THE CAPE TOWN PROTOCOL FOR THOSE OMNIPRESENT OPPORTUNISTS - CAMPYLOBACTERIACEAE AND HELICOBACTERS

Campylobacteria have come a long way since 1886 when Theodore Escherich described *Vibrio felinus* as "spiral, curved, non-culturable bacteria" seen in the intestines and faeces of children and kittens that have died of diarrhoeal disease. Due to a better understanding of the organisms and improved culture techniques this family of organisms have expanded at an unprecedented rate in the bacterial world during the past two decades.

In 1963, because of their low DNA base composition (30-40%) and their non-fermentative metabolism, Sebald and Veron transferred the micro-aerophilic vibrios to the new genus *Campylobacter*.

By the time more comprehensive studies were done 10 years later, the original cultures were no longer available and the opposing taxonomists had different views as to which organism specifically was described as *Vibrio fetus*. Veron and Chatelain's work was published first and they chose the organism causing sporadic abortion in cattle as the type species, *Campylobacter fetus fetus*. Smibert's work published in Bergey's Manual of Determinative Bacteriology a year later used the causative organism of infectious infertility as type species. According to the national rules of nomenclature his classification was invalid (and the cause of a lot of confusion).

The heterogenicity within the **CAMPYLOBACTER** group led to the reclassification of the genus. In 1989 *C. pylori* and the other campylobacteria with sheathed flagella became **HELICOBACTER** and two years later the name **ARCOBACTER** was adopted for the aerotolerant species. Strains of *Campylobacter* and *Arcobacter* are so similar that they have been grouped together in the newly created eubacterial family, **CAMPYLOBACTERIACEAE**.

According to extensive DNA and ribosomal-RNA studies all the known campylobacteria and related organisms belong to the same taxonomic group known as ribosomal superfamily VI. Many of the more elusive campylobacteria need hydrogen or formate to act as a terminal electron donor for respiration and growth. According to molecular studies the hydrogen-dependent "anaerobes" also fall into this group.

Selective inhibitory media were developed to facilitate the specific isolation of *C. jejuni jejuni* and *C. coli* and virtually nothing else - giving rise to the idea that they were the only pathogenic species apart from *C. fetus*. The high affinity of the species for placental tissue explains why *C. fetus fetus* is an infrequent isolate from other sources in humans, and according to the textbooks only from the blood cultures. No explanation is given as to how the bacteria got there in the first place, and ignores the fact that inhibitory media contains the antibiotics to which *C. fetus* is known to be sensitive.

By using the ***membrane filter technique*** for processing stool specimens, a tryptose blood agar plate (no antibiotics added) and incubating in an ***increased H_2-microaerophilic atmosphere*** (increased H_2), virtually all the campylobacteria can be isolated. Eight culture plates containing different antibiotic combinations and incubating some in CO_2-microaerophilic (CO_2) conditions and others anaerobically, will be needed to achieve the same results.

Exceptions for initial isolation are:

a) *A. cryaerophila* which requires incubation in a liquid medium at 30°C and
b) *Anaerobiospirillum* which needs anaerobic conditions.

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13% CO_2 Atmosphere

C. jejuni subsp. jejuni biotype 1
C. jejuni subsp. jejuni biotype 2
C. jejuni subsp. doylei *
C. upsaliensis *
C. lari
C. fetus subsp. fetus *
C. fetus subsp. venerealis *
C. hyoilei
C. coli

Arcobacter butzleri
Arcobacter skirrowi
Arcobacter cryaerophila

Increased H_2 Microaerophilic

C. helveticus
C. hyointestinalis
C. mucosalis
C. concisus
C. sputorum biovar. sputorum
C. sputorum biovar. bubulus
C. sputorum biovar. fecalis
C. ureolyticus
C. gracilis
C. showae
C. curvus
C. rectus

Helicobacter cinaedi
Helicobacter fennelliae
Helicobacter rappini

Anaerobiospirillum #

* Animal isolates tend to grow poorly, if at all, in CO_2
# Anaerobic conditions required for initial isolation

Gastric (mainly H_2-dependent) Helicobacter spp.

H. pylori (humans, monkeys, pigs)
H. acinonyx (cheetahs)
H. canis (dogs)
H. felis (rats, dogs, cats)
H. hepaticus (mice)
H. mustelae (ferrets)
H. muridarum (rodents)
H. nemestrinae (monkeys)
H. pamatensis (birds, pigs)
H. bilis
H. pullorum

Wolinella succinogenes (cattle)

Campylobacteria are thought to be the biggest cause of zoonosis in humans. There are certain instances, children and their pets are one of them, where it is not clear as to who is really infecting who. The abdominal pain that accompanies the gastroenteritits caused by these organisms is so severe that many unnecessary appendicectomies have been performed in the past. The disease also manifests itself as one or more of the following :

1) invasive properties (pus cells in the stool)
2) secretory diarrhoea - the enterotoxin causing the watery diarrhoea is immunologically and serologically related to cholera toxin
3) campylobacteria can also translocate whereby the organisms penetrate the intestinal mucosa to land in the blood stream and thus spread to the other organs. A fatal septicaemia can occur, especially in immunocompromised patients.

The question of pathogenicity has been raised - we have isolated all the species (except strict animal isolates, *Arcobacter* and the saprophytic *C. sputorum sputorum*) from the blood of patients with bacteraemia and septicaemia. Miscarriages due to campylobacteria, even *C. upsaliensis*, have been reported. Can the boundary of pathogenicity really be extended much further ?

PRIMARY ISOLATION PROCEDURE

1. **Specimen Preparation**:
   a) **Stools:** Prepare a ***watery*** emulsion of stool in saline. Mucoid samples should be vortexed.
   b) **Intestinal Scrapings** (PM specimens) **and sheath washings** (bulls): Shake up in saline.
   c) **Gastric Biopsy Material:** Roll gently over the surface of 2 or 3 TBA plates using a swab dipped in Tryptic Soy Broth.
   d) **Blood Cultures:** Squirt approximately 0.2 ml of the mixture taken from the blood culture bottle over the surface of a TBA plate. ***Do not use the filter method.***
   Note: all species tend to give low readings with Bactec and other automated systems.

2. Place a 0.6 micron pore-size membrane filter (Schleicher & Schuell, ME26) directly on to a TBA plate using sterile forceps.

3. Flood the central area of the filter with the emulsion using a transfer pipette.
   Do not splash or spill beyond the filter margin. Re-flood 2 or 3 times.
   The water will pass through the filter by means of gravity, carrying the very tiny organisms along.

4. Remove the filter within 15 minutes and discard and incubate the plate as soon as possible in CO_2 for the time being.
   Sterilise the forceps between specimens (heat - then cool in alcohol).

5. Incubate plate in H_2 for 6 days, examining for growth ***every 2 days*** using either an Anaerobic Gas Pack ***without catalyst*** or a 15% CO_2 and 85% H_2 gas mixture
   Do not discard or ignore the primary plate once growth has been obtained.

6. Morphologically different colonies (shape, size, time needed for growth to appear, etc.) could be indicative of a mixed infection.

IDENTIFICATION PROCEDURE

Campylobacteria colonies are mostly ***buff coloured*** or ***dirty yellow***. Exceptions are *C. helveticus*, *H. fennelliae*, *H. cinaedi* and *H. rappini*; they have a ***thin flat film-like*** growth which initially can even look like a swarming *Proteus* and can take up to 6 days to become visible to the naked eye on initial isolation, especially in a mixed culture. The gastric helicobacters form ***tiny translucent*** colonies. Colonies of *Arcobacter* spp. tend to be whiter.

H. fennelliae is the only species that has an odour - hypochlorite without the "sting".

A Gram stain will confirm any campylobacter-like organisms as either comma-, or gull-shaped, or very thin long spirals (*cinaedi* and *fennelliae*), short stubby (*mucosalis*), rather big slightly curved (*hyointestinalis* and *Arcobacter*), tiny hardly curved (*concisus, curvus, rectus, ureolyticus*), or gigantic (*Anaerobiospirillum*) gram negative bacilli. When the campylobacteria are not happy they curl up and die - but the odd comma shape can still be seen.

Campylobacteria are easily "wiped off the plate" - do not plate out "heavy handedly".

Two loops used alternatively are vital for ensuring complete cooling after flaming.

Subculturing onto 2 TBA plates should yield enough culture material to perform all the tests necessary for identification or the antibiogram can be done at the same time by putting one antibiotic disk on each isolation plate (on the streak lines). Incubate the plates in H_2 for 48 hours. A slide is prepared at the same time.

Do not leave the culture plates on the bench for a prolonged period of time - place in CO_2 until H_2 jar is put up. Time is of the essence, so leave the staining of the slides until later.

This whole procedure might have to be repeated if, at a later stage, a mixed growth becomes evident on either the primary or subsequent isolation plates .

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ONCE PURE GROWTH/S HAVE BEEN OBTAINED, PROCEED AS FOLLOWS:

1.	Two subculture plates	Incubate CO_2 Incubate H_2	for up to 4 days before scoring as "No Growth" 48 hours
2.	Antibiogram #	Incubate H_2	48 hours
3.	Indoxyl acetate	Room temp	10 minutes
4.	Nitrate reductase	Anaerobically	overnight
5.	Catalase	Room temp	immediately
6.	Pyrazinamidase	Incubate H_2	48 hrs for an IndAc positive organism 96 hrs for an IndAc negative organism
7.	Arylsulphatase	Incubate H_2	48 hrs for an IndAc positive organism 96 hrs for an IndAc negative organism
(8.)	Hippurate	Incubate CO_2	overnight (really only to confirm purity)
9.	½ plate MacConkey	Incubate H_2	48 hours
	½ plate 1.5% NaCl	Incubate H_2	48 hours
(10.)	½ plate 1% Glycine (only if needed)	Incubate H_2 (can be done CO_2)	48 hrs
11.	Rapid H_2S	Incubate	overnight
12.	Urease	Room temp	a ***few*** minutes (gastric helicobacters split urea in seconds)

From CO_2 Isolation Plate: ❖

8.	Hippurate	Incubate CO_2	overnight
13.	Lead acetate strip/TSI	Incubate CO_2	48 hours
14.	Aerobic - ¼ plate	Room temp	48 hours

Increased H_2-Microaerophilic Species:

13.	Lead acetate strip/TSI	Incubate H_2	48 hours

Never use more than 2 or 3 antibiotic discs per plate, preferably 1 per isolation plate It is more practical to incubate sensitivity plates in H_2 unless dealing with a known CO_2 organism. Larger inhibition zones are observed in CO_2

❖ Indoxyl acetate, nitrate reductase, catalase and urease can be performed from the CO_2 culture .

TESTS FOR IDENTIFICATION

Best results for all tests are obtained by using 48 hour old cultures. All enzymes are better developed in H_2.

INDOXYL ACETATE HYDROLYSIS

1. Rub a loopful of culture on to a small area of the strip.
2. Wet strip thoroughly (keep moist for up to 10 minutes) with distilled water.

Dark blue colour = positive e.g. *C. jejuni* = positive
C. fetus = negative

Some campylobacteria species produce a bacterial esterase that, in the presence of oxygen, will hydrolyse indoxyl (a breakdown product of tryptophan) to indigo white and indigo.

NITRATE REDUCTION

1. Stab and spread a large loopful of culture over a small area (no more than 4 tests per plate).
2. Incubate overnight, preferably under ***anaerobic conditions*** (essential for some species)
3. Aerate on bench before scoring.

Dark green-brown zone = positive e.g. *C .jejuni jejuni* = positive
C. jejuni doylei = negative

The formation of a green-brown zone indicates that the KNO_3 has been reduced to KNO_2 resulting in the oxidation of haemoglobin to methaemoglobin. Inoculate a known positive control onto any plate containing less than 4 tests and especially when the nitrate negative *C. jejuni doylei* or *H. fennelliae* is suspected. It is essential to incubate anaerobically without delay.
C. mucosalis and C. concisus can give very weak reactions.

CATALASE

A loopful of culture (taken carefully from a TBA plate - do not stab to cool) is "picked up" with a capillary tube containing hydrogen peroxide (20 vols). Oxygen bubbles are trapped in the capillary tube.

e.g. *C. jejuni jejuni* = positive
C. upsaliensis = neg / $+^w$

Catalase breaks down the hydrogen peroxide into oxygen and water.
A positive reaction can be so strong that large bubbles are formed - this can very successfully block the capillary tube and prevent the ascent of any bubbles. On close scrutiny the trapped bubbles can be seen stuck on the surface of the culture or forced out at the bottom.

PYRAZINAMIDASE - use a culture grown in H_2.

1. Emulsify in the top third of the substrate using a very heavy inoculum (***turbid***).
2. Incubate in H_2 - 48 hours for Indoxyl Acetate POS cultures.
   - 96 hours for Indoxyl Acetate NEG cultures.
3. Add about 1 ml freshly prepared 10% ferrous sulphate ($FeSO_4$) down the side of the tube.
4. Let stand for a few minutes.

A brown ring = positive e.g. *C. jejuni* = positive
C. fetus = negative

Pyrazinamide is hydrolysed to free pyrazinoic acid which is then demonstrated by adding $FeSO_4$.

ARYLSULPHATASE - only use a culture grown in H_2.

1. Emulsify in the top third of the substrate using a very heavy inoculum (***turbid***).
2. Incubate in H_2 - 48 hours for Indoxyl acetate POS cultures
   - 96 hours for Indoxyl acetate NEG cultures.
3. Add about 1 ml of a 15% sodium carbonate (Na_2CO_3) solution - can give the tube a shake.

A bright pink colour = positive e.g. *C. jejuni jejuni* 2 = positive
C. jejuni jejuni 1 = negative

The breakdown product phenolphthalein forms a pink colour in the presence of Na_2C0_3.

HIPPURATE HYDROLYSIS - only the *C. jejuni* subspecies are positive.

1. Thaw hippurate broth and inoculate ***heavily***.
2. Incubate overnight (more practical than 2 hours).
3. Gently add about 0.5 ml ninhydrin solution. Do not shake (aerate) the tube.
4. Read within 10 minutes (2 hour test needs 30 minutes incubation).

Positive = Purple
Negative = Colourless or light purple

Hippuricase hydrolyses hippurate to benzoic acid and glycine. Glycine is deaminated by the oxidizing agent, ninhydrin. Ninhydrin becomes reduced in the process and the end products of the ninhydrin oxidation form a purple-coloured dye.
(Sometimes H_2 cultures may give false positive results - repeat from CO_2 growth).

GROWTH ON MACCONKEY and **1.5% NaCl** (also **BILE AESCULIN**)

1. Inoculate ***lightly*** and streak out for single colonies on ½ plates.
2. Incubate in H_2 for 48 hours.

A positive result is the growth of visible ***single*** colonies. If unsure and for slow growing organisms, incubate for a further 48 hours.

AEROBIC GROWTH AT ROOM TEMPERATURE

1. Squiggle-inoculate a positive control and three tests onto a TBA plate divided in 4.
2. Leave on bench for 48 hours. Only *Arcobacter* species will grow.

1% GLYCINE - can use either CO_2 or H_2 grown culture. Use only to differentiate *C. fetus* subspecies.

1. Inoculate lightly and streak out for single colonies on 1/2 plates.
   Another plate with a positive and a negative control to compare.
2. Incubate for 48 hours in CO_2 or in H_2.

Any growth on streak lines = positive. *C. fetus fetus* = pos.
C. fetus venerealis = neg.

UREASE - really only for H_2 organisms.

Stab a loopful of culture into the agar (can utilise the same plate over a period of days).

A bright pink colour = positive. This is a very rapid reaction.

Hydrolysis of urea by the enzyme urease releases the end product ammonia. The alkalinity causes the indicator phenol red to change from yellow to pink. *Helicobacter* spp. take only seconds, *C. ureolyticus* a bit longer. *H. cinaedi* and *H. fennelliae* do not split urea.

RAPID H_2S - best results from a 48 hour culture grown in H_2.

1. A large loopful of culture is inoculated into the semisolid substrate as an intact "blob".
2. Incubate overnight.

Blackening around the inoculum = positive e.g. *C. jejuni jejuni* 2 = pos.
C. jejuni jejuni 1 = neg.

The enzyme cysteinase liberates sulphur and the resulting colourless H_2S gas reacts with the heavy iron salts to produce an insoluble black precipitate.

H_2S PRODUCTION on TRIPLE SUGAR IRON

It is imperative that a CO_2-grown culture is used for organisms that are able to grow in CO_2 to prevent false positive reactions.

1. Spread big loopful of cultures on the slant and stab-inoculate the butt of the TSI agar.
(2. Suspend PbAc strip over the slant.)
3. Incubate for 48 hours in the appropriate atmosphere.

Blackening of the media = positive. e.g. *C. hyointestinalis* = positive
C. jejuni = negative

LEAD ACETATE STRIP - to demonstrate H_2s production of weaker H_2S-producing organisms.

Carefully suspend a PbAc strip over a heavily inoculated TSI slant without touching the media.

Blackening of the strip = positive. e.g. *C. jejuni.jejuni* 1 = positive
C. jejuni jejuni 2 = negative

PbAc is extremely sensitive and will detect small quantities of H_2S.
Upon contact with PbAc, the colourless H_2S gas produces lead sulphide - a black precipitate.

Postscripts:

Because of increased incidence of resistance (up to 28% for *C. jejuni jejuni* and *C. coli*) the 30 µg nalidixic acid and cephalothin antibiotic disks should only be used as a guide-line.

C. lari - resistant to both antibiotics - can be identified by being indoxyl acetate negative and can be confirmed by the inability to grow on bile aesculin plates (Oxoid CM888), whereas *C. jejuni jejuni*, *C. coli* as well as *C. fetus* do grow on bile aesculin under H_2 conditions.

Large zones (> 3cm) round both discs usually means either *C. upsaliensis* or *C. jej. doylei* but resistance to either or both antibiotics is increasing. The "classic" 8 cm zones are getting fewer.

C. concisus is often sensitive to both nalidixic acid and cephalothin, contrary to the original description of the species.

Growth at 25° and 43° is totally impractical and a waste of time and incubators .

All 3 biovars of sputorum give a positive rapid H_2S reaction. None of them grow on bile aesculin.

In the veterinary field where it is sometimes a matter of utmost urgency to differentiate between the pathogenic *C. fetus* group and the commensal sputorums, these 2 tests have proved to be invaluable :

	Rapid H_2S	Growth on bile aesculin
C. fetus	-	+
C. sputorum	+	-

TROUBLE SHOOTING AND USEFUL HINTS

Incubate for 48 hour periods

Some of the more fastidious -species tend not to grow and can even be lost if the jar is opened after only 24 hours incubation - especially during initial isolation.

All the enzymes are at their peak and best results are obtained from 48-hour cultures. Both Arylsulphatase and Indoxyl acetate tend to give false negative results with old cultures - even a 3 day-old culture. Rapid H_2S
can also give problems, especially for *C. lari*.

Hippurate and pyrazinamidase are the only exceptions to the rule and older cultures can be used for these tests.

All the enzymes are better developed in H_2 than in CO_2

Sometimes *too* well - e.g. excess H_2S production of CO_2-species on PbAc and TSI, resulting in false positive reactions if performed from a H_2 culture.

Occasionally, otherwise obvious *C. coli* or *C. upsaliensis* cultures seem to be hippurate positive.
Repeat from a culture grown in CO_2.

C. helveticus also tends towards a false positive hippurate reaction (not dark purple, but close). Being a H_2-dependent organism it cannot be repeated from CO_2 but the spreading growth "tells it all".

False positive hippurate reactions can also occur if:

* plastic tubes are used
* the medium is defrosted for a third time (ie. re-freeze unused hippurates only once)
* the incubated tube is aerated either by shaking or if left standing for too long after the ninhydrin has been added

Rapid H_2S and arylsulphatase tests should ***always only*** be done from cultures grown in H_2 - very poor or inconsistent results are obtained from CO_2 plates.

It is just more practical to use a H_2-grown culture to do all the tests (except TSI and PbAc) while establishing if the isolate is capable of growth in CO_2.

Mixed infections

Very little variation in results occur with the tests chosen for this identification scheme. Most of the time discrepancies are indicative of a mixed infection.

Fresh TBA plates

Isolation rate of campylobacteriaceae is very poor on plates that are older than 10 days. *Helicobacter* species are even more fastidious.
Fresh plates, poured twice weekly, and using the older plates for subcultures only, is recommended.
On 3 week-old plates even established cultures are often lost.

Alternate jars

With 4 sachets in a 36 plate anaerobic jar, more H_2 is available for the organisms. (Two sachets in a small jar causes too much pressure for the bacteria to grow.) It is therefore preferable to use the large jars, otherwise alternate the culture plates - 2 days in small jar, 2 days in large jar.

New specimens

Culture plates should be incubated in H_2 without too much delay, e.g. the morning specimens by midday and the afternoon specimens by the end of the day. The H_2 -dependent organisms do not last too well in CO_2 over a prolonged period of time.

MEDIA AND REAGENTS (R*)

Tryptose Blood Agar — Biolab C48; Oxoid CM233
10% fresh Horse Blood

Membrane Filter — 47 mm diameter pore-size 0.6 µm
(Schleicher & Schuell ME26, available from LASEC).

Hydrogen atmosphere — Anaerobic sachets ***no catalyst*** (only **Oxoid BR38** or **BBL 70304**)
1 sachet per small jar (±12 plates); 4 sachets per large jar (±36 plates)
or
Evacuate jar to -560 mmHg and replace with
15% CO_2 and 85% H_2 gas mixture

INDOXYL ACETATE HYDROLYSIS

1. A 10% (wt/vol) solution of indoxyl acetate (Sigma 13500) in acetone.
2. Saturate filter paper strips with the above mixture and air dry.
3. Store in amber bottles in fridge.

NITRATE REDUCTASE

0.1% Potassium Nitrate in Tryptose Blood Agar plates

1. 0.5% KNO_3 in 3 ml dist. water.
2. Filter aseptically into 500 ml prepared TBA media.
3. Sealed in plastic packets the plates will keep for weeks.

PYRAZINAMIDASE ACTIVITY

Pyrazinamidase Medium (semi-solid)

1.	Mueller-Hinton Broth (Oxoid CM405)	11.00g
2.	Bacto-Agar (Difco 0140-01) or Technical No 3	1.75g
3.	Pyrazinamide (Sigma P7136; Merck 821050)	0.10g
4.	Dist. H_2O	500 ml

Steam to dissolve and tube in about 4 ml amounts.
Autoclave 15lb/15 mins.
Prepare small batches.

Ferrous Sulphate (R*)

Freshly prepared 10% $FeSO_4.7H_20$ in dist. H_2O

ARYLSULPHATASE ACTIVITY

Arylsulphatase Medium (semi-solid)

1.	Brucella Broth (Difco 0495-17-3)	14.00g
2.	Bacto-Agar or Technical No 3	1.75g
3.	Phenolphthalein Disulphate (Sigma P 0251)	0.325g
4.	Dist. H_2O	500 ml

Steam to dissolve, tube in about 4 ml amounts.
Autoclave 15lb/15 mins.
Prepare small batches to prevent false negatives.

Sodium Carbonate (R*)

1.	Na_2CO_3, anhydrous	15g
2.	Dist. H_2O	100 ml

Do not refrigerate, it will solidify.

HIPPURATE HYDROLYSIS

Stock = 5% hippuric acid (sodium salt; Sigma H 9380; Merck 820648) in dist. H_2O. ***Store frozen***.

Hippurate Broth

1.	Hippuric Acid stock solution	25 ml
2.	Dist. H_2O	100 ml

Filter and tube in about 1 ml amounts in ***glass*** tubes. Do not autoclave.

Ninhydrin Solution (R*). Store in deep-freeze

1.	Ninhydrin (Merck 6762, BDH 10132 4E)	3.5g
2.	50:50 Butanol in Acetone	100 ml

GROWTH ON MACCONKEY AGAR

MacConkey *without NaCl* or *Crystal violet* (***only Oxoid*** CM 7b gives consistent results)

GROWTH ON 1.5% NaCl

1.5% NaCl incorporated into a TBA plate.

GROWTH ON 1% GLYCINE

1. Dissolve 10g NH_2CH_2COOH (Sigma G7126; BDH 10119) in 100 ml dist. H_2O.
2. Filter-sterilize into 900 ml of prepared TBA media.

UREASE REACTION

Christensen's Urea Medium (5%) - poured as plates not tubes.

1. Filter-sterilize 25 ml of a 40% Urea solution (Sigma U4128; BDH 10290; Oxoid SR20)
2. Add to 475 ml prepared Urea Agar Base (Biolab C35; Oxoid CM53).

SEMI-SOLID IRON BROTH FOR RAPID H_2S PRODUCTION

1.	Bacto or Technical Agar	1.75 g	]	
2.	Nutrient Broth No. 2 (Oxoid CM67)	12.50 g	]	Steam to dissolve
3.	Dist. H_2O	400 ml	]	
4.	Sodium metabisulphite $Na_2S_2O_5$	0.5 g	]	
5.	Sodium pyruvate $CH_3COCOONa$	0.5 g	]	Dissolve
6.	Ferrous sulphate $FeSO_4.7H_2O$	0.5 g	]	
7.	Dist. H_2O	50 ml	]	

Combine the 2 solutions, bring volume up to 500 ml with dist. H_2O.
Autoclave 15lb/15 mins in 100 ml amounts - store stock at room temp. in the dark.
Dispense 2 ml amounts in small screw cap plastic tubes. Store at room temperature in the dark.

TRIPLE SUGAR IRON AGAR

TSI (Biolab C18; Oxoid CM277) tubed in such a fashion that a lead acetate strip can hang down over the length of the slope.

LEAD ACETATE STRIP

1. Soak thick filter paper strips (MAST Bacteruritests) in saturated $Pb(CH_3COO)_2.3H_2O$ solution
2. Air-dry and divide into packets or tins. Can be autoclaved.