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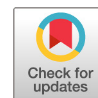

Comparison of Five Commercial Molecular Assays for *Mycoplasma* Testing of Cellular Therapy Products

Arthur H. Totten,[a*] Anna Julia Adams,[b] Hyunmi K. Halas,[c] James E. T. Gebo,[b] Amanda D. East,[b] Anna F. Lau[b]

[a]Clinical Microbiology Service, Department of Laboratory Medicine, National Institutes of Health, Bethesda, Maryland, USA
[b]Sterility Testing Service, Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA
[c]Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Arthur H. Totten and Anna Julia Adams contributed equally to this article. Author order was determined by seniority.

ABSTRACT Testing of cellular therapy products for *Mycoplasma* is a regulatory requirement by the United States Food and Drug Administration (FDA) to ensure the sterility and safety of the product prior to release for patient infusion. The risk of *Mycoplasma* contamination in cell culture is high. Gold standard testing follows USP <63> which requires a 28-day agar and broth cultivation method that is impractical for short shelf-life biologics. Several commercial molecular platforms have been marketed for faster raw material and product release testing; however, little performance data are available in the literature. In this study, we performed a proof-of-principle analysis to evaluate the performance of five commercial molecular assays, including the MycoSEQ *Mycoplasma* detection kit (Life Technologies), the MycoTOOL *Mycoplasma* real-time detection kit (Roche), the VenorGEM qOneStep kit (Minerva Biolabs), the ATCC universal *Mycoplasma* detection kit, and the Biofire *Mycoplasma* assay (bioMérieux Industry) using 10 cultured *Mollicutes* spp., with each at four log-fold dilutions (1,000 CFU/mL to 1 CFU/mL) in biological duplicates with three replicates per condition ($n = 6$) to assess limit of detection (LOD) and repeatability. Additional testing was performed in the presence of tumor infiltrating lymphocytes (TILs). Based on LOD alone, the Biofire *Mycoplasma* assay was most sensitive followed by the MycoSEQ and MycoTOOL which were comparable. We showed that not all assays were capable of meeting the $\leq$10 CFU/mL LOD to replace culture-based methods according to European and Japanese pharmacopeia standards. No assay interference was observed when testing in the presence of TILs.

KEYWORDS *Mollicutes*, cellular therapy, biologics, sterility, *Mycoplasma*, *Acholeplasma*, *Spiroplasma*

Mollicutes (or interchangeably, *Mycoplasma*) are cell-wall-lacking prokaryotic organisms of medical and industrial microbiologic importance. Unlike other bacteria, *Mollicutes* cannot be observed on a Gram stain as they lack peptidoglycan needed to retain crystal violet. Additionally, they are exceedingly small compared with "typical" prokaryotes (0.1 to 0.2 μm in size). Culture requires the use of highly enriched media and extended incubation times (>5 days) because they are auxotrophic for many biosynthetic pathways. These traits, combined with the uncultivable nature of certain *Mycoplasma* spp., makes their detection a complex and costly endeavor.

In the United States, sterility testing for *Mycoplasma* in cellular and gene therapy products is a regulatory requirement to ensure the safety of biologics for human use as prescribed by the United States Food and Drug Administration (FDA) in the Code of Federal Regulations. The FDA accepts testing standards published in the United States Pharmacopeia (USP). Other international pharmacopeia standards, such as the European Pharmacopoeia (Ph. Eur.) and the Japanese Pharmacopeia (JP), may also be applicable

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Address correspondence to Anna F. Lau, anna.lau@nih.gov.

*Present address: Arthur H. Totten, Department of Pathology, Stony Brook University Hospital, Stony Brook Medicine, Stony Brook, New York, USA.

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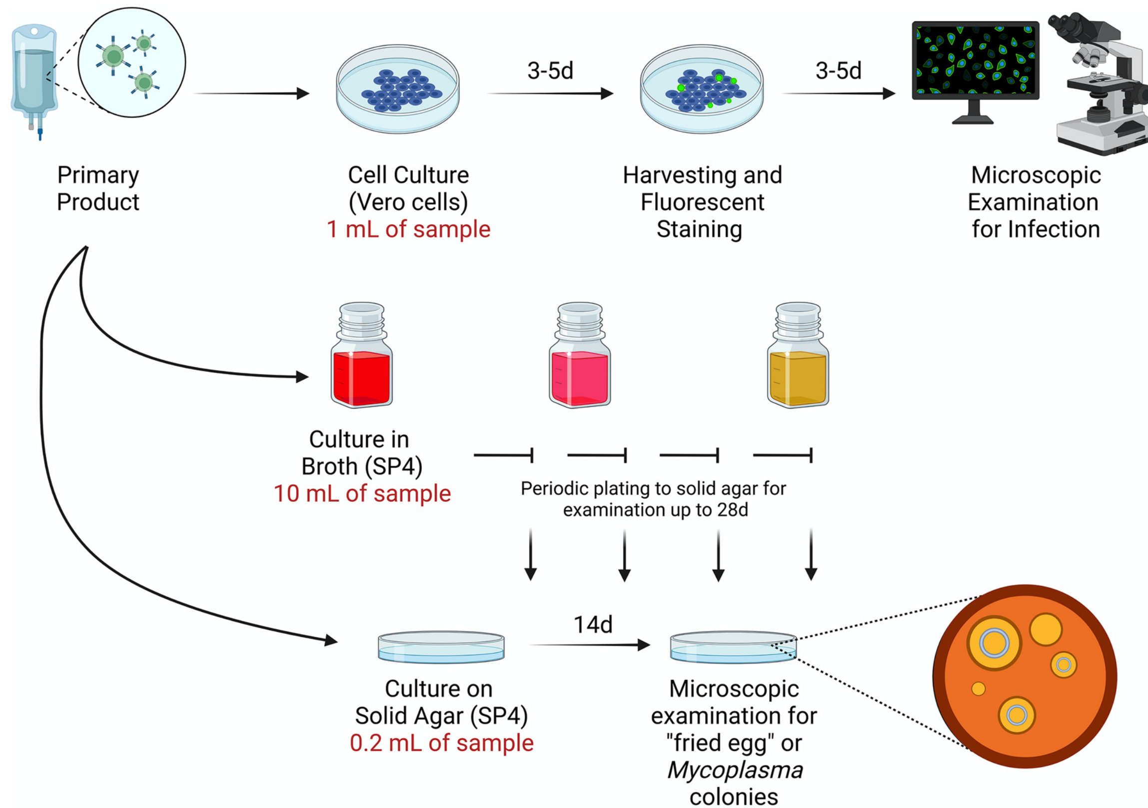

FIG 1 Compendial testing requirements for *Mycoplasma* analysis on cell and gene therapy products. Primary product is subjected to analysis by (1) culture on cells (commonly Vero cells) with analysis via fluorescence microscopy for *Mycoplasma* contamination, (2) culture of primary product in broth medium with subsequent subculture onto permissive solid agar, and (3) culture directly onto solid agar incubated for 14 days prior to examination for distinctive *Mycoplasma* colonies. Figure created with BioRender.com.

depending on geographical product distribution. Gold standard testing for *Mycoplasma* is defined in USP <63> (1), Ph. Eur. chapter 2.6.7 (2), and JP XVIII (3). Briefly, gold standard testing requires the culturing of *Mycoplasma* using a combination of three methods, including broth culture, culture on permissive solid agar, and fluorescent antibody detection of noncultivable organisms grown on a cellular monolayer (Fig. 1). The long turnaround time (28 days) and large product volume (~15 mL) required for compendial testing are incompatible with biological products that have a short shelf life (48 to 72 h).

The rapid emergence of cellular therapies over the last 2 decades has led to revolutionary treatments for a myriad of human diseases that had been previously thought untreatable (4). Historically, contamination of cell culture products with *Mollicutes* was common, with some reports estimating the presence of *Mycoplasma* in 15 to 30% of American Type Culture Collection (ATCC) cell lines and approximately 11% of all RNA sequencing (RNA-seq) data sets (Table 1) (5–7). Therefore, the detection of *Mycoplasma* in vaccines and cellular products remains a safety concern, as these organisms can go undetected easily in raw materials and cell culture due to the absence of obvious morphological/cytological changes. Molecular assays have become favorable alternatives to counter the limitations of the culture-based pharmacopeia standards. The European Pharmacopoeia and Japanese Pharmacopeia both recognize molecular testing for *Mycoplasma* as an acceptable test method if the limit of detection (LOD) is ≤10 CFU/mL compared with agar and broth culture and ≤100 CFU/mL compared with the indicator cell method (2, 3). In the United States, however, any non-USP <63> method is considered an alternative method that requires rigorous end-user validation to meet the equivalency specifications as outlined in USP <1223> (8), despite premarket and beta-testing studies that may be available by the vendor to the FDA in the form of a

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TABLE 1 *Mollicutes* type strains utilized within this study

Organism	USP <63>	Ph. Eur. 2.6.7.	JP XVIII	Reported cell culture contaminant[a]	Suitability for use
Acholeplasma laidlawii PG8 (ATCC 23206)	X[e]	X	X	Yes	Vaccines and/or cell-derived materials/cultures for human and veterinary use when an antibiotic has been used during production
Mycoplasma arginini G230 (ATCC 23838)		X	X	Yes	Core challenge organism in QCMD proficiency testing surveys 2018–2021
Mycoplasma fermentans PG18 (ATCC 19989)	X	X	X	Yes	Vaccines or cell banks for human use
Mycoplasma gallisepticum PG31 (ATCC 19610)	X	X		No	When avian material has been used during production or when the vaccine or cell culture is intended for use in poultry
Mycoplasma hominis PG21 (ATCC 23114)				Yes	Clinical relevance
Mycoplasma hyorhinis PG42 (ATCC 17981)	X	X	X	Yes	Nonavian veterinary vaccines or cell cultures
Mycoplasma orale CH19299 (ATCC 23714)[b]	X	X	X	Yes	Vaccines for human and veterinary use
Mycoplasma pneumoniae FH (ATCC 15531)	X	X	X	No	Vaccines or cell banks for human use
Mycoplasma pulmonis PG34 (ATCC 19612)				No	Core challenge organism in QCMD proficiency testing surveys 2018–2021
Mycoplasma salivarium PG20 (ATCC 23064)			X	Yes	Core challenge organism in QCMD proficiency testing surveys 2018–2021
Mycoplasma synoviae WVU 1853 (ATCC 25204)[b]	X	X	X	No	When avian material has been used during production or when the vaccine or cell bank is intended for use in poultry
Spiroplasma ixodetis Y32 (ATCC 33835)[c]	X[d]	X[d]	X[d]	No	Use of or exposure to insect or plant material during production

[a]Summarized from references 5–7.
[b]*M. orale* and *M. synoviae* failed to grow on culture and were excluded from further analysis.
[c]*S. ixodetis* was cultured in lieu of *S. citri* due to the inability to purchase the latter organism. Both organisms are genetically similar and have compatible growth requirements.
[d]*S. citri* ATCC 29747 is the listed reference organism in USP <63>, Ph. Eur. 2.6.7., and JP XVIII.
[e]X indicates that the organism is listed as an indicator organism in the respective pharmacopeia document.

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Drug Master File. And, unlike clinical *in vitro* diagnostic (IVD) assays, FDA 510(k) clearance is not an option for assays used for cGMP product release (9).

To date, several assays have been marketed for the detection of *Mollicutes* in cellular therapy products; however, little performance data are available in the literature. In this study, we conducted a proof-of-principle study to compare the performance of the following five commercially available assays marketed in the United States for *Mycoplasma* testing: the MycoSEQ *Mycoplasma* detection kit (Life Technologies), the MycoTOOL *Mycoplasma* real-time detection kit (Roche), the VenorGEM qOneStep kit (Minerva Biolabs), the ATCC universal *Mycoplasma* detection kit (American Type Culture Collection), and the Biofire *Mycoplasma* assay (bioMérieux Industry). Testing was performed using 10 *Mollicutes* type strains cultured as per the harmonized compendial methods to verify the inoculum concentration (Fig. 1). Testing was performed at log-fold dilutions and in replicates by two different analysts to assess for the limit of detection and repeatability of each platform. Additionally, three clinically relevant *Mollicutes* type strains were tested in the presence of tumor infiltrating lymphocytes (TILs) to evaluate whether this product matrix would lead to potential assay inhibition for the detection of low-level contaminants. This proof-of-principle study provides a comprehensive insight into the analytical performance of a wide variety of platforms for *Mycoplasma* product release testing from an end-user laboratory perspective.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains (Table 1) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and stored as per manufacturer's instructions prior to culture. Type strains were selected based on quality-control strains listed in international pharmacopeia standards (Table 1), reported cell culture contaminants (5–7), and organisms that have been frequently associated with human or avian origin. Isolates were initially cultured in Hayflick's broth and Hayflick's agar (Hardy Diagnostics, Santa Maria, CA). For *Mycoplasma* species, broth was inoculated at 10^{-1} to 10^{-3} (vol/vol) and incubated at 37°C until color change indicated metabolic growth (orange-yellow for glucose metabolizers and red-purple for arginine metabolizers). Agar was inoculated, wrapped in parafilm (Thermo Fisher Scientific, Waltham, MA), and incubated at 35 to 37°C with 5% CO_2 until colonies were visible under a dissection scope (×20 magnification). Subsequent subcultures were expanded and passaged as described above with no more than 15 passages from initial inoculation as per USP <63> requirements. For strains that failed to grow on Hayflick's broth or agar (*M. hyorhinis*, *M. salivarium*, *M. synoviae*, *M. orale*, *M. pulmonis*, and *M. pneumoniae*), SP4 medium and agar with arginine (Hardy Diagnostics, Santa Maria, CA) were utilized using the incubation conditions described above. For *Spiroplasma ixodetis*, cultures were incubated at 30°C (non-CO_2) utilizing SP4 broth and agar with arginine until orange-yellow color change and colony growth were observed.

When ~30 mL of culture volume had been obtained, sterile glycerol (Sigma-Aldrich, St. Louis, MO) was added at a 10% (vol/vol) concentration, and stocks were aliquoted into 0.5-mL volumes. Stocks were frozen at −80°C for further study.

Stock concentration and limit of detection quantitation. Frozen stocks of organisms were thawed on ice for determination of CFU per mL (CFU/mL), as well as color change units (CCU) as described previously (10, 11). Briefly, organism stocks were diluted, in duplicate, by 1:10 from 10^{-1} to 10^{-10} in either Hayflick or SP4 medium. For each dilution, 20 μL was cultured onto solid agar. Plates were sealed with parafilm wrap (Thermo Fisher Scientific) and incubated at 35 to 37°C with 5% CO_2. Tubes used for serial dilution were incubated at 37°C without CO_2 for 1 to 2 weeks. CCU were determined by the terminal dilution that indicated metabolic growth. Colonies were enumerated (between 30 and 300) for a single dilution. The average of two independent dilution sets was used to determine a final average CFU/mL. This CFU/mL was correlated with CCU as an internal control for organism titer within stock vials at –80°C.

Once the CFU/mL had been determined for each organism, stocks were diluted to 10^5 CFU/mL in growth medium (Hayflicks or SP4) for LOD studies. Further dilutions were performed using RPMI 1640 medium (Sigma-Aldrich) to minimize the presence of inhibitory substances (i.e., exogenous DNA and serum present in growth medium) prior to extraction. Final concentrations of 10^3, 10^2, 10^1, and 10^0 CFU/mL were completed for each strain, and all bacterial stocks were frozen in 300-μL aliquots at −80°C until the time of extraction. Dilutions were carried out in biological duplicate with three replicates per condition (n = 6) to account for repeatability studies. In addition, serial dilutions in SP4 or Hayflicks medium were performed, and 0.2 mL of each concentrate was cultured onto solid agar to ensure organism viability and to verify CFU/mL.

Due to reagent costs of the Biofire *Mycoplasma* assay, triplicate testing (rather than sextuplicate) was performed for each condition tested. Separate aliquots of thawed organism-dilution combinations were utilized for the Biofire *Mycoplasma* assay as extraction and amplification were performed in the pouch as per the manufacturer's instructions. All other assays were tested using a single eluate per organism-dilution combination to minimize potential extraction variability. Concentrations ranging between 10^2, 10^1, 10^0, and 10^{-1} CFU/mL were tested for initial evaluation and assay comparison.

Determination of the LOD for each test platform and organism was defined as the terminal dilution in which all replicates were detected.

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DNA extraction and PCR conditions. DNA was extracted using the PrepSEQ express nucleic acid extraction kit (Applied Biosystems, San Francisco, CA). DNA was stored at −20°C prior to downstream testing.

All PCR assays were performed following manufacturer's instructions. The ABI 7500 fast real-time PCR system was used for the MycoSEQ *Mycoplasma* detection kit (Thermo Fisher Scientific), VenorGEM qOneStep assay (Minerva Biolabs, Inc., Skillman, NJ), and the MycoTOOL *Mycoplasma* real-time PCR kit (Roche CustomBiotech, Basel, Switzerland). An analysis of the MycoSEQ *Mycoplasma* detection kit was performed using the AccuSEQ real-time detection software v.2.0 (Thermo Fisher Scientific). Run files for the VenorGEM qOneStep assay and the MycoTOOL *Mycoplasma* real-time PCR kit were uploaded to the Thermo Fisher connect platform and analyzed using the cloud-based relative quantitation analysis module with default analysis group settings for cycle threshold (C_T) value determination. C_T values were plotted to show dynamic ranges of each assay and analyte. Because the MycoTOOL kit was performed on an ABI 7500 fast real-time PCR system rather than a LightCycler (Roche), 18 cycles were added to the terminal C_T value following technical advice provided by the manufacturer to account for touch-down PCR steps.

Gel electrophoresis for the ATCC universal *Mycoplasma* detection kit was performed using the E-Gel 1.2% with SYBR safe and E-gel simple runner system (Invitrogen, Thermo Fisher Scientific). Amplicon bands were visualized using the GelDoc Go imaging system and Image Touch Lab Software (Bio-Rad Laboratories) at 5-s exposure with UV light.

The Biofire *Mycoplasma* assay (bioMérieux Industry) was performed following the manufacturer's instructions using a 0.2-mL sample volume. Testing was performed on the FilmArray 2.0 industry system intended for the pharma industry *Mycoplasma* panels. Note, this platform is different from the instrument and software available for clinical IVD assays, such as the respiratory, gastrointestinal, blood culture, and meningitis panels.

Tumor infiltrating lymphocyte generation and dilution experiments. Tumor infiltrating lymphocytes (TILs) were generated as described elsewhere (12). TILs were diluted to a concentration of 10^5 cells/mL in RPMI and stored on ice. Concentrated stocks of *M. hyorhinis*, *A. laidlawii*, and *M. pneumoniae* were thawed on ice and diluted in RPMI to concentrations of 10^4, 10^3, and 10^2 CFU/mL. A total of 30 μL of each dilution was added to 270 μL of TILs for each organism/dilution combination. For controls at each organism/dilution combination, 30 μL of each dilution was added to 270 μL of RPMI. Negative controls consisted of media and TILs inoculated with 30 μL of RPMI. Testing was performed in duplicate across two different users to assess for repeatability. Samples were extracted using the PrepSEQ express nucleic acid extraction kit (Applied Biosystems). PCR assays and analysis were performed as described above.

RESULTS

The LOD for each assay/organism combination and reproducibility performance are provided in Table 2 and Table S1 in the supplemental material, respectively. Overall, a wide spectrum of LODs were observed. Based on LOD alone, the Biofire *Mycoplasma* assay was the most sensitive of the assays examined. MycoSEQ and MycoTOOL performed similarly followed by the ATCC universal *Mycoplasma* detection kit and the VenorGEM qOneStep. Overall, 80% of assays failed to detect *Spiroplasma ixodetis* despite genetic similarities and growth characteristics to *Spiroplasma citri* (13–15). Additionally, some assays had a high LOD (1,000 CFU/mL) or failed to detect *Mycoplasma pulmonis* and *Mycoplasma salivarium*.

Given the differences between platforms for LOD, linearity was tracked to examine possible interassay differences for the three real-time PCR assays (Fig. 2). The MycoSEQ and MycoTOOL assays had comparable linear ranges, with most species detected across 3 to 4 $\log_{10}$ dilutions. On average, lower cycle threshold (C_T) values were reported by the MycoSEQ assay. With the exception of the Biofire *Mycoplasma* assay, none of the remaining real-time PCR assays (i.e., the VenorGEM assay) were able to adequately detect *Mollicutes* below 10 CFU/mL, with no more than 67% being detected for any species across any real-time qPCR assay (Fig. 2; Table S1). *S. ixodetis* was detected only by the MycoTOOL but in only 16.7% of replicates at any one dilution.

Similar assay performance was demonstrated for *Mycoplasma hyorhinis*, *Mycoplasma pneumoniae*, and *Acholeplasma laidlawii* at serial dilutions ranging from 1,000 to 10 CFU/mL in the presence of TILs. However, in some cases, only 50% of replicates were detected at 10 CFU/mL (Table 3).

DISCUSSION

In this study, we conducted a proof-of-principle analysis to evaluate the performance of five commercially available molecular assays marketed for the detection of *Acholeplasma*, *Mycoplasma* and *Spiroplasma* spp. using a panel of 10 type strains. All but one assay was tested in sextuplicate across two different analysts to assess for LOD and repeatability; the

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TABLE 2 Limit of detection studies carried out on *Mollicutes* type strains within this study[a]

Mollicutes spp.	ATCC Universal *Mycoplasma* Detection Kit (CFU/mL)	Biofire *Mycoplasma* (CFU/mL)	LifeTech MycoSEQ (CFU/mL)	Roche MycoTOOL (CFU/mL)	VenorGEM qOneStep (CFU/mL)
Acholeplasma laidlawii PG8	100	10	10	10	100
Mycoplasma argininii G230	100	10	100	10	100
Mycoplasma fermentans PG18	100	1	10	10	10
Mycoplasma gallisepticum PG31	100	0.1	10	10	100
Mycoplasma hominis PG21	100	1	10	100	100
Mycoplasma hyorhinis PG42	10	1	10	100	100
Mycoplasma pneumoniae FH	10	1	10	10	100
Mycoplasma pulmonis PG34	1000	1	Not detected (>1000)	100	100
Mycoplasma salivarium PG20	100	10	100	100	1000
Spiroplasma ixodetis Y32	100	Not detected (>1000)	Not detected (>1000)	Not detected (>1000)	Not detected (>1000)

[a]LOD was defined as the terminal dilution in which all replicates were detected. Green denotes an LOD of ≤10 CFU/mL to replace culture-based methods according to the European and Japanese pharmacopeia. Red denotes organisms that failed to be detected at ≥1,000 CFU/mL.

Biofire *Mycoplasma* assay was completed only in triplicate runs per test condition due to assay cost. Overall, our results indicate the superior performance of the Biofire *Mycoplasma* assay, while the MycoSEQ and the MycoTOOL assays were comparable (Table 2; Table S1). Little assay interference was observed when testing was conducted in the presence of TILs (Table 3). European and Japanese pharmacopeia standards accept molecular assays if the LOD is ≤10 CFU/mL compared with culture and ≤100 CFU/mL compared with the indicator cell method (2, 3). Based on these standards alone, our preliminary data suggest that only the Biofire *Mycoplasma* assay was able to meet the equivalency standards to replace gold standard culture, although *Mycoplasma orale* and *Mycoplasma synoviae* could not be assessed. Increased sensitivity with the Biofire assay may be attributable to the nested PCR design. However, more extensive evaluation on a larger number of clinical isolates (acquired from animal, human, and/or cell lines), beyond banked type strains, will be required for further study before definitive conclusions can be drawn. The differences in LOD between assay and organism combinations are not necessarily a limitation, as the selection for quality-control (QC) strains for testing is dependent on the origin of the raw materials used in product manufacturing as well as the intended product recipient.

Surprisingly, *S. ixodetis* failed to be detected by all assays except for the ATCC universal *Mycoplasma* detection kit, despite genetic and growth similarities to *S. citri* (13–15). In this study, *S. ixodetis* was used in lieu of *S. citri* due to our inability to purchase the *S. citri* ATCC 27556 type strain. The overall impact for failing to detect *S. ixodetis* in this study is theoretically low for biological products, unless the final product incorporated raw materials associated with ticks (such as *Ixodes pacificus*) and other arthropods (14, 15). Nonetheless, *S. ixodetis* may be of increasing medical and industrial importance, so its inclusion in this study should not be overlooked entirely (16). *S. citri*, however, is recommended by the

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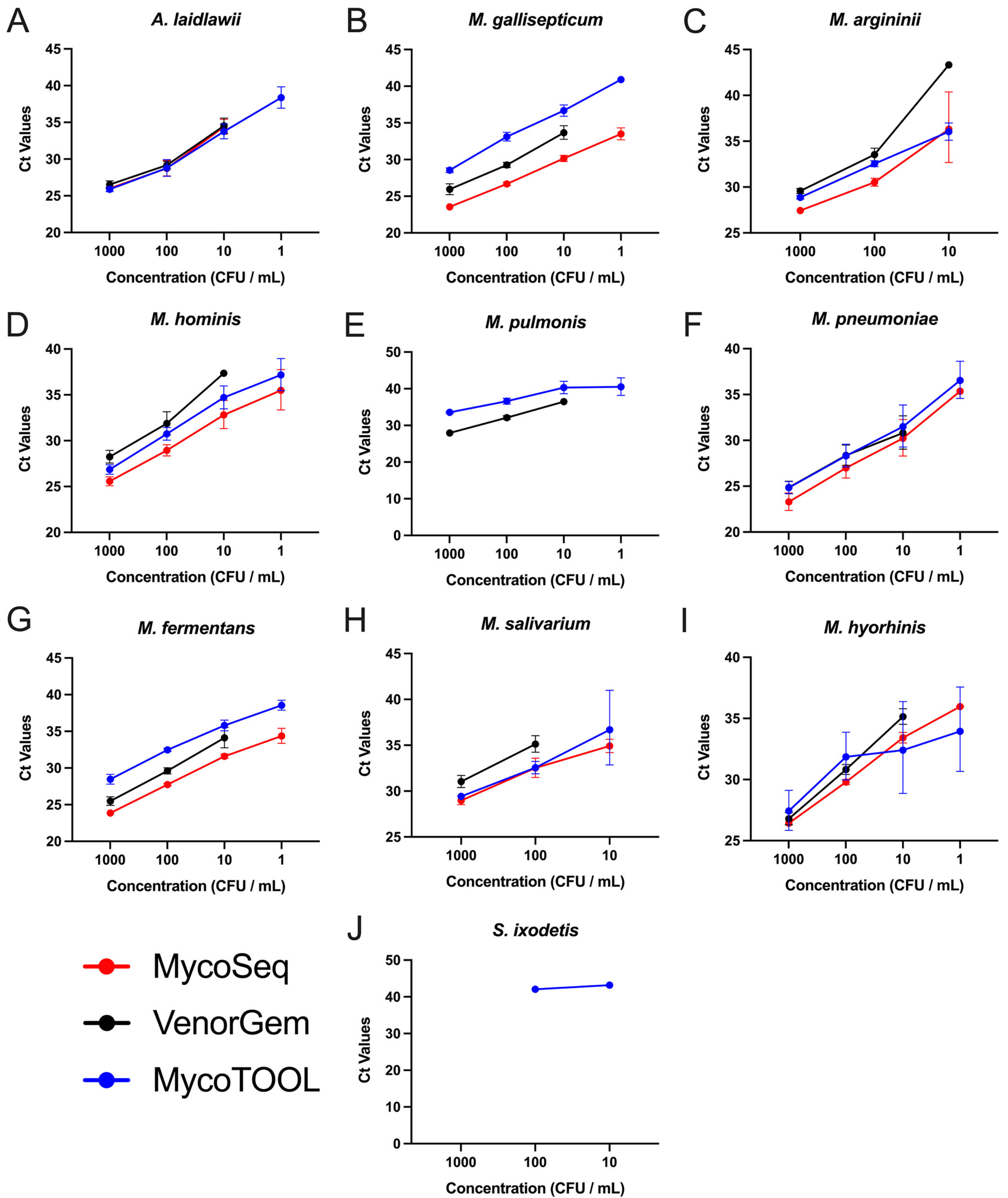

FIG 2 Linear range for limit of detection studies on *Mollicutes* spp. Linear range of MycoSEQ (red), VenorGEM (black), and MycoTOOL (blue) qPCR assay cycle threshold (C_T) values comparative to bacterial cell concentration (CFU/mL) for the following species: *A. laidlawii* (A), *M. gallisepticum* (B), *M. arginini* (C), *M. hominis* (D), *M. pulmonis* (E), *M. pneumoniae* (F), *M. fermentans* (G), *M. salivarium* (H), *M. hyorhinis* (I), and *S. ixodetis* (J). Graphs represent geometric mean and standard deviation for each test condition for all examined species.

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TABLE 3 Tumor infiltrative lymphocyte cell detection experiments carried out within this study[a]

Assay	Condition	*M. pneumoniae*		*A. laidlawii*		*M. hyorhinis*		No organism	
		Organism alone	Organism + TIL	Organism alone	Organism + TIL	Organism alone	Organism + TIL	RPMI	TIL alone
ATCC	1000	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative
	100	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative
	10	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative
Biofire	100	Not tested	Not tested	Positive	Positive	Not tested	Not tested	Not tested	Not tested
	10	Positive	Positive	Positive	Positive	Positive	Positive	Not tested	Not tested
	1	Positive	Positive	Negative	Negative	Positive	Positive	Negative	Negative
	0.1	Negative	Not tested	Not tested	Not tested	Negative	Not tested	Not tested	Not tested
MycoSEQ	1000	23.335	22.905	28.705	27.615	25.665	25.215	Negative	Negative
	100	26.465	27.58	32.855	30.45	29.215	28.61	Negative	Negative
	10	31.72	30.965	35.68	34.96	32.54	32.525	Negative	Negative
MycoTOOL	1000	25.255	24.61	29.575	27.73	26.72	26.005	Negative	Negative
	100	28.085	29.19	32.635	30.515	30.71	29.43	Negative	Negative
	10	32.885	31.44	36.46	34.96	34.465	33.05	Negative	Negative
VenorGEM	1000	25.91	25.815	30.105	29.255	27.905	27.82	Negative	Negative
	100	28.58	29.86	34.72	31.865	31.47	31.2	Negative	Negative
	10	31.77	33.555	Negative	Negative	35.3	35.7	Negative	Negative

[a]Green indicates all replicates detected. Orange (*) indicates a test combination where only 50% of replicates were detected. Red indicated no detection across all replicates.

international pharmacopeias when there is exposure to insect or plant material during production. Therefore, depending on the circumstances of product manufacturing and the intended use, further investigation would be required to determine assay suitability.

Surprisingly, proficiency testing in the cGMP setting is not a regulatory requirement. Prior to 2022, Quality Control for Molecular Diagnostics (QCMD) offered an external quality assessment (EQA) for *Mycoplasma* molecular assays. Unfortunately, low participation rates (n = 13 to 15 laboratories annually) between 2018 and 2021 have led to the discontinuation of this program. Nevertheless, a review of participant summaries between 2018 and 2021 showed consistent failures to detect *M. pulmonis*, *Mycoplasma fermentans*, *Mycoplasma gallisepticum*, and at times *Mycoplasma arginini*. Participants reported the use of commercial platforms (including four evaluated in this study, namely, MycoSEQ, MycoTOOL, VenorGEM, and ATCC universal *Mycoplasma* detection kit), as well as a few in-house developed assays. While a breakdown of each assay's performance is not disclosed, *M. pulmonis* and *M. fermentans* were consistently detected $<$80% of the time, while *M. gallisepticum* and *M. arginini* were consistently detected $<$85% of the time. Of the 12 samples included in each survey, 6 to 8 were classified as core challenges intended for positive *Mycoplasma* detection (as opposed to educational challenges). Performance for positive core challenge samples was poor, ranging between 17% and 63% for the 4 years studied. Most importantly, *M. fermentans* and *M. gallisepticum* (included in every QCMD challenge) are considered indicator organisms required by all international pharmacopeia standards. The testing of *M. fermentans* is required for vaccines and cell banks for human use, while *M. gallisepticum* testing is required for products where avian material has been used during production. Inadequate performance of certain platforms to detect *M. fermentans* and *M. gallisepticum* in the QCMD surveys highlights areas for concern. In our study, all but one assay was able to detect *M. fermentans* and *M. gallisepticum* at the LOD requirement of $\leq$10 CFU/mL to replace culture-based methods as per the European and Japanese pharmacopeias (Table 2).

All assays evaluated in this study provided generic qualitative results for the presence or absence of *Mycoplasma*. No commercial assays on the market have been

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designed to provide specific organism identification. This gap is attributed mostly to the fact that these assays (and many in-house assays) target numerous universal regions within the 16S rRNA that are specific for *Mycoplasma*, where sequence diversity is limited for the differentiation of *Mollicutes* (17). It is possible that species-specific *Mycoplasma* assays could be developed to supplement deficiencies observed with commercial platforms; however, the cost-benefit would need to be carefully balanced with regulatory requirements. Furthermore, as per the international pharmacopeias, the specificity and sensitivity of an assay should be target specific for strains directly related to the origin of materials and the intended product recipient.

LOD studies of *A. laidlawii*, *M. hyorhinis*, and *M. pneumoniae* performed in the presence and absence of TILs showed little interference with the detection of clinically relevant strains (Table 3). Testing in the presence of product, known as method suitability testing, is a regulatory requirement for any new product formulation to verify the absence of product interference for the detection of low-level (defined as <100 CFU) contaminants. Preliminary testing in the presence of TILs showed promise; however, further evaluation of other cell types and matrices is required at our institution due to the wide spectrum of investigational new drugs manufactured at the NIH for phase I and phase II clinical trials. Other institutions with a narrower product scope may find it easier to select and validate an appropriate *Mycoplasma* PCR assay to replace conventional USP <63> methods.

To date, there are limited data available in the peer-reviewed literature that describe the performance characteristics of various *Mycoplasma* assays by independent study groups, beyond work published by the manufacturers themselves. The Biofire *Mycoplasma* assay was presented recently by the manufacturer in a poster abstract format with results similar to ours (18). The MycoTOOL assay was studied by a Canadian group for rapid validation; however, only serial dilutions of *M. hominis* and *M. arginini* genomic DNA were studied (19). A subsequent study by the same group showed good performance (<10 CFU/mL detection) of *M. hominis* and *M. arginini* genomic DNA in the presence of chimeric antigen receptor T cells (20). The ATCC universal *Mycoplasma* (21–23) and the VenorGEM kits (24) have been used widely for the determination of cell line infection status, but both lacked performance data concerning assay cross-performance beyond the manufacturer's claims. The data outcomes from our study show variations in LOD based on organism, thus highlighting the importance for rigorous end-user validation based on material origin and intended product recipient.

There are several limitations to our study. In the United States, USP <1223> requires that the alternative assay demonstrate equivalency and noninferiority to the compendial method (i.e., USP <63>). The number of replicates is at the discretion of the end-user to demonstrate statistical power; this discretion contrasts with Ph. Eur. 2.6.7 which requires at least 24 replicates per test condition. This study served as a proof-of-principle analysis to evaluate overall performance using six replicates per test conditions. Further testing will be required for application of any assay in the cGMP setting. Additionally, excessive replicate testing for the Biofire *Mycoplasma* assay was not possible in this study due to cost; however, the 2-min assay setup time, open access availability, and 1-h turnaround time offer significant advantages in cost savings in time and labor compared with other assays evaluated in this study. Further replicate testing focused on product-relevant and clinically relevant strains will be required if the Biofire *Mycoplasma* assay is chosen for implementation and product release testing. Finally, culture conditions used in this study followed procedures used in clinical reference laboratories, which differ slightly to the procedures prescribed in USP <63> (10). The impact of this amendment is minimal as culture recovery was successful and colony counts were verified for each dilution during each test condition. Frozen eluates from a single extraction of each organism-concentration combination were used to evaluate all assays except for the Biofire assay, so it is possible that DNA degradation may have contributed to the LOD variations observed between assays, although the effect is deemed minimal, as testing for each organism-concentration combination was

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completed within a 1-month time frame with minimal freeze-thaw cycles. In this study, we were unable to culture *M. orale* and *M. synoviae* using commercially available media. Both these organisms are considered compendial indicator organisms, and the failure to include them is a limitation for assay comparability in this study. The use of exogenous medium additions (such as NAD^+ to enhance growth of *M. synoviae* in broth culture [25]) requires rigorous raw material testing and is generally avoided for cGMP applications.

In conclusion, we present a comprehensive comparative evaluation of five commercially available assays for the detection of *Mollicutes* spp. Our data show that not all assays were capable of meeting the $\leq$10 CFU/mL requirement to replace culture-based methods based on European and Japanese pharmacopeias. There is a critical need in the biopharmaceutical field to utilize rapid methods that offer sensitive detection of contaminants while conserving product volume. Caution must be applied to ensure that test methods are not overly sensitive, particularly in the absence of being able to confirm *Mycoplasma* viability through molecular testing alone and the questionable potential for clinical infectious risk which may jeopardize the release of a personalized, potentially lifesaving, costly product. Based on the strains studied here, our proof-of-principle analysis indicates promising broad-range sensitive detection using the Biofire *Mycoplasma* assay. The MycoSEQ and MycoTOOL assays also demonstrated good performance for selected strains. This study will help guide end-users for assay selection and further evaluation in the presence of product depending on the product type, the origin of raw materials used during manufacturing, and the intended product recipient.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

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