, but a few underwent a preliminary enrichment in MacConkey's broth. The colonies from the plates were transferred to agar slopes.

All the soil samples were enriched in MacConkey's broth, some in glucose, the majority in lactose; they were incubated at 37° C., or in a few cases first at 20° C. and afterwards at 37° C. They were plated on MacConkey's lactose agar and subcultured on agar slopes.

In all cases care was taken not to pick more than one organism of each type from the same sample of soil or faeces. The agar slope cultures were examined for purity, stained and examined microscopically. A very small quantity of the culture was inoculated into the citrate tube first, and the wire was carried on, without being recharged, to inoculate a lactose tube, so as to ensure, in the case of bacilli incapable of growing in citrate that some living organisms had been transferred to the citrate solution. The other media were then inoculated in the usual manner.

The peptone solution for indol production was made up with Bacto-peptone. Each batch was tested with an organism known to produce indol, and also with one known not to produce it.

The glucose peptone water was made according to the formula of the peptone medium for the methyl red test, p. 111, *Standard Methods for the Examination of Water and Sewage*, 1925, published by the American Public Health Association.

The citrate medium was made according to the formula given in the same book.

The time of the first appearance of turbidity in the citrate solution in 51 cultures was: 1 day in 35 cultures, 2 days in 12 cultures, 3 days in 2 cultures, 4 days in 2 cultures.

Several strains were cultured more than once, without significant alteration.

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SUMMARY.

1. A collection was made of organisms of the coli-aerogenes group; 100 from the human intestine, 50 from animals, and 50 from the soil. To these were applied the indol test, the methyl red and Voges-Proskauer tests, and Koser's citrate test. The citrate test was also applied to certain non-lactose fermenters.

2. 89·4 per cent. of 150 faecal organisms gave the combination of a failure to grow in citrate solution, the production of indol, an acid reaction with methyl red and a negative Voges-Proskauer reaction.

3. There is a probability that some faecal organisms have crept into the "soil" series, but the contamination is not heavy enough to make the results useless. The figures show that the citrate and indol tests are both useful, the citrate being a little better. The methyl red and Voges-Proskauer reactions were not helpful.

4. In the bacteriological examination of water in Shanghai it is safe to take those organisms which can grow in citrate but do not produce indol as being non-faecal.

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