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Short communication

Non-specific, agar medium-related peaks can result in false positive *Mycoplasma alkalescens* and *Mycoplasma arginini* identification by MALDI-TOF MS

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Abstract

MALDI-TOF MS is a fast and accurate tool to identify *Mycoplasma* species in liquid media. However, when trying to identify presumptive *Mycoplasma bovis* (*M. bovis*) colonies from solid medium (the “direct transfer method”) a surprisingly high occurrence of *M. arginini* and *M. alkalescens* identification was observed. It was hypothesized that agar medium components are associated with false positive identification with *Mycoplasma* spp., as *M. bovis* colonies are very small and grow into the agar. The objective of this study was to determine whether complete modified pleuropneumonia-like organism (PPLO) agar (supplemented with horse serum, sodium pyruvate, technical yeast extract, ampicillin sodium salt and colistin) and the separate components, result in false identification as *Mycoplasma* spp. by MALDI-TOF MS. A total of 100 samples were examined, of which thirty-three percent of the modified PPLO agar spots were identified as *M. alkalescens* (16%) and *M. arginini* (17%)), albeit with relatively low score values (< 1.85). No false identification of *M.*

bovis was obtained. Several medium components (unsupplemented PPLO agar, horse serum and colistin) resulted in spectra with peaks showing close matches with peaks present in the *M. alkalescens* and *M. arginini* database spectra. This study shows that the direct transfer method should be interpreted with caution, and one should strive to pick as little as possible agar when sampling *Mycoplasma*-like colonies from solid medium containing PPLO agar, horse serum and/or colistin.

Keywords: Colistin, Direct transfer method, Horse serum, PPLO agar

Cattle are subject to diseases associated with multiple *Mycoplasma* species of which *Mycoplasma bovis* (*M. bovis*) is widely accepted as a primary pathogen (Maunsell and Donovan, 2009; Maunsell et al., 2011; Haapala et al., 2019; Oliveira et al., 2019). *M. bovis* is causing pneumonia, arthritis and otitis in calves (Maunsell and Donovan 2009), but also arthritis and mastitis in adult cattle (Maunsell et al., 2011). For other *Mycoplasma* spp. literature is more contradictory. *M. alkalescens* has been associated with arthritis in calves (Bennett et al., 1978; Whithear et al., 1983) and mastitis in cattle (Jasper, 1982) in the past, whereas *M. arginini* has been described as an opportunistic pathogen (Shahriar et al., 2002; Thomas et al., 2002). *M. canadense* causes mastitis, and has been isolated from the lung (Jasper, 1977; Ball and Mackie, 1986). The pathogenicity of *M. bovirhinis* is questionable, as it is isolated from healthy and pneumonic lungs (Thomas et al., 2002). Also *M. dispar* was long thought to be pathogenic, but seems currently abundant in healthy animals (Timset et al., 2018). The impact of former *Mycoplasma* species in respiratory diseases is unknown (Thomas et al., 2002; Ayling et al., 2004), and may represent a part of the natural microbiome of the bovine respiratory tract (Klima et al., 2019; McMullen et al., 2019).

Rapid diagnosis and differentiation between *Mycoplasma* spp. is important, as *M. bovis* spreads easily, can be resistant against a lot of antimicrobials (both intrinsically and acquired)

and is difficult to eradicate (Gautier-Bouchardon, 2018; Maunsell and Chase, 2019). Isolation of *M. bovis* from clinical samples requires nutrient rich media, often based on pleuropneumonia-like organisms (PPLO) agar, supplemented with animal serum, yeast extract and various antibiotics to prevent *M. bovis* of being overgrown by other bacteria. As for many other bacterial species, MALDI-TOF MS is a fast and accurate tool to identify *Mycoplasma* species, from a liquid medium (Pereyre et al., 2013; Randall et al., 2015; Spersger et al., 2019). However, most commonly, bacteria are cultured on solid medium and identified by MALDI-TOF MS by transferring colonies from the agar to the MALDI-TOF MS target plate (“direct transfer method”)(Bizzini and Greub, 2010). In contrast to other bacteria, this direct transfer method appears to be unreliable for *M. bovis* identification (Pereyre et al., 2013). Culturing in broth and subsequent protein extraction are still necessary to achieve consistently high identification scores in *M. bovis* (Pereyre et al., 2013; Bokma et al., 2019). During attempts to identify presumptive *Mycoplasma*-like colonies obtained from broncho-alveolar lavages and pure *M. bovis* cultures with the direct transfer method, we observed a surprisingly high occurrence of *M. arginini* and *M. alkalescens* identifications (data not shown). We noticed this especially in cases with very few and/or small colonies, suggesting that medium components might play a role. Therefore, the objective of this study was to explore whether agar medium components are associated with false positive *Mycoplasma* spp. identification using MALDI-TOF MS.

Five modified PPLO agar plates were produced in-house and contained PPLO agar (Difco™) supplemented with 25% inactivated horse serum (Gibco™), 0.5% sodium pyruvate (ReagentPlus, Sigma-Aldrich®), 0.7% technical yeast extract (Bacto™), 520 µg/mL ampicillin sodium salt (Sigma-Aldrich®) and 670 I.E./mL colistin (Colistine sulfate, VMD). The non-inoculated modified PPLO agar plates were incubated for 4 days (37°C, 5% CO₂) and subsequently spotted 20 times per plate on a polished steel BC target plate with a

toothpick using the direct transfer method. Additionally, we spotted the single components (PPLO agar, horse serum, yeast extract, pyruvate, ampicillin, and colistin in duplicate to obtain reference spectra for the respective components. For colistin, both the injectable colistin sulfate (VMD) and a colistin sulfate standard (Sigma-Aldrich®) dissolved in distilled water to a concentration of 1 MIO I.E./ml, were included. Spotted samples were covered with one μ l matrix (α -cyano-4-hydroxy-cinnamic acid in 50% acetonitrile - 47.5% water - 2.5% trifluoroacetic acid; Bruker Daltonics, Bremen) and processed with an Autoflex III (Bruker Daltonics, Bremen). Successful *Mycoplasma* species identification at species level was considered for logarithmic score values ≥ 1.7 , as suggested earlier (Pereyre et al., 2013; Randall et al., 2015; Spengler et al., 2019). The standard Bruker library (server version 4.1.80 PYTH) was extended with four main spectrum profiles of recent *M. bovis* strains as described in Bokma et al. (2019). Peaks and spectra were analyzed with Flex-analysis 3.4 software after smoothing and baselining with the MBT standard method and MALDI Biotyper Compass Explorer 4.1 (Bruker Daltonics, Bremen). Bruker Bacterial Test Standard (Bruker Daltonics, Bremen) was included in every run as quality control.

Thirty-three percent of the 100 spots with pure modified PPLO agar were as best match identified as *Mycoplasma* species (16% *M. alkalescens*, 17% *M. arginini*), albeit with relatively low score values (1.70 – 1.82)(Supplement 1). In 7 out of 100 spots, both *M. alkalescens* and *M. arginini* were identified. We also observed a discrepancy between plates, as 7 out of 20 spots from plate 1 were false positive, whereas 7/20, 3/20, 5/20 and 12/20 were false positive from plate 2-5, respectively (Supplement 1). This variation might have been caused by slightly different concentrations of the agar components after solidifying the agar or the quantity of agar that was spotted on the target plate. Nonetheless, the results show that false positive identification of *M. alkalescens* and *M. arginini* was possible from every plate. No false positive identification of *M. bovis* was obtained. The latter is in line with a previous

study where false identification of *M. arginini* was detected with the direct transfer method from CHROMagarTM Orientation agar. However, in the previous study, *M. alkalescens* was never identified with relevant identification scores ≥ 1.7 (Lagacé-Wiens et al., 2019).

In order to gain information on which medium components might evoke the false positive results, the spectra of specific medium components were compared with peaks showing similar m/z [Da] values in the *M. alkalescens* 22B10 VLW and *M. arginini* 7SR10 VLW main spectrum profiles (MSP) in Bruker database records as these strains showed the highest matching identification scores with modified PPLO. As described in the metadata of the library, both MSPs of these *Mycoplasma* spp. were based on protein extraction after incubation for 3-5 days in Eaton's broth. Additionally, peaks of the entire modified PPLO agar were compared with *M. alkalescens* 22B10 VLW (Figure 1A) and *M. arginini* 7SR10 VLW (Figure 1B) MSPs.

When analyzing the separate components, only PPLO agar, horse serum and colistin (both injectable and pure form) resulted in meaningful MALDI-TOF spectra. The components together showed 39 (close) matching peaks with the investigated *Mycoplasma* spp. (7 for PPLO agar, 3 for horse serum and 32 for colistin) based on m/z [Da] values between 3000 – 15.000 (as used for identification), as shown in Table 1. Using Compass Explorer software, peaks of matched spectra are depicted as green (peak match within experimental error range), orange (close peak match, but not within experimental error range) or red (no matching peaks), as defined by the manufacturer. When comparing modified PPLO agar with *M. alkalescens* 22B10 VLW, 14 peaks matched within experimental error range (green) and 20 were close peak matches (orange), for *M. arginini* 7SR10 this was 12 and 21, respectively. These overlapping peaks might vary sometimes due to small variations in spectra caused by the sample quality or when intensity of a peak is on the borderline of detection.

No false positive *M. bovis* identification was observed with the current protocol. Nevertheless, false positive *Mycoplasma* spp. results could lead to unnecessary antimicrobial use in animals, risking increased antimicrobial resistance selection (Chantziaras et al., 2014; Tang et al., 2017).

This study showed that to a large extent, spectrum peaks originating from PPLO agar, horse serum and colistin can result in false positive identification of *M. alkalescens* and *M. arginini*. This is in contrast to an earlier study where it was hypothesized, though not investigated, that false positive *Mycoplasma* identifications were a result of yeast extract or peptone related peaks (Lagacé-Wiens et al., 2019). Nevertheless, not all PPLO agar, colistin and horse serum related peaks could explain the overlapping peaks between modified PPLO agar and the *Mycoplasma* spp. Therefore, it could be possible that yeast extract or peptones show indeed peaks, but intensity might be too low for MALDI-TOF MS to generate a distinguishable spectrum when investigating the separate agents.

One of the reasons that *M. alkalescens* and *M. arginini* were identified, while other *Mycoplasma* spp. cultivated in the same medium were not, could be the use of an imperfect protocol of generating these specific MSPs. For example, the use of *Mycoplasma* cultures with too low bacterial biomass, due to either concentration limits or phase requirements in broth culture or inefficient washing steps, may have contributed to low quality protein extractions. In addition, it was recently described that ethanol protein precipitation for *Mycoplasma* spp. may lead to unstable quality of spectra (Spergser et al., 2019). It is very probable that ethanol was used when obtaining older MSPs, even though it is not clear whether this could have contributed to differences between species. Finally, mere chance may have resulted in the fact that some *M. alkalescens* and *M. arginini* peptides/proteins share molecular masses with certain medium components, while this might not be the case for other *Mycoplasma* spp. Replacing the current library MSPs by new ones obtained with another

protocol (for example including efficient washing steps and without ethanol precipitation) might be effective. Alas, the present software does not provide the liberty to replace MSPs.

As alternative technique to reduce the amount of agar attached to the *Mycoplasma* colonies, the authors explored the use of a plastic loop instead of a toothpick. Unfortunately, it was almost impossible to gather enough material for reliable peak recognition with MALDI-TOF MS. Currently, the best option to identify *Mycoplasma* spp. with MALDI-TOF MS seems to be from broth cultures (Spergser et al., 2019., Bokma et al., 2019). Misidentifications probably do not occur starting from broth cultures, as they are usually washed and therefore medium-related proteins are removed prior to protein extraction. This results in clearer bacterial peaks and less background noise. Also *Mycoplasma* spp. concentrations are likely higher in liquid medium compared with what can be obtained from picking one colony from an agar plate. Therefore, the balance between bacterial matter and medium components is expected to be better starting from broth cultures.

The present study shows that MALDI-TOF identification results using the direct transfer method from solid media for *Mycoplasma* spp., which allows identification of bacteria within several minutes and with minimal labor, should be interpreted cautiously in diagnostic laboratories. One should strive to pick enough bacterial material with as little as possible agar, especially when colonies are small (higher risk for including agar) and when PPLO agar, colistin and/or horse serum is added to the medium. This should not only be kept in mind for identification with MALDI-TOF MS, but also when generating new MSP's to include in the library.

Declarations of Competing Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure 1A. Spectra comparison of modified PPLO agar (containing horse serum, yeast extract, pyruvate, ampicillin and colistin)(bars above the x-axis) with *Mycoplasma alkalescens* 22B10 VLW (blue bars) in MALDI Biotyper Compass Explorer 4.1 (Bruker Daltonics, Bremen). Peaks of matched spectra are depicted as green (peak match within experimental error range), orange (close peak match, but not within experimental error range) or red (no matching peaks).

Figure 1B. Spectra comparison of modified PPLO agar (containing horse serum, yeast extract, pyruvate, ampicillin and colistin)(bars above the x-axis) with *Mycoplasma arginini* 7SR10 VLW (blue bars) in MALDI Biotyper Compass Explorer 4.1 (Bruker Daltonics, Bremen). Peaks of matched spectra are depicted as green (peak match within experimental error range), orange (no peak match, but not within experimental error range) or red (no matching peaks).

Table 1. Overview of spectra (m/z)[Da] showing peaks of horse serum and colistin (injectable and standard form) similar to *M. alkalescens* 22B10 VLW and *M. arginini* 7SR10 VLW (within a deviation range of 10 Da)

PPLO agar	<i>M.</i> <i>alkalescens</i> 22B10 VLW	<i>M.</i> <i>arginini</i> 7SR10 VLW	Colistin sulfate	<i>M.</i> <i>alkalescens</i> 22B10 VLW	<i>M.</i> <i>arginini</i> 7SR10 VLW	Horse serum	<i>M.</i> <i>alkalescens</i> 22B10 VLW	<i>M.</i> <i>arginini</i> 7SR10 VLW
3024	3031	3028	3024		3028			
			3036	3031				
			3072	3073	3071			
			3087	3091	3090			
			3106	3113	3111			
			3128	3132	3129			
			3148	3151	3148			
			3170	3168				
			3187	3183				
			3195	3200	3195			
			3290	3288				
3319	3326	3324						
3334		3334						
			3412	3416	3416			
			3451	3448	3445			
			3460	3465	3462			
			3488	3489	3487			
			3507		3511			
			3679	3679	3678			
			3717		3717			
			3745	3745				
3979		3979	3980		3979			
			4010	4009				
			4043		4046			
			4065	4061				
			4085	4078	4079			
			4099	4097	4097			
			4114	4118	4115			
			4120	1118				
			4177	4176	4175			
			4178	4176				
			4232	4232	4232			
4300	4304	4305	4304	4304	4305			
			4470	4468	4467			
						4715	4717	4718
						5542	5544	5545
						5580	5574	
6804		6805						
6821	6815							

All peaks were extracted from the mass list generated by Flex-analysis 3.4 software after smoothing and baselining the initial spectra generated by MALDI-TOF MS (Bruker Daltonics, Bremen)

Highlights

- Modified PPLO agar has interfering peaks with *Mycoplasma* spp. identification
- PPLO agar, colistin and horse serum show overlapping peaks with *Mycoplasma* spp.
- Direct transfer identification with MALDI-TOF MS could result in false positives
- One should strive to pick enough bacterial material with as little as possible agar
- Identification of *Mycoplasma* spp. from agar should be interpreted cautiously