THE CLASSIFICATION OF THE COLON-AEROGENES GROUP OF BACTERIA IN RELATION TO THEIR HABITAT AND ITS APPLICATION TO THE SANITARY EXAMINATION OF WATER SUPPLIES IN THE TROPICS AND IN TEMPERATE CLIMATES.

A COMPARATIVE STUDY OF 2500 CULTURES.

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PART I. HISTORICAL SURVEY.

Introduction.

It is now close upon half a century since Robert Koch isolated the cholera vibrio from the waters of the Elbe. It is remarkable therefore that although the other pathogenic bacteria of intestinal origin are now readily cultivated, and have recently been recovered with comparative ease from sewage (Wilson, 1928), most attempts to isolate them from water supplies—at least as a routine measure—have been singularly unsuccessful. The classical Hamburg cholera epidemic of 1892 may be said to have marked the beginning of systematic water bacteriology in the form that we now know it. In the following year, Theobald Smith (1893), referring to *Bacillus coli*, wrote as follows:

"It is safe to infer that any organism which is so uniformly present in the intestinal tract, and which possesses to a slight degree pathogenic properties, really belongs there, and that its presence outside the intestines, in soil and in water, may be regarded as due to their contamination with faecal discharges of men or animals."

One might therefore assert with truth that the rôle of *B. coli* as an indicator of excretal contamination is as old as the bacteriology of water itself; yet it is a matter of considerable doubt whether the perfect bacterial indicator of faecal pollution exists even to-day.

During the present century, volumes have been written concerning the bacteriology of drinking water. Recent advances have gone far to dispel much of the confusion which was formerly attached to the subject, but there still remains a difference of opinion as to what represents the most constant index of excretal pollution. It is, however, generally conceded that for practical purposes the indicator organisms may be divided into the following well-defined groups:

(1) The colon-aerogenes group.
(2) The faecal streptococci.
(3) The sporing anaerobes.

With the last two classes we are not concerned here. Most workers are agreed that their presence in water may be a valuable confirmation of the *B. coli* test. On the other hand, it must be rare indeed to detect them in the absence of the coliform organisms. Although Wilson (1929) has recently expressed the opinion that *B. welchii* rarely occurs in the outer world apart from faecal contamination, and many workers are satisfied that the streptococci may be strong evidence of such pollution, the search for these bacteria is not in fact commonly undertaken in most routine water examinations to-day. The majority of modern methods, however they may differ in the details of their theory and their practice, are founded on the common belief that in *B. coli* we are possessed of an indicator which will satisfy most of the needs of the water bacteriologist. It may, it is true, only take us a certain part of the way: but it is at least the nearest approach to a perfect faecal indicator known

to bacteriology. The colon test, in one form or another, is still what Houston considered it to be in 1912, "the sheet-anchor of the bacteriologist."

THE COLON GROUP AS FAECAL INDICATORS.

(1) *The* B. coli *test, its uses and limitations.*

In England and other temperate climates, the *B. coli* test, despite many vicissitudes, has undergone few permanent changes in its essentials since its inception. The modified form in which it is now commonly employed aims at simplicity; to minimise confusion, a broad interpretation has been put upon the term "*B. coli*," by taking into account only the more constant features of the group. There are certain permanent characters of the colon family upon the importance of which all observers are agreed. Savage (1907) pointed out that different characters have not an equal value: that, for example, failure to produce indol would not immediately exclude an organism from the group, whilst inability to ferment lactose would cause one gravely to doubt whether it were *B. coli* at all. It is accepted that in order to justify its claim to the title of *B. coli*, an organism must fulfil certain primary obligations. It must be a short bacillus with rounded ends: it must be Gram-negative, non-sporing, and a facultative anaerobe: it should be capable of fermenting glucose and lactose at a temperature of 37° C. with the formation of acid and gas, of coagulating milk, failing to liquefy gelatin, and producing indol in a medium containing peptone.

It was on the basis of this grouping that Houston formulated his now well-known "lactose positive-indol positive" index. Houston (1912), whilst admitting the value of the more comprehensive tests for the purposes of research and classification, regards his standard as being "common ground for water bacteriologists." His position, simply stated, is that organisms answering to his definition of *B. coli* are in England abundant in the intestine and rare outside it: that their presence is in itself adequate evidence of faecal pollution: and that a water containing them ought properly to be condemned. Savage (1912) expressed a somewhat similar view. He contended that by the use of lengthy classifications, the additional work involved and the rigid standards produced had no justification in practice unless it could be shown that a real natural differentiation was thereby demonstrated.

The water bacteriologist of temperate climates will, in practice, regard with suspicion a water containing Houston's *B. coli*, and in the majority of instances he will be right. Recent work has gone to show that in a proportion of cases, varying according to local conditions, there may be a fair margin of error. Usually, however, this error will be in the direction of safety and can do no harm. In the Tropics, a very different state of things exists. The sanitary bacteriologist frequently finds himself called upon to adjudge the safety of a water which may well be the only supply for many miles. In these circumstances he cannot afford to apply unnecessarily rigid standards. On the other

hand, he must leave no doubt as to the potability of a water supplying perhaps all the needs of a large community. Tropical workers have long ago observed that to apply the bacteriological standards of temperate climates to their local water supplies was to condemn almost every sample. It was at one time assumed that in the absence of specific differentiation between excretal organisms of human and animal origin, a large number of waters were being condemned because of the influence of that gross pollution with animal excreta which is so widespread in the East. This was obviously not the whole explanation, and the relative infrequency of water-borne disease in many tropical countries remains all out of proportion to the bacteriological findings.

Daniels and Finlayson (1908), in the Federated Malay States, observed that *B. coli* was rarely absent from 2 c.c. of any water sample, as judged on English standards. Archibald (1911) and Balfour (1911) remarked upon the inconclusive results obtained with the use of standard methods in the Sudan. The first, and the most far-reaching effort to establish the bacteriology of waters in the Tropics upon a rational basis was that attempted by Clemesha in 1912. Clemesha, after a careful study in India, discussed the subject in great detail. He criticised Houston and the British school on the grounds that in classifying *B. coli* they applied to a group of bacteria the characteristics which properly belonged to a species. He showed furthermore that different species did not react equally to the forces of nature. He pointed out that in India one at least of these forces—namely insolation—could be readily studied, and he was able to divide the colon group into different classes according to their reaction to the effects of sunlight. In this way, he devised three classes, the susceptible, the less susceptible, and the resistant. He maintained that the presence in surface waters of the more susceptible organisms could be regarded as undoubted evidence of recent contamination. He also showed that the bacterial flora of human and animal excreta in India not only differed from those in England in the relative proportions of the organisms which they contained, but that they were subject to definite local and seasonal variations. In this connection he referred to the comparative rarity of true *B. coli communis* in India, even in faeces, a point which had already been discussed by Castellani (1910) in Ceylon.

Clemesha, in criticising the failure of European standards in the Tropics, attempted to explain their success in England and other temperate climates. He asserted that water contamination in England was largely manurial in nature, human in origin and urban in source: that the true *B. coli communis* was the common excretal organism, and that it was therefore the bacillus most frequently encountered in polluted water. He corroborated what had already been put forward by McConkey (1905) and Savage (1907), namely that the fermentation reactions were of great value in separating species, but only when by such a separation they threw some light on the natural history of the organisms concerned. Clemesha wrote as follows:

"Of course, classification of organisms according to their fermentation

reactions is necessary in order to describe them...but we wish it to be understood clearly from the very outset, that we never for a moment suppose that all organisms which ferment the same sugar possess similar characteristics in other respects, or behave similarly when passed into water and subjected to natural conditions."

Clemesha's observations found general acceptance in India, and his methods are still widely employed in that country. Thus, Hirst in Ceylon and Morison in Poona have found them to be of practical value. On the other hand, many observers meeting with less success, have been unable to confirm all Clemesha's views. Glen Liston in Bombay and McCombie Young in Assam could not agree that the presence in water of Clemesha's susceptible class of organisms was proportionate to the degree of pollution found: indeed the latter worker was able to isolate them from waters where no possible risk of contamination could be demonstrated. It would appear that Clemesha's assertions have not as yet received practical confirmation to an adequate extent in other countries. Even though the principles involved should be found to apply to certain parts of India, where so many supplies are derived from large rivers and lakes, there appears to be no *a priori* reason why they should have the same significance in other parts of the Tropics, as for example where shallow wells predominate. In such circumstances, their propriety could only be proved after a lengthy series of observations.

However much Clemesha's assertions may be contested, there is little doubt that his main propositions represented a distinct advance in our conceptions of the rôle of *B. coli* as an indicator of faecal pollution. It is agreed that in the criteria of excretal contamination employed in temperate climates, there are included several species, many of which are not of faecal origin. As these species presumably do not occur with any frequency in the soil and waters in Europe, the error due to their inclusion is not a serious one. The general opinion in the Tropics, however, is, on the other hand, that the appearance of non-faecal species in water must be by no means uncommon; and that therefore much water must be condemned which might well be drunk with impunity. It is evident that since both the "coli" method and the "separate species" method of classification have proved of doubtful value, the position of water bacteriology in the Tropics has been far from satisfactory.

Recent researches, developing since 1914, have thrown a new light on the significance of the colon-aerogenes group of organisms in their relation to the bacteriology of water. There is abundant evidence to suggest that the coliform bacteria can be assigned to their proper environmental categories without having resort to the identification of a large number of individual species. Tests have been devised which, it is claimed, are capable of separating faecal from non-faecal organisms. The originators of these tests have shown that they may be applied to water analysis, and that in such an application correlate closely with the sanitary considerations. These tests have attracted little attention in England, presumably because the need for them is not great. The

results of many workers in other countries, particularly in America and to some extent in the Tropics, suggest that the newer methods may be of considerable practical value to the sanitary bacteriologist in general and the tropical worker in particular.

A review of the literature would appear to indicate that a sufficient amount of work has not yet been described in the Tropics in connection with modern methods. It is realised that different localities must be surveyed separately and individually before any new methods can be included in routine bacteriological standards. Scientific authorities, ever conservative, are naturally slow to adopt any principles which have not been founded upon adequate experimental data. Savage in 1912, referring to differentiation tests for the coliform organisms, wrote:

"The value of their extensive employment for the differentiation of *B. coli* found in water must depend upon the light they shed upon the origin of the organisms to which they are applied. Unless their use differentiates organisms so as to show a different distribution in Nature, and a different significance, it cannot be said that they are of any special value."

The American school claims, with considerable support, that these conditions have been definitely fulfilled. Whether such claims can be substantiated throughout the Tropics is, in the light of our present limited experience, a matter of some speculation. At least a reason is suggested for investigating them: for as Topley has remarked in this connection: "There is nothing to be gained by the deliberate neglect of scientific methods in routine bacteriology."

Such investigations have been carried out in great detail in some tropical countries, such as India (Cunningham and Raghavachari, 1924; Taylor, 1926), Trinidad (Pawan, 1925, 1926), Shanghai (Hicks, 1927) and Colombia (Brewster, 1929). A survey of the local water supplies by the use of more recent methods has not, however, been reported hitherto from East Africa. It is on a consummation of evidence from different sources that the value of new methods can best be ascertained, and the present work was therefore undertaken with a view to contributing something further to our knowledge of water bacteriology in the Tropics.

Much careful work has already been done in other countries, and several valuable summaries of latest advances are available. Notable amongst these are the reports of Levine (1916), Chen and Rettger (1920), Bardsley (1926), Pawan (1927), and Singer (1929). Although one hesitates to add anything further to these excellent surveys, it is believed that before describing the experimental work it would be advantageous to review briefly the principal investigations which have led up to our present knowledge, the better to follow its application.

(2) *Differentiation methods for the colon-aerogenes organisms.*

A. *Distribution of the group in nature.*

Escherich (1885) recognised the existence of two distinct types of *B. coli*, and in 1886 described a second variety to which he applied the name *B. lactis aerogenes*. Contemporary workers (Laruelle, 1899; Levy and Bruns, 1899) advocated the employment of pathogenicity tests for distinguishing between the species isolated from water, but Savage (1903) showed that such tests, as a measure of water pollution, yielded no important information. For a time it was believed that Escherich's bacilli represented the perfect indicators of faecal pollution. It was not long before a number of observers were able to establish that bacilli apparently indistinguishable from those described by Escherich were widely distributed in nature. Such organisms were isolated in typical or atypical forms from the faeces of various animals (Dyar, 1894), grain, cereals and tubers (Laurent, 1899; Klein and Houston, 1899; Prescott, 1902), from soil and water where the possibility of faecal pollution appeared to be remote (Jordan, 1903; Houston, 1912; McConkey, 1905), and more recently from the dust of streets, grass, trees, plants, and the washers of pumps and wells (Schobl and Ramirez, 1925). The behaviour of the coli-typhoid organisms in soil attracted the attention of many workers (Savage, 1907; Mair, 1908; Laybourn, 1920), and beyond establishing that these organisms could live for some time outside the body, did not prove of any special value for the purposes of differentiation. They did, however, throw some light on the biology of the organisms, for it was established by various workers that different species did not react similarly to the forces of Nature (Savage, 1907; Clemesha, 1912; Skinner and Murray, 1926). Savage noted that some alteration of character occurred in *B. coli* in soil, but not to any great extent. He believed that there was no evidence to show that aberrant types represented typical *B. coli* in an unfavourable environment. For the typical *B. coli* of Escherich he proposed the name "excretal *B. coli*." He found that a large proportion of coliform bacilli from surface wells were atypical in one or more particulars. In discussing whether aberrant forms represented decadent *B. coli* in a non-intestinal habitat, or were merely the survivors under more suitable conditions of the few atypical forms normally present in excreta, he wrote:

"The more nearly an organism isolated resembles an 'excretal' *B. coli*, the greater its significance as an indicator of pollution. Consequently the fewer required to condemn a sample of water in which they occur. Stated as a working proposition, the more the characters of the coli-like organisms deviate from that which for convenience may be spoken of as the typical form, the greater the proportionate number of them required to condemn the water."

It is not remarkable that the number of views held at one time cast a serious doubt upon the reliability of *B. coli* as an indicator of faecal pollution. The finding of coliform bacilli where excretal contamination appeared to be absent

or remote tended to the belief that the group was saprophytic and widely distributed in Nature, so much so that at one period the German school of which Kruse was the leader, actually discarded the *B. coli* test as being of no practical value. The observations of Savage were, however, a definite step in the direction of separating species more in terms of their natural history than of their cultural characteristics. His belief that "atypical" *B. coli* was of less sanitary significance than "typical" *B. coli* was the basis upon which many of the more modern methods were founded.

B. *The fermentation of carbohydrates and indol production.*

Most of the earlier differentiation methods of the coliform organisms were concerned with their growth in media containing fermentable carbohydrates, or containing peptone. Theobald Smith (1893) made the important observation that the majority of *B. coli communis* strains were unable to ferment saccharose, whilst *B. lactis aerogenes* fermented this substance with the production of acid and gas. Subsequent classifications on the basis of carbohydrate metabolism were made by Refik (1896), Durham (1901), Ford (1901), and McConkey (1905–1909). It is to the last-named observer that we owe the most satisfactory systematic classification of this nature. McConkey divided the coliform bacilli according to their fermentative action upon lactose, saccharose and dulcite. He was able to show that whilst the group represented by *B. coli communis* was able to ferment dulcite, it did not ferment saccharose. The *B. lactis aerogenes* type was able to ferment saccharose, but not dulcite, whilst the *B. coli communior* variety fermented both of these substances. Members of the group represented by *B. acidi lactici* had no fermentative action upon either carbohydrate. This classification, and modifications of it, was employed by numerous subsequent workers (Winslow and Walker, 1907; Bergey and Deehan, 1908; Clemesha, 1912), most of whom concluded thereby that *B. lactis aerogenes* was uncommon in faeces. Kligler (1914) believed that salicin offered better advantages than dulcite, whilst Levine (1916) considered that saccharose alone formed a more reliable basis of classification.

Later workers were soon to dispute the value of acid production from carbohydrates as a factor in differentiation. Rogers, Clark and Evans (1914, 1915) contested its significance, claiming that it was frequently obscured by secondary alkali fermentation. Laybourn (1920) failed to differentiate intestinal from soil strains of *B. aerogenes* by means of polysaccharide fermentation. Berry and Montford (1921) drew attention to the fallacies attached to the preliminary isolation of lactose fermenters from water by means of liquid media, owing to overgrowth of the aerogenes type. Schobl and Ramirez (1925) attempted to differentiate between *B. coli* of human faeces and of the faeces of terrestrial and water-living animals. They concluded: "that there is no single carbohydrate or any group of them which would enable us to differentiate lactose fermenters giving a *B. coli*-like colony of human origin from those of

animal origin. On the other hand, there is a distinct difference between all of the faecal strains and the strain isolate from the experimental fish pond."

Bardsley (1926) regarded detailed fermentation reactions as having a limited value, to be used only for taxonomic purposes. Thompson (1927) remarked upon the frequent failure of *B. coli* to grow in glucose broth owing to the development of a lethal H-ion concentration during preliminary enrichment, and suggested the elimination of this error by the use of dipotassium phosphate as a buffer. Greer (1928) and his fellow-workers, in an extensive study of lactose-fermenting organisms, discussed the fallacies occasioned by the isolation of lactose fermenters not belonging to the colon group. He showed that many fallacious results were brought about by the presence of spurious gas or acid formation, owing to symbiosis, synergism or antagonism on the part of a number of different bacteria.

The production of indol has been studied by all workers interested in the colon-aerogenes group. The nitroso-indol reaction of Bayer (1870), introduced to bacteriology by Kitasato (1889), had long been known. Blumenthal (1895) showed that *B. coli* during its growth in peptone media was capable of forming indol amongst other products. Böhme (1906) applied to bacteriology the test produced by Ehrlich (1901) for indol in urine. Other tests are employed, notably the oxalic acid test (Gnezda, 1899), vanillin (Steensma, 1906). The subject of indol production has been studied with great thoroughness by Holman and Gonzales (1923), Fellers and Clough (1925), Kulp (1925) and Koser and Galt (1926). Most of these workers are agreed upon the satisfactory results given by the Erlich-Böhme reaction. It would appear that the variety of methods in use has probably contributed in no small way to the lack of agreement regarding the significance of indol production. It is generally held that whilst true *B. coli* almost invariably produces indol from peptone members of the aerogenes group are also frequently able to do so. Hence a persistently negative result may be regarded as considerable confirmatory evidence against the presence of "excretal" *B. coli*.

From the foregoing, it may be concluded that many authorities have found the production of acid from carbohydrates and the production of indol from peptone inadequate means of differentiating the coliform bacilli. Their value for the purpose of general classification is undoubted: but their apparent inconstancy precludes their use as a sole means of distinguishing members of the group, at least from the standpoint of the sanitary bacteriologist. In applied bacteriology, where a method of differentiation based upon the natural occurrence of the organisms is so essential, other tests have been found more reliable. Most of these are dependent upon a recognition of the products of glucose metabolism by the bacteria themselves.

C. *The gas ratios, the methyl-red test and the Voges-Proskauer reaction.*

These reactions have now been so completely described in the past, that only the briefest reference to them is required. The reaction first described

by Voges and Proskauer (1898)—the production of an eosin-red colour in a glucose-broth culture to which a solution of caustic potash had been added and the tube exposed to the air for 24 hours—was studied by Durham (1901), who noted that the "polysaccharide factors" gave a positive result. Howe (1904) applied the reaction to water bacteriology, and observed that a positive result only occurred with strains which produced a large amount of gas from glucose. When a negative result was obtained a correspondingly large amount of glucose remained unused. The chemistry of the reaction was carefully studied by Harden and Walpole (1906). These workers were able to show that the characteristic fluorescence was due to a reaction between some constituent of the peptone and an oxidation product of glucose, namely diacetyl—itself, an oxidation product of acetyl-methyl-carbinol. It was soon demonstrated by several observers that the Voges-Proskauer reaction was rarely positive in the case of lactose fermenters derived from faeces (McConkey, 1905–1909; Ferreira, Horta and Paredes, 1908; Clemesha, 1912; Kligler, 1914). Levine first collated the reaction with the source of the organisms, and obtained entirely negative results in the case of faecal strains of *B. coli*, but a proportion of positive reactions with strains derived from sewage. These results were confirmed by Burton and Rettger (1917) and Chen and Rettger (1920) with cultures from faeces and soils, and by Robinson (1920), who only obtained 6·1 per cent. of positive results with 2100 colon cultures from faeces.

The classical work upon the gas ratios by Th. Smith (1895), Harden (1905), Harden and Walpole (1906) and Keyes (1909) is well known. Rogers and his colleagues (1914), after accurate gas determinations, expressed the belief that we had a ready means whereby the organisms of the colon group could be differentiated, in the different proportions of $CO_2 : H_2$ produced by the various species. They maintained that the gas ratios were exceedingly constant. They were able to divide the coliform organisms into two main groups, which they designated "high ratio" and "low ratio," the figure for the latter group being about 1, and the former about 1·5–2·0 or more. They observed that, judged on McConkey's grouping, the species *B. coli communis* and *B. acidi lactici* belonged entirely to the low-ratio group, whilst half the *B. coli communior* and 79 per cent. of the *B. lactis aerogenes* types belonged to the high-ratio group. Later, they reported that only about 8 per cent. of faecal organisms isolated by them were high ratio, whilst 95 per cent. of non-faecal organisms were of this variety.

For some time the difficulty of measuring the gas ratios precluded their general application; hence the correlation between them and the Voges-Proskauer reaction was not widely considered. Clark and Lubs (1915) demonstrated that the $CO_2 : H_2$ ratio correlated closely with the limiting H-ion concentration brought about in the medium used, and that the change in *p*H could be readily determined by use of the indicator methyl-red. This was soon confirmed, and the correlation between the methyl-red and Voges-Proskauer reactions was investigated by many workers. It was found that a considerable

degree of agreement existed between the two tests, and that by their use it could be shown that the majority of true faecal *B. coli* were methyl-red positive and Voges-Proskauer negative (Levine, 1916; Johnson, 1916; Rogers, Clark and Lubs, 1918). Numerous other investigators followed with reports as to the use of the tests in the identification of coliform organisms from soil and water. These included Burton and Rettger (1917), Winslow and Cohen (1918), Chen and Rettger (1920), Koser (1923), Bardsley (1926), Hill (1929), Perry (1929), and, in the Tropics, Pawan (1925), Cunningham and Raghavachari (1926), Taylor (1926), and Hicks (1927). Most of these workers found a fair degree of agreement between the tests, and Wood (1920) summarised the then current opinion as follows:

"The results of various investigators show that the lactose-fermenting bacilli can be divided into two main groups by the methyl-red and Voges-Proskauer tests, that the MR− VP+ type are rare in the faeces of man and animals, are more common in surface water and sewage, and are the predominant type in grain and soils. These findings are in favour of the view that they are either the natural survivors of the lactose fermenters present in excretal matter, or are derived from soil or possibly grain, and consequently their presence in water and food products is to be regarded as of less sanitary significance than the presence of excretal *B. coli*."

Unfortunately, discrepancies were found which were higher than could be explained by ordinary slight errors. Thus, Koser found that 80 per cent. of cultures from polluted waters, and 73 per cent. from unpolluted waters were MR+ VP−. This type of result was reported by other workers (Chen and Rettger, Perry and Montford, Pawan, Hicks). Winslow and Cohen found the proportion of MR+ VP− to be practically the same in polluted and unpolluted water. Most observers noticed that disagreement between the tests themselves was least noticeable in the case of organisms of the faecal type, thus confirming the opinion of Burton and Rettger (1917) that whilst correlation was complete or almost complete for the low-ratio organisms, the high-ratio types varied so considerably as to cast a serious doubt upon the biometric method of classification.

Apparently agreement between the two tests was in itself imperfect, or else some modification of their interpretation was required. The latter hypothesis involved the recognition of a natural existence of one or more groups other than the simple faecal and non-faecal. The difficulty in proving the existence of such organisms lay in the fact that no independent criterion was at the time available, whereby the results of the methyl-red and Voges-Proskauer tests could with accuracy be controlled. It was at about this time that a revived interest in the fermentation of the salts of organic acids by bacteria drew attention to a means by which the results of existing tests might possibly be checked.

D. *Utilisation of organic acids and their salts.*

Koser (1918) studied the deportment of certain bacteria towards uric acid as a sole source of nitrogen. He found that *B. coli* was unable to obtain therefrom sufficient nitrogen for its uses, whilst *B. lactis aerogenes* was able to grow profusely in a medium containing uric acid and simple salts. Other workers compared the uric acid utilisation with the methyl-red and Voges-Proskauer reactions, and found it to give a better indication of the source of the organisms (Chen and Rettger, 1920; Perry and Montford, 1921). Bardsley (1926) was unable to confirm this in the case of organisms isolated from water.

Brown (1921) observed a similar phenomenon, using sodium citrate. *B. aerogenes* grew more luxuriantly in a citrated broth than in plain broth, whilst *B. coli* was unable to grow in such a medium. Koser (1923) made a detailed study of the utilisation by bacteria of the salts of organic acids. He confirmed Brown's findings, and showed that *B. coli* was incapable of using sodium citrate as its only source of carbon. He devised a simple synthetic medium, by means of which he was able to show a close agreement between citrate utilisation and other differential tests. He concluded that the ability to utilise citrate was a definite and constant character of *B. aerogenes*.

Brown, Duncan and Henry (1924) in the main confirmed Koser's work. Koser, in the same year, collated his citrate test with the source of the organisms and with other criteria. He reported a close correlation between the citrate, methyl-red and Voges-Proskauer reactions in the case of cultures from faeces. Examining soil cultures, he obtained very different results. Of 72 such strains, although 34·7 per cent. were persistently MR+ VP−, and therefore presumably of the faecal type, all but two developed in citrate. Soil cultures thus adjudged to be faecal on the basis of the MRVP tests were shown to be non-faecal by the citrate test. Koser concluded that not all the MR+ organisms of nature were of faecal origin, and that the MR+ type of faeces and the MR+ of soil could be differentiated by means of the citrate test. This observation went far to explain the anomalous results previously reported by those workers who had obtained MR+ organisms from apparently non-polluted soil. Koser believed that as the MR+ organisms which also used citrate were encountered rather frequently, they might conceivably find their way into water and cause confusion. He therefore adapted his test to the sanitary analysis of water (1924). He isolated 107 coliform bacilli from polluted and 90 from unpolluted water, and found that whilst approximately 80 per cent. of the former and 75 per cent. of the latter were MR+ VP−, only 64·5 per cent. of cultures from polluted water and 16·7 per cent. from unpolluted were citrate negative. In other words, the faecal type in polluted and unpolluted samples as judged by the MRVP were approximately the same percentage, but the proportion of faecal organisms in the unpolluted samples was considerably reduced by the citrate test. Koser believed that the character of using citrate was stable and in no way altered by unfavourable conditions of environment.

Jones and Wise (1926) found that the organisms which used citrate were usually able to ferment cellobiose. Koser, in the same year, confirmed this finding, and considered that it added strength to his belief that the "intermediate" (MR+ K+) strains were more closely allied to the aerogenes than to the coli group.

In the same year, Koser examined 41 soil samples from cultivated fields, presumably remote from faecal pollution, and 11 samples from pasture lands frequently grossly contaminated. 104 cultures from the first group and 33 from the second were tested. He found that 67·3 per cent. of cultures from the first were of the aerogenes type (MR− VP+ K+), whilst 63·6 per cent. from the second were of the coli type. In the cultivated fields 7·7 per cent. and in the pasture lands 3 per cent. of cultures were "intermediate" (MR+ K+). Koser was satisfied that the MR+ K+ organisms which could not previously be detected by the methyl-red and Voges-Proskauer tests alone, were commonly present in unpolluted soil and water and could not be regarded as having the same significance as the MR+ type of faeces, at least in relation to water supplies.

Simmon's (1926) modified Koser's medium by solidifying it with agar, and adding brom-thymol blue as an indicator. He reported that it gave results which corresponded closely with the original, but the reading of which was greatly simplified.

Koser's citrate test was soon adopted by water analysts, particularly in the Tropics, where a need for a test on these lines had long existed. Pawan (1925) in Trinidad studied the citrate test in relation to a number of cultures from faeces, soil and water. He had previously found that by using the standard index of Houston (lactose+ indol+), a proportion of 42 per cent. of the organisms isolated from apparently unpolluted soil had to be regarded as excretal *B. coli*. Employment of the MRVP tests was found to reduce considerably the number of bacilli of this type in non-faecal samples, but the results still failed to agree completely with the sanitary considerations. Pawan obtained the following results:

	Faeces	Unpolluted soil	Polluted water	Unpolluted water
Number of cultures	740	120	220	240
MR + VP − per cent.	94	15	87·3	42·5

Still obtaining an undesirably high proportion of MR positive organisms from unpolluted sources, he applied the citrate test, with the results shown:

	Faeces	Unpolluted soil	Polluted water	Unpolluted water
Number of cultures	432	214	210	240
Citrate negative per cent.	96·3	10	90·9	18·7

Pawan concluded that although the use of the MRVP tests reduced considerably the number of strains from non-faecal sources to be regarded as "excretal *B. coli*," the citrate test almost eliminated them. He continued this work in relation to water analysis in 1926, making use, in addition to Koser's

citrate medium, the citrate and tartrate media employed by Brown, Duncan and Henry. He confirmed his previous results, noting in addition a close agreement between Koser's test and the test of indol production.

Raghavachari (1926) in India studied the application of the MRVPK tests to Indian water supplies. He also found a large number of MR+ K+ strains in unpolluted soils and waters, but did not regard it as safe to ignore this intermediate type in assessing the sanitary quality of a water sample. He made the interesting observation that all his intermediate strains were unable to produce indol, and that no indol producer was capable of utilising citrate, thus confirming Pawan's later findings. Taylor (1926) in Burma, compared the citrate test with Clemesha's method. Whilst recording a fair agreement between the MRVPK tests and the sanitary findings, he believed that Clemesha's classification gave a better indication of the source of the strains as regarded the remoteness or otherwise of pollution. On the other hand, he considered that the MRVPK combination might bring an opinion a stage farther, since these tests were capable of giving some clue as to the ultimate source of the organisms. With regard to the intermediate MR+ K+ group, he suggested tentatively that they might be regarded as indicating remote pollution. Hicks (1927) in Shanghai observed that 89·4 per cent. of 150 cultures from faeces might be regarded as being members of the "excretal *B. coli*" group on the basis of the MRVPK and indol tests. 76 per cent. of strains from soil were MR+ VP− but 80 per cent. of them were able to utilise citrate. He believed that the citrate and indol tests used conjointly were of considerable sanitary value. Perry (1929) studied lactose-fermenting bacilli isolated from faeces, water and oysters. He reported that 90·3 per cent. of 93 cultures from faeces were *B. coli* as judged by the MRVPK and indol tests. On the same basis, only 11·2 per cent. of strains isolated from water and oysters were true *B. coli*. He also drew attention to the relative frequency with which the "intermediate" type was encountered in water samples. He considered that the citrate and cellobiose tests were both of great value in the determination of bacterial types. Hill (1929), with Seidman and Stadnichenko, obtained a close agreement between the MRVPK reactions in the differentiation of lactose-fermenting bacilli isolated from the genito-urinary tract. They drew attention to the high percentage of strains belonging to the aerogenes group occurring in this region. Brewster (1929) in Colombia made use of the citrate test in water analysis and found that, even when used alone, it was a valuable criterion of the sanitary condition of the water.

It would seem that the test of citrate utilisation has proved itself a valuable means of checking the results of other differential criteria, particularly in relation to the identification of those organisms in soil and water which might by means of other tests be regarded as faecal strains. Many workers have adopted it as a routine test. There remains a more cautious proportion who are unwilling to accept it in the place of methods based upon many years of scientific experience.

In general, therefore, it may be said that the division of colon-like bacilli into faecal and non-faecal groups may be said to have been established by use of the methyl-red, Voges-Proskauer and Koser tests. Despite a certain lack of correlation, as yet imperfectly explained, between the tests themselves, there seems to be no doubt that this subdivision is a justifiable one. Whether by such a grouping the sanitary quality of water supplies may at all times and under all circumstances be accurately ascertained is a proposition only to be solved after a lengthy series of observations.

E. *Growth of coliform organisms at a temperature above* 37° *C. and its significance.*

Eijkman (1904) claimed that faecal *B. coli* of warm-blooded animals was capable of fermenting glucose at a temperature of 46° C. Interest in this phenomenon has been revived within recent years, owing to the increasing doubt as to whether organisms isolated at 37° C. in fluid media are numerically representative of their disposition in the original water sample. Opinions as to the value of Eijkman's test are divided. Konrich (1910) reported that a proportion of true faecal *B. coli* of mammalian origin, when incubated in glucose broth at 46° C. failed to develop. Hehewerth (1911) found that not more than 38·8 per cent. of *B. coli* strains were able to ferment glucose at this temperature and concluded that the test was only valid in its positive aspect. Eijkman (1912) agreed that a negative result alone could not be regarded as proof of the absence of faecal *B. coli*. Flu (1915) considered that the test was valuable as a presumptive indication of recent contamination, but that where pollution was more remote it required a larger amount of water to produce a positive result. De Graaff (1922) found that incubation at 46° C. repressed all the aerogenes organisms, and Minkewitsch (1928) considered that it eliminated false positive results. Leiter (1929) believed that in water analysis a better agreement was obtained between the citrate and indol tests and incubation at 46° C. than between these tests and incubation at 37° C. Brewster (1929) in Colombia expressed the opinion that incubation at 46° C. encouraged rather than suppressed the growth of the aerogenes group. Barth (1930) confirmed Hehewerth's findings. Recently Brown and Skinner (1930) in a careful study report that only a small percentage of human faecal *B. coli* fermented glucose at 46° C. Many true *B. coli* failed to grow and a number of citrate utilisers were not eliminated.

Evidently the Eijkman fermentation test alone has not been found a sufficiently constant presumptive procedure for differentiation purposes. Wilson (1929) offers an ingenious solution to the difficulties encountered in its use. He advocates primary incubation in lactose-bile-salt-peptone tubes at 37° C., with early sub-culture on McConkey or Endo plates, and subsequent incubation of one plate at 37° and one at 46° C. In this way cultures can always be obtained for further differentiation if required.

FALLACIES IN THE APPLICATION OF THE FOREGOING METHODS TO THE BACTERIOLOGICAL EXAMINATION OF WATER.

It will be readily appreciated that almost all the differential criteria for the colon-aerogenes group have been employed in water bacteriology, and that almost all of them, in one or other particular, have been found imperfect. An examination of the methods used for the isolation and identification of the lactose-fermenting bacilli shows that fallacies may be encountered in almost every stage of almost every process. The truth of this statement was abundantly proved by Greer and his confrères (1928). The present tendency is to choose from all the characters of differentiation those which in the hands of the majority have proved the most stable and permanent, discarding those in which correlation has been least.

Whatever the criterion adopted, there is abundant proof that *B. aerogenes* is rare in the intestines of men and animals and common outside the body, whilst *B. coli* is overwhelmingly faecal. The number of anomalous results reported have not been sufficiently large to negative the principle whereby grouping of coliform organisms into faecal and non-faecal may be effected. It seems probable that with a better understanding of the existing tests, their interpretation may be so adjusted that what are now regarded as atypical results may be found to have a definite significance.

The discussion of fallacies may be conveniently considered under three headings:

A. Initial isolation;
B. Subsequent identification;
C. Interpretation of results from a sanitary standpoint.

A. *The fallacies encountered during initial isolation.*

Preliminary enrichment in lactose broth is the basis of most methods of primary isolation. Variations are to be found in the composition of the media used according to the individual choice of selective or inhibitory substances, but the principle involved is the same in all. The fermentation of lactose is, as Houston has said, the common ground upon which all water bacteriologists can meet, and it is to this character that Dodgson (1928) has applied the apt mnemonic "lag" (lactose acid gas).

The advantages of liquid media for primary isolation are, as regards water bacteriology, somewhat overshadowed by the difficulties encountered in their use. The possibility of an alteration in the numerical disposition of the different organisms as regards their presence in the original samples has for long been appreciated. McConkey in this connection remarked (1905):

"The preliminary incubation might therefore alter the relative proportions by favouring the growth of the lactis-aerogenes group: but if *B. coli communis* is found, then it is not at all likely that any of the other organisms have been overlooked."

It is debatable whether the converse is true—*i.e.* whether failure to isolate *B. coli* may be taken as proof of its absence from the original sample. Gaertner (1910), finding a 30 per cent. error in the employment of liquid media, considered that for the purposes of water analysis, solid media were preferable for the purposes of isolation. Thompson, as already noted, showed that a lethal *p*H inhibitory to its own growth is developed by *B. coli* in glucose broth. The careful work of Greer and his colleagues demonstrated clearly how many fallacies were to be met with during the preliminary processes. Their results, in the cases of anaerobes producing spurious positive presumptive tests, confirmed those of Muer and Harris.

It is evident that when liquid media are used at this stage, both false positive and false negative results are liable to be encountered. The possible solution offered by the Eijkman fermentation test does not appear to have been fully substantiated by sanitary bacteriologists. It might with truth be asserted that if a specific direct plating medium for differentiation were available, many of the difficulties of water bacteriology would be at an end.

B. *Limitations of differentiation methods.*

Since the power to ferment lactose is accepted as an inseparable feature of any conception of *B. coli*, it is evident that Houston's index is largely concerned with whether or not indol is produced. That this is a variable factor has been amply demonstrated; the fallacy of this method appears to lie in the fact that a proportion of manifestly non-faecal strains are capable of indol production. On the other hand, failure to produce indol might be taken as strong negative evidence against the presence of an excretal organism. The "lactose+ indol+" index is the starting-point from which other differentiation methods may be developed if required, and the only justifiable criticism against it is that under certain conditions it may not bring an opinion far enough. Houston himself (1912) adequately summarised the position thus:

"The *B. coli* test ought primarily to be regarded as a quantitive decimal enumeration of 'lactose+ indol+' microbes, but the subsequent grouping according to certain attributes of the organisms thus obtained, into fairly stable or apparent varieties, may be of real practicable or diagnostic importance."

The further division into specific groups by McConkey or similar classifications has proved more satisfactory; but such groups again may be variable owing to alterations in the proportions of the groups found under a variety of conditions. By extending the number of fermentable substances employed, the number of species might be increased indefinitely, but such a procedure offers no practical advantages. Many workers have from time to time referred to the relative rarity of Escherich's true *B. coli* in the Tropics (Castellani, 1910; Clemesha, 1912; Butler, 1921). Although of undoubted value for taxonomic purposes, and even for provisional identification, it is doubtful whether the separate species method can yield any useful information to the water

bacteriologist. Clemesha's classification being based upon McConkey's is open to the same objections; it has, however, the distinct advantage that it attempts to correlate the bacteriological findings with the natural history of the organisms—an entirely rational procedure, which, under certain conditions, has given satisfactory results in the hands of many workers. The recognition of high and low ratio groups, and allied principles, would appear to bring this conception a stage farther. Most observers are agreed that these tests form a reliable means of subdividing faecal coliform organisms: their frequent failure to do so in the case of soil and water has been the subject of much discussion, and the significance of the "intermediate" group is still a matter of speculation. There is considerable evidence to show that by the use of the Koser citrate test the various types of organisms may be assigned to their proper aetiological categories. This is the view of Pawan, Hicks and Brewster, but many others, such as Cunningham, Raghavachari and Taylor cannot accept it in its entirety. The consensus of opinion appears to be in favour of the view that the organisms of the intermediate group represent nothing more dangerous than evidence of remote faecal pollution.

Although the subject is not concerned with *B. coli*, and hence has no bearing upon the objects of this paper, one cannot complete a survey of water bacteriology without referring to the sulphite reduction test of Wilson and Blair (1924). This test is perhaps the nearest approach yet presented to a method of measuring directly the pathogenicity of a water. Many workers would perhaps hesitate to admit that the absence of reducing organisms, such as *B. welchii*, would justify them in passing a water to which the test had been applied, but Wilson (1929) writes:

"Further experience has impressed me so much with its value that if I found no reducing organisms in 40 c.c. of the water, I would be disposed to consider the supply reasonably free from excretal contamination, even though coliform organisms were present in 1 c.c."

Even though unassailable tests for excretal *B. coli* be established, there yet remains one obstacle common to all methods of water bacteriology. By no existing means are we able to distinguish with certainty between human and animal excretal bacteria. The claims of Eijkman to have partially solved this difficulty remain controversial. Clemesha originally drew attention to the quantitative and qualitative seasonal changes in the bacterial flora of human and animal intestines. It is difficult to conceive such differences as being sufficiently constant to serve as a basis of classification.

This failure to recognise *B. coli* of animal origin, serious though it may be in temperate climates, is infinitely more serious in the Tropics, where the protection of water supplies is often at best primitive and frequently absent. It has been shown by numerous authorities that, apart from the flora of the larger domestic animals, *B. coli* may be present in the faeces of gulls and fish (Houston, 1923), frogs (Raju, 1922), small birds (Emmel, Minkewitsch, 1930), lizards and bats, in quantities sufficiently large to produce positive results

with very small volumes of water which may contain them. That pollution of tropical water supplies can and does occur from the presence in wells, pipes and storage tanks of the excreta of these creatures, sanitarians in Eastern countries are fully aware. The present writer had occasion some years ago to examine the water supply of a public institution. The supply was derived from an excellently protected well, from which the water was pumped to a storage tank on the roof of an outhouse, thence being distributed to a standpipe in the compound. Examination of water from the well resulted in a negative presumptive test with 25 c.c. of water. Water from the standpipe showed the presence of "excretal" *B. coli* in less than 10 c.c. The tank was examined, when it was discovered that the wire-gauze cover was deficient in several places, and was admitting the faeces of bats to the tank. The tank was cleaned out, the gauze repaired, and thereafter no *B. coli* was found in the water upon subsequent examination.

C. *Difficulties in interpreting results.*

One final difficulty still remains. Even though it be proved beyond doubt that faecal bacilli are present in a water sample, in what manner is their presence to be interpreted? Does it signify that faecal pollution is, or was recently, present? Is that pollution continuous, and whence has it been derived? And finally, what are the numerical standards of bacteriological purity, and on what are they to be based? These are all questions which bacteriology unaided cannot but fail to answer. Attempts to establish general standards of water purity have been in the past frankly disappointing. The most that different workers have been able to accomplish has been to produce local standards based upon a lengthy series of personal experiments. It is not in fact possible to lay down standards for all waters—but it is possible to lay down a working standard for one water or series of waters. Houston has said that "standards are worse than useless if they are not interpreted with discretion and in relation to local and other conditions. Instances could be quoted where even similar *B. coli* results ought properly to lead to dissimilar conclusions as regards quality and safety." It has been shown that *B. coli* can develop in the immediate neighbourhood of a well without any increase of pollution having taken place. Thresh and Beale (1926), referring to a growth known as Enteromorpha intestinalis, which favours the development of *B. coli* in reservoirs and filter beds in the absence of manurial pollution, state that "*B. coli* can grow and multiply profusely on the surface of decaying growths of this character." Raju (1922) believed that stasis in pipes could account for an increase in the number of *B. coli* isolated, although no further pollution was possible. Havens and Dehler (1923) demonstrated the presence of *B. pyocyaneus* in the intestines of *Gambusia affinis*, a small fish commonly employed for the destruction of mosquito larvae in water supplies. They concluded that in waters to which this *Gambusia* had gained access, *B. coli* would disappear, owing to the antagonism existing between it and *B. pyocyaneus*; and that in

the absence of a bacterial indicator, a water might be passed which ought properly to be condemned. Schobl and Ramirez (1925) have found that the hemp packings and washers of pumps of wells may harbour lactose-fermenting organisms in great numbers.

Frequent and regular examination is essential if the bacteriology of water supplies is to be of any value, and the limited significance of a single analysis has been stressed by many workers. Morison (1920) considered that the important factor to be kept in mind regarding the bacterial state of a water is its constancy: that, for example, the presence of excretal *B. coli* in 10 c.c. of a water might not necessarily be an indication of danger, provided that the 10 c.c. margin be not exceeded. Beattie (1930), in a recent discussion, referred to the need for systematic and regular bacteriological examination of supplies to enable the water to be kept at a "minimum point of risk."

It is observations such as these which impress upon the tropical worker to what extent the interpretation of his bacteriological results must be made subject to other considerations. It is obvious that no standard of water purity, unless founded upon a knowledge of local conditions, can be more than arbitrary. A standard, eminently suited to the needs of one locality, might well be worse than useless when applied to another. The problem for the sanitary bacteriologist in a newly inhabited tropical district thus lies in the investigation of all available methods in relation to the particular locality in which he is situated; the adoption of those measures which appear to be most practicable; and the proper interpretation of results with due regard to local topographical and seasonal conditions. Probably no single test is adequate for the judgment of a water sample, any more than a single analysis is adequate for a judgment of the supply from whence it has been derived. Evidently a complete survey of local biological conditions becomes essential before any standards of purity can with accuracy be established for previously uninvestigated water supplies in tropical countries.

One cannot delve deeply into the history of water bacteriology in search for an apposite doctrine without having frequent recourse to the writings of Sir Alexander Houston. The foregoing survey might be appropriately summarised by quoting verbatim his now well-known dictum (1912):

"With the establishment of too severe standards, there is the danger of condemning reasonably safe supplies, and with too lax standards, there is the possibility of passing waters which ought properly to be condemned. Always to steer safely between the Scylla of the one and the Charybdis of the other may be difficult or impossible without a pilot familiar with the local conditions."

PART II. EXPERIMENTAL.

INTRODUCTION AND SCOPE OF THE WORK.

The purpose of the present investigations may be said to be threefold:

(1) To determine, by means of certain reactions, the local prevailing types of coliform bacilli, and the habitat in which each type is commonly found.

(2) To investigate the occurrence in water supplies of the various types thus determined.

(3) From the results so obtained, to examine the application of the methods employed to the sanitary analysis of local water supplies.

Previous available records go to show that no comprehensive study of modern bacteriological methods has hitherto been undertaken in connection with the local waters. Butler (1921), from the Dar-es-Salaam Laboratory, commented upon the methods then in use:

"It has been a constant source of worry to decide what should be taken as a bacteriological standard whereby the potability of Dar-es-Salaam water could be judged. Probably this can only be decided upon by a long series of observations. If one were to judge a water supply in the Tropics strictly on the standards employed at home, there would probably be no water fit to drink. Waters of this nature are being constantly drunk without ill effects, and either the home standards are unsuitable as tropical standards, or their interpretation should be considerably modified. Escherich's true *B. coli* I have not yet met with in tropical waters, but its variants are undoubtedly present in numbers that at home would be considered very serious. The standard that I employ is one I believe commonly in use, and that is to regard lactose-fermenters—indol-formers as indicators of danger if present in 50 c.c. of water —but it may be eventually found necessary to modify this again."

For some years, no change was made in the above methods. Clearkin (1926) commenced a study of well samples, using the methyl-red, Voges-Proskauer, and the citrate utilisation tests as performed by Brown, Duncan and Henry. He reported that the number of samples examined was not sufficient to warrant the drawing of conclusions: unfortunately before the series was completed the work had to be postponed.

The present writer (1928) undertook an investigation of the Dar-es-Salaam water supplies, studying all available methods for the detection of the colon-aerogenes group. The work was carried out between October, 1928 and August, 1930. During that time 84 samples from 20 wells, 20 samples from other waters, and 36 samples from various sources were examined. A total of 1923 organisms of the colon-aerogenes group was studied. A further series of 577 cultures was studied from a comparative point of view upon the writer's return to Europe on leave this year. These will be discussed separately at the end of this section.

(1) *Sanitary considerations.*

Local conditions are eminently suitable for a comparative study of water supplies. Every gradation of water, from the reasonably pure to the grossly polluted, may be found in the immediate neighbourhood of Dar-es-Salaam. The present supply of the town is derived from a system of deep borings, excellently protected, and providing a water which is credited with a high degree of purity. Up to comparatively recent years, however, Dar-es-Salaam was served by shallow wells: the greater number of these are still in existence, and many are in daily use. Situated for the most part in compounds or in public places, these wells are often to be found in close proximity to cesspits and other sources of pollution. They are being closed in increasing numbers, but a sufficient proportion of them are still commonly used in the native quarter of the town to constitute a potential source of danger. It is with these shallow wells that the present investigation is principally concerned.

(2) *Rainfall.*

In view of the recognised variations occurring in the tropics regarding the bacteriological content of faeces, soil and water at different periods of the year, care was taken to space the collection of samples suitably so as to include all seasonal changes.

Although rain falls intermittently throughout the year, there are two more or less well-defined rainy seasons in Dar-es-Salaam. The "Long Rains" fall about February to May and the "Short Rains" in November and December. The amount of rain falling at any one period is subject to wide and frequent fluctuations, varying from a short torrential shower to a steady downpour which may continue for several days. After a heavy fall, it is not uncommon, even in the township, for the ground to be several inches under water, and at such times the effect upon the subsoil of this flooding must be very considerable.

During the long rains, the average fall for the three months is about 20 inches, and in the short rains about 11 inches. The average yearly rainfall for the five years 1925–1929 has been 40·1 inches. Water samples were taken in the months of October, May and August, that is immediately before and after heavy rains and during an intermediate period. Samples of colon bacilli from other sources were collected at corresponding periods of the year.

(3) *Choice, collection and grading of samples.*

Well samples were chosen in such a way as to obtain waters from every degree of sanitary quality. All the samples were taken in the same way, and every effort made to ensure that they were representative. Two samples from the main borings and one from a frankly polluted well were used as controls. It is considered that no useful purpose will be served by a detailed description of each well: this would tend to obscure the main issue, which in this case is less an investigation of the sanitary state of the wells than an examination of

the utility of the methods employed. In the description of experiments, the well samples are grouped under three headings, according as whether the source of supply could be regarded as good, suspicious or bad, on the basis of a careful sanitary survey.

(4) *Methods and media.*

Preliminary enrichment in lactose-bile-salt broth was used for all water samples, except in the case of Exp. G. Samples other than water were plated directly on lactose-bile-salt neutral red agar. The quantities of water employed were $\frac{1}{10}$th, 1, 10 and 25 c.c. After 24 or 48 hours' incubation at 37° C. a sub-culture was made from each tube of lactose broth in which acid and gas had formed. McConkey's agar was employed. After further incubation, lactose-fermenting colonies were picked from each plate. As a rule, 16 colonies were thus obtained from each sample. These colonies were further sub-cultured in peptone water, from which subsequent inoculations were made into the special media employed, and motility and staining reactions carried out. Only those cultures were subjected to further examination which were Gram-negative rods, non-sporing, and fermenting lactose at 37° C. in 24–48 hours. Positive presumptive tests were obtained with every well sample in the $\frac{1}{10}$th c.c. quantities, with the exception of those taken from the main deep wells.

The media used were prepared according to standard formulae. In this connection the following authorities were consulted: Standard Methods, American Public Health Association (1925), McConkey (1908), Koser (1923). The carbohydrate media were 1 per cent. solutions in peptone water, except dulcite which was 0·5 per cent. Simmon's modification of Koser's citrate medium was used in parallel and found to correlate perfectly with the original. The Erlich-Böhme method of demonstrating indol production was employed.

(5) *Tests employed.*

The following separate methods were used at various stages of the work:

(1) Houston's "coli test" (lactose+ indol+).
(2) McConkey's groups (saccharose-dulcite fermentation).
(3) The methyl-red and Voges-Proskauer reactions.
(4) Koser's citrate test.
(5) Eijkman's fermentation test.
(6) A direct-plating method devised by the writer.

A word of explanation is required regarding the Voges-Proskauer reaction. For reasons for which no explanation appeared this test only gave a correlation of 83 per cent. with the methyl-red test. After 730 cultures had been examined, and this average of agreement existed, it was considered that more useful information would be obtained by the use of two closely agreeing methods than by three discordant ones. The Voges-Proskauer reaction was therefore discontinued, and in order to make the completed results comparable, it will not be considered in the discussion of results. It might, however, be added that, believing the unsatisfactory results obtained with such a well-known test to

have been due to prolonged incubation, the writer on returning to Europe performed it in parallel with the methyl-red reaction in a series of 577 cultures. In this case the test was carried out after 24 hours' incubation, and a 97 per cent. correlation with the MR was obtained.

(6) *Expression of results.*

In the discussion of results, a constant method of notation is employed. The fermentation reactions are divided into the four groups proposed by McConkey, and the methyl-red and citrate tests into those used by Koser:

McConkey's groups.

	Lactose	Saccharose	Dulcite	Type
1	AG	—	—	*B. acidi lactici*
2	AG	—	AG	*B. coli communis*
3	AG	AG	AG	*B. coli communior*
4	AG	AG	—	*B. lactis aerogenes*

Methyl-red citrate groups.

1	MR + K −	Coli
2	MR − K +	Aerogenes
3	MR + K +	Intermediate
4	MR − K −	Atypical

The results are stated in percentages, to the nearest whole number.

Discussion of experimental findings.

(1) *Tropical series.*

Exp. A.

As a preliminary, a number of colon-aerogenes organisms were isolated from faeces, urine, pus, cesspits, grossly polluted water and samples from various main water taps. After the usual preliminaries, 154 colonies were isolated and examined by the fermentations, the indol test, and the MR and K tests.

The results thus obtained are shown in Table I.

Table I.

			Fermentation groups				MRK groups			
Source	No.	Indol	1	2	3	4	Coli	Aero-genes	Inter-mediate	Atypical
Faeces	33	60	12	37	27	24	90	7	3	—
Urine	10	50	—	40	30	30	70	10	10	10
Pus	6	100	83	17	—	—	100	—	—	—
Cesspits	30	43	26	4	40	30	47	43	—	10
Polluted water	6	83	—	83	—	17	83	17	—	—
Main water	69	23	6	—	44	50	—	93	6	1

It will be seen that if reliance were placed upon the lactose+ indol+ index alone, some 23 per cent. of waters believed to be pure would have been condemned. Furthermore, a number of cultures manifestly of excretal origin would have to be regarded as non-faecal.

McConkey's group 2 organisms were the commonest in faeces, urine and polluted waters, group 3 the commonest in cesspits, and group 4 in unpolluted

waters. This would suggest the tendency of *B. coli communis* to die outside the intestine. It is interesting to note that in every case except that of polluted water the proportion of groups 3 and 4 were approximately the same.

The MRK combination gave a good agreement with the source of the organisms. It will be noted that the intermediate types were commonest in urine, and the atypical in urine and cesspits. The balance between the coli and aerogenes types in cesspits is of interest.

The citrate test alone agreed almost perfectly with the sanitary considerations.

Exp. B.

Three hundred and four cultures of lactose-fermenting bacilli were isolated from twenty samples of well water, and subsequently examined in a manner identical with that of Exp. A.

Table II shows the percentage results obtained:

Table II.

				Fermentations				MRK			
Source	Samples	Cultures	Indol	1	2	3	4	Coli	Aero-genes	Inter-mediate	Atypical
Good	8	112	20	4	1	28	67	12	78	2	8
Suspicious	6	96	43	9	1	45	45	27	63	4	6
Bad	6	96	63	2	14	37	47	61	23	5	11

Here group 2 is found largely in samples from bad wells. Group 3 is commonest in the suspicious samples, whilst group 4 predominates in all three.

The indol test agrees fairly well with the sanitary features. By means of the MRK tests the agreement is satisfactory. It will be seen that the atypical varieties of organisms increased from the good to the bad samples. A better opinion on the sanitary state of the wells would appear to be obtainable by the combined MRK than by the citrate test alone.

Table III shows the smallest volume of water from each sample in which *B. coli* was found, as judged by the MRK tests.

Table III.

		B. coli present in				*B. coli* absent in
Wells	Samples	0·1 c.c.	1·0 c.c.	10 c.c.	25 c.c.	25 c.c.
Good	8	5	—	—	—	3
Suspicious	6	5	1	—	—	—
Bad	6	5	—	1	—	—

Here may be noticed one serious anomaly which repeated itself in subsequent experiments. Although the actual number of *B. coli*, as judged by the citrate test, found in each type of sample approximates to the sanitary findings, the qualitative results are very different. An equal number of samples belonging to each grade showed the presence of excretal *B. coli* in 0·1 c.c. Furthermore, when individual well samples were considered, it was found that in a fairly large number of cases, *B. coli* was found in 0·1 c.c., but not in the

higher quantities. It is evident that such findings cannot but obscure the interpretation of results from a sanitary standpoint. So long as the element of speculation is present in sub-culturing from McConkey plates, it would seem that this kind of result is inevitable. The use of liquid media in the preliminary enrichment process probably increases the chance of this fallacy occurring. Some method of primary isolation is desirable which can be guaranteed to ensure the growth of colonies in proportions reasonably approaching to their disposition in the original water sample. This subject is discussed at greater length in a subsequent experiment.

Exp. B was conducted in October, 1928—that is before the heavy rains. The next experiment was carried out identically but was performed in the following May. The rainfall for the months February to May was 23 inches.

Exp. C.

From eighteen of the twenty wells, two hundred and seventy-two coliform organisms were isolated. Subsequent examination was carried out in the same way as that of Exp. B.

The results are shown in Table IV.

Table IV.

				Fermentations				MRK			
Source	Samples	Cultures	Indol	1	2	3	4	Coli	Aero-genes	Inter-mediate	Atypical
Good	8	112	41	11	3	40	46	12	72	16	—
Suspicious	5	80	42	6	3	52	39	15	62	18	5
Bad	5	80	67	5	30	45	20	30	35	35	—

These results are at first sight confusing. In the first place, the lactose-indol test suggests that the proportion of excretal *B. coli* in the good samples is doubled as compared with Exp. B: on the other hand, on the same basis, the other two types of samples are little changed.

McConkey's groups show a very different result. In this case *B. coli communis* is only slightly increased in the good and suspicious samples, whilst it is doubled in the bad. The communior group is increased in all samples, and the aerogenes relatively decreased.

The MRK combination produces yet another result. *B. coli* is unaltered in the good samples, and decreased in the other two. The intermediate variety is increased in all three from the good to the bad, whilst the aerogenes type only shows a marked difference in the bad samples, where it is increased.

Two important observations may be gleaned from the above results. In the first place, the percentage of *B. coli* in the bad samples by both the fermentation reactions and the MRK tests is exactly the same, whilst in the other samples the percentage has shown little change from that of Exp. B. Secondly, there is a general increase of the communior and intermediate types in all three samples.

In other words true excretal pollution of recent origin is only evident to a serious extent in the bad samples, and then in a relatively small amount. Indol

production is increased out of proportion to *B. coli* in the good samples, and to a lesser extent in the other two.

If one considers the effect of continuous heavy rain upon surface wells, some light is thrown upon the results. The first part of the rain will probably wash into the wells whatever pollution exists upon the surface layers of the soil. This will presumably affect principally those wells which are least well-protected. Later, increasing amounts of soil, in which pollution is relatively less prominent, will be swept into the wells as the heavy rain continues, producing a diluting effect. One would therefore at first expect an increase of excretal contamination of the wells, with the finding of a correspondingly larger proportion of excretal *B. coli* of recent excretal origin. An excess of washings from the deeper layers of the soil would probably be associated with a relative increase in the number of organisms derived from soil and from excretal pollution of remote origin. To the last two classes, according to most workers, belong the aerogenes type and the intermediate group of Koser.

In the present experiment, the increases in the number of organisms of this intermediate group is accompanied by a similar increase in *B. coli communior*. May one assume that these two classes have the same significance, namely that they represent those bacteria which have survived from remote excretal-contamination, and that a large percentage of them producing indol, the indol test tends to raise the percentage of *B. coli* isolated? It would therefore appear that as a result of the heavy rains, surface pollution of the poorly protected wells by excretal *B. coli* takes place, whilst later all wells are polluted by *B. coli* not of recent excretal origin which is contained in the deeper layers of the soil. It would seem that the MRK combination in this way confirms the essential parts of Clemesha's theory, although approaching the subject from a different angle. One might regard the MR+ K− group as susceptible organisms, the MR+ K+ as less susceptible, and the MR− K+ as resistant.

Table V shows the smallest volume of water in each grade of well from which *B. coli* (MR+ K−) was isolated.

Table V.

		B. coli present in				*B. coli* absent from
Wells	Samples	0·1 c.c.	1·0 c.c.	10·0 c.c.	25·0 c.c.	25·0 c.c.
Good	8	—	1	1	2	4
Suspicious	5	1	—	—	2	2
Bad	5	2	—	2	—	1

These results are more rational than those obtained in Exp. B. No good sample contained *B. coli* in 0·1 c.c. and only one suspicious sample contained them in less than 25 c.c.

Exp. D.

In order to ascertain whether initial enrichment in lactose broth at a temperature of 37° C. favoured the overgrowth of *B. aerogenes* and the production of false positive results, it was decided to compare the incubation of

water samples at this temperature with incubation of the same samples at 46° C., as recommended by Eijkman.

In this experiment, which was carried out in May, 1930, two hundred and eighty-five cultures were isolated from fifteen of the well samples. In order to test the value of the indol, methyl-red and Koser reactions alone, the fermentation tests were omitted.

The percentage results are shown in Tables VI and VII:

Table VI. *Standard incubation at* 37° C.

Source	Samples	Cultures	Indol	Coli	Aerogenes	Inter-mediate	Atypical
Good	6	90	19	13	78	9	—
Suspicious	4	60	33	23	64	13	—
Bad	5	75	60	55	40	—	5

Table VII. *Incubation at* 46° C.

Source	Samples	Cultures	Indol	Coli	Aerogenes	Inter-mediate	Atypical
Good	6	15	60	—	60	—	40
Suspicious	4	15	—	—	100	—	—
Bad	5	30	60	50	50	—	—

In the case of those samples incubated at 37° C. a favourable agreement was shown between the MRK combination and the sanitary opinion. The indol test showed a somewhat higher proportion of *B. coli* in the better samples.

The results of Eijkman's test were unsatisfactory. Acid and gas were produced in only four samples: of these, one was from a good well, and contained no *B. coli*, one from a suspicious well contained no *B. aerogenes*, whilst two were from bad wells, of which only one showed the presence of *B. coli*. The indol test was of little value. Actually more organisms of the aerogenes than of the coli type developed at 46° C. The inhibitory effect seems to be actually more pronounced in the case of the latter organisms. This is in accordance with the experiences of Brewster (1929) and Barth (1930), and has since been confirmed by Brown and Skinner (August, 1930).

In Tables VIII and IX it will be seen that the disposition of the smallest volumes showing *B. coli* is, in the case of samples incubated at 37° C., more satisfactory than in previous experiments. Table IX clearly indicates the suppression of *B. coli* at 37° C.

Table VIII. *Incubation at* 37° C.

		B. coli present in				*B. coli* absent from
Wells	Samples	0·1 c.c.	1·0 c.c.	10·0 c.c.	25·0 c.c.	25·0 c.c.
Good	6	1	—	1	—	4
Suspicious	4	1	—	—	—	3
Bad	5	4	—	—	—	1

Table IX. *Incubation at* 46° C.

		B. coli present in				*B. coli* absent from
Wells	Samples	0·1 c.c.	1·0 c.c.	10·0 c.c.	25·0 c.c.	25·0 c.c.
Good	6	—	—	—	—	6
Suspicious	4	—	—	—	—	4
Bad	5	—	—	1	—	4

Exp. E.

As no benefit was derived from raising the temperature of incubation as a means of suppressing non-faecal *B. coli*, a search was made for another method for differentiating species. Taking into consideration the findings of previous writers regarding the causation of false positive presumptive *B. coli* tests, it would appear that available evidence is in favour of a solid direct plating medium. On such a medium, the *B. coli* and *B. aerogenes* groups should not only grow readily and be easily isolated, but they should develop on the medium in approximately the proportions in which they existed in the original water sample: furthermore, each type of colony should have a distinctive appearance by means of which they might readily be differentiated. The only medium of this type which would appear to have a supported claim to differentiating *B. coli* and *B. aerogenes* on a single plate is Levine's eosin-methylene-blue. Not every worker has been able to confirm its originator's observations.

As the methyl-red test had proved itself a reasonably accurate technique as applied to water analysis, it was decided to investigate the application of its principles to direct isolation of coliform organisms from water. It was believed that a medium based upon the test might with advantage be employed for presumptive tests, to be subjected to confirmation at a later stage. Most glucose-fermenting aerobes found in water also ferment lactose: the glucose-phosphate-peptone medium therefore offers a suitable basis for a solid medium, the results obtained from glucose fermentation being afterwards confirmed by the inoculation of lactose broth. A solid medium on these lines has already been described by Salle (1927), but this worker did not prepare his medium for the purposes of preliminary isolation, and made no provision for the inhibition of other organisms.

As a preliminary, the glucose-phosphate-peptone medium as used in the methyl-red test was solidified with agar. With the idea of conforming as far as possible to the original test, methyl-red was added as an indicator, but it was found that no satisfactory colour differences could be obtained in this way. Brom-thymol blue, as recommended by Baker (1922), and Simmons (1926) was next employed, with very satisfactory results. This was added to the basic medium. The reaction being fixed by the buffer salt at a constant *p*H of 6·8–7·0, the resultant medium was of an olive green colour resembling that of Simmons' citrate agar. The composition of the finished medium was as follows:

Glucose	0·5 grm.
K_2HPO_4	0·5 ,,
Agar	2·7 ,,
Peptone	0·5 ,,
Brom-thymol blue (0·4 per cent. aqueous)	2·0 ,,
Distilled water to 100 c.c.	

The medium was sterilised fractionally for 20 minutes on three successive days, and poured in tubes for slanting and pouring of plates.

Some known colon-aerogenes cultures were inoculated on this medium, and incubated at 37° C. for 24 hours. At the end of this time, growth was considerable in all the tubes. The coli colonies were discrete lemon-yellow discs on a paler background, and the aerogenes type showed a luxuriant green growth on a green background. As the period of incubation increased, the colour distinctions became more pronounced. In four days, the coli colonies were a rich orange colour on an orange medium, and the aerogenes a grass green on a deep green background.

Two hundred cultures from various sources were obtained in order to confirm the differential properties of the medium. These had been previously grouped into coli and aerogenes by means of the MRK tests. These cultures were sub-cultured upon peptone, citrate medium and the new glucose-agar-brom-thymol blue medium. The results (Table X) are classified according to the MRK principle in order to conform with previous records. This has been done by substituting the new medium for methyl-red test in recording of results. Thus, the "coli" and "intermediate" organisms were orange-coloured colonies, and the "aerogenes" and "atypical" were green.

Table X.

Type	Source	No.	Coli	Aero-genes	Inter-mediate	Atypical	Indol
Aerogenes	Water	50	—	96	4	—	—
Coli and aerogenes	Water	50	58	34	8	—	94
Coli and aerogenes	Faeces	100	76	9	15	—	58

No double negative results were obtained, so that the agreement with the citrate test and with source was reasonably satisfactory.

The use of the medium was next studied in relation to the original methyl-red test, the object being to determine whether all the green colonies were MR− and all yellow colonies MR+. One hundred lactose-fermenting bacilli from five samples were used. As before, these had been classified previously into coli and aerogenes. The cultures in peptone water were plated directly upon the new medium. Twenty colonies from each plate were picked off, and subjected to the MRK tests. The differentiation upon the plates containing mixed cultures was quite clear. The percentage results are shown in Table XI:

Table XI.

Type	Source	No.	Colour	Coli	Aero-genes	Inter-mediate	Atypical
Coli	Faeces	20	Yellow	100	—	—	—
Aerogenes	Water	20	Green	—	100	—	—
Coli and aerogenes	Faeces / Water	20	Green	—	100	—	—
Coli	Faeces	20	Yellow	100	—	—	—
Coli	Urine	20	Yellow	100	—	—	—

Agreement was perfect. All the yellow colonies were methyl-red positive, and all the green were methyl-red negative.

The final step was to introduce some inhibitory substance in order to adapt the medium to the needs of direct plating of water samples. Bile salt was

found satisfactory, but tended to lessen the pronounced colour differences. Crystal violet, 1/100,000, as recommended by Skinner and Murray (1924), was then used, and found to inhibit non-lactose fermenters and spreading organisms, without interfering with the specificity of the medium.

Exp. F.

The medium described in the last experiment was employed for the analysis of water samples. Sixteen of the 20 wells were sampled, and 208 cultures isolated.

The water was inoculated directly into melted "stabs" of the medium, and plates were then poured after mixing of the contents. The usual quantities were used, the 10 c.c. volumes being distributed in two portions of 5 c.c., and the 25 c.c. in five 5 c.c. quantities. McConkey plates were inoculated with corresponding amounts of water to confirm the presence of lactose-fermenters. An equal number of green and yellow colonies was picked from those plates in which both were present. Subsequent confirmation was established by means of sub-culture in lactose, and by the indol and citrate tests. All the glucose fermenters were lactose fermenters. No growth on the new medium occurred in a dilution lower than that which developed red colonies on the McConkey plates.

The percentage results, expressed as in the last experiment (MR corresponding to the new medium), are shown in Table XII:

Table XII.

Source	Samples	Cultures	Indol	Coli	Aero-genes	Inter-mediate	Atypical
Good	7	80	11	14	35	36	15
Suspicious	4	64	53	15	23	35	27
Bad	5	64	51	30	42	20	8

These figures do not of course show the actual proportion of each type of organism isolated from water samples, and are not therefore a true criterion of the sanitary state of the water, as a known number of green and yellow colonies was deliberately chosen. The points to be considered are the correlation between the colour of the colonies and the citrate test, and between the same standard and the source of the organisms.

The agreement with the citrate test was not good. A fair number of green colonies was citrate negative. On the other hand, the medium served its essential purpose of eliminating anomalies in the presumptive test for the amounts of water showing excretal *B. coli*, and may thus be said to have been an advantage over liquid media. Table XIII shows the smallest volume of water from each grade in which yellow colonies were found by direct plating:

Table XIII.

Wells	Samples	Yellow colonies in 0·1 c.c.	1·0 c.c.	10·0 c.c.	25·0 c.c.	Yellow colonies absent in 25·0 c.c.
Good	7	—	—	4	1	2
Suspicious	4	—	1	3	—	—
Bad	5	2	1	1	—	1

It is suggested that, in the practical use of this medium for direct plating of water samples, the proportion of yellow to green colonies in the plate showing yellow colonies from the smallest volume of water should be recorded, together with the subsequent identification by confirmatory tests of all yellow indol-producing cultures.

Exp. G.

The not infrequent occurrence of the "intermediate" MR+ K+ type of organisms suggested a further study of colon bacilli from various sources, in order to determine if possible the origin of the members of this group. In previous experiments, the acceptance of the intermediate organisms as a definite type which approximated to *B. aerogenes* was entirely presumptive and based upon the experience of Koser and other observers. Before the combined use of the methyl-red and citrate tests could be accepted as a basis for the establishment of local standards, it became necessary to confirm this presumption from local experiments.

Four hundred cultures were isolated from samples of faeces, urine sewage, and from soil believed to be recently polluted, remotely polluted and free from pollution. All samples were inoculated on McConkey plates directly, from which sub-culture in peptone water was made, and further sub-cultures carried out for the purpose of performing the indol, methyl-red and citrate tests.

The percentage results obtained by means of these tests are shown in Table XIV:

Table XIV.

Source	No.	Indol	Coli	Aerogenes	Inter-mediate	Atypical
Unpolluted soil	40	8	—	100	—	—
Remotely polluted soil	24	42	4	67	29	—
Recently polluted soil	36	90	81	11	8	—
Faeces	100	95	92	2	4	2
Urine	100	44	11	79	9	1
Cesspits	100	88	47	30	5	18

The correlation between the source and the MRK tests is in the main excellent. Atypical reactions were few, and almost entirely confined to samples from cesspits, where such results had been previously obtained. No intermediate types were isolated from soil believed to be unpolluted, and only 4 per cent. from faeces. Urine, cesspits, and recently polluted soil showed a number, whilst in remotely polluted soil they appeared to be relatively common, amounting to 29 per cent. This result differs somewhat from the results of Koser and others, who found a high percentage of intermediate organisms in unpolluted soil. In the present instance one concludes that this type suggests pollution of remote origin rather than its complete absence, as believed by many workers. It is of interest to note that in the case of samples where excretal *B. coli* is presumed to be infrequent, the number of indol producers is relatively large.

In this connection, another remarkable feature may be noted. Of 100 organisms isolated from urine, only 12 per cent. were proved to be of the coli

type, and 9 per cent. were intermediate. This interesting phenomenon has already been reported by Hill and his co-workers (1929). The ultimate origin of these cultures is a matter of some speculation. Hitherto the majority of studies on the source of coliform organisms have been confined to samples of faeces on the one hand, and soil water and non-excretal sources on the other. It has been assumed, on abundant evidence, that all *B. coli* in urine was of immediately faecal origin. The manner and the route by means of which this organism reaches the urinary tract is still controversial, but as Walker (1930) has recently stated, most workers are agreed that it is originally derived from faeces.

The finding of *B. aerogenes* and the intermediates as the predominant types in infected urine, throws a new light, in the writer's opinion, on the relation of these organisms to excretal *B. coli*. In the case of atypical organisms isolated from soil, it is almost impossible to decide for certain, on sanitary grounds, from what source they were immediately derived. With organisms from urine, on the other hand, the possible sources of origin are limited. It is difficult to believe that colon bacilli could reach the urine in anything like large numbers from any source other than the faeces, in which it is agreed that *B. aerogenes* are few. If, then, the organisms are derived directly from faeces, how is this factor to be reconciled with the cultural reactions already described? Hill suggests that in the urine the aerogenes type respond to some selective action during their sojourn in the urinary tract, or that they survive the coli type and therefore ultimately outnumber them. In the case under review, the cultures were not all typical *B. aerogenes*. An unusually high percentage of them produced indol, and 9 per cent. were of the intermediate type. What then is the position of these intermediate organisms? In the present experiments they were entirely absent from unpolluted soil, rare in faeces, and relatively common in sewage and polluted soil. If one adds to this the fact that in samples from polluted sources, both this intermediate group and the aerogenes group retained the power to produce indol, it would seem that a definite chain of events was taking place. Is one entitled to assume that the power to utilise citrate, the retention of indol-producing capacity, and the occasional production of a positive methyl-red reaction are all cultural manifestations of an environmental change? It is suggested that the farther it is removed from its normal intestinal habitat, the less does *B. coli* retain its faecal characters: and that non-faecal characters are developed before the latter are entirely lost. Does *B. coli* in fact, when placed in unfavourable surroundings, adopt a wider metabolic activity as a biological necessity, and in the urine, divorced from its natural habitat, has the transition already begun? Such a theory might explain the occurrence of the intermediate group, and the true significance of the aerogenes type. It would seem that the question is an ecological one, to be solved by a better knowledge of the metabolic needs of the colon-aerogenes organisms. At least, the subject of coliform organisms in urine is worthy of further study.

Summary 1. *Total well samples.*

In this summary, the total results obtained by the examination of well samples by the MRK and indol tests are shown. The figures in Table XV represent the percentage number of organisms belonging to each group isolated from the different grades of water.

Table XV.

Source	Samples	Cultures	Indol	Coli	Aerogenes	Inter-mediate	Atypical
Good	35	409	25	12	67	14	7
Suspicious	23	315	41	20	56	15	9
Bad	26	345	63	46	35	13	6

It is remarkable that in the grand total of well samples, the percentage number of intermediate and atypical reactions in each grade of well sample is approximately the same. The correlation between the tests and the sanitary findings is on the whole most satisfactory. The best agreement is perhaps that shown by the MR and K tests together: the citrate test alone tends to increase the number of *B. coli* in the samples believed on sanitary grounds to be free from pollution. Indol production as a single criterion would cause some 25 per cent. of samples from good wells to be condemned. The use of the MRK combination reduces that number by more than half. This adds strength to the view that by the use of the indol and citrate tests, organisms not of recent excretal origin may be detected.

Summary 2. *Total general cultures.*

The total number of cultures other than those from well samples are shown in this summary. Here is found a very fair agreement with the origin of the organisms on the part of all the tests. The indol reaction on the whole is positive in a higher number of cases than is suggested by the sanitary considerations, particularly in the case of samples from unpolluted sources.

The percentage results are shown in Table XVI:

Table XVI.

Source	Number	Indol	Coli	Aerogenes	Inter-mediate	Atypical
Unpolluted waters	204	17	—	87	13	—
Polluted waters	6	83	83	17	—	—
Faeces	278	84	96	1	2	1
Pus	6	100	100	—	—	—
Urine	130	34	29	63	7	1
Unpolluted soil	40	8	—	100	—	—
Remotely polluted soil	24	42	4	67	29	—
Recently polluted soil	36	90	81	11	8	—
Cesspits	130	78	47	33	4	16

It is of interest to compare the aerogenes type from non-polluted sources with those from polluted sources. Sixty-seven per cent. from remotely polluted soil and 63 per cent. from urine belong to this type, yet 42 per cent. of the former and 34 per cent. of the latter are indol producers. On the other hand, of the aerogenes type from unpolluted soil and water—100 per cent. in the case

of the former, and 87 per cent. of the latter—only 8 per cent. of the soil and 17 per cent. of the water cultures are of this nature. This suggests at once a very different character of the *B. aerogenes* of polluted and unpolluted sources.

Summary 3. *Grand total of results.*

Finally, the results of examination of all the 1923 cultures are presented. It is on this summation of evidence that the claims of the methyl-red and citrate tests to differentiate between excretal and non-excretal *B. coli* may best be judged.

Table XVII shows the percentage results in terms of the ultimate source of the organisms:

Table XVII.

Source	Number	Indol	Coli	Aero-genes	Inter-mediate	Atypical
Non-faecal: unpolluted water, unpolluted soil	653	23	8	76	12	4
Partly faecal: polluted water, polluted soil, cesspits, urine	986	54	36	45	12	7
Wholly faecal: faeces ...	284	91	96	1	2	1
Grand total	1923					

The correlation between the MRK combination and the source of the organisms is excellent. It will be noted that in the case of the samples from faeces, the intermediate and atypical results are almost entirely absent, whilst in the other samples they comprise 16–19 per cent. of the whole. On the basis of indol production alone, a large number of false positive results would be obtained for excretal *B. coli.*

General discussion.

From the foregoing investigations, it may be concluded, as far as local conditions are concerned, that the general belief as to the existence of two natural groups of colon bacilli has in the main been confirmed. These groups have been variously described as typical and non-typical, high ratio and low ratio, excretal and non-excretal and now more generally as coli and aerogenes. Whether this division into groups is also a division into separated species is another question, and at least from the standpoint of the research bacteriologist, must be regarded with considerable reserve. From the etiological point of view—which is that of the sanitary bacteriologist—such a classification would appear to be of considerable practical value. It may reasonably be concluded that locally the aerogenes type is rare in faeces and common outside the intestine, whilst the coli type is overwhelmingly faecal.

In the study of colon bacilli from faeces, sewage and soil, and from waters from the two extremes of sanitary quality, the propriety of this differentiation can be regarded as proven. In the study of water samples from wells, various anomalous results have been obtained. Hence the method of differentiation may be said to have lacked some of its delicacy when applied to the very conditions in which it was hoped that it might prove a useful criterion. One

would, however, hesitate before indicting the tests employed upon a charge of non-specificity when applied to water samples, in view of the high degree of agreement obtained between them and the origin of organisms isolated from other sources: rather would one attribute the anomalous results to the difficulty in the quantitative isolation of the respective types in the first instance. Failure to agree with the sanitary considerations in the case of water samples is believed to be traceable to two main factors, the one dependent upon the isolation methods employed, the other upon the natural history of the organisms themselves. These factors are primarily associated with the greater fermentative activity and the greater resistance of the aerogenes type. On the one hand, the use of liquid media in the initial stages tends to encourage the overgrowth of *B. aerogenes* at the expense of *B. coli*: on the other, even though isolation methods prove to be satisfactory, the relative proportions of each type isolated from ground waters is controlled from time to time by various natural forces. Heavy rains, with the subsequent flooding of wells with soil and surface washings, must increase tremendously the relative number of the aerogenes organisms with a corresponding difficulty in isolating the coli type.

It would appear that *B. aerogenes* of faeces differ substantially from those of unpolluted soil. In this connection arises the question already discussed of the origin of the aerogenes and "intermediate" groups. It has been shown that the aerogenes type predominated in urine, and were very commonly present in soil and water; furthermore, many organisms isolated from these sources were of the intermediate type. Practically no faecal strains were intermediate. Again, a high proportion of aerogenes organisms from polluted sources produced indol. These observations might suggest that *B. coli* in its transition to a non-intestinal habitat had lost some of its faecal characters whilst retaining others, and might serve to explain the existence of the intermediate group and indol-forming citrate utilisers, and their significance in remotely polluted sources.

The contention of Koser, therefore, that three distinct broad groups of colon bacilli exist—namely the "coli," "intermediate" and "aerogenes" types is supported in the present work. Probably this distinction—at least in temperate climates—is less of practical than of taxonomic interest. Whether one regards the citrate utilisers as representing both remote faecal and non-faecal organisms is largely of academic interest, and does not affect the main proposition that sanitarians attach less significance to the aerogenes type than to the coli type. The water bacteriologist of temperate climates is prepared to dismiss as being of little practical importance the organism that is not proved to be of recent excretal origin, whatever the method of proof employed, for he knows that pollution, unless it be recent, may be discounted.

The position in the Tropics is different. However perfect the differentiation tests, and however fixed the characters of *B. coli* and *B. aerogenes*, this grouping must have a limited practical value as a sole criterion of water purity. That *B. aerogenes*—the soil organism—should be less an indicator of pollution than

B. coli would require more confirmation than it has so far received. It has already been shown that the flooding of wells during heavy rains might result—as the figures suggest that it does result—in a relative increase of the number of aerogenes organisms isolated. In this connection Houston (1925) has sounded a note of warning which cannot be lightly disregarded:

"Some bacteriologists are a little too eager to deny recognition to the aerogenes group of organisms because they are apt to be associated with the 'washings' from grain and soils. Yet it is in times of flood when all sorts of 'unchartable' pollutions are swept into watercourses that these soil microbes may be perhaps specially noticeable, and few will deny that floods are periods of epidemiological danger. Take the case of Poona (India) as an example. Before chlorination was practised, the advent of flood water was inevitably followed by water-borne epidemics of a most serious kind. Presumably at these periods the presence of the aerogenes group of microbes and such perfectly harmless soil bacteria as *B. mycoides* might be considered indicators not of safety but of danger.

"The writer ventures to think that bacteriologists should think first of epidemiology. It would be too much to ask epidemiologists to reverse the position. In striving after commonsense both schools have a common playground."

Taking into account the results of Exps. C and D of the present series, it becomes apparent that the presence of the aerogenes type in excess cannot be taken as an unqualified indication of freedom of the wells from pollution.

As regards individual tests and groups of tests, varying degrees of success have been recorded. The standard "lactose positive-indol positive" of temperate climates did not prove a true criterion of water quality. It tended to cast suspicion upon a large number of samples which on the basis of other standards appeared to be of good quality. This, as already suggested, was doubtless due to the presence of indol-forming, citrate-utilising organisms associated with remote pollution. The negative aspects of the indol test proved more valuable than the positive, even though it involved the use of a somewhat rigid standard.

The division of McConkey's groups into "coli" and "aerogenes" frankly failed. This was largely due to the appearance of group 3 organisms in more or less equal proportions in excretal and non-excretal samples. The use of the four groups individually produced more significant results. Group 2 was commonly found in excretal, and group 4 in non-excretal samples. They proved at least of this value, that the presence of group 2 could be usually accepted as evidence of recent pollution. Its absence did not, however, have the same significance. There appeared to be some degree of relationship between saccharose fermentation and the utilisation of citrate. In the writer's opinion, the fermentation reactions of McConkey are not entirely without their value in water bacteriology, and the inclusion of the saccharose-fermentation test might with advantage be included in routine methods.

If one recognises the existence of the "intermediate" group, it may be said that the methyl-red and citrate tests in combination agreed well with the sanitary considerations. So long as the significance of this intermediate group of organisms remains unsettled, it is believed that the use of the methyl-red and citrate tests together will give more informative results than the latter alone. It is desirable in the writer's opinion to employ both reactions in water analysis, the one checking rather than supplanting the other.

Difficulty was experienced with the use of lactose-bile-salt broth as a means of primary enrichment and isolation, a difficulty which the employment of Eijkman's incubation method did nothing to dispel. The direct-plating medium suggested by the writer tended to lessen the ambiguous findings regarding the volume of water containing excretal *B. coli*, but the colour reactions of the coli colonies were not uniformly constant. It is believed nevertheless that recognition of the presence and relative number of yellow colonies isolated might prove of aid as a presumptive test.

So far, therefore, as the present investigation is concerned, the following is suggested as a technique for the bacterial examination of local water supplies:

(1) Direct inoculation of McConkey plates and of brom-thymol-blue-glucose-peptone-phosphate plates with different volumes of water; the number of plates to be adjusted according to the volume of water to be examined; incubation at 37° C. for 48 hours.

(2) Counting the proportion of yellow to green colonies which develop on the plate containing the smallest volume of water from which lactose fermenters were isolated on the McConkey plates.

(3) From this volume on McConkey plates to pick off a number of lactose-fermenting colonies. Twelve colonies is suggested as a suitable number. Sub-inoculation of peptone water tubes.

(4) Transfer of the peptone cultures into lactose and saccharose-peptone water, glucose-phosphate-peptone, brom-thymol-blue-phosphate agar slopes, and citrate medium.

(5) On this basis to regard as excretal *B. coli* all lactose fermenters which grow as yellow colonies on brom-thymol-blue-phosphate agar, and which produce indol, give a positive methyl-red reaction and fail to develop in citrate medium: saccharose fermenters to have less significance as regards recent pollution than saccharose non-fermenters: MR+ K+ to have less significance than MR+ K− and MR− K+ to have less significance than either. Indol non-production by itself to suggest absence of recent pollution.

Unfortunately the opinion on the purity of the water does not end with the interpretation of the tests. The differentiation between human and animal *B. coli* is still unsolved, and in the Tropics the presence of the latter is difficult to exclude. Further, the amount of water in which the presence of excretal *B. coli* is to be regarded as undesirable is a matter only to be fixed by a long experience of local conditions. It is well known that surface wells may vary in their bacterial content from time to time for no obvious reason, so that it is

difficult to lay down rigid numerical standards unless they be founded upon an adequate series of observations. It is hardly necessary to repeat what is common knowledge to all sanitarians, that no bacteriological methods can be interpreted with accuracy unless their limitations are kept constantly in mind.

General summary and conclusions.

(1) A study of coliform bacteria from various sources, and by means of a number of bacteriological methods, was undertaken in Dar-es-Salaam, East Africa, with the primary object of ascertaining in what way local standards of water purity might best be established.

(2) A total of 1923 cultures was studied; 1069 were taken from 84 chosen well samples, 210 from other water samples, 130 from urine, 100 from soil, and 414 from faeces and sewage. The samples were graded into three classes according as to whether they were obtained from faecal, partly faecal or non-faecal sources.

(3) The methods employed have been described. These consisted of the indol test, the fermentation reactions of McConkey, the methyl-red and citrate tests, the Eijkman fermentation test, and a direct-plating method suggested by the present writer.

(4) The "lactose+ indol+" index failed to give a perfect indication as to the sanitary quality of the water. The failure was in the direction of a high percentage of *B. coli* isolated on this basis from wells believed to be free from excretal contamination. It is believed that a large number of the indol positive strains were due to the presence of remote pollution. It is suggested that the negative aspects of this index are of value in the tropics.

(5) Primary isolation by enrichment in lactose-bile-salt broth was employed. This was found to encourage the overgrowth of *B. aerogenes* and lactose-fermenting bacteria not belonging to the *B. coli* group. Eijkman's method of incubation at 46° C. failed to eliminate these fallacies.

(6) It is suggested that a direct-plating medium can best overcome these difficulties. Such a medium is described. It is submitted that by the use of this medium, presumptive evidence of the presence of excretal *B. coli* in given volumes of water may be obtained with a reasonable degree of certainty.

(7) The fermentation groups of McConkey did not prove sufficiently specific for differentiation purposes. Group 2 predominated in faeces and group 4 outside the intestine. The presence of group 2 in water is very probably an indication of recent pollution of faecal origin. The significance of its absence could not with certainty be adjudged. Group 3 was encountered with great frequency in every type of sample. It is, however, considered that saccharose fermentation is not a character of *B. coli* recently derived from faeces. Some relation between this character and the intermediate type of methyl-red citrate result was apparent.

(8) The methyl-red and citrate tests agreed closely with each other and with the source of the organisms. By means of these two tests the existence in

nature locally of at least two distinct types of colon bacilli was confirmed. The organisms could be divided into high and low ratio groups. The former were relatively uncommon in faeces, common in water and were predominant in soil.

(9) Such a subdivision was not so well defined when the tests were applied to water analysis. An intermediate type was found in apparently unpolluted soil and water. This could only be recognised by means of the citrate test in addition to the methyl-red. It is believed that the methyl-red positive organism of soil has not the same significance as the methyl-red organism of faeces, and that it represents at most remote pollution.

(10) A high proportion of citrate positive organisms from polluted sources were indol producers. This finding, together with the predominance of the aerogenes and intermediate types in urine, suggests the existence of a transition phase of *B. coli*. It is submitted that in this way the different reactions obtained by use of the methyl-red and citrate tests might be explained.

(11) By the methyl-red and citrate tests, 96 per cent. of organisms from excretal sources, 36 per cent. from partly excretal, and 8 per cent. from non-excretal were shown to belong to the "coli" type. The principal advantage of this combination over the "lactose-indol" method appears to be its power to distinguish between recent and remote pollution.

(12) Heavy rainfall increased the relative number of the aerogenes type isolated. It cannot be concluded therefrom that, at least during the earlier part of the rains, the predominance of this group is necessarily an indication of safety.

(13) In a tropical country, hitherto unexplored by the sanitary bacteriologist, a survey of the predominating coliform organisms in faeces, soil and waters ought to be made before a standard of water purity can with safety be established. In the preparation of such a standard, due consideration must be paid to local seasonal and topographical variations.

(14) The interpretation of results must be dependent upon sanitary, chemical and other considerations. The constancy of bacteriological findings may in the tropics be considered as a more important factor than arbitrary numerical standards.

(15) The bacteriological analysis of water in the Tropics can be a useful addition to other standards, so long as its limited value is constantly kept in mind. It is to be considered as a means of controlling rather than of replacing other criteria.

(16) Water bacteriology in other parts of the Tropics is worthy of further and fuller investigation.

(2) *European series.*

Upon the writer's return to Europe on leave early in 1931, a further series of cultures was studied, with a view to obtaining a comparative estimate of the types of coliform organisms prevailing in these countries.

This work was carried out in the Bacteriological Laboratories in the

Department of Pathology, Trinity College, Dublin, through the courtesy of Prof. J. W. Bigger.

Only 577 cultures could be studied in the available time. The methods and technique employed were similar in every way to those described in the tropical experiments. In every case the indol test, the fermentation tests of McConkey, and the methyl-red, Voges-Proskauer, and citrate tests were carried out. The correlation between the methyl-red and Voges-Proskauer reactions, as already stated, was 97 per cent.

The samples were obtained from four sources, namely faeces, urine, water and soil. In the expression of results, the last two are grouped according to the possible degree of pollution on the basis of the sanitary considerations. Primary isolation in lactose-bile-salt broth was employed, in order to conform with the original work.

The first noticeable difference between the results and those obtained in Africa was to be found in the appearance of positive presumptive tests. It will be recalled that in the case of water samples examined in the Tropics, a positive acid gas result was obtained in 0·1 c.c. of every sample, with the exception of one from the main water supply. In the case of samples of good or fair sanitary quality, many of the organisms were subsequently shown to be of the aerogenes type. In the present instance, a different type of result was encountered. In the case of water samples believed to be free from pollution, coliform organisms were not isolated in a single instance in 60 c.c., the highest quantity examined: in the case of unpolluted soils, coliform organisms were never isolated at all. Such coliform bacilli as were obtained were only isolated from samples in which faecal pollution was recent or remote. Furthermore, the true aerogenes type was rare. This at once points to the fallacies encountered in the presumptive stages in the Tropics, and confirms the finding that the aerogenes type of organism being frequently present, helped to account for a large number of the false positive results obtained in small quantities of waters believed to be free from pollution.

The percentage results obtained are shown in Table XVIII.

Table XVIII.

Source	No.	Indol	McConkey's groups				Methyl-red—citrate groups			
			1	2	3	4	Coli	Aero-genes	Inter-mediate	Atypical
Faeces	145	91	50	22	16	12	87	9	3	1
Urine	144	87	20	60	12	8	74	8	18	—
Water recently polluted	84	83	44	46	5	5	97	3	—	—
Water remotely polluted	60	13	36	7	5	52	15	11	67	7
Soil recently polluted	120	76	41	25	—	34	80	3	6	11
Soil remotely polluted	24	75	4	59	37	—	29	4	58	9

The principal difference from the tropical series will be seen to consist of the relatively large number of the "intermediate" organisms found, particularly in remotely polluted samples. Forty-two per cent. of intermediates were indol positive and 59 per cent. fermented saccharose. They accounted for the greater

number of citrate users, for it will be noted that the true aerogenes type was rare. It is somewhat remarkable, therefore, that this aerogenes type should have proved more common in faeces than it did in the Tropics, and yet was less common in other sources, with the exception of samples from remotely polluted water. It is of interest to note that most of the latter samples were from pools or roof pipes, where the chance of human excretal pollution was remote. The number of cultures not proved to be excretal *B. coli*, though not as high as was found in the Tropics, was nevertheless sufficiently high to confirm their previous findings in urine.

McConkey's fermentation groups proved of little value, except that the fermentation of saccharose was rather more commonly associated with cultures not recently of faecal origin.

The practical feature of the results would appear to be that a positive "lactose-indol" result was more frequently confirmed by the methyl-red and citrate tests than in the tropics. This is more clearly indicated by expressing the cultures in terms of their ultimate origin as shown in Table XX.

Table XIX.

Source	Experiments	No.	Indol	Coli	Aerogenes	Inter-mediate	Atypical
Faecal	European	145	91	87	9	3	1
	Tropical	284	91	96	1	2	1
Partly faecal	European	432	72	70	6	20	4
	Tropical	986	54	36	45	12	7
Non-faecal	European	—	—	—	—	—	—
	Tropical	653	23	8	76	12	4
Total		2500					

It will be seen that whilst in the European series, the correlation between indol and the methyl-red and citrate tests is almost perfect, in the tropical series, a large percentage of indol-producing organisms were proved, in the case of samples believed not to be recently polluted, to be citrate utilisers. In addition it may be seen that whilst some 75 per cent. of all cultures isolated in the Dublin experiments were citrate non-utilisers—*i.e.* true "excretal" *B. coli*, only 59 per cent. of the Dar-es-Salaam strains were of this nature.

The above results would appear to indicate that the failure of the "lactose-indol" test in the Tropics, and its success in temperate climates, is largely due to the fact that in Europe a positive test indicates the presence of true excretal *B. coli* in the majority of cases, this being the commonest organism encountered, whilst in the Tropics, the aerogenes and associated types are commonly present, and give confusing false positive results. The "intermediate" results obtained in the European series were almost entirely obtained from waters and soils which would not ordinarily be examined in routine sanitary bacteriology. It is suggested that whilst Houston's index is a satisfactory bacterial indicator of water purity in these countries, and an inadequate one in the Tropics, the true explanation of its adequacy is to be explained more on sanitary than on pure bacteriological grounds. Water supplies in these

civilised countries are adequately protected and carefully controlled. Pollution when it does occur is largely chance and occasional pollution of human origin. In tropical countries, such protection and such control are always difficult and frequently impossible. Sanitation is in many cases exceedingly primitive, and hence frequent, gross, and permanent pollution of soil and waters by the excreta of both man and animals is only too often present. It is easy to understand, therefore, why indications of remote faecal pollution should be so often encountered, in addition to recent pollution. It is also easy to understand why in the Tropics, such indications should so readily be made manifest by a positive "lactose-indol" test which can be subsequently proved not to be evidence of the presence of recently excreted *B. coli*. This approaches to Clemesha's original dictum that the index of Houston was representative of a species, and that as such it served its purpose where that species predominated. Although approaching the subject from a different angle, there appears to be abundant evidence in the foregoing results to support Clemesha's assertion that it is only by a study of the biology of the coliform bacteria in relation to their natural occurrence that their rôle as indicators of water pollution in the Tropics can be safely ascertained.

FINAL SUMMARY.

(1) The "lactose+ indol+" index as employed in temperate climate is usually an adequate criterion of water purity.

(2) Where a positive test does not appear to be substantiated by the sanitary findings, a further differentiation of the organisms isolated becomes necessary. The methyl-red and citrate tests have been found to supply that differentiation in a satisfactory manner.

(3) In the Tropics, false positive "lactose-indol" tests are commonly encountered, owing to the presence in large numbers in soil and water of organisms derived from sources other than recently excreted faeces.

(4) In the Tropics, the lactose-indol test should always be confirmed by the methyl-red, citrate and saccharose tests or such other reactions or groups of reactions as may be found by a local survey to be applicable.

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