International Testing Methods for food relevant microorganisms

Wolfgang E. Schmidt , MERCK KGaA , Darmstadt , Germany

Standard Methods

ISO

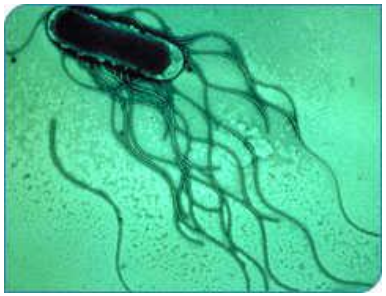
FDA / BAM

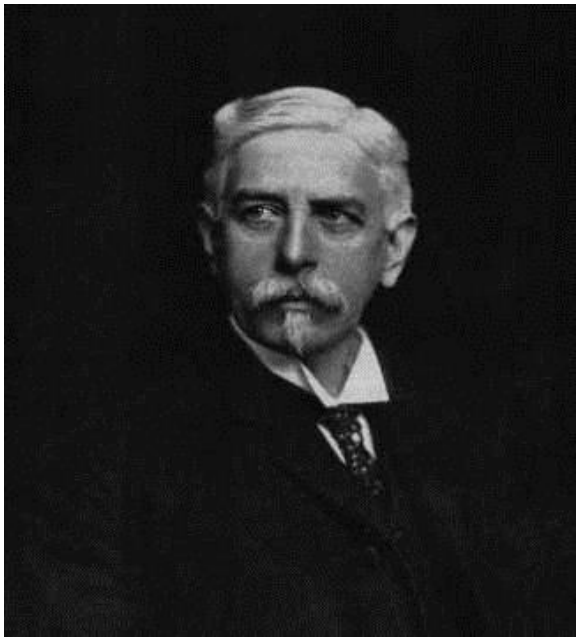
- Salmonella
- Listeria
- Campylobacter
- pathogenic E.coli
- Cronobacter

Bacillus cereus
Clostridium perfringens
Coliforms / E.coli
Enterococci
Legionella

Salmonella

- Rod shaped
- Gram negative enterobacteria
- Salmonella species are motile and produces hydrogen sulfide
- Usually not able to ferment Lactose
- Near related to Escherichia coli
- Can be found world wide





DANIEL ELMER SALMON, D.V.M. (1850-1914)

Salmonella

- Leading cause of food borne bacterial diseases in many countries

- ~2500 Serovars
- Divided in 2 species:
  - S. *enterica* - non-typhoid serovars
    - 6 Subspecies
  - S. *bongori* - typhoid serovars

Salmonella

- High environmental resistance (can survive 73 days at 2°C or at pH 3.5)
- Deep freezing don't kill Salmonella
- Infections result from contaminated meat (poultry!!!), eggs, milk products (cheese, cream, ice cream, etc.), spices, etc.

Salmonella

Major outbreaks of Salmonellosis:

Year	Country	Food	Serovar	No. Infection	No. Deaths
1984	Canada	Cheddar Cheese	*S. typhimurium*	2,700	0
1985	USA	Pasteur. Milk	*S. typhimurium*	16,284	11
1988	Japan	Cooked eggs	*Salmonella spp.*	10,476	N.S.
1993	Germany	Paprika Chips	*S. saint-paul*	1,000	0
2007	Germany	Cream	./.	over 260	8

Salmonella

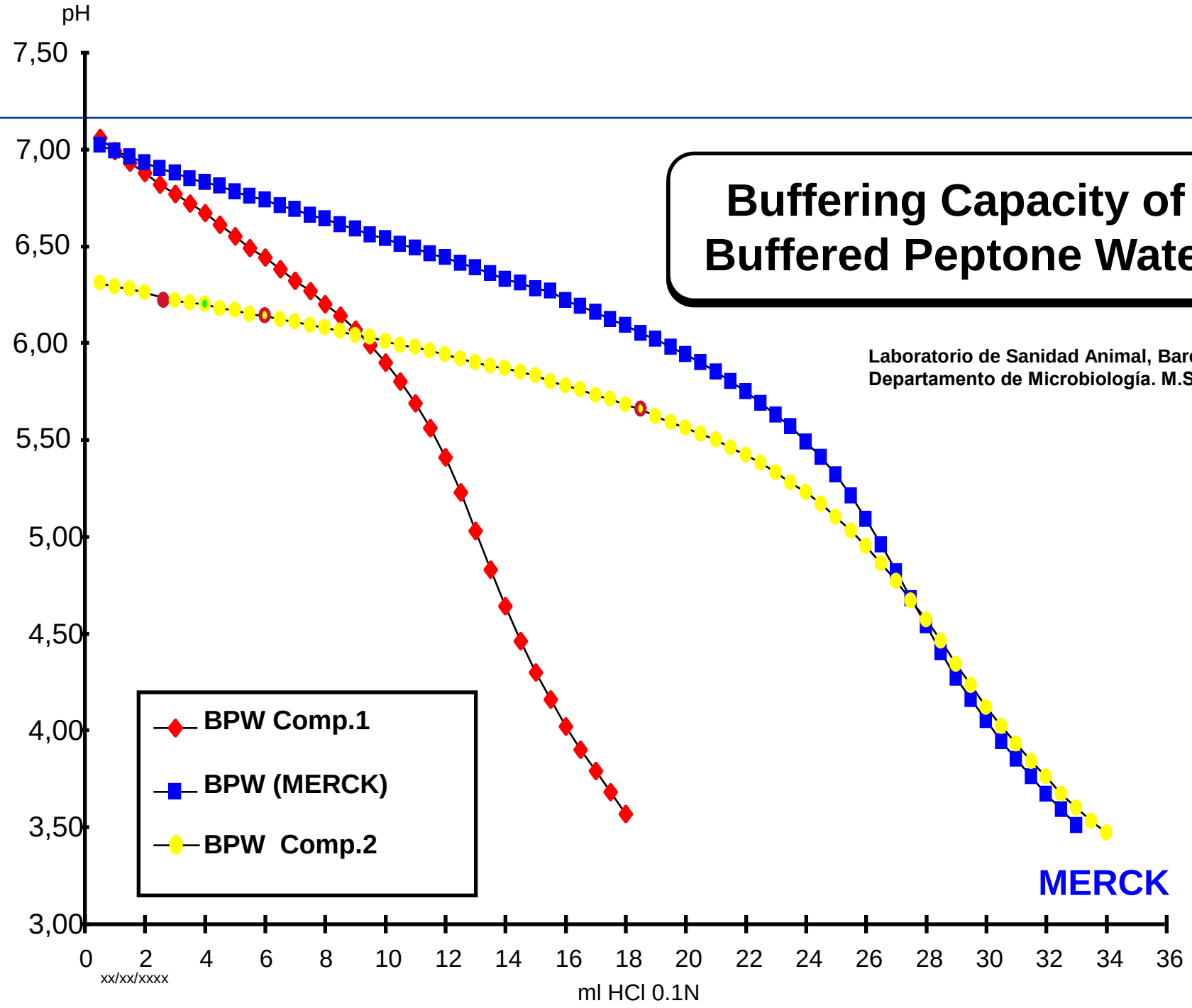
Enrichment culture

- Buffered peptone water
- Tetrathionate broth
- Rappaport Vassiliadis

DIRECT OR PRE-ENRICHMENT ?

TYPE OF SAMPLE	RECOVERY % ENRICHMENT PRE-	DIRECT	REFERENCE
RAW MEAT (1983)	83	56	EDEL & KAMPELMACHER
	90	58	VASSILIADIS ET AL. (1981)
	91	62	GABIS & SILLIKER (1974)
	60	55	EDEL ET AL. (1976)
CHICKEN	96	10	VASSILIADIS ET AL. (1972)
CARCASSES	43	47	COX ET AL (1978)
	99	99	COX ET AL. (1979)
ANIMAL FEED	43	36	JUVEN ET AL. (1984)
	40	33	

Buffering Capacity of Buffered Peptone Water

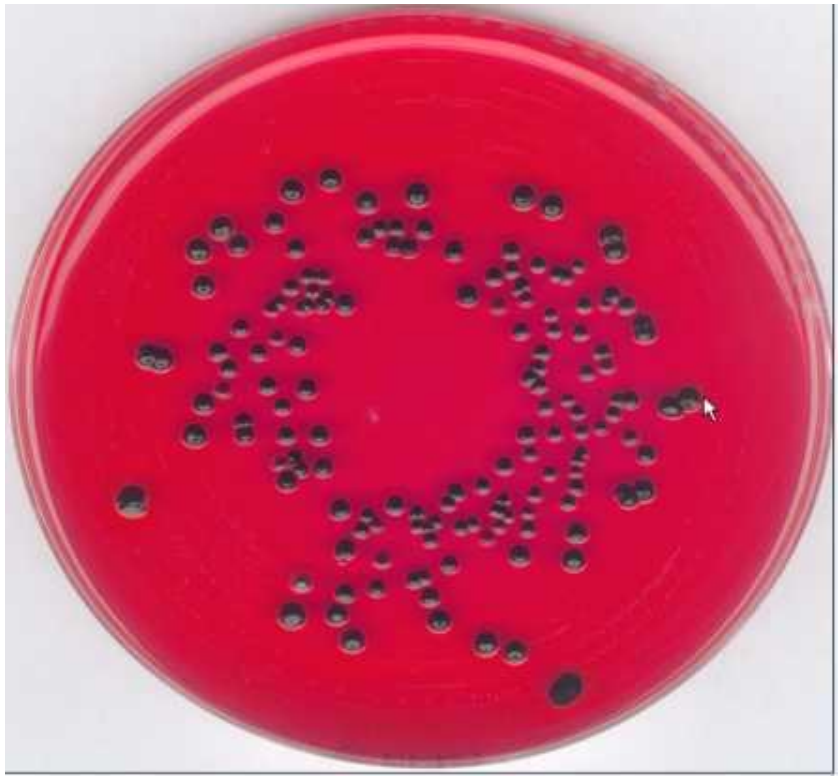


Laboratorio de Sanidad Animal, Barcelona.
Departamento de Microbiología. M.Saco.

Salmonella

Isolation media

- XLD Agar
- Rambach
- Any other agar



ISO Standard 6579 for Detection of Salmonella

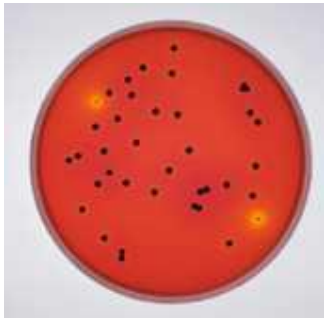
Day 1 — **25 g/ml test sample in 225 ml BPW 16 - 20 h / 35 - 37 °C**

4 days

Day 2 — **0.1 ml BPW to 10 ml RVS Broth 24 h at 41.5°C h** | **1 ml BPW to 10 ml MKTTn Broth 24 h at 37°C**

Day 3

XLD Agar 24 h / 35-37°C 1 plate 14 cm / 2 plates 9 cm	Any other Salmonella Agar 1 plate 14 cm / 2 plates 9 cm	**XLD Agar 24 h / 35-37°C 1 plate 14 cm / 2 plates 9 cm**	Any other Salmonella Agar 1 plate 14 cm / 2 plates 9 cm



Day 4 — Interpretating of Growth on Plates

11 media

Day 5 — **For confirmation take 5 suspected colonies from each plate and streak onto Nutrient agar 18 - 24 h / 35 - 37 °C**

Day 6 — **Biochemical / Serological Confirmation Tests TSI / Urea / Lysin / β-Gal / VP / Indol**

Discover the new Readybag pouches with dehydrated culture media for fast , flexible and easy pathogen testing

Readybag concept

Speed up and simplify your workflow!

- Pre-weighed
- Gamma-irradiated
- No media preparation needed!
- Eliminates autoclavation
- Sterile lab water provided with ELIX advantage

Food sample in stomacher bag + Readybag® pouch content + Sterile lab water = Ready to incubate!

Pathogen enrichment - Traditional workflow

1

1. Media preparation

Weighing + Dissolving media — 5 min

2

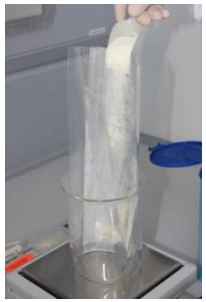
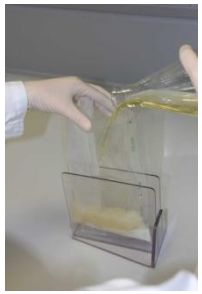
•Add water	01 min	98 min
•Stir and dissolve	05 min	
•Load autoclave	02 min	
•Autoclaving	25 min	
•Cool down autoclave	30 min	
•Unload autoclave	05 min	
•Cool down media	30 min	

3

Supplementation Selective Media — 6 min

Pathogen enrichment - Traditional workflow

2. Food sample handling

4		Weighing food sample	3 min
5		Adding media	3 min
6	 	Homogenization	2 min --- **117 min**

Pathogen enrichment – Readybag Workflow

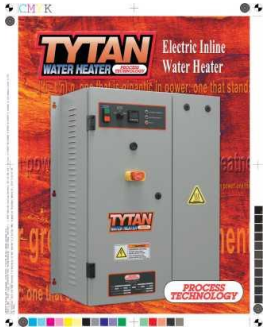
1		Weighing food sample	3 min
2	 	Add Readybag content Add sterile water	1 min 2 min
3		Homogenization	2 min --- **8 min**

High throughput sample solution – ELIX + Heated Water + Readybag Workflow

1

Weighing food sample

2



1. ELIX with tank and Pump

2. Commercial available heater to heat water to approx.35-37 C

3. Dispenser for eg 225 or 3375 ml water

3

<u>Homogenization</u>

1. Add Readybag to food sample
2. Add 225 ml or 3375 ml water
3. Blend sample
4. Incubate

FDA-BAM Standard for Detection of Salmonella

Day 1 — 25 g/ml test sample in 225 ml Lactose Broth, 16 - 20 h / 35 - 37 °C

4 days

Day 2 — 1 ml BPW to 10 ml SC Broth, 24 h at 35°C h | 3 plates from each selective broth | 1 ml BPW to 10 ml TT Broth, 24 h at 35°C

Day 3

- **Hektoen Agar** 24± 2 h at 35 °C
- **XLD Agar** 24± 2 h at 35 °C
- **Bismuth Sulfite Agar** 24± 2 h at 35 °C



Day 4 — Interpretating of Growth on Plates

9 media

Day 5 — For confirmation take 5 suspected colonies from each plate and streak onto Nutrient agar 18 - 24 h / 35 - 37 °C

Day 6 — Biochemical / Serological Confirmation Tests
TSI / Urea / Lysin / β-Gal / VP / Indol

Immunological Rapid Test Method -

Singlepath® Salmonella for the Detection of Salmonella in Food

Day 1

25 g/ml TEST SAMPLE
In 225 ml Buff. Peptone Water
35-37 °C for 16-20 h

Day 2

only 2 days

0.1 ml BPW to 9.9 ml RVS Broth
42°C for 24 h

only 2 media

Day 3

NO ***Salmonella*** **present**

YES ***Salmonella*** **present**

Day 4

If positive, confirmation by culture and biochemical tests required

STREAK OUT
ON RAMBACH
37 °C FOR 24 h

Salmosyst ®

Salmosyst is a 2-step enrichment system designed for sublethally damaged Salmonella.

Salmosyst is TT-Broth devided in :

a.) Non-selective Broth

b.) Selective Enrichment
as slow-release tablets

Salmosyst ®

a.) Non-selective Broth

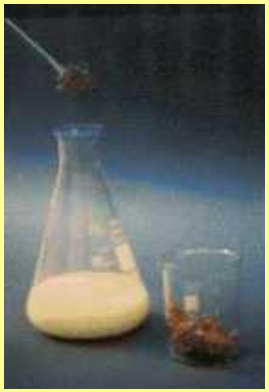
Tryptone	5.0 g
Meat-Peptone	5.0 g
NaCl	5.0 g
Calciumcarbonate	10.0 g

b.) Selective Enrichment as slow-release tablet

Potassium-tetrathionate	0.2
Oxbile	0.08
Brillantgreen	0.0007
Calciumcarbonate	0.1

Salmosyst ® 24 h Method

Day 1



25 g/ml TEST SAMPLE
IN SALMOSYST® BROTH
6 h / 35 - 37 °C

ADD 1 SALMOSYST® SELECTIVE
TABLET TO 10 ml SALMOSYST®
BROTH AND CONTINUE INCUBATION
18 h / 35 - 37 °C

Day 2

SUBCULTURE ON
SALMONELLA AGAR`S

1 Medium only

Salmonella Detection from Naturally Contaminated Food Samples by ISO Method in Comparison to Salmosyst® - Rambach® Agar Method

Food Samples	Number of Samples	No. of positive samples (%) ISO Method	Salmosyst® - Rambach® Method
Poultry	85	18 (21.2)	26 (30.5)
Meat	246	14 (5.7)	14 (5.7)
Eggs	188	7 (3.7)	7 (3.7)
Sensitivity:		81.2 %	97.9 %
Total Analysis Time:		4-6 days	2 days
No. of media:		5	2

Study published in Applied and Environmental Microbiology, May 1995, p. 1996 - 1999 by Giuseppe Giammanco et al.

Detection of Salmonella using Rambach® Agar

Traditional Salmonella media are based on H_2S reaction:

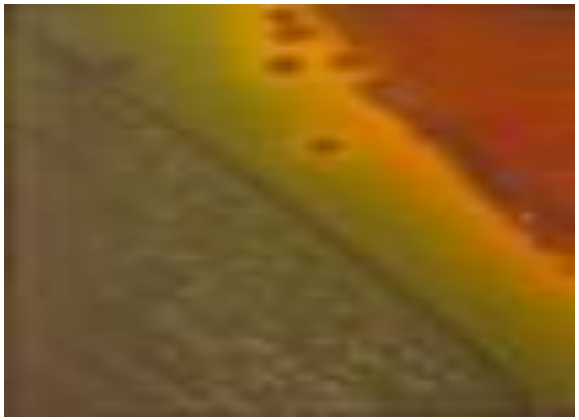
Disadvantage:

Citrobacter and Proteus mimic Salmonella

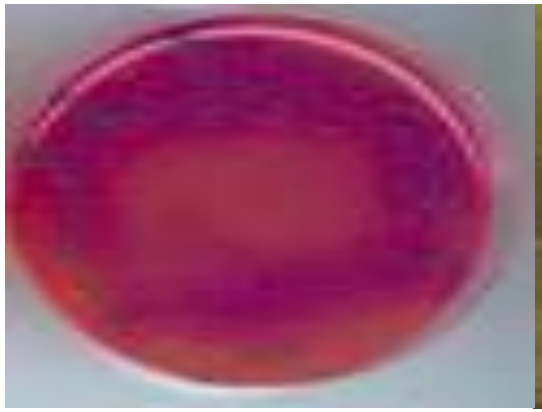
High number of false - positives

Citrobacter and Proteus mimic Salmonella

High number of false - positives



Hektoen



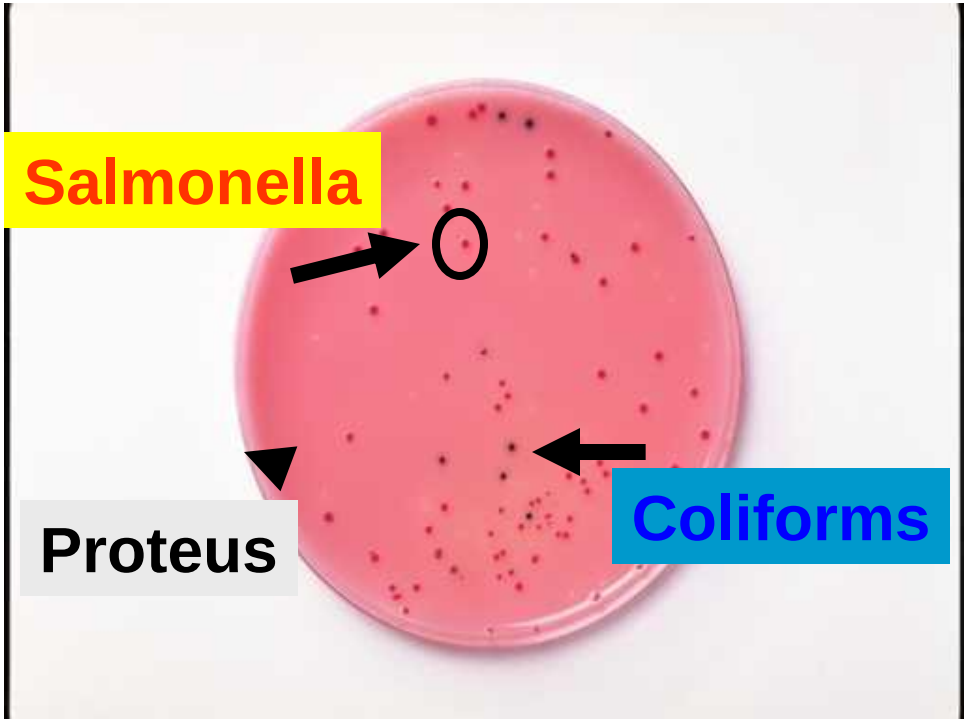
XLD

BSA

SALMONELLA
RAMBACH AGAR

Differential system

Red	Salmonella
Violet	Coliforms
Colourless	Other bacteria



Selective system

Sodium deoxycholate

Principle

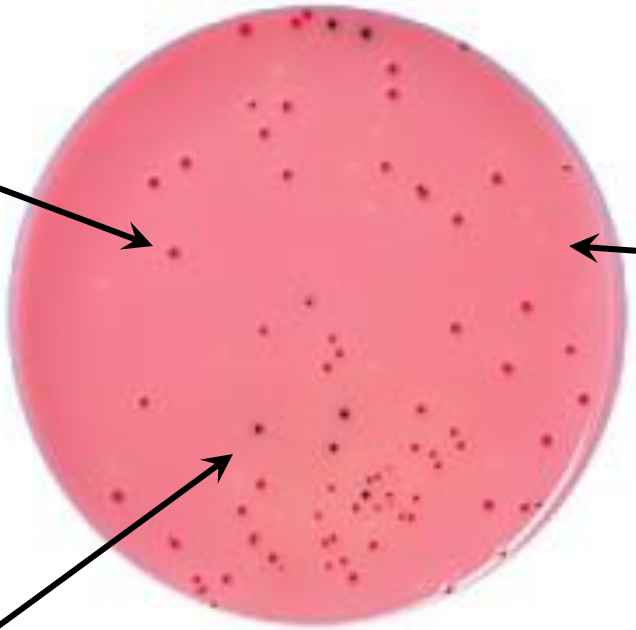
1. Salmonellae dissimilate
   propylene glycol producing acid. Neutral red gives colonies red colour

2. β galatosidase positive organisms
   split chromogenic substrate producing blue-violet colonies

Rambach® Agar

Clear, Easy and Reliable Identification of Salmonella in Food Samples

Salmonella
red



Proteus
yellowish / colourless

Coliforms
blue - green

Rambach® Agar # 1.07500

Rambach® Agar

ISO Standard 6579 for Detection of Salmonella

0 h

25 g test sample
in 225 ml **BPW**
18 h / 37°C

24 h

0.1 ml BPW to
10 ml **RVS Broth**
24 h at 41.5°C h

1 ml BPW to
10 ml **MKTTn Broth**
24 h at 37°C

48 h

XLD Agar	Rambach Agar	XLD Agar	Rambach Agar
24 h / 37°C	24 h / 37°C	24 h / 37°C	24 h / 37°C
1 plate 14 cm or	1 plate 14 cm or	1 plate 14 cm or	1 plate 14 cm or
2 plates 9 cm	2 plates 9 cm	2 plates 9 cm	2 plates 9 cm

72 h

Interpretating of Growth

Biochemical / Serological Confirmation Tests

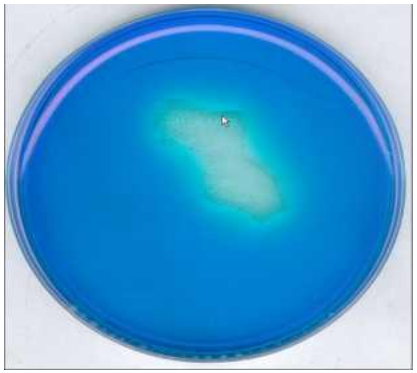
Salmonella

ISO 6579:2002, Amendment 1, Detection of Salmonella spp. in animal faeces and in samples from primary production stage

- Animal faeces (such as from poultry pigs cattle)
- Environmental samples in the area of primary production stage (such as dust)

MSRV Medium

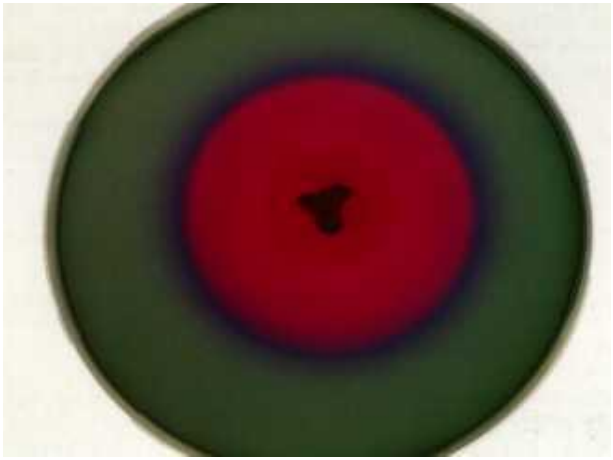
- the detection principle is based on the motility of *Salmonellae* to migrate into the semi-solid medium
- the motility of other organisms is largely inhibited by magnesium chloride, malachite green and Novobiocin and the enhanced incubation temperature of 41.5°C

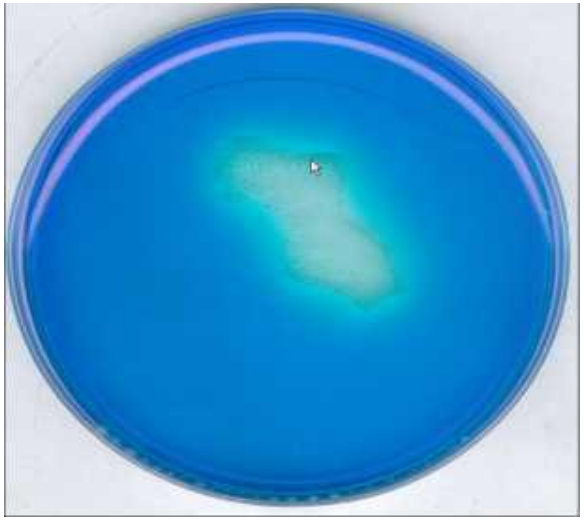


Salmonella

Recommended media

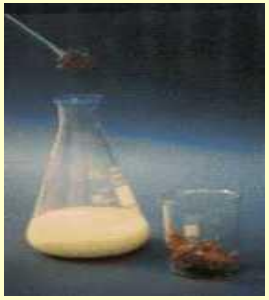
- MSRV-Agar
- DIASALM



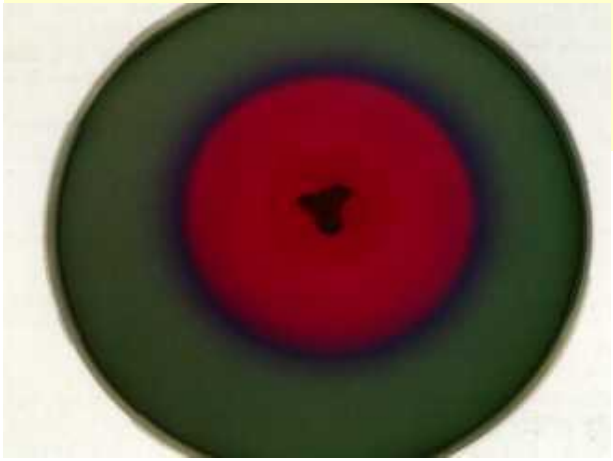


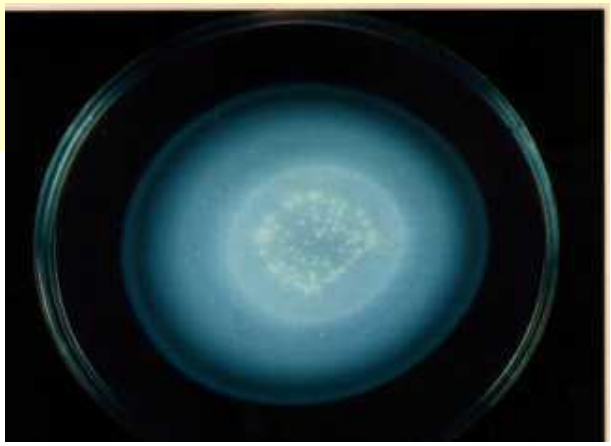
Semi-solid testing Method acc.to ISO 6579

Day 1



25g/ml TEST SAMPLE
IN 225 ml BPW
16 - 20 h / 35 - 37 °C





Day 2

DIASALM OR MSRV
24 h / at 41.5 °C

SALMONELLA DETECTION FROM NATURALLY CONTAMINATED RAW MEAT AND MEAT PRODUCTS BY ISO METHOD IN COMPARISON TO DIASALM METHOD

No. / TYPE of samples	NO. OF POSITIVE SAMPLES MODIFIED ISO (BPW+RVS+XLD)	**DIASALM**
136 POULTRY	101	134
SENSITIVITY	(74.3 %)	**(98.5 %)**
102 PORK + BEEF	97	101
SENSITIVITY	(95.1 %)	**(99 %)**
TOTAL ANALYSIS TIME	3 days	2 days
NO. OF MEDIA	3	2

STUDY PUBLISHED IN CULTURE MEDIA, DE WARE(N) CHEMICUS 28 (1998) 34 - 43 BY L. DE ZUTTER *et al.*

SALMONELLA DETECTION FROM NATURALLY CONTAMINATED RAW MEAT AND MEAT PRODUCTS BY ISO METHOD IN COMPARISON TO MSRV METHOD

No./TYPE of Samples	NO. OF POSITIVE SAMPLES		
	ISO + MSRV	**ISO**	**MSRV**
913 (80 % CHICKEN MEAT)	309 (33.8 %)	251 (27.5 %)	291 (31.8 %)
SENSITIVITY		81.2 %	**94.5 %**
TOTAL ANALYSIS TIME		4 - 6 days	2 days
NO. OF MEDIA		5	2

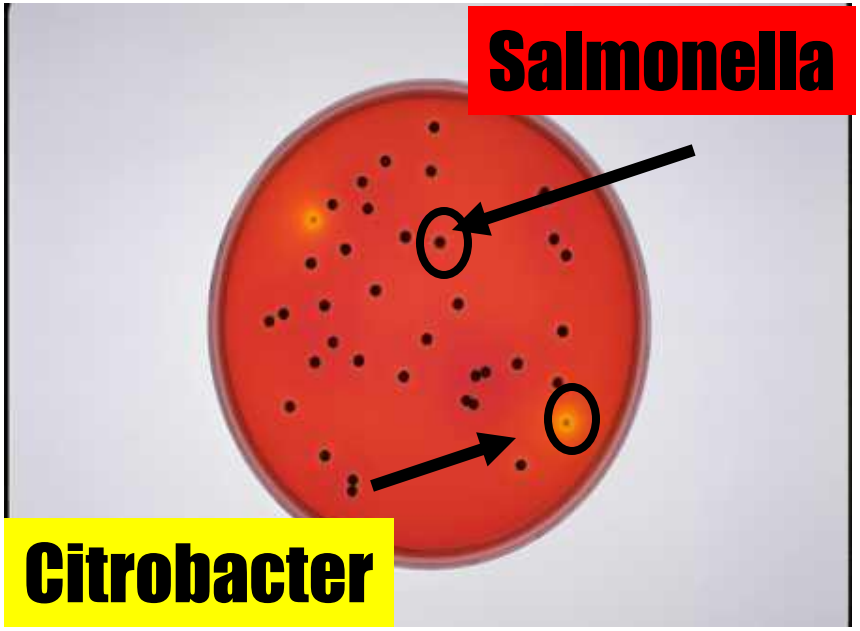
XLT4-AGAR

Differential system

Black	Salmonellae
Black with yellow halo	Citrobacter
Colourless with yellow halo	Xylose-Lactose-Sucrose positive bacteria
Colourless	Other bacteria e.g. Shigella

Selective system

Tergitol 4 inhibites Proteus



Principle

H_2S formation via thiosulphate
and iron produces black colonies
Dissimilation of sugars produces acid resulting in phenol red shift of red to yellow

XLT4 Agar:
An Improved Selective Agar Medium for the Isolation of Salmonella
R.C. Miller, USDA, 1991 Poultry Science 70

Salmonella isolation rates observed on each medium examined from 91 Salmonella-positive drag-swab samples collected from 7 table egg layer flocks.

Variable	Medium				
	XLT4	XLD	XLDN [1]	BGA	BGAN [1]
Number positive out of 91	89	27	76	65	77
Percentage positive	98 %	30 %	84%	71 %	85 %
Average purity of Salmonella colonies [2]	3+ to 4+	1+	2+	1+ to 2+	2+ to 3+

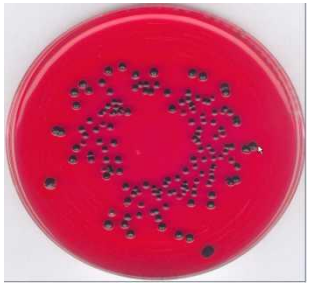
[1] = Medium supplemented with Novobiocin

[2] = 1+ = 1 to 25 % were Salmonella
= 2+ = 25 to 50 % were Salmonella
= 3+ = 51 to 75 % were Salmonella
= 4+ = 76 to 100 % were Salmonella

XLD-Agar , XLT 4 Agar and Rambach Agar

Interpretation of colony colours :

	Colony color		
Organisms	XLD Agar	XLT4 Agar	Rambach Agar
Citrobacter freundii			
Proteus			
Salmonella			

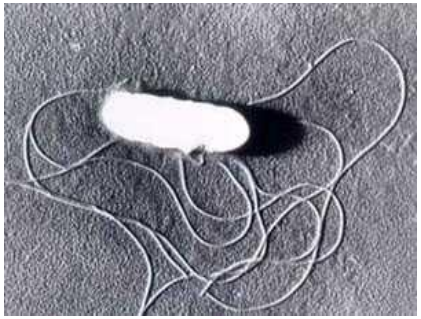


Detection Methods for Listeria and L.monocytogenes

Established Pathogen: *Listeria*

- Genus *Listeria* comprises 6 different species
- Only *L. monocytogenes* can be pathogenic for humans and animals
- **Disease:** Systemic Infections: Meningitis, Encephalitis, Septicemia
  Local Infections: Gastroenteritis
- **Frequency:** Only 7 / 1 Mio. individuals suffer from Listeriosis
  But: Mortality rate is extremely high (up to 25% of patients die)
- **Resistance:** Multiply at 2°C, survive at many preservation methods (10% osmolarity, pH 5.0, 55°C)
- All *Listeria* species serve as indicator for improper hygiene conditions

Listeria characteristics



Genus Listeria comprises 10 different species

- Listeria monocytogenes
- Listeria ivanovii
- Listeria innocua
- Listerai seelegeri
- Listeria welshimeri
- Listerai grayi

Listeria marthii

Listeria rocourtiae

Listeria weihenstephanensis

Listeria fleischmannii

Listeria disease - Listeriosis

- Listeriosis is an invasive infection caused when contaminated food is ingested.
- Symptoms vary, ranging from flu-like symptoms to meningitis and encephalitis.
- Mortality rate for Listeriosis is 23%.
- The infection usually occurs sporadically.

Media for enrichment of Listeria

- Half FRASER broth
- FRASER Listera Enrichment broth
- Listeria Enrichment broth
- Buffered Listeria Enrichement Broth
- UVM Listeria Selective Enrichment broth
- PALCAM Selective Enrichment broth

Listeria Enrichment Media

* Esculin hydrolysis = blackening broth in the presence of Esculin positive organisms like Listeria = is used in UVM , FRASER and PALCAM as presumptive indicator for Listeria spp.

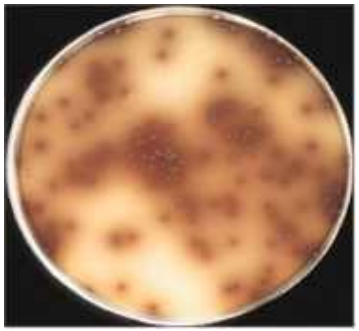
* **Colour development is depending on level of Listeria present in the sample . To use colour development only is not reliable , therefore all broth cultures must be sub-cultured to plating media . Usually a level of 10.000 c.f.u./ml does not result in blackening of broth**

Media for enumeration of Listeria

- PALCAM Agar
  - Listeria spp.
- OXFORD Agar
  - Listeria spp.
- Listeria selective agar acc. to AGOSTI & OTTAVIANI
  - Listeria monocytogenes
  - **New mandatory isolation medium in the revised ISO Standard 11290 (2004)**

Listeria Plating Agars

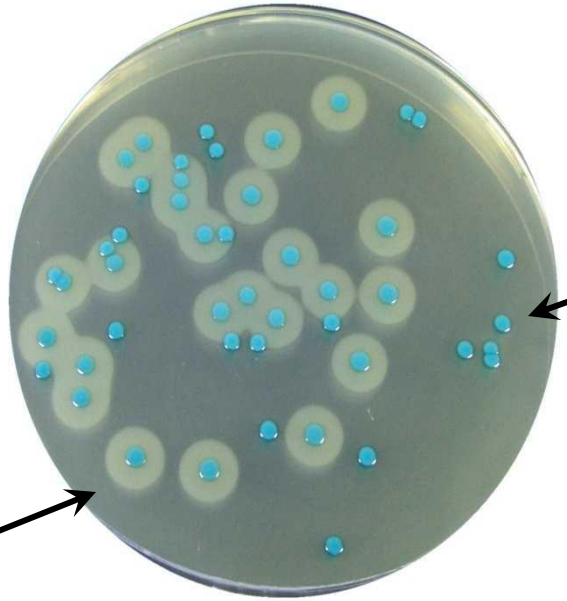
* Common used plating media are PALCAM and OXFORD Agar . Both media are based on the colour development of hydrolysed esculin .



* PALCAM Agar contains Mannitol to differentiate Enterococci from Listeria by Mannitol fermentation

Chromocult® Listeria Selective Agar

Chromogenic Culture Medium for the Detection and Enumeration of *Listeria monocytogenes* in Foods.



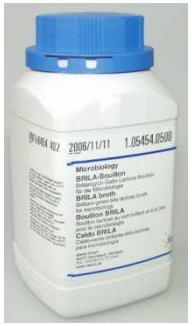
L. innocua

L. Monocytogenes
L. ivanovii

Chromocult® Listeria Selektiv Agar # 1.00427

Chromocult® Listeria Selective Agar

Chromocult® Listeria Selective Agar complies with Agar Listeria according to Ottaviani and Agosti (ALOA®) in line with the recommendations of ISO 11290 (2004) and FDA / BAM (2003)



1.00427 Chromocult®
Listeria Selective Agar Base

1.00432 Chromocult®
Listeria Selective Supplement

1.00439 Chromocult®
Listeria Enrichment Supplement

ISO 11290-Part 1: Detection Method (*L. monocytogenes* in Food)

Day 1

25 g Test samples
in 225 ml 1/2 strength FRASER
30°C for 24 h

Streak out
on Chromocult ® +
PALCAM
37°C for 24 - 48 h

Day 3 - 4

0.1 ml in 10 ml FRASER
35 or 37°C for 48 h

Day 3 - 5

Biochemical Confirmation

Streak out
on Chromocult ® +
PALCAM
37°C for 24 - 48 h

Day 5 - 7

Biochemical Confirmation

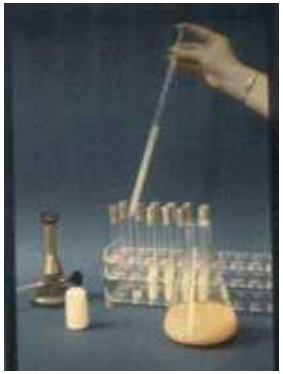
ISO 11290-Part 2: Enumeration Method (*L. monocytogenes* in Food)

Day 1

Test sample

↓ **0,1 ml**

Surface spreading onto Chromocult ®



↓ **37°C** | **24 - 48 h**

Day 2 - 3

Count all colonies showing a blue-green colour with opaque halo

↓

Day 4 - 5

Biochemical Confirmation

FDA-BAM Standard for the detection of Listeria

Day 1

25 g/ml TEST SAMPLE
IN 225 ml Buffered LEB
30 °C for 4 h

Day 2-3

add selective agents
30 °C for 44 h

After 24 h

STREAK OUT ONTO Chromocult ® 37 °C FOR 24 - 48h	STREAK OUT ONTO OXFORD 35°C FOR 24 - 48h

After 48 h

STREAK OUT ONTO Chromocult® 37 °C FOR 24 - 48h	STREAK OUT ONTO OXFORD 35°C FOR 24 –4 8h

Day 3-5

PICK 5 CHARACTERISTIC COLONIES AND SUBCULTURE ON TSYE AGAR 35 OR 37°C FOR 24 h

Day 6

BIOCHEMICAL CONFIRMATION: GRAM (+), CATALASE (+), MOTILITY (+), CAMP (+), β HAEMOLYSIS (+), GLUCOSE (+), RHAMNOSE (+), XYLOSE (-)

USDA Standard for testing of Listeria in Red Meat, Poultry, Eggs, Environmentals

Day 1

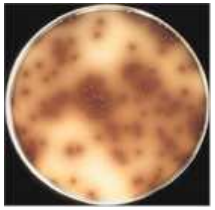
25 g/ml TEST SAMPLE
IN 225 ml UVM 1
30 °C FOR 24 h

Day 2

0.1 ml to 10 ml to
UVM II or FRASER Broth
35 °C for 24 h

Day 3-5

STREAK OUT
ONTO OXFORD AGAR
35 °C FOR 24-48h



PICK OFF 5 -20 CHARACTERISTIC COLONIES AND SUBCULTURE ON HORSE BLOOD AGAR 35°C FOR 24 h

Day 6

BIOCHEMICAL CONFIRMATION: GRAM (+), CATALASE (+), MOTILITY (+), CAMP (+), β HAEMOLYSIS (+), GLUCOSE (+), RHAMNOSE (+) , XYLOSE (-)

Singlepath® L' mono - Screening

Day 1

25 g/ml TEST SAMPLE
IN 225 ml 1/2 STRENGTH FRASER
30°C FOR 24h

Day 2

0,1 ml IN 10 ml LEB or
Full Fraser OR UVM
30˚ / 37°C FOR 21 - 24 h

150µl

Day 3

L. monocytogenes
not present

L. monocytogenes
present

Confirmation

ALOA®
37°C for 24 - 48h

Listeria Identification Tests

*

Characteristic	Reaction
Motility (at 20 - 25 °C)	+
Oxygen requirement	facultative
Growth at 35 °C	+
Catalase activity	+
Hydrogen sulphide production	–
Acid from glucose	+
Methyl red reaction	+
Voges-Proskauer reaction	+
Indole production	–
Citrate utilisation	–
Urease activity	–

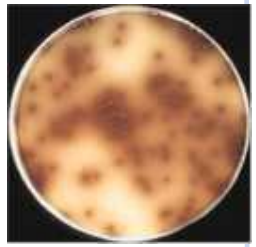
Singlepath® L' mono - Confirmation

ALOA® Agar

suspend 1 - 3 presumptive colonies in 250 µl BHI or CASO or Fraser or bLEB Broth

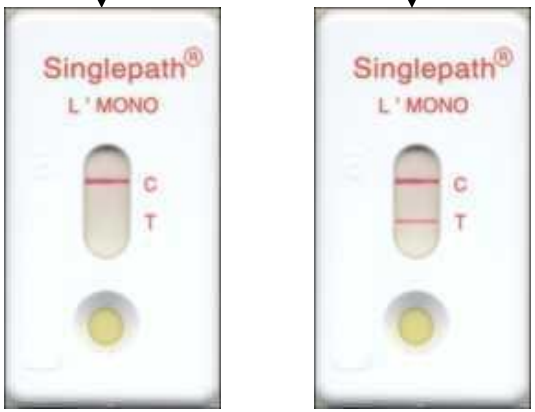
1 h at 37°C

PALCAM Agar



OXFORD Agar

150 µl



L. monocytogenes not confirmed

L. monocytogenes confirmed

Fastest manual confirmatory test for Listeria monocytogenes

Upcoming Pathogen: *Campylobacter*

- Found in birds, wild and domestic animals
- Most common bacterial cause of food-borne diseases
- Thermophilic *C. jejuni* and *C. coli* cause fever and gastroenteritis
- Duration: 2 - 4 days; incubation period: 1 - 7 days

- 1/1000 patients develop Guilian Barré Syndrom (Paralysis for weeks - months)
- Infections result from consumption of contaminated poultry, raw milk products
- Grows under microaerophilic conditions (difficult to culture in a lab)

→ Problem: Many food labs cannot analyse *Campylobacter* in foods because of no knowledge of handling of these pathogens

Detection of Campylobacter in Food acc. to FDA-BAM and ISO

Day 1

BOLTON Broth & Supplement

25g/ml Test Sample in 225 ml Bolton Broth
37°C for 4 h
41,5°C for 44 h

Day 2

Day 3 & 4

CCDA Agar & Supplement

Streak out on CCDA, 37°C for 48 h

Day 5

If positive, confirmation required by culture and biochemical tests

Immunological Rapid Test for Detection of Campylobacter in Food by Singlepath® *Campylobacter*

Day 1	only 2 days	25g/ml Test Sample in 225 ml Bolton Broth 37°C for 4 h 41,5°C for 44 h	only 1 medium
Day 2			
Day 3 & 4	NO Campylobacter present		YES Campylobacter present
Day 5	If positive, confirmation required by culture and biochemical tests		Streak out on CCDA, 37°C for 48 h 

Enterobacter sakazakii

Enterobacter sakazakii

- **Considered as a clear species since 1980**
- ***E. sakazakii* is an opportunistic pathogen**
- **Risk for new-borns, especially low-birth weight infants**
- **Causes servere neonatal sepsis, meningitis**
- **Infant formula food has been a vehicle and cause of *E. sakazakii* illness**

Enterobacter sakazakii

- Powdered infant milk formulas is not a sterile product and may occasional contain pathogen.

- One of the pathogen can be *Enterobacter sakazakii*

- Normally Enterobacter are not harmful and in the past it was not easy to detect this specific Enterobacter

Chromocult® Enterobacter sakazakii Agar

ISO TS 22964

Day 1

Test sample
in BPW (1:10)
37°C for 16 - 20 h

↓ **0,1 ml**

Day 2

10 ml Modified LST Broth – Vancomycin Medium
45°C for 22 - 26 h

↓

Day 3

Streak onto
Enterobacter sakazakii Isolation Agar
44°C for 22 - 26 h

Day 4

Biochemical Confirmation

Enterobacter sakazaki Agar

During the past decade there has been a significant increase in the use of chromogenic substrates in isolation media (7).

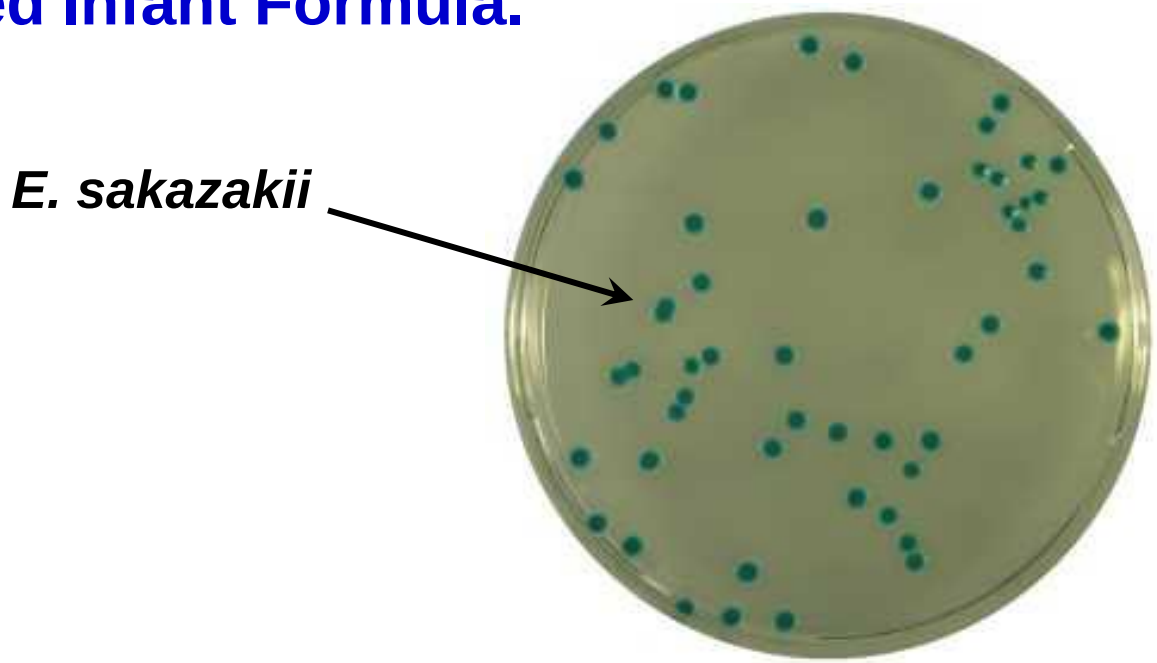
It has been reported that 100% of *E. Sakazakii* were positive for á-D-glucosidase and that 100% of other *Enterobacter* species were negative for this enzyme (8).

Based on this observation 5-bromo-4-chloro-3-indolyl-á-D-glucopyranoside (X-á-Glc, 6) and 4-methylumbelliferyl-á-D-glucoside (9) was added to a basal medium to differentiate *E. sakazakii* strains from other members of the *Enterobacteriaceae.*

The enzyme á- glucosidase hydrolyses X-á-Glc giving blue coloured colonies on DFI agar (Oxoid, UK), ESIA agar (AES, France) or Chromocult ES agar (Merck, Germany), which are commercially available.

Chromocult® Enterobacter Sakazakii Agar

Chromogenic Culture Medium for the Detection and Enumeration of *Enterobacter sakazakii* in Milk Powder and Powdered Infant Formula.



Chromocult® Enterobacter Sakazakii Agar # 1.00873

Bacillus cereus

Bacillus cereus – Sources

Humans are not a significant source of food contamination by B.cereus. This organism already exists on many foods and can therefore be transiently carried in the intestine of healthy humans.

Animals can carry B.cereus on parts of their body. May cause mastitis in cows.

Raw Foods of plant origin are the major source.
The ability of spores to survive dried storage and the thermal resistance of spores, means that most ready-to-eat foods will contain B.cereus.
Strains producing Emetic toxin grow well in rice dishes and other starch foods whereas strains producing diarrhoeal toxin grow in a wide variety of foods from vegetables and salads to meat and casseroles.
Numerous dried herbs and spices and dehydrated foods have been shown to contain B.cereus

Bacillus cereus – Sources

Environment

B.cereus is widely distributed in nature and can be found in soil, dust, Air,water and decaying matter. Its ability to form spores allows survival through all stages of food-processing, other than retorting.

Transmission Routes

Ingestion of contaminated food

Treatment

Usually no treatment is given. Fluids may be administered when

Diarrhoea and vomiting are severe

Bacillus cereus – Foodborne illness

2 Types of Infections:

1. **Diarrheal illness** **= Toxicoinfection**

Enterotoxin(s) produced during vegetative growth of B. cereus in small intestine

Associated with ingestion of B. cereus producing heat-labile toxins
(occurs within 8-12 hours)

2. **Emetic illness (Vomiting)** **= Intoxication**

Cereulide Toxin preformed in food

Usually associated with the ingestion of a heat-stable toxin from contaminated rice
(occurs within 1-6 hours)

Potential food hazards / Risk factors

- INADEQUATE REFRIGERATION for several HOURS
- PREPARATION OF FOODS IN ADVANCE
- POOR PERSONAL HYGIENE
- INADEQUATE HEATING OR COOKING
- PROLONGED USE OF WARMING PLATES (PROMOTES Toxine PRODUCTION)

FOODS AT RISK

FISH	Boiled cod, Lobster pate
MILK	Pasteur.milk, Infant cream
MEAT	Meat loaf, boiled sausage, beef stew, veal broth
CEREALS	Fried rice, cooked rice and pasta dishes
POULTRY	Turkey loaf, roast turkey, barbecued chicken
EGG	Omelette
DESSERTS	Vanilla slices, vanilla pudding, sauce
VEGETABLES	Vegetable sprouts, pea soup, mashed potatoes
HERBS/SPICES	Many foods

Bacillus cereus: Sources of infections

Meat products, soups, milk & milk products, vegetables, puddings & sauces

– for **diarrhoeal syndrome**

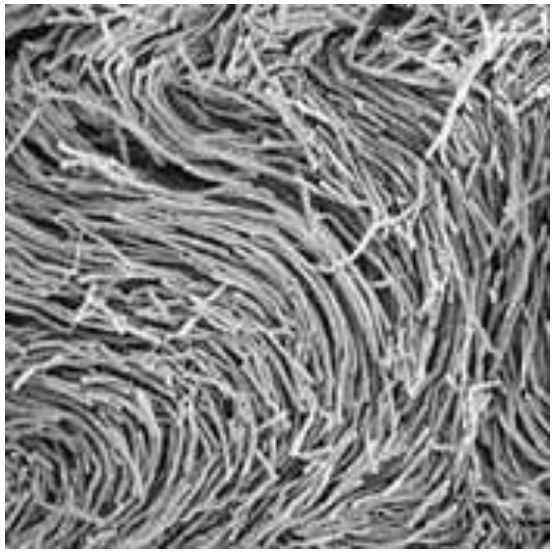
Rice, pasta, noodles, pastry, starchy products

– for **emetic syndrome**

Outbreaks / infections due to B. cereus

Most Susceptible Populations

- Young people (<19 years)
- Elderly (>60 years)



Infectious dose:

- 5 x 10^4 to 10^{11} cells / dose
- 10^5 to 10^8 viable cells or spores – cause Illness
- >10^3 cells/g food – not safe for consumption

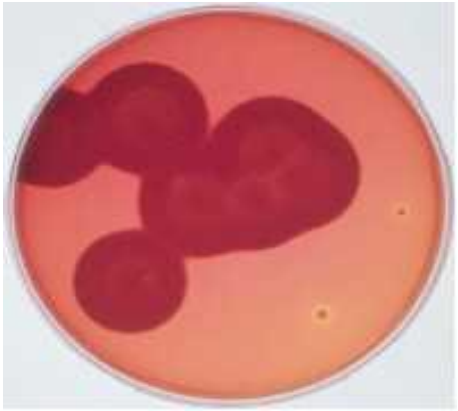
Infections / outbreaks due to B. cereus are underreported because of ususally mild disease (lasts < 24 h)

ISO 7932
Colony count of Bacillus cereus at 30°C

Day 1

25 g/ml TEST SAMPLE IN 225 ml BPW
MAX. RECOVERY MEDIUM,
RINGERS OR PEPTONE SALINE

0.1 ml
(MYP Agar)
30 °C FOR 18-48h



Day 2,3

PICK OFF 5 CHARACTERISTIC COLONIES
AND SUBCULTURE ON NUTRIENT AGAR
35 OR 37 °C FOR 18-24 h

Day 3,4

BIOCHEMICAL CONFIRMATION:
GLUCOSE FERMENTATION (+), VOGES PROSKAUER (ACETOIN +),
NITRATE (+)

B. cereus by Duopath® Cereus Enterotoxins
Emetic B. cereus by Singlepath Emetic TOX MRK

Day 1

suspend
1 - 3 presumptive colonies into 1 ml CGY Broth + 1% Glucose,

4 h at 37°C

M.Y.P. Agar

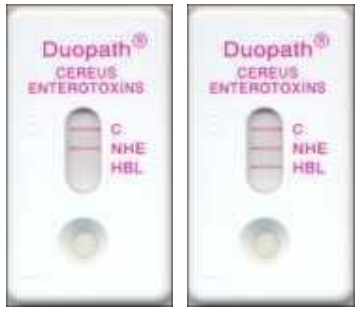
150 µl

NO

B. cereus **not present**

Emetic *B. cereus* not present

YES



B. cereus **present**

Emetic *B. cereus* present

Clostridium perfringens

INDICATOR ORGANISMS
Clostridium perfringens

- In human faeces
- Sulphite reducing Clostridium
- Gram-positive, rod-shaped

- Anaerobic spore-forming bacteria
- Spores that are resistant to environmental conditions (temperature, pH, UV, water treatment processes, disinfection)
- Occurs smaller numbers than *E. coli*.

C. perfringens

- ***C. perfringens*** **is an indicator for fecal contamination**

- ***C. perfringens*** **spores indicate effectiveness of water purification processes**

- **Possible index parameter for occurence of persistent pathogenes e.g. viruses and oocysts (cryptosporidia)**

ISO 7937:
Colony count of Clostridium perfringens

Dilute food sample and add 1 ml of sample to 15 ml TSC agar

TSC-Agar, anaerob (37 °C, (20 ± 2) h

Select plates with less than 150 colonies with black or brown colonies

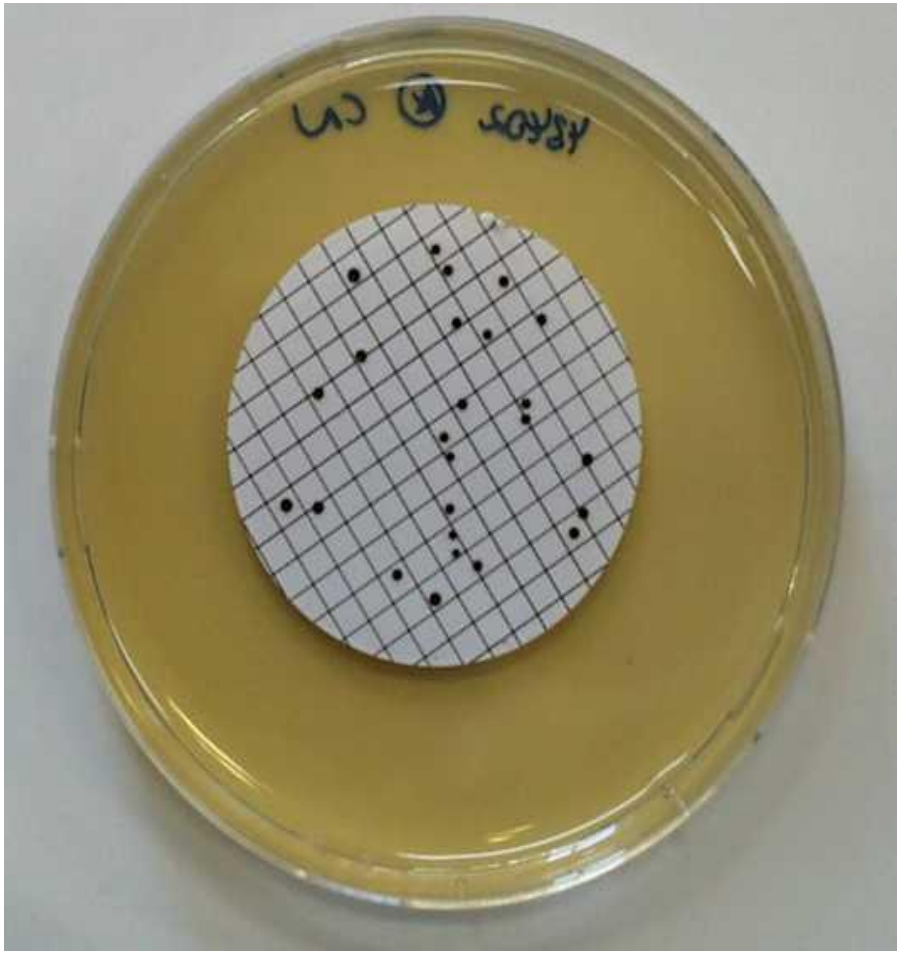
Confirmation test

Thioglycollate medium

LS medium

Nitrate motility medium

Lactose gelatine medium



ISO 7937: Alternative Identification procedure Colony count of Clostridium perfringens

Dilute food sample and add 1 ml of sample to 15 ml TSC agar

TSC-Agar, anaerob (37 °C, (20 ± 2) h

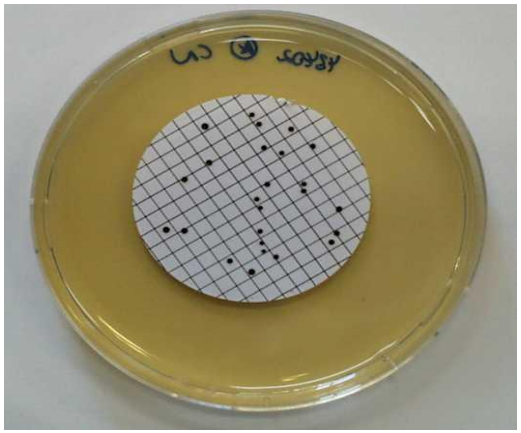
Select plates with less than 150 comonies with black or brown colonies

Confirmation test

Fluorescence

reading

TSC-agar base



TSC agar MERCK supplemented with Clostridium Perfringens Selective Supplement: Cycloserine & MUP

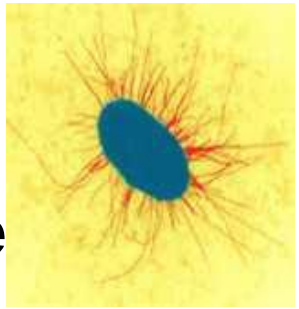
Coliforms and E. coli

Coliforms

- **The coliform group consists of several genera of bacteria belonging to the family of Enterobacteriaceae. The historical definition of this group has been based on the method used for detection of lactose fermentation .**

Indicator and index concept

- **Mossel (1982) defined the term 'marker organism' which refers to two different functions, index and indicator.**



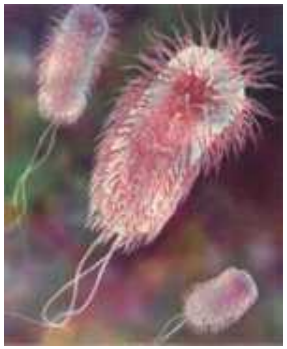
- **'Index organisms' are related, directly or indirectly, either to the health hazards or to the presence of pathogens.**

- **On the other hand, 'indicator organisms' are related only to the effects of treatment processes or control of water quality.**

Alternative Microbiological Methods

Total Coliforms

(Marker Organisms)



Indicator Organisms Coliforms

indicate insufficient hygiene

during a process/production

Index Organisms *E.coli*

indicates a potential hazard

to health due to fecal contamination

Gas and acid production from lactose

- ***Escherichia coli***
- ***Klebsiella***
- ***Enterobacter***
- ***Citrobacter***

- **Gas + Acid from Lactose at 37°C: 95 % of Coliforms**
- **Gas + Acid from Lactose at 44°C: ONLY 90 % of E.coli**
- **Tryptophanase: 99 % of E.coli are Indole positive**

Fecal Coliforms / E. coli

- **The presence of fecal coliforms in drinking water indicates that an urgent public health problem probably exists, since human pathogens of co-exist with fecal coliforms.**

- **Therefore each total coliform-positive sample has to be analyzed if it contains fecal coliforms.**

Reference Method: ISO 4831
General guidance for coliforms – MPN technique

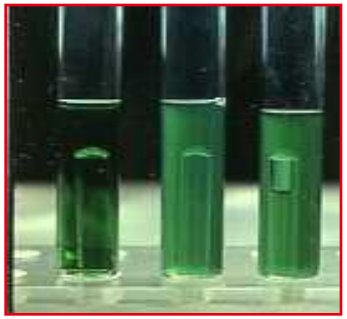
3 tubes of LST broth
Single strenght

3 tubes of LST broth
Double strength

Incubation:
24 to 48 hours at 30°C or 35-37°C

Check tubes for gas formation

MPN
24-48 h



Subculture into BRILA Broth

Incubation:
24 to 48 hours at 30°C or 35-37°C

Confirmation
24-48 h

Coliforms → Check for Gas formation

Reference Method: ISO 7251 & ISO 11866-1
Horizontal method for E.coli - MPN

3 tubes of LST broth
Single strenght

3 tubes of LST broth
Double strength

Incubation:
24 to 48 hours at 37°C +/_ 1°C

MPN
24-48 h

Check tubes for gas formation

Subculture in Peptone water
24-48 h at 44 ± 1°C

Subculture in EC Broth
24-48 h at 44 ±1° C

Indole

Gas

Coliform → -

E.coli → +

- +

Confirmation
24-48 h

Reference Method: ISO 11866-1
E.coli – MPN-MUG Foods

3 tubes of LST broth
Single strenght

3 tubes of LST broth
Double strength

Incubation:
24 to 48 hours at 30°C

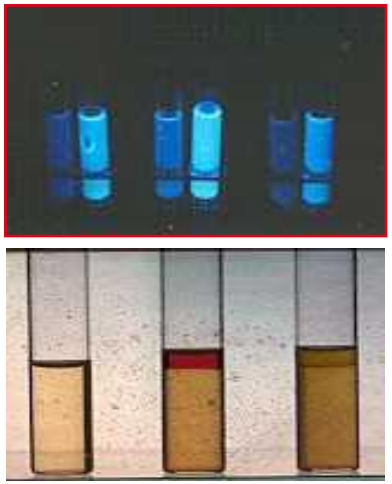
MPN
24-48 h

Check tubes for gas , fluorescence
and indole formation

E.coli

fluorescence

Indole



Coliform

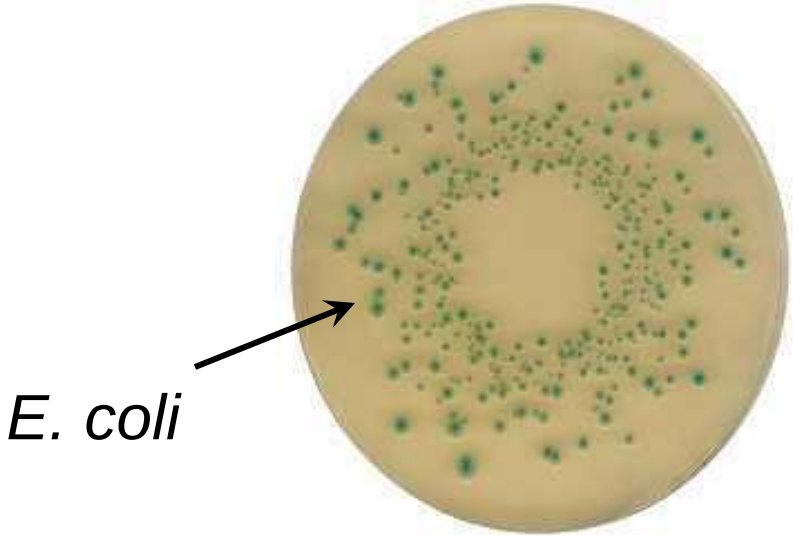
Gas

Confirmation
24-48 h

Chromocult® TBX Agar

Chromogenic Culture Medium for the detection and enumeration of β-glucuronidase-positive *Escherichia coli* in foods.

The culture medium complies with the recommendations in ISO 16649



Chromocult® TBX Agar # 1.16122

Chromocult® TBX Agar

Chromocult® TBX Agar -

New EU Food Regulation Law 2073/2074/2075/2076

Chromocult® TBX Agar is used in the regulation for the detection of :

- Living shells, snails
- Cooked crabs
- Fruits and vegetables
- Minced meat, Meat preparations
- Milk from cheese thermally treated
- Butter and cream not pasteurized

Chromocult® TBX Agar

Mineral - Modified Glutamate Agar (MMGA)

Culture medium for the resuscitation of severely stressed cells (for example by heating, drying, chemical preservation, freeze-drying, acidification) of *E. coli* from food samples (ISO 16649 - Part 1)

In combination with Chromocult® TBX Agar, the enumeration of damaged *E. coli* is possible.

MMGA overlayed with a cellulose membrane

MMGA Agar # 1.09045

Chromocult® TBX Agar

E. coli Enumeration in Processed Foods (ISO 16649-1) Colony-count Technique using Membranes

Resuscitation

Test sample

↓ 1 ml

Spread over cellulose membrane
Mineral - Modified Glutamate Agar (MMGA)

37°C | 4 h ± 1 h

Selective medium

Transfer membrane from **MMGA** to **TBX Agar**

44°C | 18 - 24 h

Counting

Count all blue-green colonies

Chromocult® TBX Agar

E. coli Enumeration in Fresh Foods (ISO 16649-2)
Colony-count Technique using Pour Plate Method

Selective medium

Test sample

↓ 1 ml

Carefully mix the inoculum with 15 ml **TBX Agar**

↓ 44°C | 18 - 24 h

Counting

Count all blue-green colonies

Reference method : ISO 4832
General guidance for coliforms – colony count

2 plates of VRB agar
(1 ml sample + 15 ml agar)
24 h at 30° or 35-37 °C

Counting
Confirmation
24 h

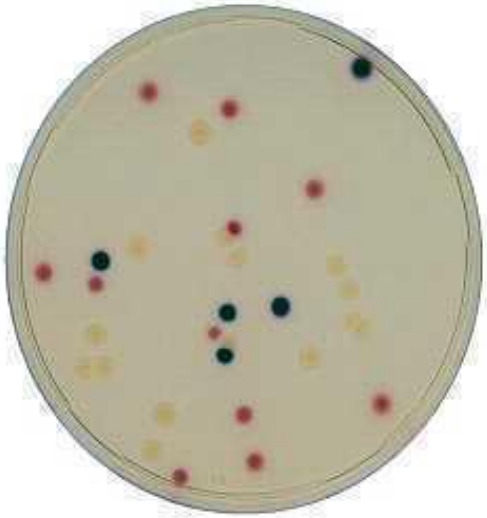
Coliforms
red colonies , diameter > 0,5 mm
reddish zone of precipitated bile

Rapid Methods for Water and Food

Approved
Alternative Methods
Chromocult Coliform Agar

USEPA
for water

ISO 9308
for water

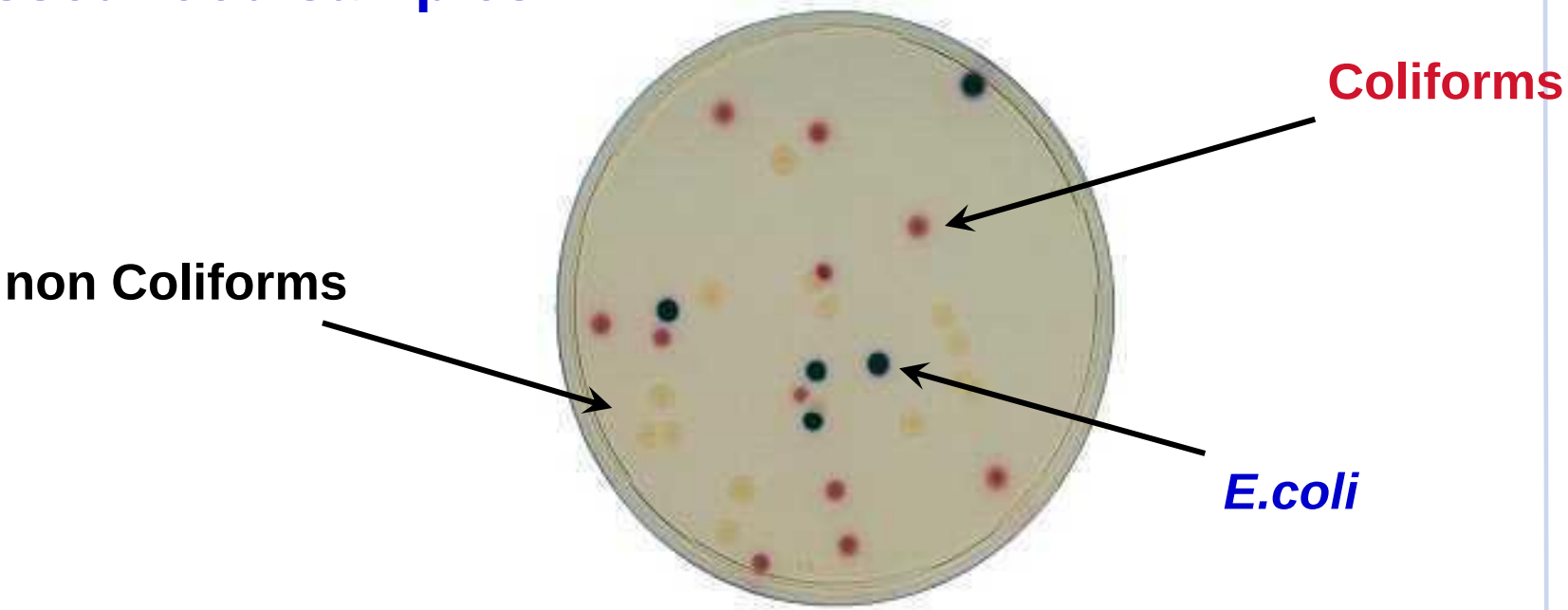


AOAC-RI
for food

PERFORMANCE TESTED
AOAC
RESEARCH INSTITUTE
LICENSE NUMBER 060401
Cat. No. 1.10426.0500
(500 g)

Chromocult® Coliform Agar

Chromogenic Agar for the simultaneous detection of total coliforms and *E. coli* in drinking water and processed food samples.



Chromocult® Coliform Agar # 1.10426

Chromocult® Coliform Agar

Water Testing: Approved Method (USEPA/ ISO 9308

Filtration of 100 ml water
0,45 µm Filter

Chromocult® Coliform Agar + Supplement
24 h at 36 ± 1°C

Non coliforms
light- blue, turquoise or colourless colonies

Coliforms red colonies

E. coli violet colonies

Counting/Confirmation 24 h

Chromocult® Coliform Agar

Food Testing: AOAC™ Validated Method (Processed Food)

Test sample

↓ 1 ml

Carefully mix the inoculum
with 15 ml **Chromocult® Coliform Agar**

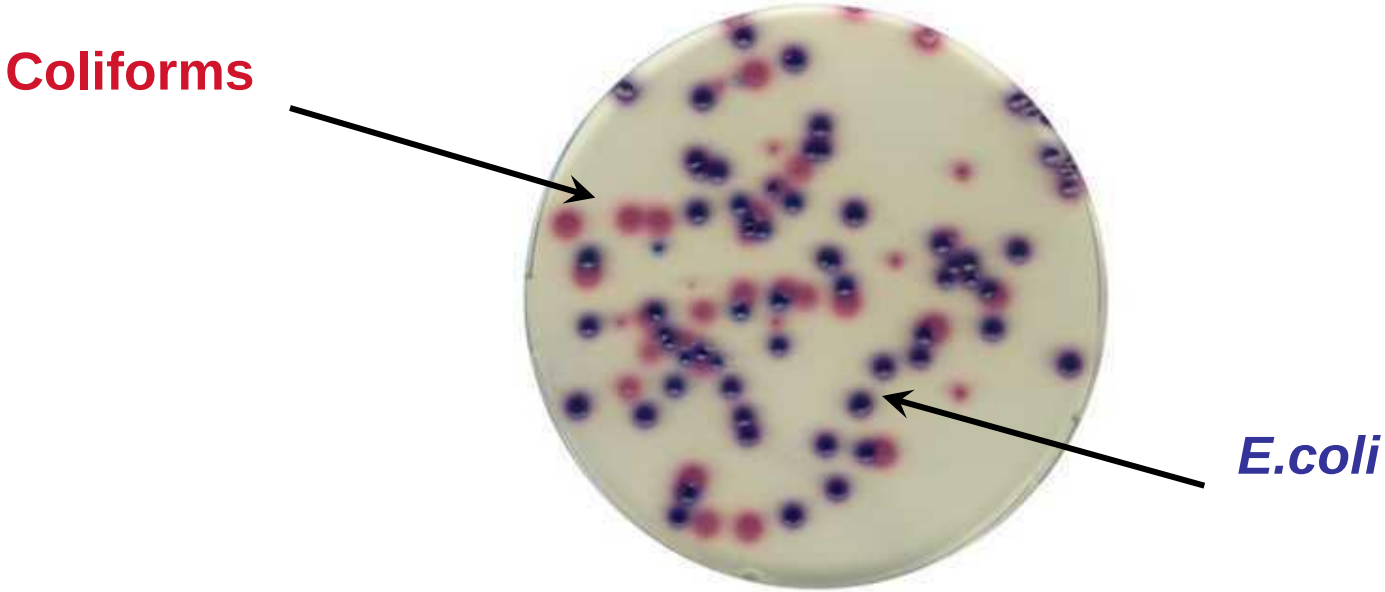
↓ 35 - 37°C | 18 - 24 h

Non coliforms	**Coliforms**	***E. coli***
light- blue, turquoise or colourless colonies	**red colonies**	**violet colonies**

Counting/Confirmation 24 h

Chromocult® Coliform Agar ES

Chromogenic Agar for the simultaneous detection and colony count of total coliforms and *E. coli* in food samples.



Chromocult® Coliform Agar ES # 1.00850

Chromocult® Coliform Agar ES

Food Testing

2007: AOAC™ validated method for the detection of total coliforms and *E. coli* in fresh food (raw ground beef, raw ground chicken, and raw milk)

Chromocult® Coliform Agar ES

Food Testing: AOAC™ Validated Method (Fresh Food)

Test sample

↓ 1 ml

Carefully mix the inoculum
with 15 ml **Chromocult® Coliform Agar ES**

↓ 35 - 37°C | 18 - 24 h

Non coliforms	**Coliforms**	***E. coli***
light- blue, turquoise or colourless colonies	red colonies	**violet colonies**

Counting/Confirmation 24 h

Simplified Microbial WaterTesting

Chromogenic Substrate Coliform Test

- **The chromogenic substrate coliform test is recommended for the analysis of drinking and fresh source water samples acc. to U.S. Standard Methods , 19th Edition 1995**

- **Readycult Coliforms is US-EPA approved since October 2002**

Readycult® Coliforms Procedure

- Add one Readycult® snap pack to 100 ml of water in a sterile 120 ml to 150 ml bottle.
- Seal vessel and shake completely to dissolve granules.
- Incubate 24 hr +/- 1 hr at 35-37°C

- Interpretion of results

Interpretation of Results

NEGATIVE RESULTS (no coliforms)

Broth Stays Clear After Incubation for 24 Hours

NEGATIVE

If Broth Becomes Turbid After Incubation the Sample is Still Negative. (Possible Growth of Non-coliforms)

NEGATIVE

Interpretation of Results

POSITIVE TOTALCOLIFORMS

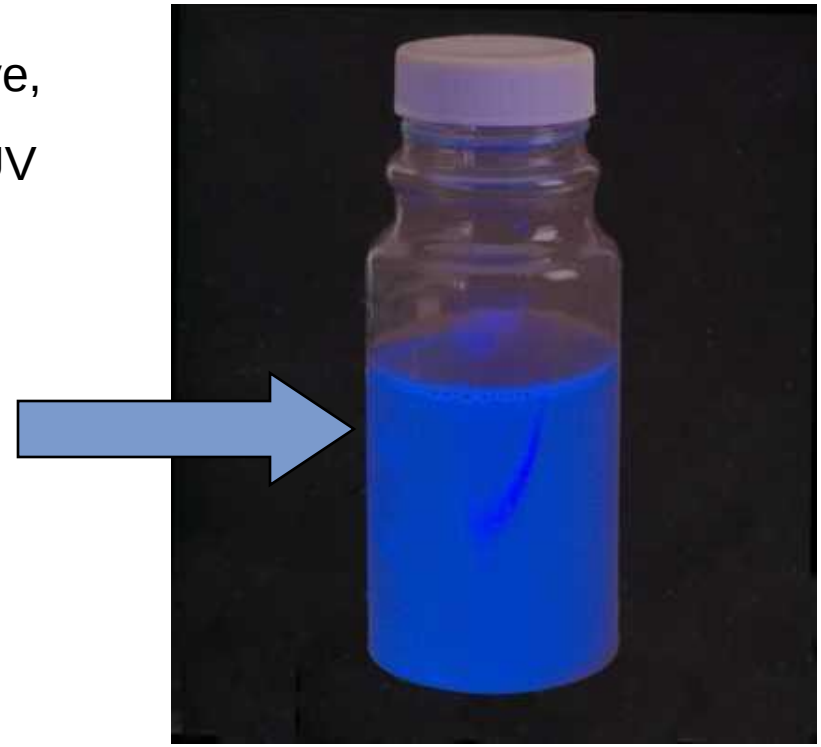
- **Coliforms Present**
- **β–D-Galactosidase Positive**

Distinct Color Change to Blue-Green

Interpretation of Results

E. coli POSITIVE

If the Broth is Total Coliform Positive, (Blue-Green in Color) Check with UV Light for FLUORESCENCE.



If the Broth Fluoresces then:

- ***E. coli* is Present**
- β-D-Glucuronidase Positive

Interpretation of Results

E. coli

BIOCHEMICAL CONFIRMATION WITH THE INDOLE REACTION

- **Indole Reaction 99% Accurate.**
- **Tryptophanase Positive.**

Overlay the Total Coliform or E..coli Positive Sample with Kovac's Indole Reagent. The Formation of a Red Ring Indicates a Positive Indole Test

INDOLE POSITIVE

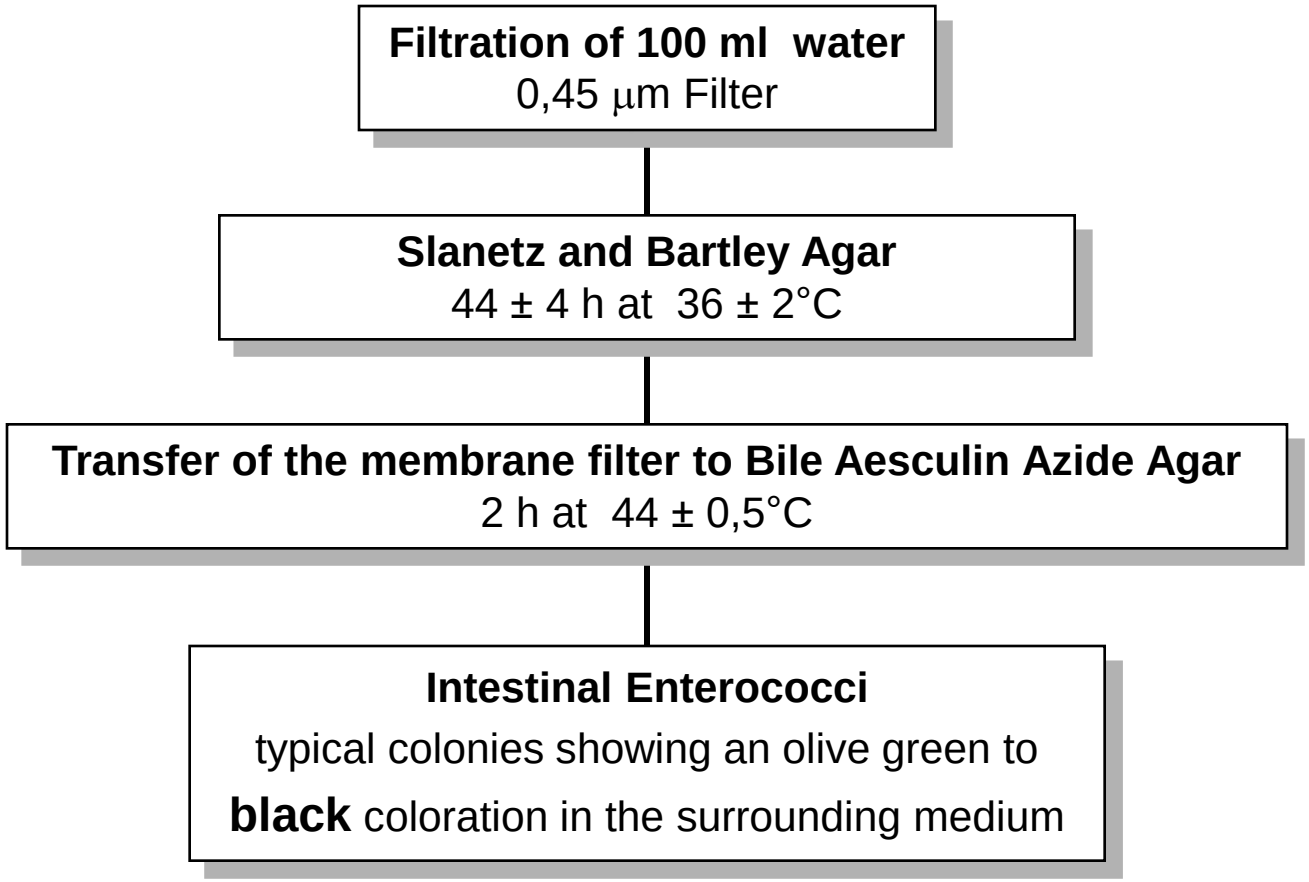
E. Coli Confirmed 99%

Enterococci

Index Organisms

- Intestinal Enterococci like *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* indicate a potential hazard to health due to fecal contamination
- Lower concentration than *E. coli* in faeces
- Survive longer than *E. coli* in environment

Reference Method: Slanetz and Bartley Agar (ISO 7899-2)



Counting Confirmation 48 h

SLANETZ and BARTLEY Agar # 1.05262

Enterococcus faecalis
Red colonies

Bile Aesculin Azide Agar # 1.00072

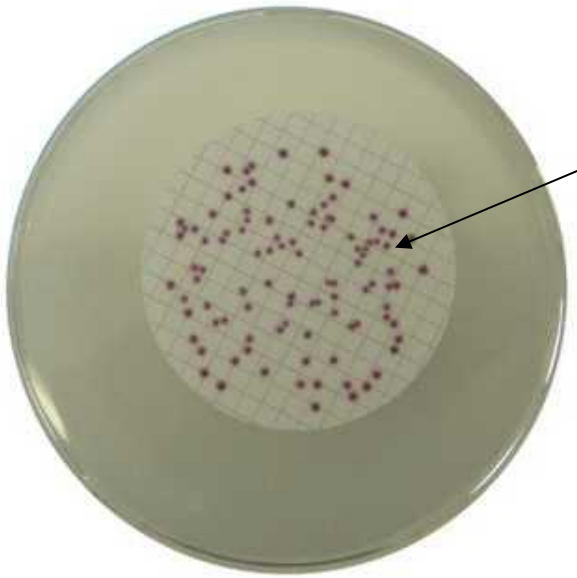
Enterococcus faecalis

Red colonies with black stained medium

Black color in the medium below the membrane

Chromocult® Enterococci Agar

Chromogenic Culture Medium for the Detection and Enumeration of Enterococci in Water.



Enterococcus faecalis
RED colonies

Chromocult® Enterococci Agar # 1.00950

Chromocult® Enterococci Agar

Water Testing

2006: Accepted and approved

as alternative method according to

the EU Directive on Drinking Water in Germany

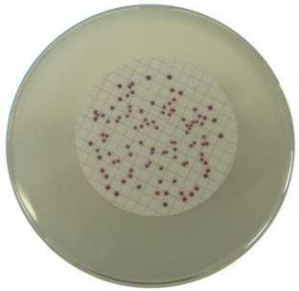
Validated method ISO 17994

Chromocult® Enterococci Agar

Water Testing: Approved Method (EU Directive)

Filtration of 100 ml water
0,45 µm Filter

Chromocult® Enterococci Agar
24 h at 36 ± 1°C



Non Enterococci
blue/violet, turquoise or colourless colonies

Enterococci **red colonies**

Counting/Confirmation 24 h

Rapid Detection of *E. coli* O157 *and* EHEC's

EHEC‘s: Emerging Pathogens

- Variety of *E. coli* present in nature, usually found in the intestines of healthy humans and animals and offer beneficial properties.

- Highly pathogenic E. coli (EHEC) strains exist.

- *E. coli* O157:H7 is most prominent EHEC strain which can cause severe foodborne illness in a VERY low dosage (10 CFU can cause bloody diarrhea and abdominal cramps).

- Complication: The elderly and children <5 years are highly susceptible to Hemolytic Uremic Syndrome (HUS: destruction of red blood cells and kidneys failure).

- Disease is mainly caused by production of Verotoxins (= Shiga-like toxins)

EHEC - Relevance to Public

ca. 100,000 people in the US are annually affected

- Fecal contaminated meats (most frequent outbreaks)
- undercooked beef (HAMBURGERS!!!!!)
- water
- raw milk
- unpasteurized apple juice/cider
- sandwiches
- lettuce
- dry cured salami
- produce from manure-fertilized gardens
- radish sprouts, alfalfa sprouts

EHEC disease also known as *Hamburger Disease*

ISO Standard for testing of *E. coli* O157 in Food

Day 1

25 g/ml TEST SAMPLE
225 ml m-EC or m-TSB
37 °C FOR 24 h

Day 2

Sorbitol (+) are non pathogenic E. coli

Caution: Some E.c. O157 are Sorbitol (+) -> false-neg. result

CT-SMAC Agar 37°C / 24 h Fluorocult E. coli O157 Agar

Day 3

Confirmation by culture and biochemical tests required

Singlepath® *E. coli* O157 for the Detection of *E. coli* O157 in Food

Day 1

25 g/ml TEST SAMPLE
225 ml in m-TSB
37 °C FOR 8 h

OR

25 g/ml TEST SAMPLE
in 225 ml mEC+N or mTSB+N
37- 42 °C FOR 24 h

NEW

Day 2

No *E. coli* O157 present

only 8 h

YES *E. coli* O157 present

only 1 medium

Day 3

If positive, confirmation by culture
and
biochemical tests required

STREAK OUT ON
CT-SMAC Agar
37 °C FOR 24 h

Detection of E. coli O157:H7 in drinking water

The first 24 hours Rapid Method for the detection of E. coli O157:H7 in water using ReadyCult Coliforms for screening and Singlepath E. coli O157 for confirmation

Rapid Detection of *E. coli* O157 in WATER using Singlepath® *E. coli* O157

100 ml water sample
+ Readycult Coliforms 100
Enrichment 18-24 h, 35-37°C

Blue-Green colour
Fluorescence negative
***E. coli* O157:H7/:H- may be present**

Red ring formed (Indole pos.)
***E. coli* O157:H7/:H- probably present**

Blue-green colour
No Fluorescence
Indol positive

150 µl of Indole-pos. sample immediately transferred to Singlepath E. coli O157;
<20 min RT
***E. coli* O157:H7/:H- is present**

YES
***E. coli* O157**
present !

Duopath® Verotoxins

Unique Lateral Flow Test for Verotoxin Identification

- Confirmation test for the detection of Verotoxins (Shiga-like Toxins) 1 and 2 from pathogenic *E. coli* isolates (VTEC‘s or STEC‘s)
- 6 h enrichment in CAYE Broth required
- No centrifugation step
- No boiling
- 10 min. treatment with Polymixin recommended to free cell-associated Verotoxins
- read results within 20 min

  (most results within 5-10)
- Lower detection limit for toxins: 10^7 cfu/ml
- Approvals: AOAC-RI, CE, FDA 510(k), IVD (Japan)

Confirmation of VTEC‘s using Duopath® Verotoxins

day 1

SMAC Agar

CT-SMAC Agar

Streak out on BHI Agar + Carb. at 37°C for 24 h, suspend in 250µl PBS, Polymyxin 10 min

OR

Suspend 1 – 5 colonies into CAYE Broth + Carbadox at 37°C for 6 h, Polymyxin 10 min

NO Verotoxin present

YES
Verotoxins detected and presence of VTEC confirmed !

Duopath® Legionella

Natural habitat of Legionella

Legionella are frequently found in aquatic environments and some species have been recovered from soil.

Temperature is a critical determinant for Legionella proliferation.

Colonization of hot water tanks is more likely if tank temperatures are between 40 and 50°C.

Detection and enumeration of Legionella
Direct membrane filtration method (ISO 11731-2)

Filtration of 10 – 1000 ml water

↓ Acid buffer treatment

BCYE / GVPC Agar

↓ 36 ± 2°C | up to 10 days

Presumptive Legionella
Typical colonies

↓

Confirmation

Confirmation of presumptive Legionella colonies (ISO 11731-2)

Presumptive Legionella
Typical colonies on BCYE / GVPC Agar

BCYE / GVPC Agar

BCYE with Cystein

BCYE without Cystein

48 h / 36 ± 2°C

Growth

No growth

Legionella

Unsafe Method as some Legionellae can grow on BCYE without Cys: Leads to false-negative results

Thank you for your attention