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CHROMagar™
The Chromogenic Media Pioneer

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Performance of CHROMagar™ Mycoplasma

Chromogenic Culture Medium for Detection of Mycoplasma bovis.

Laboratory

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This report contains 11 pages, including 1 page of annexes

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1. Introduction

Mycoplasma bovis is an emerging bovine pathogen accounting for significant economic and production losses in the cattle and dairy industries worldwide. Infection can result in a variety of clinical signs, such as pneumonia, arthritis, mastitis and keratoconjunctivitis, which can be caused by several animal pathogens. Laboratory diagnosis is therefore important.

CHROMagar™ Mycoplasma is a selective chromogenic medium, intended for use in the qualitative direct detection of *Mycoplasma bovis*. Other animal *Mycoplasma* species can also be detected on this medium.

Veterinary samples like nose swabs and broncho-alveolar lavage samples can be processed by direct streaking onto CHROMagar™ Mycoplasma plates. Medium plates are incubated under a CO_2 atmosphere at 37 °C for 3 to up to 7 days. The detection of typical fried-egg aspect of the colonies of *M. bovis* with a red coloration allows an easier detection than on current culture media.

The product is composed of a powder base medium and three supplements. The powder base and supplement S1 are stored at 15 to 30 °C, the storage temperature of supplement S2 and lyophilized supplement S3 is 2 to 8 °C (see annex 1).

This document compiles CHROMagar™ Mycoplasma evaluations at two stages:

- In-house evaluations of the chromogenic formula with pure strains.
- Independent laboratory evaluations of the CHROMagar™ Mycoplasma formula using *Mycoplasma* strains, respiratory tract swabs, respiratory organ specimens, and bovine milk samples.

2. Performance of the chromogenic formula

2.1. Pure strains

A large number of bacterial strains was tested on CHROMagar™ Mycoplasma plates, incubated under a CO_2 atmosphere at 37 °C for 3 to up to 7 days to evaluate the inclusivity and exclusivity of the chromogenic formula. In these studies, plates of Oxoid™ Mycoplasma were used as reference medium. Results obtained in November 2020 and January 2022 are shown in Tables I-II.

Table I. Bacterial strains tested to evaluate inclusivity of *Mycoplasma* culture media.

Bacterial Species	Strain #	Oxoid™ Mycoplasma Agar		CHROMagar™ Mycoplasma	
		3 days	7 days	3 days	7 days
Mycoplasma bovis	AR6348	unc., 0.1 mm	unc., 0.5 mm	R+, 0.2 mm / DZ	R++, 0.5 mm
M. bovis	AR6349	unc., 0.1 mm / DZ	unc., 0.2-0.4 mm	R-/+, 0.15 mm	R++, 0.6 mm
M. bovis	ATCC® 25523 / AR6350	unc., 0.15 mm	unc., 0.6 mm	R++, 1 mm	R++, 0.8 mm
M. bovis	AR6351	unc., 0.15 mm	unc., 0.4 mm	R+/-, 0.1 mm	R++, 0.5 mm
M. bovis	AR6352	unc., 0.15 mm	unc., 0.6 mm	R++, 0.8 mm	R++, 0.5 mm
M. bovis	AR6353	unc., 0.15 mm	unc., 0.5 mm	R++, 0.5 mm	R++, 0.5 mm
M. bovis	AR6354	unc., 0.15 mm / DZ	unc., 0.5 mm	R++, 0.6 mm	R++, 0.7 mm
M. bovis	AR6355	unc., 0.1 mm	unc., 0.6 mm	R++, 0.5-0.8 mm	R++, 0.8 mm
M. bovis	AR6356	unc., 0.1 mm	unc., 0.5 mm	R++, 0.6 mm	R++, 0.8 mm
M. bovis	AR6357	unc., 0.15 mm	unc., 0.6 mm	R++, 1 mm	R++, 0.8 mm
M. bovis	AR6358	unc., 0.1 mm	unc., 0.5 mm	R++, 0.6 mm	R++, 0.6 mm
M. bovirhinis	AR6359	unc., 0.15 mm	unc., 0.6 mm	R+/++, 0.6 mm	R++, 0.8 mm
M. bovirhinis	AR6360	-	unc. col., 0.4 mm / DZ	col. DZ, R+/-	R++, 0.6 mm / DZ
M. bovigenitalium	AR6361	unc., µcol	unc., 0.5 mm	R+, µcol., 0.3 mm	R++, 0.6 mm
M. bovigenitalium	AR6362	unc., 0.15 mm	unc., 0.5 mm / DZ	unc. col. / DZ	R++, 0.2 mm / DZ
M. alkalescens	AR6363	unc., 0.15 mm	unc., 0.5 mm	unc., 0.15 mm / DZ	R++, 0.4 mm
M. alkalescens	AR6364	unc., 0.15 mm	unc., 0.5 mm	unc., R--/+, 0.15 mm	R++, 0.4 mm
M. arginini	AR6365	unc., 0.15 mm / DZ	unc., 0.4 mm	unc., R--/+, 0.15 mm / DZ	R++, 0.5 mm / DZ
M. arginini	AR6366	unc., 0.15 mm / DZ	unc., 0.2 mm / DZ	unc., R-/+, 0.15 mm / DZ	R++, 0.3 mm / DZ

R, red colour; unc., uncolored ; DZ, dense zone ; +,- colour intensity ; -, growth absence ; µcol., microcolony ; colony size in mm ; AR, CHROMagar™ strain collection ; ATCC®, American Type Culture Collection.

Mycoplasma strains used were both purchased from ATCC® and received from the French Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'environnement et du travail, Anses – Réseau Vigimyc or the Faculty of Veterinary Medicine - University of Liège, Dept. of Bacteriology and Pathology of Bacterial Diseases. Thus, these strains were integrated to the CHROMagar strain collection.

The chromogenic formula showed 91 % and 100 % inclusivity after 3 and 7 days of incubation, respectively for eleven strains of *Mycoplasma bovis*. The typical fried-egg appearance of the *M. bovis* colonies with a red coloration was observed (See Section 2.2). The inclusivity for eight *Mycoplasma* spp. strains was 88 % and 100 % after 3 and 7 days of incubation, respectively.

Table II. Bacterial strains tested to evaluate exclusivity of *Mycoplasma* culture media.

Bacterial Species	Strain #	Oxoid™ Mycoplasma Agar		CHROMagar™ Mycoplasma	
		3 days	7 days	3 days	7 days
Staphylococcus aureus	ATCC® 43300	DZ, Y	DZ, Y	-	-
S. aureus	ATCC® 25923	-	-	-	One col.
Mould	AR4585	-	DZ, W	-	-
Candida albicans	AR3927	-	T	-	T
Escherichia coli	AR3740	unc., 0.5-1 mm	unc., 2 mm	-	-
Acinetobacter	AR5193	-	0.5-4 mm, DZ	-	One col. 10 mm
Proteus vulgaris	AR3919	SW++	SW+++	-	R, T
Enterococcus faecalis	AR6622	unc., 2 mm	unc., 2 mm	Bm +, 2 - 4 mm	MB++, 10 mm, R halo
P. mirabilis	AR3022	SW+++	SW+++	-	R, T, two col. R
Pseudomonas	ATCC® 10145	unc., 0.2 mm	DZ, 0.5-1 mm	-	-

unc., uncolored ; Y, yellow ; W, white ; MB, metallic blue ; R, red ; DZ, dense zone ; col., colony ; size in mm ; T, trace ; SW, swarming ; AR, CHROMagar™ strain collection.

The chromogenic formula showed 100 % exclusivity for ten bacterial and fungal species which were either inhibited or showed a metallic blue counter-coloration.

2.2. Confirmation tests

Examination of the morphological appearance of *Mycoplasma* colonies. *Mycoplasma* colonies grown with a red coloration on CHROMagar™ Mycoplasma do conserve their characteristic fried-egg appearance which can be observed under a stereoscopic microscope (Figure 1).

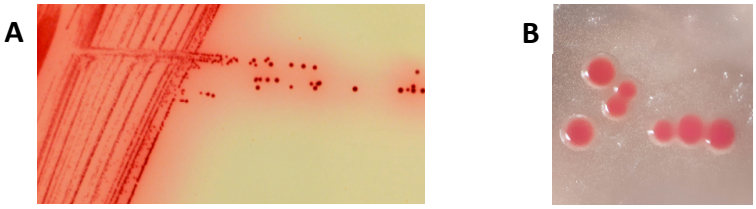


Fig. 1. With the naked eye (**A**) and stereomicroscopic (**B**) views of *M. bovis* colonies on CHROMagar™ Mycoplasma media after a 7-day incubation at 37 °C, under CO_2 atmosphere.

Sensitivity to digitonin of *Mycoplasma*. *Mycoplasma* bacteria require an external source of sterol for growth. The sterol requirement can be determined using an indirect method, the digitonin disk diffusion assay (Alluotto *et al*., 1970), which differentiates the sterol-requiring *Mycoplasma* species from the nonsterol-requiring species like *Acholeplasma* (Freundt *et al*., 1973).

M. bovis colonies (strains ATCC® 25025 and ATCC® 25523) grown 6 days at 37 °C, under CO_2 atmosphere, on CHROMagar™ Mycoplasma medium were sampled to evenly streaking them on a CHROMagar™ Mycoplasma plate surface. A red bacterial lawn can also be obtained on the medium plate surface by evenly streaking from a 3-day *Mycoplasma* PPLO Broth culture at 37 °C, under CO_2 atmosphere. A 1.5 % digitonin disk was placed in the center of the inoculated area.

After incubation at 37 °C, under CO_2 atmosphere during 3 days, clear inhibition zones around the digitonin disks indicated the sterol-requiring *Mycoplasma* feature (Figure 2).

According to the literature, when the growth inhibition zone is <3 mm or nonexistent, the assay is considered as negative, indicating that the strain belongs to the genus *Acholeplasma* (Boonyayatra *et al.*, 2012).

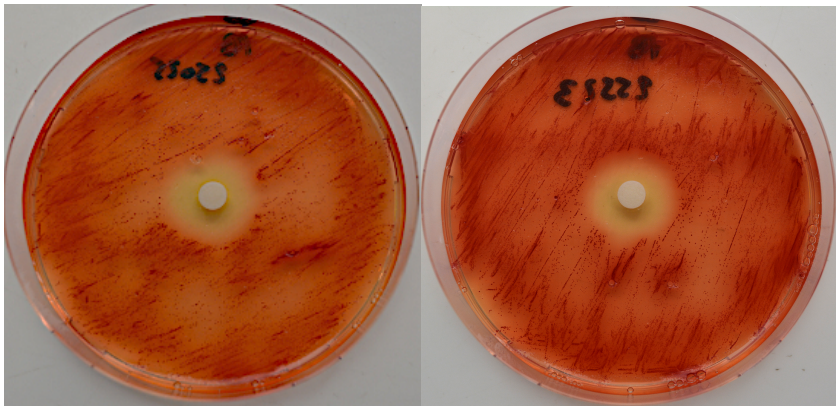


Fig. 2. Digitonin disk diffusion assay. Growth inhibition zones (diam. 2 cm) of *M. bovis* ATCC® 25025 and *M. bovis* ATCC® 25523 on CHROMagar™ Mycoplasma, after a 3-day incubation at 37 °C, under CO_2 atmosphere.

3. Medium performance with *Mycoplasma* strains

Culture medium plates were prepared in the laboratory using different batches of CHROMagar™ Mycoplasma base and CHROMagar™ Mycoplasma supplements, including different batches and suppliers of heat-inactivated horse serum. These pre poured medium were sent to different French departmental laboratories of analyses, including the Anses – Réseau Vigimyc. All the participating laboratories reported a good sensitivity and the widespread red coloration of the characteristic fried-egg colonies facilitated the detection of *Mycoplasma* strains on plates.

Laboratories in different countries also tested the chromogenic formula in its dehydrated form, using either their own horse serum employed in routine, or the lyophilized horse serum provided by CHROMagar™. The results obtained are shown in Table III.

Table III. Bacterial strains to evaluate sensitivity and specificity of CHROMagar™ Mycoplasma.

Laboratory, Country / Medium form	# of strains	Sensitivity	Specificity	Colony identification	Comments
Veterinary Medical Research Institute, Hungary / Dehydrated + tester serum	8 *Mycoplasma* strains from bovines	100 %	ND	8 ATCC strains used, no identification needed.	*Mycoplasma* spp. strains were also detected in red coloration
Kanto Chemical, Japan / Dehydrated + tester serum	8 *Mycoplasma* strains (5 of them from mastitis) 8 annex flora strains	100 %	75 %	PCR from colony grown on CHROMagar™ Mycoplasma	Incubation under microaerophilic atmosphere (CO_2 atmosphere is more often used)

ND, not determined

4. Medium performance with field samples

4.1. Detection of *Mycoplasma* species in cattle

Laboratories in different countries poured plates from dehydrated medium, using either their own horse serum employed in routine, and either liquid or lyophilized horse serum validated and provided by CHROMagar. The results obtained with cattle samples between July 2021 and January 2022 are shown below.

Table IV. Cattle samples tested on CHROMagar™ *Mycoplasma*.

Laboratory, Country / Medium form	# of samples	Sensitivity	Specificity	Colony ID	Comments
Royal GD, Netherlands / Dehydrated + liquid serum (CHROMagar)	26 bovine (pathologic) lung and lung lavage samples	82 %	100 %	ND	Use in micro aerophilic conditions Less annex flora compared to PPLO
Mediamage. Vetdiagnostix, South Africa / Dehydrated + tester serum & lyophilized serum (CHROMagar)	15 bovine lung samples	100 % (2 positives)	100 %	ND	-
APEK. Arbilim Biyoteknoloji, Türkiye / Dehydrated + tester serum	50 samples, bovine respiratory disease suspected calves	Absence of *M. bovis* in samples	Very little or no annex flora	PCR positive, *M*. spp	Viral transport medium w/o antibiotics
Arbilim Biyoteknoloji, Türkiye / Dehydrated + tester serum	28 nasopharyngeal swab samples	*Mycoplasma* like colonies (n=6)	Counter-colored colonies of non-targeted organisms	PCR, on going	Results to be confirmed
ARSIA asbl, Service de bactériologie, Belgium / Dehydrated + liquid serum (CHROMagar)	3 (ear swabs)	100 % (2 *M. bovis*)	100 %	Mass spectro metry	Directly from colony
	10 (lung samples)	100 % (2 *M. bovis*)	87,5 % (*M. arginini* was detected)		
	46 (nasal swabs)	95 %	81 % (5 *M.* spp. were detected)		
LABOCEA. Anatomie Pathologique Microbiologie Vétérinaire, France / Dehydrated + lyophilized serum (CHROMagar)	2 nasopharyngeal swab samples	100 % (1 sample with *M. bovis* and *M. bovirhinis*)	100 %	Mass spectro metry	Oxoid™ Mycoplasma Agar as reference. Lung samples without or counter-coloured annex flora. *M. arginini* grew white colonies
	5 bovine lung samples	100 % (1 sample with *M. bovis*)			
	1 bovine arthritis sample	100 % (*M. bovis*)			

ND, not determined

According to the laboratories results, CHROMagar™ Mycoplasma is a very useful tool to detect *Mycoplasma* species, notably *M. bovis* from cattle, testing respiratory tract swabs and respiratory organ specimens.

4.2. Detection of *Mycoplasma* species in milk samples

Laboratories in different countries poured plates from dehydrated medium, using their own horse serum employed in routine. The results obtained with bulk tank milk samples are shown below.

Table V. Milk samples tested on CHROMagar™ Mycoplasma.

Laboratory, Country / Medium form	# of milk samples	Sensitivity	Specificity	Colony identification	Comments
School of Veterinary Medicine and Animal Sciences. U. of São Paulo, Qualileite. Dept. de Nutrição e Produção Animal-VNP, Brazil / Dehydrated + tester serum	2	100 %	100 %	PCR from milk sample, 2 PCR + plated	*Hayflick* agar medium as reference, anaerobic conditions
BIO Diagnóstico Veterinario, Argentina / Dehydrated + tester serum	12	1 suspect *M. bovis* on both media	Typical colony morphology	Confirmed by PCR	*Hayflick* agar medium as reference.
Greenfields – dairy company, Indonesia / Dehydrated + tester serum	95	1 suspect but uncolored	Typical colony morphology	-	Oxoid™ Mycoplasma Agar as reference. Digitonin resistant = *Acholeplasma*

Although the number of true positive samples was low in these evaluations, CHROMagar™ Mycoplasma showed good performance with milk samples. Additional results from on-going studies are expected to strength the intended use to monitor the presence of *Mycoplasma* in bulk tank milk..

5. Conclusion

The performance of the CHROMagar™ Mycoplasma medium has been validated by a series of evaluations. These evaluations included inclusivity and exclusivity studies, as well as analyses of respiratory tract swabs, respiratory organ specimens, and bovine milk samples (on-going evaluation).

Parameter	**Performance of CHROMagar™ Mycoplasma**
Inclusivity	100 % with *Mycoplasma bovis*
Exclusivity	100 % with bacterial strains
Detection of *Mycoplasma bovis*	Sensitivity 100 %
Morphological appearance of colonies	Characteristic fried-egg appearance with red coloration
Confirmation test on colony	Sensitivity to digitonin differentiates from *Acholeplasma*
Microbial identification directly from colony	By PCR or mass spectrometry

In appropriate storage, the shelf life of the powder base and supplement S1 and S2 is 24 months. Supplement S3 shelf life is 18 months at 2-8 °C. Advantages in the sensitivity and visualisation of *Mycoplasma* colonies on CHROMagar™ Mycoplasma plates compared to Oxoid™ Mycoplasma Agar plates, were reported from laboratories. Good preparation of the medium can be verified by isolating recommended ATCC strains for Quality Control testing (see annex 1).

Thallous acetate is a dangerous poison used in standard *Mycoplasma* selective media. CHROMagar™ Mycoplasma does not contain thallous acetate to avoid this hazard. For security, other agents warrant the selectivity of CHROMagar™ Mycoplasma medium.

The results on CHROMagar™ Mycoplasma plates are easy-to-read with the naked eye, a confirmation can be carried out by examination of the morphological appearance of suspected colonies under a stereoscopic microscope, by a digitonin disk diffusion assay, PCR or by mass spectrometry.

This medium has very good performances but one limitation can be pointed out:

- Colonies of *M. bovis* are detected with the same chromogenic features and morphological appearance of other *Mycoplasma* species.

Hugo CRUZ RAMOS, PhD.

Scientific Expert

6. Literature

1) Alluotto, B. B., Wittler, R. G., Williams, C. O., and Faber, J. E. 1970. Standardized bacteriologic techniques for the characterization of *Mycoplasma* species. *Int. J. Syst. Bacteriol.* **20:** 35-58.

2) Freundt, E. A., Andrews, B. E., Erno, H., Kunze, M. and Black, F. T. 1973 The sensitivity of *Mycoplasmatales* to sodium-polyanetholsulfonate and digitonin. *Zentralbl. Bakteriol. Parasitenkd. Infektioskr. Hyg. Abt*. 1 Orig. Reihe A. **225:** 104-112.

3) Boonyayatra, S., Fox , L. K., Gay, J. M., Sawant, A., and Besser, T. E. 2012. Discrimination between *Mycoplasma* and *Acholeplasma* species of bovine origin using digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase chain reaction. *J. Vet. Diagn. Invest*. **24:** 7-13.

Annexes

Annex 1. Website information about CHROMagar™ Mycoplasma.

Veterinary Microbiology

Veterinary Microbiology is concerned by bacterial infections in domesticated vertebrate animals that supply food or companionship. Among the current critical challenges, veterinarians need to obtain precise species identification of organisms in a timely manner. CHROMagar™ has elaborated a range of tools, based on our pioneering technology of chromogenic culture media for the veterinary field.

INOCULATION

Related samples are inoculated by direct streaking on the plate.

- If the agar plate has been refrigerated, allow to warm to room temperature before inoculation.
- Streak sample onto plate.
- Incubate at 37 °C for 3-7 days in a CO_2 atmosphere.

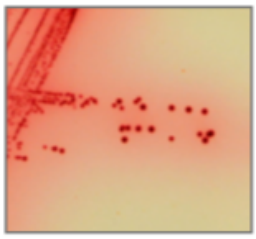
Typical samples
e.g. nasal swab, broncho-alveolar lavage samples

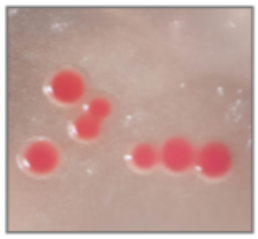
INTERPRETATION

Qualitative reading and interpretation of the Petri dishes.

Microorganism	Typical colony appearance
Mycoplasma bovis	→ red with a fried egg morphology
Gram (+) bacteria	→ mostly inhibited
Gram (-) bacteria	→ inhibited
Yeast and mould	→ inhibited

Typical colony appearance





The pictures shown are not contractual.

LIMITATIONS AND COMPLEMENTARY TESTS

- Some particular resistant strains from annexe flora may grow as metallic blue colonies.
- Some strains of *Mycoplasma* spp. may appear red with a fried egg morphology.
- The fried egg morphology is visible under a microscope.

QUALITY CONTROL

Please perform Quality Control according to the use of the medium and the local QC regulations and norms. Good preparation of the medium can be tested, isolating the following ATCC strains:

Microorganism	Typical colony appearance
Mycoplasma bovis ATCC® 25523	→ red with a fried egg morphology
Mycoplasma bovis ATCC® 25025	→ red with a fried egg morphology
Staphylococcus aureus ATCC® 25923	→ inhibited
Klebsiella ATCC® 13883	→ inhibited

Pack Size		Ordering Reference		Base (B)		Supplement (S1)		Supplement (S2)		Supplement (S3)
1000 mL	= 125 Tests of 8 mL	MB641	=	MB641(B) Weight: 43.8 g	+	MB641(S1) Weight: 1 g	+	MB641(S2) Weight: 1 g	+	MB641(S3) 10 vials (1 vial for 100 mL of final media)