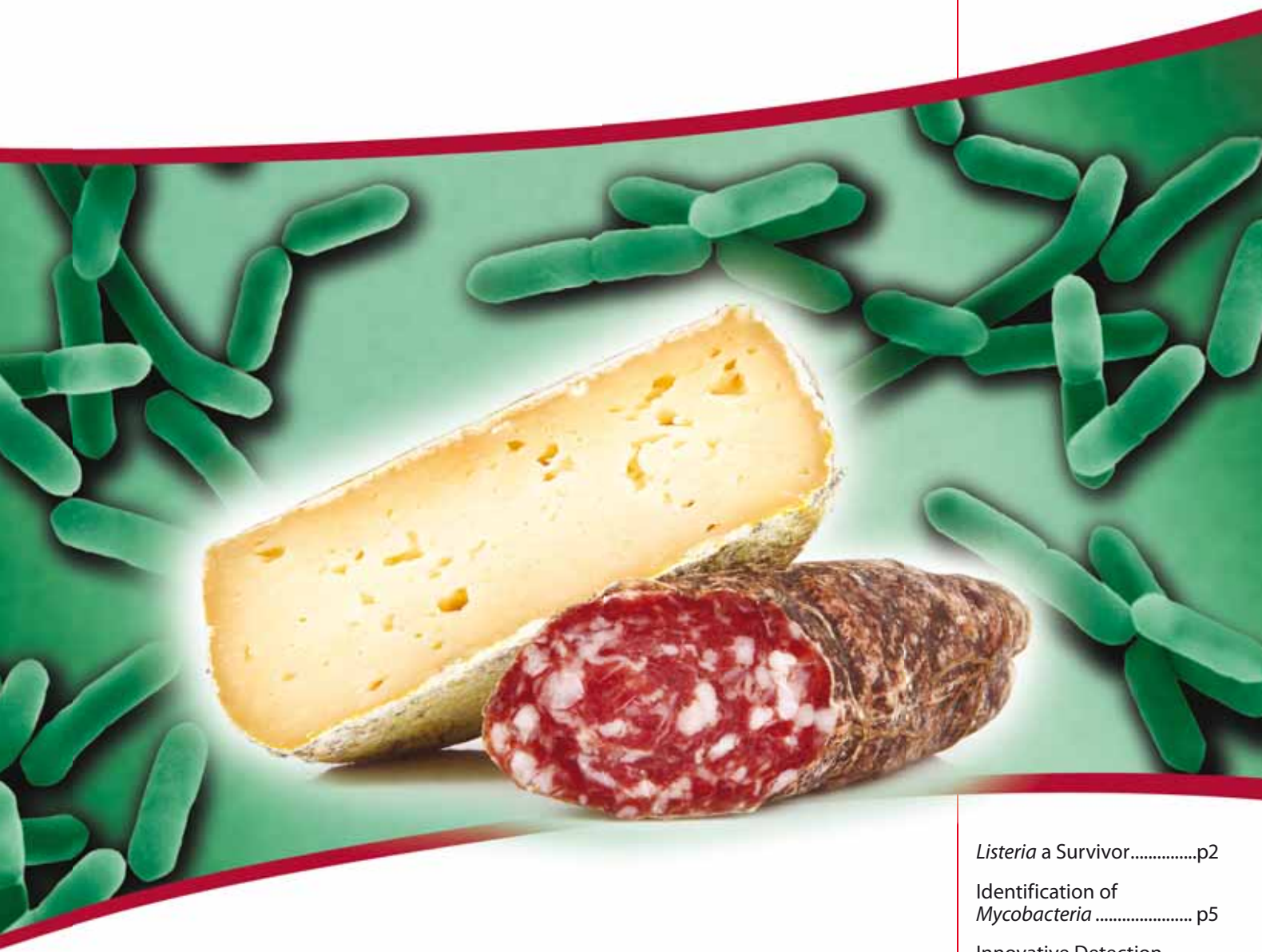
Microbiology Focus

Volume 2.2, 2010

Fluka Analytical

Listeria a Survivor



Raw milk cheese and salami are some of the favourite substrates of Listeria monocytogenes.

Listeria a Survivor

By Jvo Siegrist, Product Manager Microbiology…. *ivo.siegrist@sial.com*

A bacteria on the rise, profiting from today's trends in food product types

Listeriosis is a serious infection caused by *Listeria monocytogenes*. In recent years it has been recognized that *Listeria* is an important public health problem. The disease affects primarily people of advanced age, pregnant women, newborns, and adults with weakened immune systems.

Listeriosis manifests in flu-like symptoms, fever, muscle aches, and sometimes gastrointestinal symptoms such as nausea or diarrhea. If infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance, or convulsions can occur. A severe bout of the disease may lead to blood poisoning, encephalitis and meningitis.

Infected pregnant women may experience only a mild, flu-like illness but this can lead to miscarriage or stillbirth, premature delivery or infection of the newborn.

A lot of research was done in the last years concerning mechanism which this pathogen is using to invade into the host and was found that *L. monocytogenes* is replicated rapidly in the cytosol of host cells like macrophages and lymphocytes (4).

Why have *Listeria* infections increased recently?

Today's major problem is a change in food consumption patterns and the increased demand for longer shelf life. An increasing variety of food products and the trend for "ready-to-eat" and "ready-to-cook" products are some of the reasons for such problems as well as longer storage at cool temperatures (4–8 °C). New preparation technologies, like "Cook&Chill" and "sous vide" and new processes to extend shelf life have led to increasing problems with *Listeria*.

Did you know …

the L-form of *Listeria monocytogens*?

ETH Zurich researchers have discovered a new life form of *L. monocytogenes*. The bacteria are in so-called L-forms and are able to reproduce and proliferate. The cell wall deficient cells are only surrounded by a single membrane, they are spherical and greatly enlarged. These cells cannot be detected with classical plating media.

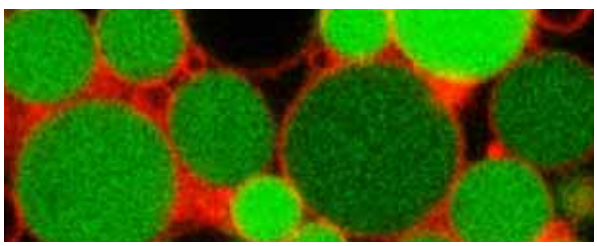


Figure 1: L-form of *Listeria monocytogens* (source: M.Loessner and Y. Briers, ETH Zurich)

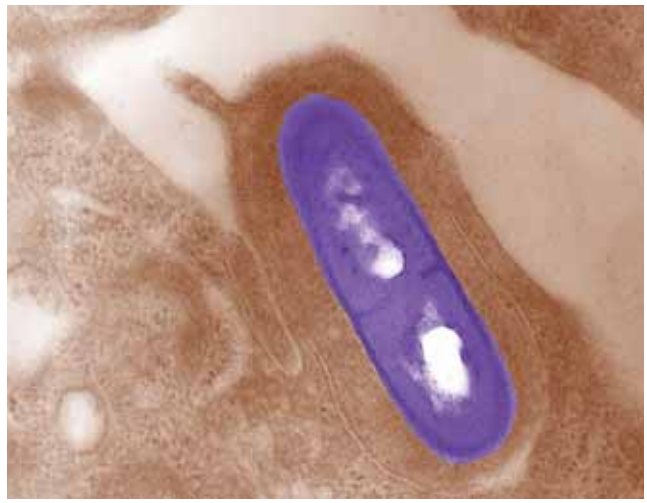


Figure 2: Electron micrograph of a *Listeria monocytogenes* bacterium in tissue.

Listeria monocytogenes is the infectious agent responsible for the food borne illness Listeriosis. In the United States, an estimated 2,500 persons become seriously ill with listeriosis each year. Of these, 500 die. (source: Dr. Balasubr Swaminathan; Peggy Hayes; CDC - Division of Bacterial and Mycotic Diseases: Listeriosis, 2002)

The Nature of *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, non spore forming, rod-shaped flagellate (see **figure 2**). It is an ubiquitous organism, it exists in plants, soil and the guts of birds, fish, shellfish and some mammals, including humans. Some studies suggest that 1-10% of humans may be intestinal carriers of *L. monocytogenes*. Special risk materials are raw or processed meat, raw milk products, raw or smoked fish, ready prepared salad and long stored vacuum packed food.

Listeria species are killed by heating steps, but the bacterium is relatively insensitive to high concentration of salts and acids. It also is able to multiply at fridge temperatures and inside vacuum packaging.

Biochemical Tests and Cultural Methods

The biochemical profile of *Listeria* includes: catalase positive, oxidase negative, fermentation of carbohydrates to acid but not to gas, hydrolysis of esculin and sodium hippurate, methyl red positive, ammonia production from arginine, negative reaction for hydrogen sulfide production, indole negative, nitrate reductase negative, no gelatin liquefaction, no hydrolysis of starch and no urea hydrolysis.

Further differentiation of *Listeria ssp.*, specially for *L. monocytogenes*, by phenotypic properties is possible with additional biochemical test. All of them start with the β-hemolysis test

(*L. monocytogenes* is positive) followed then by detection of carbohydrate fermentation ability. One possibility is to test positive for rhamnose and methyl α-D-mannopyranoside fermentation and a positive CAMP-test. In the CAMP-test some *Listeria* species shows the ability to enhance the haemolysis of *Staphylococcus aureus*. More details about this first method can be found online on the Rhamnose Broth data sheet (Fluka 80547, see also **table 2d,** page 5). Another possibility for phenotype identification is the testing of the fermentation ability of rhamnose, xylose and mannitol (see identification flow chart **figure 7,** page 4).

An interesting topic and a smart solution for confirmation of *L. monocytogenes* are the chromogenic media. There are diverse commercial available chromogenic media like the Agar Listeria Ottavani and Agosti (ALOA) and most of them use the following systems for differentiation:

1. Detection of β-glucosidase activity (by X-glu = 5-bromo-4-chloro-3indolyl-β-D-glucopyranoside) and also Rhamnose fermentation (by indicator phenol red) on a selective media. *Listeria monocytogenes* and *Listeria innocua* results in blue colonies with yellow background, while *Listeria ivanovii* shows only blue colonies.
2. Screen for the presence of β-glucosidase (by X-glu) and phosphatidylinositol specific phospholipase C on a selective media. *Listeria monocytogenes* and *Listeria ivanovii* results in greenish-blue colonies with an opaque halo, while *Listeria innocua* shows only greenish-blue colonies (recommended by ISO 11209-2)

There is more information about the detection systems of the chromogenic and other confirmation media in **table 2d**. To give the media selectivity, phenyl ethanol and a high concentration of lithium chloride and sodium chloride are added to the media. As well antibiotics like moxolactam, nalidixic acid, polymyxin B sulphate, ceftazidime, amphotericin B, acriflavine, cycloheximide, colistin sulphate, cefotetan and fosfomycin are taken to inhibit growth of fungi, Gram-negative and Gram-positive bacteria.

EN ISO 11290-1 and EN ISO 11290-2: (Microbiology of Food and Animal Feeding Stuffs) describe a horizontal method for the detection and enumeration of *Listeria monocytogenes*. A flow chart of the process appears in **figure 3**. The method involves a general four-step process: enrichment, identification, isolation and confirmation.

Sigma-Aldrich, through the innovations of chemists at its Fluka-brand, developed and commercialized reliable media and biochemical tests for many pathogens, including *Listeria* according to EN/ISO methodologies. The media contain the elements necessary to selectively grow and identify Listeria in food substances according to recommended and established methods. The biochemical tests are designed to get easy, quick, and reliable results.

Common media, tests and related products are more detailed and sorted in **tables 2a-d and 3,** pages 4-5.

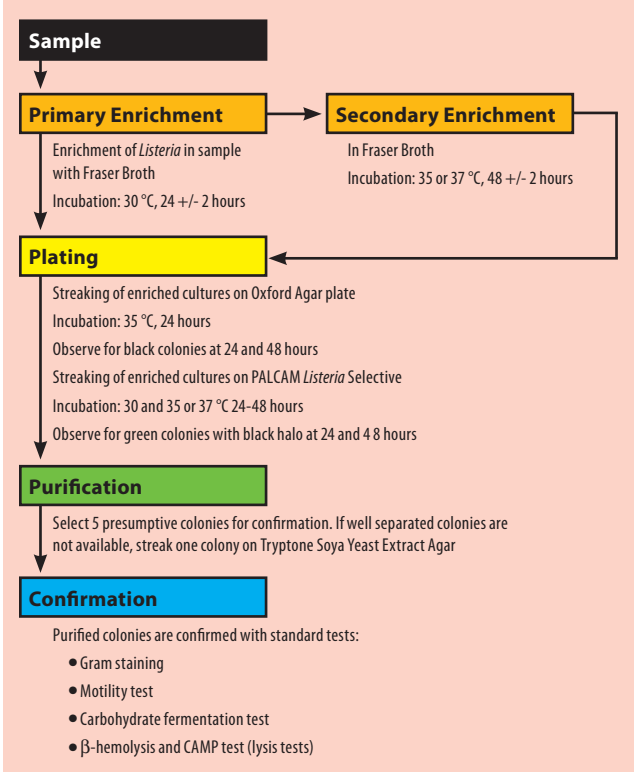


Figure 3: ISO Protocol (EN-ISO 11290-1:1996) for detection and enumeration of *Listeria monocytogenes*

Recommended Media and Tests used for the ISO Method

Description	Brand	Cat. No.
Products for Enrichment Steps		
Fraser Broth, Base	Fluka	69198
Fraser Selective Supplement	Fluka	18038
Fraser Supplement	Fluka	90836
Products for Plating		
Oxford Agar	Fluka	75805
Oxford-Listeria Selective Supplement	Fluka	75806
PALCAM Listeria Selective Agar Plate	Fluka	75977
PALCAM Listeria Selective Supplement	Fluka	03396
Purification Medium		
Tryptone Soya Yeast Extract Agar	Fluka	93395
Products for Confirmation		
Gram Staining Kit	Fluka	77730
Listeria Motility Medium	Fluka	55265
Carbohydrate Consumption Broth	Fluka	07410
Blood Agar base No. 2	Sigma	B1676

Table 1: Products used for ISO Method

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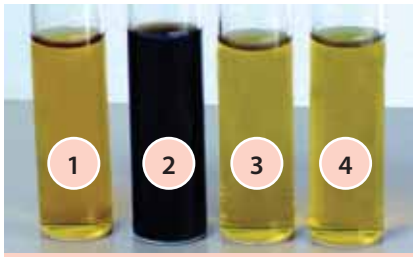
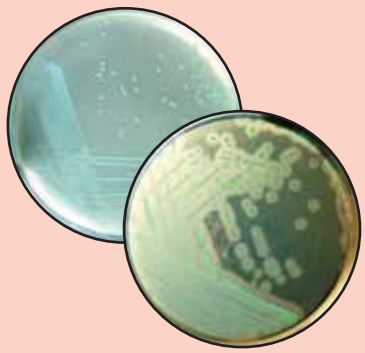


1. Control **2.** *Listeria moncytogenes* **3.** *E. coli* **4.** *E. faecalis*

Figure 4: Fraser Broth (Fluka 69198)

Figure 5: IS *Listeria mono* Confirmatory Agar Fluka 92302 In front *Listeria moncytogenes*



Listeria innocua* and *Listeria moncytogenes

Figure 6: *Listeria mono* Differential Agar (ALOA, Fluka 77408

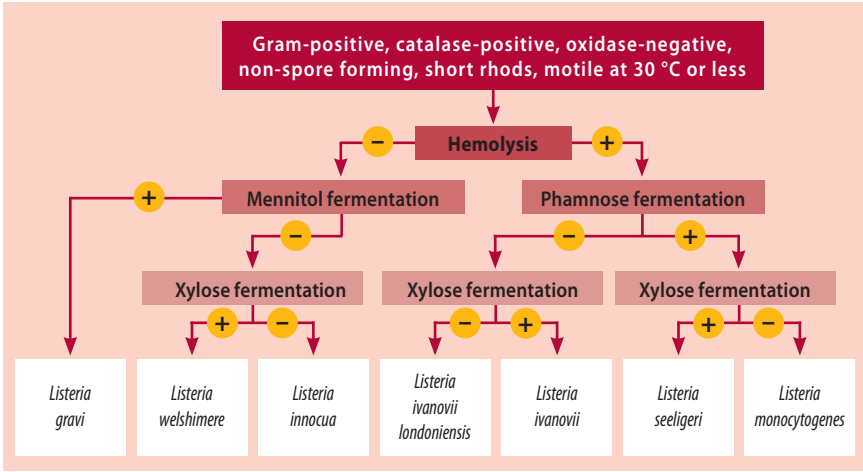


Figure 7: Schematic of biochemical identification for *Listeria spp.* based on carbohydrate fermentation tests and hemolysis (Source: Handbook of *Listeria monocytogenes*, 2008)

Selective Enrichment Media

Cat. No.	Brand	Description	Package Size
69198	Fluka	Fraser Broth, Base (see **Figure 4**)	500 g
18038	Fluka	Fraser Selective Supplement	5 vials
90836	Fluka	Fraser Supplement	10 vials
F6672	Fluka	Fraser secondary enrichment broth base	500 g
F2674	Sigma	Fraser enrichment supplement	5 vials
62353	Fluka	Listeria Enrichment Broth according to FDA/IDF-FIL	500 g
62351	Fluka	Listeria Selective Supplements according to IDF-FIL	16 vials
62348	Fluka	Listeria Selective Supplement according to FDA	16 vials
59859	Fluka	PALCAM Listeria Selective Enrichment Broth, Vegitone (see **Figure 5**)	500 g
91986	Fluka	PALCAM Listeria Selective Supplement according to Van Netten et al.	10 vials
94485	Fluka	UVM Listeria Selective Enrichment Broth, modified	500 g

Table 2a: Selective Enrichment Media

Identification Media

Cat. No.	Brand	Description	Features	Package Size
62355	Fluka	Listeria Selective Agar	Selective media	500 g
62653	Fluka	LPM Agar	Selective media	500 g
43963	Fluka	Moxalactam Supplement		5 vials
75805	Fluka	Oxford Agar	esculin hydrolysis, selective media	500 g
51352	Fluka	Oxford-Listeria Selective Supplement	(uses with 75805)	10 vials
75977	Fluka	PALCAM Listeria Selective Agar	esculin hydrolysis, selective media	500 g
15776	Fluka	PALCAM Listeria Selective Agar, Vegitone	"	500 g
91986	Fluka	PALCAM Listeria Selective Supplement according to Van Netten et al.	(uses with 75977 and 15776)	10 vials

Table 2b: Identification Media

Purification Media

Cat. No.	Brand	Description	Package Size
93395	Fluka	Tryptone Soya Yeast Extract Agar	500 g

Table 2c: Purification Media

References

1. Food-Borne Pathogenic Microorganisms and Natural Toxins Handbook: The "Bad Bug Book" U.S. FDA/CFSAN. Center for Food Safety and Applied Nutrition, Food and Drug Administration, College park, MD (2003)
2. Cossart, P.; Bierne, H.; The use of host cell machinery in the pathogenesis of Listeria monocytogenes. Curr. Opin. Immunol. (England), 13(1), 96-103 (2001)
3. Verbrauchertipps: Schutz vor lebensmittelbedingten Infektionen mit Listerien, Bundesinstitut für Risikobewertung (2008)
4. C.L. Birmingham et al., Listeriolysin O allows Listeria monocytogenes replication in macrophage vacuoles, Nature 451: 350-354 (2008)
5. L. Dongyou, Handbook of Listeria monocytogenes, CRC Press (2008)

Confirmation Media (for Differentiation)

Cat. No.	Brand	Description	Features	Package Size
B1676	Sigma	Blood Agar Base No. 2	Lysis test (β-hemolysis)	500 g
07410	Fluka	Carbohydrate Consumption Broth	Fermentation ability	500 g
53707	Fluka	HiCrome™ Listeria Agar Base, modified (chromogenic media)	β-glucosidase activity, rhamnose fermentation, selective media	250 g
59688	Fluka	HiCrome™ Listeria Selective Supplement	(use with 53707)	5 vials
92302	Fluka	Listeria mono Confirmatory Agar, Base (see **Figure 5**)	Presence of phosphatidylinositol specific phospholipase C and fermentation of α-methyl D-mannoside, selective media	38.5 g, 500 g
15895	Fluka	Listeria mono Enrichment Supplement II	(Use with 92302)	5 vials
92301	Fluka	Listeria mono Selective Supplement I	"	5 vials
91603	Fluka	Listeria mono Selective Supplement II	"	5 vials
77408	Fluka	Listeria mono Differential Agar, Base (ALOA, chromogenic media acc. ISO, see **Figure 6**)	Presence of β-glucosidase and phosphatidylinositol specific phospholipase C , selective media	500 g
03708	Fluka	Listeria mono Enrichment Supplement I	(Use with 03708)	5 vials
92301	Fluka	Listeria mono Selective Supplement I	"	5 vials
91603	Fluka	Listeria mono Selective Supplement II	"	5 vials
55265	Fluka	Listeria Motility Medium	Motility test	500 g
80547	Fluka	Rhamnose Broth / Methyl α-D-mannopyranoside Broth	Rhamnose and methyl α-D-mannopyranoside fermentation	500g
80301	Fluka	Rhamnose Broth Supplement	(uses with 80547)	25 mL
02046	Fluka	Methyl α-D-mannopyranoside Supplement	"	5 mL

Table 2d: Confirmation Media

Cat. No.	Brand	Description	Testing features	Package Size
88597	Fluka	Catalase Test (H_2O_2, 3% solution)	Presence of catalase	100 mL
77730	Fluka	Gram Staining Kit	Cell wall properties	1 Kit
40405	Fluka	Hippurate Disks	Hydrolysis of hippuric acid	25 Disks
01869	Fluka	Hippurate Strips Kit	Hydrolysis of hippuric acid	50 Strips
94438	Fluka	Mannitol disks	Fermentation abilities	10 x 25 Disks
07345	Fluka	Oxidase Reagent acc. Gaby-Hadley A	Presence of oxidase	100 mL
07817	Fluka	Oxidase Reagent acc. Gaby-Hadley B	Presence of oxidase	100 mL
18502	Fluka	Oxidase Reagent acc. Gordon-McLeod	Presence of oxidase	100 mL
40560	Fluka	Oxidase Strips	Presence of oxidase	100 Strips
70439	Fluka	Oxidase Test	Presence of oxidase	50 Disks
93999	Fluka	Rhamnose disks	Fermentation abilities	10 x 25 Disks
07411	Fluka	Xylose disks	Fermentation abilities	10 x 25 Disks

Table 3: Biochemical tests

Identification of Mycobacteria

Anandi Martin, PhD, Institute of Tropical Medicine, Mycobacteriology Unit, Belgium .... *amartin@itg.be*

Figure 1: Typical colony of *Mycobacterium tuberculosis* seen under a microscope with 10x magnification.

The diseases produced by species of the genus *Mycobacterium* are important causes of morbidity and mortality in the world. The identification of mycobacteria to the species level is important because of the clinical significance; some species are pathogenic while others are not.

Traditionally, mycobacteria are identified by phenotypic methods, based on culture, such as morphological characteristics, growth rates, preferred growth temperature, pigmentation and on a series of biochemical tests. Testing is laborious, difficult and time-consuming, requiring several weeks for adequate growth, and sometimes misidentification may occur because different species may have indistinguishable morphological and biochemical profiles. Different culture media are in use for the isolation of mycobacteria. The most common are based on eggs called "Löwenstein-Jensen medium" and contain high concentrations of malachite green to overcome contamination with other bacteria.

In the last decade, several commercial systems for mycobacterial culture

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based on liquid media have been introduced. Liquid culture media have been proven to be significantly more sensitive than egg-based solid media for the isolation of mycobacteria from clinical specimens. *M. tuberculosis* bacilli are slow-growing mycobacteria which means that in primary isolation they hardly show any visible growth during the first week of culture. On egg-based media they produce characteristic non-pigmented colonies, with a general rough and dry appearance simulating breadcrumbs. On agar based media, the colonies appear flat, dry and rough with irregular edges. *M. tuberculosis* is niacin positive, is inhibited by p-nitrobenzoic acid and display nitratase activity. Additional tests that confirm an isolate as *M. tuberculosis* are susceptible to pyrazinamide, growth on thiophene carboxylic acid hydrazide (TCH), absence of catalase production at 68 °C and absence of iron uptake.

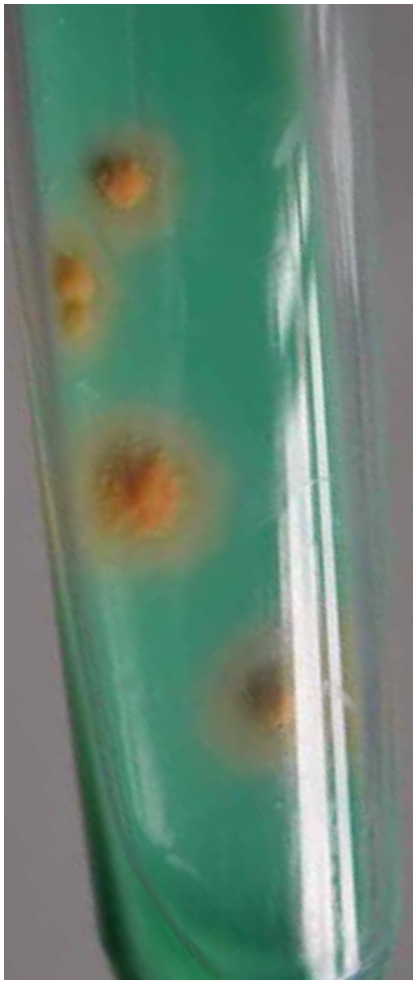

Figure 2: Mycobacteria colonies on TB-Medium Base according to Löwenstein-Jensen

The Ziehl-Neelsen staining for the direct detection of mycobacteria by microscopy is used to identify acid fast bacilli. The lipid rich cell wall of mycobacteria makes it resistant to Gram stain. It can also be used to stain few other bacteria like Nocardia. The reagents used for the staining are carbolfuchsin, acid-alcohol and methylene blue. Acid fast bacilli appear bright red after staining.

Non-tuberculous mycobacteria (NTM) are ubiquitous organisms that are frequently isolated from environmental sources, including surface water, tap water, and soil. The NTM species most frequently associated with pulmonary disease are *M. avium, M. kansasii and M. abscessus*. Injury cutaneous/ subcutaneous infections have been attributed to rapidly growing mycobacteria. *M. fortuitum, M. abscessus*, and *M. chelonae* are thought to be caused by local environmental strains or contaminated commercial surgical materials, devices or solutions for injection. Rapidly growing mycobacteria often grow on classical bacterial culture media, especially on blood agar plates, however, due to their delay in forming visible colonies (up to 10 days), they are usually not detected in the routine bacteriology laboratory. They can also be isolated on most media available for the isolation of mycobacteria. Although the optimum temperature for most species is 30-32 °C, they also grow at 36-37 °C, the standard temperature for isolation of the *M. tuberculosis*.

In the last decade, advances in molecular methods have facilitated the rapid and reliable identification of many mycobacterial species. Nucleic acid probes, species-specific PCR, reverse hybridization and 16S rRNA sequencing have been evaluated for application in clinical laboratories. The first method commercially available was the AccuProbe (Gen-Probe Inc.), based on species-specific DNA probes that hybridize to rRNA for the identification of several important mycobacteria, including the *M. tuberculosis* complex, *M. avium, M. intracellulare*, the *M. avium* complex, *M. kansasii* and *M. gordonae*. More recently, other molecular commercial systems have also been introduced for the rapid identification of *M. tuberculosis* complex: the INNO-LiPA MYCOBACTERIA v2 (Innogenetics NV, Ghent, Belgium), and the GenoType MTBC and GenoType Mycobacterium (Hain Lifesciences, Nehren, Germany). INNO-LiPA MYCOBACTERIA v2 is a line probe assay that simultaneously detects and identifies the genus Mycobacterium and 16 different mycobacterial species. It is based on nucleotide differences in the 16S-23S rRNA gene spacers. The GenoType MTBC and GenoType Mycobacterium are also based on the reverse line probe hybridization assay and are intended for the differentiation of members of the *M. tuberculosis* complex and for the identification of 35 species of mycobacteria including *M. tuberculosis*, respectively. The GenoType MTBC is based on a 23S rRNA gene fragment specific for the *M. tuberculosis* complex, together with gyrB sequence polymorphisms, and the RD1 deletion for identification of *M. bovis* BCG. Several 'in-house' techniques are also available with sequencing of the 16S rRNA gene as the reference standard to which all other new techniques are generally compared.

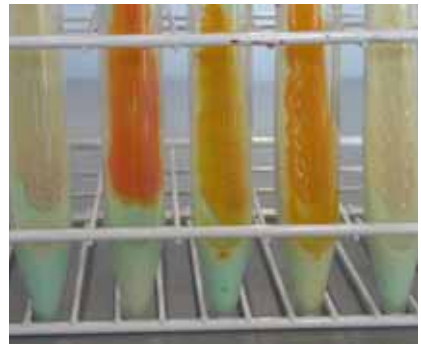

Figure 3: Different mycobacteria species grown on TB-Medium Base according to Löwenstein-Jensen

Since NTM's are present everywhere in the environment and sometimes colonize healthy individuals mycobacteria should be identified at the species level before starting treatment, because different species display different antibiotic resistance patterns. Certain NTM are fastidious and special culture conditions or growth requirements should be observed for their isolation.

References

1. Tortoli E. Clinical manifestations of nontuberculous mycobacteria infections. Clin Microbiol Infect. 2009 Oct;15(10):906-10.
2. Palomino JC. Molecular detection, identification and drug resistance detection in Mycobacterium tuberculosis. FEMS Immunol Med Microbiol. 2009 Jul;56(2):103-11.

Sigma-Aldrich Products for Mycobacteria

Brand	Cat. No.	Media & Supplements
Fluka	63237	TB-Medium Base according to Loewenstein–Jensen
Fluka	51803	Gruft Mycobacterial Supplement
Fluka	M0178	Middlebrook 7H9 Broth Base
Fluka	M0303	Middlebrook 7H10 Broth Base
Fluka	M0428	Middlebrook 7H11 Broth Base

Table 1: Media for detection, isolation, differentiation of mycobacteria

Brand	Cat. No.	Media & Supplements
Fluka	21820	Carbol-Fuchsin solution according to Ziehl–Neelsen
Fluka	21819	Carbol-Fuchsin solution according to Kinyoun
Fluka	05151	Fluorescent Stain Kit for Mycobacteria
Fluka	56694	Acid Alcohol solution
Fluka	30503	Phenolic auramine solution

Table 2: Fluka products for staining of mycobacteria

Innovative Detection of *Listeria*

By Jvo Siegrist, Product Manager Microbiology…. *ivo.siegrist@sial.com*

Today, Real Time PCR is the standard for modern microbiology, but there are also other easier methods.

Our **HybriScan®** test system is able to detect *Listeria* ssp. and specifically *Listeria monocytogenes*. PCR is not needed but nevertheless the result is based on the genetic information, the target molecule being ribosomal RNA. Each bacterial cell posseses several hundred copies of ribosomal RNA and therefore no PCR is needed. The test method is designed on simple 96-well microplate with 12 strips of 8 wells and is sandwich hybridization. The test is not sensitive to sample matrix and detects only living cells. No special expensive equipment is needed and the test is done in approximately 2 to 2.5 hours. A positive result is visible to the naked eye, but can also be read by a standard microplate reader to quantify the number of cells at 450 nm. With a pre-enrichment step (24-30 hours in Half Fraser and Fraser broth) a sensitivity of 1 CFU/25 g can be reached.

The system was validated according to the German § 64 LFGB method. 25 g sample was taken and enriched in 225 mL ONE-Broth for 24 h at 30 °C. Then the broth was streaked out on PALCAM agar incubated for 48 h at 37 °C and all *Listeria* appears as grayish-black, caved in, colonies. The colonies are then confirmed as Gram-positive rods using biochemical differentiation with BD BBL CRYSTAL. As the HybriScan system missed three *L. monocytogenes* positive samples, the single colonies grown on PALCAM agar, sequenced by 16S rDNA and the three undetected species turned out to be *L. innocua, L. seeligeri* and *L. welshimeri*. So the cultivation based method (§ 64 LFGB method) lead to false positive results due to a wrong biochemical identification (see more in **table 1**).

		Number of *Listeria monocytogenes* Positive Samples	
food category	n	§ 64 LFGB	HybriScan
meat products	72	42* (39)	39
fish	36	15	15
milk products	108	42	42
fruits & vegetables	72	24	24
sum	288	123* (120)	120

Table 1: HybriScan*D* *Listeria monocytogenes* test validation (*subsequently identified as false positive result with cultivation based; § 64 LFGB method)

Figure 1: Microbiologist performing a simple test

HybriScan®*D* *Listeria monocytogenes* has a relative accuracy of 99.0%, a relative specificity of 98.2% and relative sensitivity of 100%. For more information about the **HybriScan**-System and the range of yeast and bacteria detection kits, visit ***www.sigma-aldrich.com/hybriscan***.

Fluka No.	Name	Tests/kit
55661	***HybriScanD*** *Listeria*	96
49699	***HybriScanD*** *Listeria monocytogenes*	48
49712	***HybriScanI*** *Listeria monocytogenes*	96

Table 2: HybriScan *Listeria* Kits

Sigma-Aldrich® Worldwide Offices

Argentina
Free Tel: 0810 888 7446
Tel: (+54) 11 4556 1472
Fax: (+54) 11 4552 1698

Australia
Free Tel: 1800 800 097
Free Fax: 1800 800 096
Tel: (+61) 2 9841 0555
Fax: (+61) 2 9841 0500

Austria
Tel: (+43) 1 605 81 10
Fax: (+43) 1 605 81 20

Belgium
Free Tel: 0800 14747
Free Fax: 0800 14745
Tel: (+32) 3 899 13 01
Fax: (+32) 3 899 13 11

Brazil
Free Tel: 0800 701 7425
Tel: (+55) 11 3732 3100
Fax: (+55) 11 5522 9895

Canada
Free Tel: 1800 565 1400
Free Fax: 1800 265 3858
Tel: (+1) 905 829 9500
Fax: (+1) 905 829 9292

Chile
Tel: (+56) 2 495 7395
Fax: (+56) 2 495 7396

China
Free Tel: 800 819 3336
Tel: (+86) 21 6141 5566
Fax: (+86) 21 6141 5567

Czech Republic
Tel: (+420) 246 003 200
Fax: (+420) 246 003 291

Denmark
Tel: (+45) 43 56 59 00
Fax: (+45) 43 56 59 05

Finland
Tel: (+358) 9 350 9250
Fax: (+358) 9 350 92555

France
Free Tel: 0800 211 408
Free Fax: 0800 031 052
Tel: (+33) 474 82 28 88
Fax: (+33) 474 95 68 08

Germany
Free Tel: 0800 51 55 000
Free Fax: 0800 64 90 000
Tel: (+49) 89 6513 0
Fax: (+49) 89 6513 1160

Hungary
Ingyenes telefonszám: 06 80 355 355
Ingyenes fax szám: 06 80 344 344
Tel: (+36) 1 235 9063
Fax: (+36) 1 269 6470

India
Telephone
Bangalore: (+91) 80 6621 9400
New Delhi: (+91) 11 4358 8000
Mumbai: (+91) 22 2570 2364
Hyderabad: (+91) 40 4015 5488
Kolkata: (+91) 33 4013 8003
Fax
Bangalore: (+91) 80 6621 9550
New Delhi: (+91) 11 4358 8001
Mumbai: (+91) 22 4087 2364
Hyderabad: (+91) 40 4015 5488
Kolkata: (+91) 33 4013 8000

Ireland
Free Tel: 1800 200 888
Free Fax: 1800 600 222
Tel: (+353) 402 20370
Fax: (+ 353) 402 20375

Israel
Free Tel: 1 800 70 2222
Tel: (+972) 8 948 4100
Fax: (+972) 8 948 4200

Italy
Free Tel: 800 827 018
Tel: (+39) 02 3341 7310
Fax: (+39) 02 3801 0737

Japan
Tel: (+81) 3 5796 7300
Fax: (+81) 3 5796 7315

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