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STUDY OF THE TECHNIQUES FOR THE DETERMINATION OF CERTAIN MICROBIOLOGICAL PARAMETERS IN DRINKING WATER

(Salmonella – Pathogenic Staphylococci – Coliphages)

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(Salmonella - Pathogenic Staphylococci - Coliphages)

by

KAI GRUNNET
Institute of Hygiene, University of Aarhus, Denmark

Environment and Consumer Protection Service
and Directorate-General Employment and Social Affairs

1978

EUR 6020 EN

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ABSTRACT.

The aim of this paper is to select suitable methods for demonstrating Salmonella, pathogenic staphylococci and coliphages in drinking water. The methods mentioned are applicable to all kinds of water, but can be used even for examination of bottom samples, sludge and food.

PRINCIPLE.

Salmonella

The 5-litre sample is membrane filtered, and the filter is placed in peptone water for resuscitation, followed by enrichment in a selective enrichment broth, and by secondary seeding on selective agar plates.

From these plates the Salmonella-like colonies are picked out and the diagnosis Salmonella confirmed or excluded after a few biochemical tests. Subsequently the Salmonella strains can be sero-fermentatively typed, and in some cases phage typed, too.

Pathogenic staphylococci.

The sample of 100 ml is filtered through a membrane filter, and the filter is placed on a selective enrichment agar plate. Staphylocuccus-like colonies are picked out and the diagnosis of pathogenic staphylococci is confirmed by gram-staining and coagulase and DNase test.

Coliphages.

The sample of 100 ml is enriched in a selective enrichment broth (medium + host organism), followed by secondary seeding on an E.coli agar plate, on which coliphages will produce a plaque.

DEMONSTRATION OF SALMONELLA

1. INTRODUCTION.

In almost any isolation of Salmonella from drinking water, sewage and receiving waters, these organisms are present in a concentration so low that an enrichment is needed before they can be isolated. This enrichment, with subsequent isolation and identification, is carried out as described below.

2. FIELD OF APPLICATION.

This method is applicable to all kinds of water (drinking water, fresh- and marine waters and sewage). Furthermore, the method can be used in examining bottom samples, sludge and foods (for solid foods after suitable mincing). Only the examination of drinking water will be considered below.

3. CLASSIFICATION.

Salmonella is a gram-negative non-sporogenic rod, about 0.6 x 3 µm. With few exceptions it is motile by peritrichal flagella. It makes up an independent genus belonging to the family Enterobacteriaceae, order Eubacteriales.

Salmonella grows readily on usual media, ferments glucose and a number of other sugars mostly under gas and acid production, but does not ferment lactose or sucrose. Salmonella does not produce indole or hydrolyze urea.

4. PRINCIPLE.

After resuscitation in peptone water, Salmonella is enriched in a selective enrichment broth, followed by secondary seeding on selective agar plates.

From these plates the Salmonella-like colonies are picked out and the diagnosis Salmonella confirmed or excluded after a few biochemical tests. Subsequently the Salmonella strains can be sero-fermentatively typed, and in some cases phage typed, too.

The demonstration may be qualitative, semiquantitative or quantitative.

(a) Qualitative Demonstration.

This is performed according to the pad method (Moore 1948) which will not be mentioned in detail here. This method is most valid in tracing Salmonella excretors and contaminated places (Grunnet 1975).

(b) Semiquantitative and Quantitative Demonstration.

The semiquantitative method is based upon qualitative demonstration of Salmonella in a certain volume, e.g. 5 litres.

The method becomes quantitative, if a dilution series is made, e.g. decimal dilutions, and the samples are cultivated in triplet or quintuplet.

These demonstrations can be carried out by direct inoculation of samples by the MPN procedure or by

inoculation of samples after filtration, flocculation, and centrifuging. These methods have been described in detail by Grunnet (1975).
Of the three methods filtration is recommended below.

5. SAMPLING AND TRANSPORTATION.

As the volume to be investigated for Salmonella is 5 litres (cf. Official Journal 1975), a minimum of 5 litres are sampled in sterile glass flasks or containers of equal quality. The bacteriological examination must be commenced less than 6 hours after sampling. If this is impossible, samples should be cooled to 0-4°C immediately after collection and examined before 24 (-30) hours.

Tap water may be filtered on the sampling location (cf. paragraf 6).

6. PROCEDURE.

The different media and methods are discussed in Appendix II.

6.1. Filtration.

6.1.1. Filtration in the laboratory.

Prior to filtration pour off the uppermost part of the sample and turn bottle upside down 10 times to resuspend settled material.

(a). Waters with a high content of suspended solids.

The easiest way of filtration is to combine the speed of

a large-pore filter with the efficacy of a small-pore filter by initial filtering of the 5-litre sample through a normal paper filter followed by filtration through one (or more) membrane filter(s). All filters are placed in 30-40 ml peptone water for resuscitation.

(b). Waters with a low content of suspended solids.

Filter the 5-litre sample through one (or more) membrane filter(s), and place filter(s) in 30 ml peptone water for resuscitation.

6.1.2. Filtration of tap water on the sampling location.

After cleaning the tap by the usual procedure (cf. direction no. -) furnish it with sterile filter equipment.

As filter type, cylinder filters (e.g. Whatman filter tubes 10-03) are suitable, as they have a very great capacity (Grunnet 1975). Membrane filters can be used when the content of suspended solids is low. Measure volume of filtrate and stop filtration when the filtrate is 5 litres $\pm$ 50 ml. Then place filter in sterile container. Transportation, cf. 5. On arrival in the laboratory the filters are covered with peptone water, membrane filters with about 30 ml, and cylinder filters with about 100 ml.

6.2. Resuscitation.

Resuscitation is not commonly included in the isolation procedure of attenuated Salmonella in waters, while it is a routine procedure for demonstrating Salmonella in foods.

For waters with a low concentration of Salmonella it has been shown (Grunnet 1975) that the isolation frequency was almost doubled by a 6-hour resuscitation in peptone water. It is consequently suggested to use resuscitation in the routine procedure for demonstrating Salmonella in waters, as is already recommended in food bacteriology (accepted as a routine method in the "Community" (in press)).

6.2.1. Membrane filters.

Cover filter with 30-40 ml buffered peptone water (cf. 9.3.) and place at room temperature for about 6 hours.

6.2.2. Cylinder filters.

Cover filter with buffered peptone water and place at room temperature for about 6 hours.

6.3. Enrichment.

After resuscitation place filters in a selektive enrichment medium (tetrathionate broth, cf. 9.9., or Rappaport's medium, cf. 9.6.).

6.3.1. Membrane filters.

Mix filter and resuscitation broth with 300 ml enrichment broth and incubate at $42^{\circ}C \pm 1^{\circ}$.

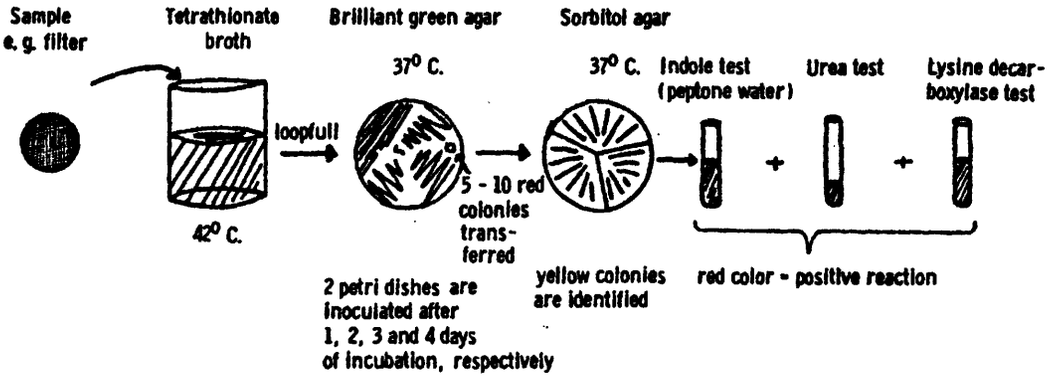
6.3.2. Cylinder filters.

Transfer filter together with 30 ml resuscitation broth to 300 ml enrichment broth and incubate at $42^{\circ}C$.

6.4. Isolation Technique.

During the subsequent four days inoculate sub-cultures daily on two brilliant green agar plates (cf. 9.2.) and incubate at 37°C for 16-24 h. The sugars lactose and sucrose are not split by Salmonella. Transfer from each plate 5-10 Salmonella-like colonies (red) to a sorbitol-agar plate (cf. 9.8.). After incubation for 16-24 h at 37°C test sorbitol-splitting bacteria (yellow) for ability at 37°C to form indole in peptone water (cf. 9.3.) and to split urea (cf. 9.10.) and lysine (cf. 9.4.) and fig. 1 (Grunnet 1975). Indole- and urea-negative but lysine positive bacteria are considered Salmonella.

Fig. 1. SALMONELLA AND PS.AERUGINOSA ISOLATION PROCEDURE.



The Salmonella strains are finally typed at a specialized laboratory by serological and biochemical properties. Some types may even be phage typed.

7. EXPRESSION OF RESULTS.

Results of this semiquantitative examination are recorded as:
Salmonella detected/not detected in 5 litres.
For quantitative demonstration cf. 8.2.

8. SPECIAL CASES.

8.1. Isolation of S.typhi.

When demonstrating S.typhi the standard procedure (cf. 6) is also used, but with selenite broth (cf. 9.7.) incubated at 37°C+) and bismuth sulphite agar (cf. 9.1.) as enrichment broth and primary plates, respectively.

On the bismuth sulphite agar S.typhi form "rabbit eye" colonies (black centre with clear translucent edge after 24 h - uniform black after 48 h).

From the bismuth sulphite agar transfer suspect colonies either to brilliant-green-agar, where isolation procedure continues as usual (cf. 6.4.) or start serotyping of S.typhi directly.

Results are recorded as S.typhi present/not present in 5 litres.

+) It has lately been claimed (Kampelmacher 1977 in press) that incubation at 22°C increases the isolation frequency, but this has to be studied more before it can be accepted as a standard method.

8.2. Quantitative demonstration.

For this demonstration take a sample of 6 litres and determine the Most Probable Number (MPN) using the series: 5 times 1 litre, 5 times 100 ml and 5 times 10 ml.

Filter samples of 1 litre and of 100 ml (cf. 6.1.). (The 10 ml samples are inoculated directly). Each sample (filter or 10 ml) is resuscitated and enriched separately, and isolation performed as described in 6.4.

From the combination of positive and negative part-samples of each volume look up the number of Salmonella in the MPN-tables (Table 1, WHO 1971). If the concentration of Salmonella is between 160 and 1600 per litre, use 5 x 100 ml, 5 x 10 ml, and 5 x 1 ml. At higher concentrations dilute further. At lower concentrations use 5 x 1000 ml, 5 x 100 ml, and 5 x 10 ml, cf. example 3.

Table 1. MPN INDEX per 100 ml.

5 x 10 ml (1)	5 x 1 ml (1)	5 x 0,1 ml (1)	MPN Index (2)
0	0	1	2
0	1	0	2
0	2	0	4
1	0	0	2
1	0	1	4
1	1	0	4
1	1	1	6
1	2	0	6
2	0	0	5
2	0	1	7
2	1	0	7
2	1	1	9
2	2	0	9
2	3	0	12
3	0	0	8
3	0	1	11
3	1	0	11
3	1	1	14
3	2	0	14
3	2	1	17
3	3	0	17
4	0	0	13
4	0	1	17
4	1	0	17
4	1	1	21
4	1	2	26
4	2	0	22

Look up in column (1) of the table the combination of figures that corresponds to the numbers of positive tubes in the three consecutive series which include the smallest quantities of water that have had growth of Salmonella (see, however, example 1). Column (2) then directly gives the MPN number per 100 ml looked for the series 10-1-0.1 ml, while for the series 100-10-1 ml, 1000-100-10 ml, etc. the figures in column (2) must be divided by 10,100, etc.

MOST PROBABLE NUMBER OF BACTERIA PER LITRE.

Example 1				Example 2				Example 3			
10ml	1ml	0.1ml	0.01ml	100ml	10ml	1ml	0.1ml	1000ml	100ml	10ml	1ml
+	+	+	-	+	+	+	-	+	+	+	
+	+	-	-	+	+	-	-	+	+	-	-
+	-	-	-	+	-	-	-	+	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
170				170:10=17				170:100=1.7			

The results are recorded as MPN for Salmonella per litre.

9. APPENDIX I. MEDIA AND REAGENTS.

When available dehydrated media may be used.

9.1. Bismuth sulfite agar (Wilson Blair-Modified).

Dissolve 5 g beef extract, 10 g peptone, 5 g dextrose, 4 g disodium phosphate, 0.3 g ferrous sulfate, 8 g bismuth sulfite indicator, 0.025 g brilliant green, and 20 g agar in 1 litre distilled water and sterilize by streaming for 30 minutes, cool to 50-55°C, agitate to disperse the precipitate evenly and pour the plates.

9.2. Brilliant green agar.

Dissolve 10 g peptone, 5 g beef extract, 5 g sodium chloride in 920 ml distilled water, and add 20 g agar, and adjust pH to 7.2.

Dissolve 80 mg phenol red, 10 g lactose, and 10 g sucrose in 80 ml of 0.005 ml sodium hydroxide. Add this solution together with 2 ml of a 5 per mille solution of brilliant green to the medium. Mix and sterilize at 115°C for 15 minutes.

9.3. Buffered peptone water (Resuscitation broth).

Dissolve 10 g peptone, 5 g sodium chloride, 9 g disodium hydrogen phosphate ($Na_2HPO_4,12H_2O$) and 1.5 g potassium dihydrogen phosphate (KH_2PO_4) in 1000 ml distilled water. Adjust pH to 7.0-7.2 and sterilize at 121°C for 15 minutes.

9.4. Indole reagent (Kovacs).

Dissolve 5 g paradimethylaminobenzaldehyde in 75 ml n-pentanol and 25 ml concentrated hydrochloric acid.

9.5. Lysine decarboxylase broth.

Dissolve 5 g peptone, 3 g yeast extract, 1 g dextrose, 5 g L-lysine and 0.2 g brom cresol purple in 1000 ml distilled water. Adjust pH to 6.8 and dispense in 5 ml amounts into test tubes and sterilize at 121°C for 15 minutes.

9.6. Rappaport's medium.

Solution A: Dissolve 0.5 g Bacto tryptone, 0.8 g NaCl and 0.16 g KH_2PO_4 in 100 ml bi-distilled water.

Solution B: Dissolve 40 g of $MgCl_2$ (C.P.) in 100 ml water (it is advisable to dissolve the entire contents of $MgCl_2$ from a newly opened container according to the formula, as this salt is hygroscopic).

Solution C: A 0.4% solution of malachite green in distilled water is required.
For use, to each 100 ml of solution A i added 10 ml of solution B and 3 ml of solution C. The final medium is autoclaved for 20 minutes and can be kept at room-temperature for 1 month.

9.7. Selenite broth (Raj 1966).

Dissolve 4 g proteose-peptone, 1.5 g yeast extract, 4 g dulcitol, 5 g sodium selenite, 1.25 g Na_2HPO_4, and 1.25 g KH_2PO_4 in 1000 ml distilled water. Adjust pH to 6.9. Sterilize in flowing stream for 30 minutes. The medium can be kept in dark at 4°C until a red sediment appear.

9.8. Sorbitol agar.

Procedure as for brilliant green agar (cf. 9.2.) except that lactose and saccharose are replaced by 10 g of sorbitol.

9.9. Tetrathionate broth (Kauffmann).

Basal medium: Dissolve 5 g proteose peptone and 1 g sodium taurocholate in 1 liter of distilled water. Add 30 g calcium carbonate (insoluble). Sterilize at 121°C for 15 minutes.
Immediately before use this basal medium is added sodium thiosulfate 30 g, brilliant green 11 ml of an 0.1% aqueous solution, and iodine solution 25 ml (iodine 25 g and potassium iodide 20 g dissolved in 100 ml of distilled water), and the medium is ready for use.
NB! No sterilization.

9.10. Urea broth.

Basal medium: Dissolve 1 g peptone, 5 g sodium chloride, 2 g potassium dihydrogen phosphate (KH_2PO_4), and 5 ml phenol red of an 0.2% solution in 100 ml distilled water. Adjust pH to 6.8-6.9. Sterilize at 121°C for 20 minutes. Before use, the basal medium is added 1 g dextrose, and 20 g urea. After the medium has been sterilized by filtration the medium is aseptically tapped on sterile test tubes (appr. 2 ml in each).

10. APPENDIX II: DISCUSSION OF METHODS FOR DEMONSTRATION OF SALMONELLA IN WATERS.

For isolating a bacterial species from a mixed flora two conditions have to be fulfilled. First, the species concerned must be liberated, if enveloped in conglomerates of organic matter, other microorganisms, etc. Secondly, the selectivity of the medium used must favour the growth of the bacterium concerned and/or inhibit that of other microorganisms.

Initially, when attempts were made to isolate Salmonella from sewage and receiving waters, the methods (solid media) used in human medicine were applied (Drigalski & Conradi 1902, Loeffler 1903, Lentz and Tietz 1903, Houston 1913). On these plate media it was not possible to isolate Salmonella from mixed floras originating from sewage and receiving waters, as could be done from patients with salmonellosis, presumably due to a far higher Salmonella concentration in the latter. On solid media one Salmonella from the inoculum will form on the plate only one colony which is easily overlooked. On the other hand, one Salmonella, after enrichment in a selective medium, will result in several colonies on secondary seeding. Accordingly, a liquid medium should be used for isolating Salmonella from materials having low Salmonella concentrations.

SELECTIVE LIQUID ENRICHMENT MEDIA

Selenite Broth

Guth (1916) was the first to succeed in producing a liquid, selective Salmonella enrichment medium, similar to the already existing E.coli enrichment medium (MacConkey & Hill 1901, MacConkey 1905). Guth utilized Klett's (1900) finding that 0.1% sodium selenite solution inhibited S.typhi to only a slight extent, whereas it inhibited the greater part of the competing microflora.

However, selenite broth did not come into general use until Leifson (1936) had further developed this medium and worked out various modifications according to the origin of the sample, e.g. a selenite S broth, S signifying sewage. During their growth, a number of bacteria, including Salmonella, reduce selenite to free selene (Levine 1925). At the same time the pH rises. This increase in pH reduces the toxic action of selenite, and thereby the selective properties of the medium. This was compen-

sated for by adding 0.4% lactose which is fermented by enterococci and "colon bacilli", whereby the pH is kept natural or faintly acid. Leifson (1936) reported that lactose might perhaps be substituted by other sugars, but that this was rarely an advantage. The inhibitory effect of the medium varied, so that even closely related types of Salmonella, such as S.paratyphi A and S.paratyphi B, can be distinguished by their growth, the former growing in a 1% sodium selenite solution, the latter not.

As Leifson's selenite broth did not inhibit the growth of Proteus and only slightly that of E.coli, Stokes & Osborne (1955) modified the medium by adding brilliant green (5 mg/litre) and taurocholate (1 g/litre) and by replacing lactose with mannitol. According to Miller & Banwart (1965), however, this modification also had an inhibitory effect upon small inocula of Salmonella. Osborne & Stokes (1955) demonstrated that the inhibitory action upon Proteus and E.coli disappeared on addition of egg products, but that these microorganisms could be inhibited instead by the addition of sulpha preparations (500 µg sodium sulphapyridine per ml) which, however, also inhibit certain types of Salmonella.

Knott, Kutzsche & Walter (1961), studying the effect of various sulpha drugs upon Salmonella and E.coli, found a greater inhibition of Salmonella than of E.coli. Thus, the value of sulpha drugs in enrichment media is questionable.

Taylor, Silliker & Andrews (1958) tested int.al. Stokes & Osborne's modified selinite broth which they found to be less effective for isolating Salmonella in egg products than the selenite-F broth and a modification containing cysteine (10 µg/ml) (North & Bartram 1953). Like Leifson (1936), Taylor, Silliker & Andrews (1958) were unable to demonstrate any increase in the frequency of Salmonella isolation when lactose was replaced with mannitol or dulcitol in the selenite-F broth.

For isolating Salmonella from fish products, Raj (1966) modified the selenite-F broth by replacing lactose with dulcitol, peptone with proteose peptone, and by adding extract of yeast. By this modification he reported finding 2-7 Salmonella in a mixed flora of 10^4-10^6 bacteria.

From a theoretical point of view it seems correct to replace lactose or mannitol with dulcitol or sorbitol, as these sugars are split by practically all types of Salmonella, but only by some of the competing microorganisms. On the other hand, it must be a matter of dispute whether the selenite broth, as emphasized by several authors, is the best enrichment medium for isolating the Salmonella group as a whole. As demonstrated by Leifson and later confirmed by many others, e.g. Wundt (1953-54) and Kauffmann (1966 b), selenite broth is particularly well-suited for isolating S.typhi and S.paratyphi A. As S.typhi can withstand high selenite concentrations, the concentration of selenite in the medium may be increased, with advantage, from Leifson's 0.4% (in the selenite-F medium) or Kauffmann's 0.6% to 0.8% (Harvey & Price 1964) or even up to 1-1.5% as in Leifson's selenite-S broth. This affords a highly selective medium for the demonstration of these two types. This enrichment can take place at 40°C (Metropolitan Water Board 1963-64).

Iveson & Mackay-Scollay (1972) recommend addition of Strontium chloride to the medium.

Tetrathionate Broth

Tetrathionate broth was developed by Müller (1923) who tested various tetrathionate compounds and arrived at the result that ammonium, potassium, magnesium, and calcium tetrathionate broths were equally suited for isolating Salmonella from faeces. Sulphuric acid forms in the enrichment medium. To keep the pH at a faintly alkaline or neutral level, calcium carbonate was added. This medium was further developed by Kauffmann (1930 and 1935) who combined Müller's tetrathionate broth and brilliant green broth (Killian 1924) into one medium, added bile, and demonstrated that the selective effect was further increased by omitting heating.

Since then, several modifications of the medium have seen the light of day. Bierbrauer's (1939) medium with malachite green, Knothe's (1949) medium with potassium chromate, and Preuss' (1949) medium with crystal violet-metachrome-yellow. Now, Kauffmann's and Preuss' modifications are in most common use, but the latter without metachrome-yellow. Comparing these two media, Lang (1959) found largely identical results. He demonstrated also that unlike potassium tetrathionate broth, sodium tetrathionate broth influenced the formation of the flagella and that Kauffmann's medium inhibited the growth of E.coli as well as of "Aerobacter" (Enterobacter) aerogenes, while Preuss' medium inhibited only E.coli. On the other hand, none of the media inhibited the growth of Proteus vulgaris.

Kauffmann (1966 b) reported that tetrathionate broth was better suited than selenite broth for isolating Salmonella, with the exception of S.typhi and a few other types. This has been confirmed by int.al. Edel & Kampelmacher (1968). After sending artificially contaminated samples of faeces to 8 major laboratories, they found the combination of tetrathionate broth and elevated enrichment temperature (41-42°C, cf. temperature selection, p.25) to result in the highest recovery percentage.

Rappaport's Medium

Rappaport, Konforti & Navon (1956) introduced a new enrichment medium (Rappaport's broth) which they said was better suited than selenite or tetrathionate media for isolating Salmonella from human faeces. This medium was supposed to possess the further advantage of inhibiting the coliform bacteria. Iveson, Kovacs & Laurie (1964) confirmed this finding by isolating Salmonella from dried coconut.

Collard & Unwin (1958) also found the use of Rappaport's medium to increase the frequency of Salmonella isolation from human faeces, but less so than claimed by Rappaport, Konforti & Navon (1956). On the other hand, Sen (1964) and Raj (1966) were unable to find this effect of Rappaport's medium. Grunnet (1975) and Brest Nielsen & Grunnet (1976) found tetrathionate broth and Rappaport's medium to give the same results. As Rappaport's medium is simpler and cheaper, this is in favour of Rappaport's medium.

Simultaneous Use of Several Enrichment Media

As the simultaneous use of several media has proved to increase the isolation frequency, a number of workers have ended in using routinely two media (int.al. Slanetz, Bartley & Stanley 1968, Harper & Shortridge 1969).

Suppression of Proteus in Enrichment Media and on Solid Media

The growth of Proteus is not inhibited, either by selenite broth (1%) (Leifson 1936), tetrathionate broth (Lang 1959), or Rappaport's medium (Rappaport, Konforti & Navon 1956). These media even afford enrichment possibilities for Proteus which on inoculation is often transferred to the secondary plates which they "swarm" and thus prevent the demonstration of Salmonella or at least render it difficult.

A large number of substances have been tried for inhibiting swarming of Proteus on solid media: phenol, metal ions, surface-active substances, alcohol, chloral hydrate, etc. (Krämer & Koch 1931, Kauffmann 1935, Sedlák & Rische 1968). Lominski & Lendrum (1942) demonstrated that lauryl sulphate can temporarily alter Proteus to a non-flagellate bacterium, but without any change in their growth or other properties. Lauryl sulphate has a similar effect upon S.paratyphi B among others.

Döll (1956) introduced these anion-active substances (alkyl sulphate, alkyl lauryl sulphonate, and alkyl benzol sulphonate) in the Salmonella isolation procedure by adding them to solid media. In this country the substances have been tested and introduced by Andersen (1957) who also employed them in liquid media. Kristensen (1969) using 1% teepol 515 (alkyl benzene sulphonate) in the enrichment medium, reported that this increased the isolation frequency of Salmonella.

As these anion-active substances also influence the formation of flagella on several types of Salmonella, it seems wrong to use them in liquid media, if it is a case of acting only upon the flagella. On solid media, on the other hand, it is a gain to immobilize Salmonella as well as Proteus, so that their colonies can be differentiated on the basis of other properties. This is obtained e.g. by adding 0.1-0.3% teepol 515 to the solid media.

Sompolinsky (1948) and Sedlák & Rische (1968) prevented Proteus swarming on solid media by means of immune sera (H antigen).

Sulpha drugs (Stokes & Osborne 1955, cf. p.22) and albomycin (60 µg/ml, Slanetz, Bartley & Stanley 1968) have also been used for suppressing Proteus in the enrichment media. As demonstrated by Knott, Kutzsche & Walter (1961) the value of sulpha drugs is very doubtful, since frequently they also inhibit Salmonella. Something similar presumably applies to most other chemotherapeutics.

From what has been stated above, it may be concluded that suppression of Proteus swarming by adding anion-active detergents to the diagnostic agar plates is of great importance in isolating Salmonella from sewage

and receiving waters. On the other hand, this addition seems less well-founded in enrichment media. Addition of sulpha drugs also appears to be of limited value, as they inhibit Salmonella too.

Suppression of E.coli in Enrichment Media

This has been tried int.al. by Stokes & Osborne (1955, cf. p.22) who inhibited the growth of E.coli in selenite broth by adding sulpha drugs. This seems of questionable value, as Salmonella is often inhibited as well (Knott, Kutzsche & Walter 1961).

Suppression of Pseudomonas

Pseudomonas is also not essentially inhibited in the enrichment media, but by covering the medium with a layer of mineral oil Slanetz, Bartley & Stanley (1968) were able to prevent the growth of these obligate aerobic bacteria.

Temperature Selection

As early as 1908 it was pointed out by MacConkey, in his efforts at isolating S.typhi and E.coli, that enrichment at 42°C of course had a more selective effect than enrichment at 37°C.

Harvey & Thomson (1953), Harvey & Price (1968), and Spino (1966) introduced enrichment at about 42°C, except in culturing S.typhi, as this temperature is unfavourable for S.typhi (vide supra). However, Burman (1967) recommended a compromise, viz. using Rappaport's medium at 37°C and the others at 40°C, in order to be able to isolate also S.typhi.

Summary on Enrichment Media

The varying views on the mutual sensitivity of the media in isolating Salmonella are often due to insufficient attention to the fact that the sensitivity varies with the nature of the sample.

Selenite broth (about 1%), possibly with cysteine, is particularly well-suited for isolating S.typhi and S.paratyphi A (at about 37°C). Most other types of Salmonella are best isolated by using tetrathionate broth, either in the modification of Kauffmann or of Preuss (at about 42°C), but even though there seems to be very little difference between the modification of Kauffmann and Preuss, the modification of Kauffmann is recommended, because this already is the accepted method in the "Community" for demonstration of Salmonella in foods. Rappaport's medium is a good alternative to tetrathionate broth.

SOLID MEDIA AND ESTABLISHING THE DIAGNOSIS OF SALMONELLA

Among the solid media a distinction is made between plates to be seeded from the enrichment media (secondary plates) and plates to be seeded direct from the sample (primary plates). The solid media will be mentioned only briefly, as unlike the enrichment media they are not of the same importance in isolating Salmonella from sewage and receiving waters.

Secondary Plates

As a rule seeding is done on two plates from the enrichment medium, the first time 18-24 hours after the primary inoculation and usually two times in all at intervals of about 24 hours. Slanetz, Bartley & Stanley (1968), however, routinely used daily seeding for up to 14 days.

The two secondary plates in most common use are: (1) brilliant green-lactose-phenol red agar plates (BLSP) (Lang 1959, Schönberg & Widdik 1937) which seem well-suited for the cultivation of all Salmonella, and (2) bismuth sulphite agar plates (Wilson & Blair 1927-28) which are said to be particularly well suited for the cultivation of S.typhi and S.paratyphi.

Galton, Hardy & Mitchell (1950) and Dunlop, Twedt & Wang (1952) found BLSP plates to be more selective, and therefore better suited, for isolating Salmonella than were the bismuth sulphite agar plates which again were more selective than Salmonella-Shigella-(SS-) agar plates. The latter are used mostly for direct seeding.

From the secondary plates the Salmonella-like colonies are transferred to a new selective plate whence the diagnosis of Salmonella may be confirmed by a few biochemical tests: examination for sorbitol fermentation, splitting of urea and lysine, indol formation in peptone water, and growth in KCN medium. A few laboratories, however, still prefer selecting Salmonella from secondary plates with polyvalent anti-immune sera.

Finally, the serofermentative type (Kauffmann 1963 b) is determined, while phage typing is not yet a routine procedure in Denmark.

Primary Plates

This method is of importance mainly in isolating microorganisms from faeces, in which the absolute as well as relative concentration of Salmonella is high. In sewage and in receiving waters the concentration of Salmonella is usually too low for direct demonstration. Let it be mentioned, however, that they were demonstrated for the very first time on primary plates (Wilson 1928).

The most common medium for direct seeding is Salmonella-Shigella (SS-) agar (Difco Manual 1953). Isenberg, Kominos & Siegel (1969) compared this SS agar with a xylose-lysine-desoxycholate (XLD) agar (Taylor 1965) and with hectoenenteric (HE-) agar (King & Metzger 1968). They reported that although the Salmonella isolation frequency could be increased by [illegible] the use of selective [illegible] by these media were inter-

Drigalski & Conradi's (1902) medium is also employed in many laboratories for direct seeding in identifying Enterobacteriaceae, but as this medium too is rather less specific than SS agar, it is hardly indicated in cases where interest is centred on the isolation of Salmonella (Brest Nielsen, personal communication 1972).

Wilson & Blair's (1927/28) medium is used both for primary and secondary seeding of the plates in studies of S.typhi.

As a number of the agar plates for primary seeding mentioned above are not particularly specific (selective), the choice of primary plates must depend to a marked extent also upon the nature and origin of the sample.

Pure culture and isolation from the primary plates is carried out analogously to that from the secondary plates.

OTHER METHODS OF ISOLATION

Selective "Motility Medium"

On the basis of Craigie's technique (1931) Harper & Shortridge (1969) developed a "motility medium" in which semi-liquid SS agar was placed in a U tube.

A mixed culture is added to one limb of the U tube, and Salmonella is isolated from the other. This method afforded an appreciably higher isolation frequency than that obtained by inoculating selenite F broth (Leifson 1936). Among the Salmonella positive samples, Salmonella was isolated by the selective "motility medium" in 90%, by selenite F broth in 50%, and by simultaneous inoculation of both media in 100%.

As this technique of isolation (Harper & Shortridge 1969) has been compared with isolation from selenite broth which, as already mentioned, generally affords lower isolation frequencies than those obtained by the use of tetrathionate broth, it is difficult to estimate whether the motility medium is better or poorer than tetrathionate broth. A drawback of the motility medium is the need for more equipment and the fact that it is more difficult to handle then the current enrichment media.

It has not been stated, whether Proteus is able to move through this medium. If so, addition of anion-active detergents in concentrations which do not prevent the motility of Salmonella may be tried.

Fluorescence

Abrahamsson, Patterson & Riemann (1968) used a decrease of the pH in Raj's (1966) dulcite-selenite broth as indicator of the presence of Salmonella and demonstrated the bacteria by a fluorescence-antibody reaction.

Bissett, Powers & Wood (1969) also used a fluorescence-antibody technique for screening suspect persons in a typhoid epidemic. Thereby they found 20% false positive reactions, due in three cases to reaction by a Citrobacter strain which had antigen in common with S.typhi. These authors therefore pointed out the necessity of further bacteriological confirmation (isolation and identification) of all positive reactions.

Accordingly, it is hardly possible to conclude, as did e.g. Janssen & Meyers (1968), that fish are infected with Salmonella from a demonstration of serum antibodies.

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DEMONSTRATION OF PATHOGENIC STAPHYLOCOCCI.

1. INTRODUCTION
2. FIELD OF APPLICATION
3. CLASSIFICATION
4. PRINCIPLE
5. SAMPLING AND TRANSPORTATION
6. PROCEDURE
   - 6.1. Presumptive staphylococcus organisms
   - 6.2. Confirmation
     - 6.2.1. Gram stain
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     - 6.2.3. DNase test
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9. APPENDIX: MEDIA AND REAGENTS

DEMONSTRATION OF PATHOGENIC STAPHYLOCOCCI.

1. INTRODUCTION.

Direct demonstration of pathogenic staphylococci in waters by the aid of selective media gives rise to certain problems, as the indicative principle is based on properties which are not always associated with staphylococci and as the selective principle is inhibitary to the growth of attenuated staphylococci.

2. FIELD OF APPLICATION.

This method is applicable to all kinds of water, i.e. drinking-water, fresh- and marine waters, and sewage.

Furthermore, the method can be used in examining foods (in the case of solid foods after suitable mincing). Only the examination of drinking water will be considered below.

3. CLASSIFICATION.

Staphylococcus is a gram-positive spherical coccus, diameter 0.8-1.0 µm. The cells occur singly, in pairs, and divide in more than one plane, forming irregular clusters.

The Staphylococcus is an independent genus belonging to the family Micrococcaceae.

4. PRINCIPLE.

Even though a resuscitation of the often attenuated staphylococci in waters might improve the sensitivity

of the method, this is not included in the isolation procedure.

A sample of 100 ml is filtered through a membrane filter, and the filter is placed on a selective enrichment agar plate. Staphylococcus-like colonies are picked out and the diagnosis of pathogenic staphylococci is confirmed by gram-staining and coagulase- and DNase test.

5. SAMPLING AND TRANSPORTATION.

The samples are drawn into sterile glass flasks or containers of equal quality.

Tap water is drawn after the usual cleaning procedure (cf. direction No.). The bacteriological examination must be commenced less than 6 hours after sampling.

If this is possible, samples should be cooled to 0-4°C. immediately after collection and examined within 24 (-30) hours.

6. PROCEDURE.

6.1. Presumptive staphylococcus organisms.

Filter the sample of 100 ml through a membrane filter (0.45 um), and place filter on a Baird-Parker or a Chapman agar plate, and incubate at 37°C for 18-24 hours.

Count the number of staphylococcus-like colonies i.e. black, shiny convex 1-1.5 mm in diameter or yellow, on Baird-Parker agar and Chapman agar, respectively.

If no staphylococcus-like colonies are seen incubate for another 24 hours.

6.2. Confirmation.

Subculture each of five typical colonies and perform a

6.2.1. Gram stain

6.2.2. coagulase test

Mix 0.5 ml citrated rabbit plasma, diluted 1:1 with sterile saline (0.85% aqueous solution of sodium chloride) with an equal volume of an 18-24 hour broth culture and incubate at 37°C. Examine after 1 and 4 hours for coagulum. Negative tests should be examined again after 24 hours.

It is preferable to include, as controls, tubes containing strains of staphylococcus known to be coagulase positive (S.aureus) and coagulase negative (S.epidermidis).

6.2.3. DNase test

Transfer the colonies to a DNase agar and incubate the plate at 37°C for 24 hours.
Flood the DNase agar plate with 1 molar hydrochloric acid and look for clearings around the colonies (DNase positive). DNase positive staphylococci are considered pathogenic.
The gram positive cocci, that are coagulase and DNase positive are considered pathogenic staphylococci.

7. EXPRESSION OF RESULTS.

The result of this quantitative examination is recorded as the number of pathogenic staphylococci per 100 ml.

8. SPECIAL CASES.

None.

9. APPENDIX:

MEDIA AND REAGENTS.

9.1. Baird-Parker agar.

Dissolve 10 g tryptone, 5 g beef extract, 1 g yeast extract, 10 g sodium pyruvate, 12 g glycine, 5 g lithium chloride in 1000 distilled water, adjust pH to approx 6.8, add 20 g agar and sterilize at 121°C for 15 minutes. Cool to 45-50°C and add aseptically 50 ml concentrated egg yolk emulsion and 3 ml potassium tellurite 3.5%, mix well and pour the plates. The plates must be used within 24 hours of preparation.

9.2. Chapman agar.

Dissolve 2.5 g yeast extract, 100 g tryptone, 2 g lactose, 10 g mannitol, 75 g sodium chloride, 5 g dipotassium hydrogen phosphate, 30 g gelatin and 15 g agar in 1000 ml distilled water, adjust pH to approx 7.1, and sterilize at 121°C for 15 minutes. Disperse the precipitate by gentle agitation before pouring.

9.3. DNase agar.

Dissolve 20 g tryptose, 2 g deoxyribonucleic acid, 5 g sodium chloride in 1000 ml distilled water, adjust pH to approx. 7.3, ad 12 g agar and sterilize for 15 minutes at 121°C.

Mix well and pour plates.

9.4. Hydrochloric acid 1 molar.

Dilute 80 ml concentrated hydrochloric acid with 920 ml distilled water.

DEMONSTRATION OF COLIPHAGES

1. INTRODUCTION.

Bacteriophages have been reported for various groups of enteric bacteria, e.g. Salmonella, Shigella, Vibrio, Escherichia coli, Proteus, etc.

As a total examination for all faecal bacteriophages is too laborious in daily routine, the degree of contamination may be assessed instead on the basis of the content of indicator bacteriophages, i.e. a group of intestinal bacteriophages which are present simultaneously with the other faecal bacteriophages, which occur in greater number, and whose resistance is greater than or equal to that of the other faecal bacteriophages (according to definition of indicator bacteria, Bonde 1963).

For this purpose coliphages were chosen. The other faecal bacteriophages may be used in typing bacteria and in epidemiological investigations.

2. FIELD OF APPLICATION.

This method is applicable to all kinds of water, i.e. drinking water, fresh and marine waters, and sewage. Furthermore, the method can be used in examining sludge and soil. Only the examination of drinking water will be considered below.

3. CLASSIFICATION.

Coliphages are a heterogeneous group of RNA and DNA viruses parasitic on Escherichia coli (E.coli),

here defined by their ability to grow on the chosen host organism, a strain B of E.coli.

4. PRINCIPLE.

The coliphages are enriched in a selective enrichment broth (medium + host organism), followed by secondary seeding on an E.coli agar plate, on which coliphages will produce a plaque.

5. SAMPLING AND TRANSPORTATION.

The samples are drawn into sterile flasks or containers of equal quality.
Tap water is drawn after the usual cleaning procedure (cf. direction No.). The bacteriological examination must be commenced less than 6 hours after sampling.
If this is impossible, samples should be cooled to 0-4°C immediately after collection and examined within 24 (-30) hours.

6. PROCEDURE.

6.1. Preparation of host culture.

One day prior to the examination of the drinking water, prepare the host culture by inoculating the E.coli B strain in peptone water (cf. 9.3.) and incubate at 37°C.

6.2. Enrichment.

Measure a 100 ml sample into a sterile graduated cylinder and pour the sample into an Ehrlenmeyer flask containing 100 ml of double-strength MacConkey broth (cf. 9.1.),

Add two drops of an 18-hour-old host culture of an E.coli B strain and incubate at 37°C for 16-20 hours.

6.3. Secondary plating.

Transfer a loopful from each of the enriched samples to a freshly seeded E.coli B MacConkey agar plate (cf. 9.2.) and incubate at 37°C. Read the results after 6 hours or/and 24 hours. Samples with coliphages have then produced a plaque on the plate.

7. EXPRESSION OF RESULTS.

Results of this semiquantitative examination are recorded as:

Coliphages detected/not detected in 100 ml water.

For quantitative demonstration cf. 8.1.

8. SPECIAL CASES.

8.1. Quantitative demonstration.

For this demonstration take a sample of 105 ml and determine the Most Probable Number (MPN) using series: once 50 ml, 5 times 10 ml, and 5 times 1 ml.

Incubate each part sample separately (50 and 10 ml in broth of double-strength and 1 ml in single-strength). Incubation and secondary seeding as described in 6.2. and 6.3.

From the combination of positive and negative part samples of each volume look up the number of coliphages in the MPN tables (Table 1, WHO 1971). At higher concentration dilute with decimal dilutions and look up the

results in table 2.

Table 1. MPN Index.

1 x 50 ml (1)	5 x 10 ml (1)	5 x 1 ml (1)	MPN Index (2)
0	0	1	1
0	0	2	2
0	1	0	1
0	1	1	2
0	1	2	3
0	2	0	2
0	2	1	3
0	2	2	4
0	3	0	3
0	3	1	5
0	4	0	5
1	0	0	1
1	0	1	3
1	0	2	4
1	0	3	6
1	1	0	3
1	1	1	5
1	1	2	7
1	1	3	9
1	2	0	5
1	2	1	7
1	2	2	10
1	2	3	12
1	3	0	8
1	3	1	11
1	3	2	14
1	3	3	18
1	3	4	21
1	4	0	13
1	4	1	17
1	4	2	22
1	4	3	28
1	4	4	35
1	4	5	43
1	5	0	24
1	5	1	35
1	5	2	54
1	5	3	92
1	5	4	161

Look up in column (1) of the table the combination of figures that corresponds to the number of positive tubes in the three consecutive series which have resulted in plaque formation.

Column (2) the directly gives the MPN number per 100 ml for the series 50-10-1 ml.

Table 2. MPN Index.

5 x 10 ml (1)	5 x 1 ml (1)	5 x 0,1 ml (1)	MPN Index (2)
0	0	1	2
0	1	0	2
0	2	0	4
1	0	0	2
1	0	1	4
1	1	0	4
1	1	1	6
1	2	0	6
2	0	0	5
2	0	1	7
2	1	0	7
2	1	1	9
2	2	0	9
2	3	0	12
3	0	0	8
3	0	1	11
3	1	0	11
3	1	1	14
3	2	0	14
3	2	1	17
3	3	0	17
4	0	0	13
4	0	1	17
4	1	0	17
4	1	1	21
4	1	2	26
4	2	0	22

Look up in column (1) of the table the combination of figures that corresponds to the number of positive tubes in the three consecutive series which have resulted in plaque formation. Column (2) then directly gives the MPN number per 100 ml for series 10-1-0.1 ml, while for series 1-0.1-0.01 ml, 0.1-0.01-0.001 ml, etc. the figures in column (2) must be multiplied by 10,100, etc.

Example 1				Example 2				Example 3			
10ml	1ml	0.1ml	0.01ml	1ml	0.1ml	0.01ml	0.001ml	0.1ml	0.01ml	0.001ml	0.0001ml
+	+	+	-	+	+	+	-	+	+	+	-
+	+	-	-	+	+	-	-	+	+	-	-
+	-	-	-	+	-	-	-	+	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
		17				17 · 10 = 170				17 · 100 = 1700	

9. APPENDIX: MEDIA.

9.1. MacConkey broth.

	single-strength	double-strength
sodium taurocholate	5 g	10 g
lactose	10 g	20 g
peptone	20 g	40 g
distilled water	1000 ml	1000 ml

Dissolve by shaking. Adjust pH to 7.4, add 2 ml of an alcoholic solution of bromo-cresol purple (1%) to the single-strength medium and 4 ml to the double-strength medium.
Sterilize at 110°C for 15 minutes.

9.2. MacConkey agar.

To single-strength MacConkey broth (cf.10.7) add 1% agar. pH and sterilization as for MacConkey broth.

9.3. Peptone water.

Dissolve 10 g peptone and 5 g sodium chloride in 1000 ml distilled water.
Adjust pH to 7.0-7.4 and sterilize at 121°C for 15 minutes.