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Mycoplasma detection in a historical arbovirus repository: Commercial kit comparison and implications for improved repository management

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ABSTRACT

The Centers for Disease Control and Prevention, Arbovirus Reference Collection (ARC) contains viral isolates from both environmental and human sources that are maintained in the laboratory through passage in suckling mouse brain and/or vertebrate and invertebrate cell culture. There has been increased concern regarding the effect of mycoplasma contamination on virus growth and its impact on research and phenotypic analysis. Therefore, quality control testing of virus preparations has become a routine part of the ARC quality assurance program. We compared the performance of three kits - the PCR Mycoplasma Detection Kit (ABM), the VenorGem Mycoplasma Detection Kit (Sigma), and the MycoAlert Mycoplasma Detection Kit (Lonza) - against a reference mycoplasma detection assay from the American Tissue Culture Collection (ATCC) using 744 virus preparations in the ARC, representing 721 unique viruses comprising twelve families and unclassified viruses. We found the ABM kit had the highest sensitivity and specificity, followed by the Sigma kit and Lonza kit, when compared to the ATCC kit. An increase in false positives was observed for the Lonza kit for preparations recently passaged in suckling mouse. Our data supports previously reported observations; that once introduced a specific species of mycoplasma is maintained within a lab.

1. Introduction

The Centers for Disease Controland Prevention (CDC), Division of Vector-Borne Diseases (DVBD), Arboviral Diseases Branch (ADB) Arbovirus Reference Collection (ARC) is a key element of DVBD's activities as a World Health Organization (WHO) Collaborating Center. The ARC was established in 1958 in Atlanta, Georgia, and was relocated to what is now the DVBD in Fort Collins, Colorado, in 1973. The ARC contains arboviruses isolated from environmental, animal, and human sources dating back to the 1920s. The ARC also contains clonal recombinant viruses developed by researchers. Virus stocks are maintained by passage in suckling mouse brain (SMB) and/or vertebrate and invertebrate cell culture.

Mycoplasma are small self-replicating organisms belonging to the class Mollicutes. A contaminated eukaryotic cell line can contain 10^6-10^8 organisms/ml (Drexler and Uphoff, 2002). The most common cell culture contaminants are *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. fermentans*, *M. hyorhinis* and *M. orale*. Fetal bovine serum (FBS) is the primary source of *M. arginini* and *A. laidlawii* contamination (Nikfarjam and Farzaneh, 2012). From the 1960s to 1970s, 25%–40% of FBS used in cell culture was contaminated with mycoplasma (Barile et al., 1973). Laboratory personnel are the primary source of *M. orale* and *M. fermentans* contamination. Historically, these contaminants were introduced as a result of mouth pipetting. *M. hyorhinis* is introduced from porcine derived Trypsin. Contaminated cell lines are estimated to make up 5–30% of the total population of cell lines in use today (Nikfarjam and Farzaneh, 2012).

In recent years there has been increased concern regarding the effect of mycoplasma contamination on virus growth and its impact on research and phenotypic analysis. Viruses contaminated with mycoplasma may show no observable effects or their growth may be altered. Contaminated cell lines can exhibit cytopathic effects (CPE), making it difficult to determine if the CPE is being caused by the contaminant or the viral infection. Both enhanced and inhibited virus growth have been reported when using contaminated cell lines for virus replication (Chernov et al., 2014; Hargreaves and Leach, 1970; Lidsky et al., 2009; Netto et al., 2014). If the virus preparation is contaminated with mycoplasma, the mycoplasma is maintained along with the virus during passage. The impact of contamination on research is difficult to predict but using virus or cell lines which have been shown to contain a mycoplasma contaminant may lead to questionable results. As a result of the increased concern for mycoplasma contamination, quality control

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(QC) testing of virus preparations has become a routine part of the ARC quality assurance (QA) program. The ARC is a large repository, and routine testing can be cost prohibitive and time consuming. There are various mycoplasma detection kits available commercially, and they differ by the number and types of species they detect and cost. The available commercial kits are marketed for testing cell lines, not virus preparations. We compared the performance of three mycoplasma detection kits - the PCR Mycoplasma Detection Kit (ABM), the VenorGem Mycoplasma Detection Kit (Sigma), and the MycoAlert Mycoplasma Detection Kit (Lonza) - to the ATCC reference assay using 744 virus preparations from the ARC, representing 721 unique viruses comprising twelve families and unclassified viruses. The goals for this comparison were to determine if the kits on the market would be effective for this application, and if a more cost-effective and less time-consuming kit was available compared to the ATCC kit.

2. Materials and methods

2.1. Virus preparation selection

Virus preparations from the ARC were selected for mycoplasma species contamination testing based on routine maintenance and QC testing prior to distribution to researchers' requests submitted to the ARC. For this reason, there is not an equal representation among the viral families nor the cell types in which they were propagated. Historically, some virus preparations have been passaged through multiple cell types or animals, including SMB, non-human primate, African green monkey kidney cells (Vero, Vero E6), hamster kidney cells (BHK-21, CER), *Aedes albopictus* cells (C6/36, C7/10, RML-12), rhesus monkey kidney cells (LLC-Mk2), pig kidney cells (PK-15, PS) *Aedes pseudoscutellaris* cells (AP61), duck embryo cells (DE), human lung cells (MRC-5), hepato cellular carcinoma (HuH-7). Prior to freezing and storage, 20% fresh FBS (VWR, Radnor, PA) was added to viruses passaged in cell culture to maintain stability.

2.2. RNA and DNA extraction

Viral RNA and mycoplasma species DNA were co-extracted using the QIAamp viral RNA minikit (Qiagen, Germantown, MD) following the manufacturer's recommendations. Extractions were performed using sample volumes of 140 µl and the final product was eluted in 100 µl. The eluted RNA/DNA mixture was stored at -70 °C until evaluation by the ATCC, Sigma, and ABM assays.

2.3. Commercial Mycoplasma detection kits

The characteristics of the commercial mycoplasma detection kits are summarized in Table 1.

2.3.1. Universal mycoplasma detection kit (ATCC (30 − 1012 K), Manassas, VA))

The ATCC kit detects 62 species of mycoplasma and other mollicutes, including the most common cell culture contaminants and *Spriroplasma species;* the manufacturer's product insert includes a list of the species detected by this kit. The primers used in the kit are specific to the 16S rRNA coding region of the mycoplasma genome using a touchdown program to increase sensitivity. PCR was performed on the extracted RNA/DNA mixture following the manufacturer's recommendations. For step three, the manufacturer recommended a minimum of 12 and maximum of 20 cycles; 15 cycles were used in this comparison. The kit contained enough material for 40 reactions.

2.3.2. PCR mycoplasma detection kit (ABM (G238), Richmond, BC, Canada)

The ABM kit detects 95 species of mycoplasma, but other mollicutes that are common cell culture contaminants such as *A. laidlawii* are not included; the manufacturer's product insert includes a list of the species detected by this kit. The primers used in the kit are specific to the 16S and 23 s rRNA coding region of the mycoplasma genome (personal communication, Erica Tang, ABM, 2017). PCR was performed on the extracted RNA/DNA mixture following the manufacturer's recommendations. The manufacturer's recommendation is to use 30–40 cycles; 35 cycles were used in this comparison. The kit contained enough material for 100 reactions.

2.3.3. MycoAlert mycoplasma detection kit (Lonza (LT07-418 and LT07-518), Walkersville, MD)

The Lonza kit is not species dependent and does not detect genomic DNA but measures an enzymatic reaction to detect viable mycoplasma and other mollicutes. One hundred microliters of each live virus preparation was evaluated using a Junior LB95096 luminometer (Berthold Technologies, Oak Ridge, TN). Modifications to the manufacturer's recommendations were as follows: all additions of buffers to live virus were carried out in a biosafety cabinet under appropriate containment. Results were calculated and interpreted according to the manufacturer's instructions. The kit came in various sizes for 10–100 samples.

2.3.4. VenorGeM mycoplasma detection kit (Sigma (MP0025), St. Louis, MO)

The Sigma kit detects 24 species of mycoplasma and other mollicutes, including the most common cell culture contaminants; the manufacturer's product insert includes a list of the species detected by this kit. The primers used in the kit are specific to the 16S rRNA coding region of the mycoplasma genome. PCR was performed on the extracted RNA/DNA mixture following the manufacturer's recommendations; an internal control was added to every sample tested. The kit contained enough material for 25 reactions.

2.3.5. Visualization of PCR product

Ten microliters of each PCR amplification product was evaluated by electrophoresis using a 2% ethidium bromide e-gel (Thermo Fisher, Carlsbad, CA). DNA bands were visualized using UV transillumination. A sample was classified as positive if there was a band of the correct size present as specified by the kit.

2.4. Mycoplasma species identification via sequencing

2.4.1. Sample selection

Mycoplasma species identification was attempted on 52 of the 57 samples that were positive by either the ATCC or ABM kits. Five of the samples were depleted in volume and unable to be tested further. An additional 28 samples that were negative by both the ATCC and ABM

Table 1
Summary of kits used in comparison.

	ABM	ATCC	Lonza	Sigma
Method of Detection	PCR	PCR	Enzymatic Reaction	PCR
Sample Type	Extracted DNA	Extracted DNA	Live culture material	Extracted DNA
Sample Volume	2.5 µl	2.5 µl	100 µl	2 µl
Species Detected	95	62	All viable mycoplasma	24
# Samples Tested per Kit	100	40	10–100	25

kits, but positive by at least one of the other kits, were also selected for attempted amplification and sequencing.

2.4.2. DNA amplification

PCR was performed on the extracted RNA/DNA mixture using primers directed at the tuf gene of *Mollicutes* (tuf-064 F and tuf-681R; Schnee et al., 2012) and 16S rDNA of *Spiroplasma* species (TKSSsp and 16SA1; Fukatsu et al., 2001). PCR reactions were performed with 10 µl of the extracted RNA/DNA mixture and 50 pmol of each forward and reverse primer in a 50 µl total reaction volume using the Taq PCR Core kit (Qiagen). PCR reactions were amplified using the following cycling conditions; tuf gene primers: initial denaturation at 95 °C for 60 s, 40 cycles (95 °C for 30 s, 52 °C for 30 s and 72 °C for 60 s) and final elongation at 72 °C for 60 s; 16S rDNA primers: initial denaturation at 94 °C for 2 min, 30 cycles (94 °C for 1 min, 55 °C for 1 min, and 70 °C for 1 min). Five microliters of the RT-PCR amplification products were evaluated by electrophoresis using a 2% ethidium bromide e-gel (Thermo Fisher). DNA bands were visualized using UV transillumination. A sample was classified for sequencing follow-up if there was a band of the correct size present for the primer set chosen.

2.4.3. DNA purification and nucleotide sequencing

Gel extraction was performed using a 2% agarose gel in Tris-acetate-EDTA (TAE) buffer (Thermo Fisher). DNA bands were visualized using gel green (Biotium, Fremont, CA) and UV transillumination. DNA bands of appropriate size were excised from the agarose gel and purified using the QIAquick gel extraction kit (Qiagen). Gel-extracted DNA was sequenced with the same forward and reverse primers used for the PCR amplification step. A total of 5 pmol of primer and 8 − 10 ng of template was added to each reaction using the ABI BigDye Terminator V3.1 ready reaction cycle sequencing mix (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using BigDye Xterminator (Thermo Fisher). Nucleotide sequences were determined by running the purified sequencing reactions on the ABI 3130XL genetic analyzer (Applied Biosystems). Sequence fragments were generated for the region targeted by the selected primers.

2.4.4. Sequence analysis

The nucleotide sequences generated from both the forward and reverse primers were assembled using SeqMan Pro 14 software (DNASTAR). The consensus sequence was queried using the NCBI BLAST function of the SeqMan software. Sequences that matched the consensus sequence with the highest overall maximum value were considered to be the closest identity match, which ranged from 96.2%–99.8%.

2.5. Statistical analysis

Sensitivities (true positive rates) and specificities (true negative rates) were calculated for the Sigma, ABM and Lonza kit results against the ATCC standard, along with 95% confidence intervals (CIs). For additional evaluation of results, a likelihood ratios graph was produced, which plots the true positive rate against the false positive rate for each kit (Biggerstaff, 2000). Because not all samples were tested with all kits, multiple imputation and bootstrapping methods were used before comparing sensitivities. Multiple imputation was utilized to predict an independent plausible value for missing values (percent missing ranged from 24.3 to 31.2%) using generalized linear regression on non-missing variables to create 40 imputed complete data sets (Rubin, 1987). The differences in sensitivities among the three kits were calculated with each imputed data set, then bootstrapped 999 times to calculate appropriate standard errors in order to create 95% confidence intervals (Schomaker and Heumann, 2018). The Bonferroni correction was used to account for the simultaneous multiple comparisons. The baseline number (N) for each kit is provided in Fig. 1.

3. Results

There were 512 virus preparations tested by all four kits. Due to limited volume of low passage stock viruses, there were an additional 51 samples that were not tested by the Lonza kit. The Lonza kit requires a separate sample for testing compared to the other assays, which use the same RNA/DNA extracted mixture. Based on the initial results from the 563 samples, the ABM kit was selected for testing an additional 181 virus preparations. The total samples tested by each kit are as follows: 744 by ATCC, 744 by ABM, 563 by Sigma, and 512 by Lonza. The total number of positives by each kit are as follows: 55 by ATCC, 52 by ABM, 65 by Sigma, 189 by Lonza.

The ATCC kit detected mycoplasma contamination (positive result) in 55 of the 744 virus preparations tested. Of those 55 preparations, the ABM kit detected 50 (Sens: 90.9%, 95% CI: 80.4%–96.1%), the Sigma kit detected 38 (N = 43, Sens: 88.4%, 95% CI: 75.5%, 94.9%) and the Lonza kit detected 21 (N = 39, Sens: 53.8%, 95% CI: 38.6%, 68.4%). The ATCC kit detected no mycoplasma contamination (negative result) in 689 samples. Of these 689 samples, the ABM kit had a positive result for two (Spec: 99.7%, 95% CI: 98.9%, 99.9%), the Sigma kit had a positive result for 27 (N = 520, Spec: 94.8%, 95% CI: 92.6%, 96.4%) and the Lonza had a positive result for 168 (N = 473, Spec: 64.5%, 95% CI: 60.1%, 68.7%) (Fig. 1). The difference in sensitivity between the Sigma kit and ABM kit was -2.1% (95% CI: -15.4%, 11.1%). The difference in sensitivity of the Lonza kit compared to the ABM kit was -32.5% (95% CI: -49.1%, -16.0%) and was -30.4% (95% CI: -47.6%, -13.3%) compared to the Sigma kit.

The likelihood ratio graph, which plots the true positive rate against the false positive rate, further illustrates the results from Fig. 1. The ABM kit had the highest true positive rate and lowest false positive rate versus the Lonza kit, which had the lowest true positive rate and highest false positive rate (Fig. 2).

For the 744 preparations tested, the range of viruses tested by family is shown in Table 2. To evaluate if the host, animal, or cell type in which the virus was most recently cultured influenced the results, the data was also examined by culture type (Table 3). There are 14 known cell lines and two animals represented in the data, including one preparation with unknown passage history. The only host that appeared to have a noticeable impact on the results was suckling mouse brain. Of the 331 mouse brain preparations, zero were positive by either the ATCC or ABM kits, but 161 were positive by either the Lonza or Sigma kits.

Mycoplasma species identifications were successful for 48 of the 57 samples positive by either the ATCC or ABM kits. Of the 48 preparations, 28 were positive for *M. arginini*, four were positive for *A. laidlawii,* one was positive for *M. yeatsii*, and 15 were positive for *M. orale*. No *Spriroplasma* spp. were detected in the positive samples (Fig. 3). For the nine preparations where identification was unsuccessful, three were PCR negative with both primer sets (tuf gene and 16S rDNA), one produced a very faint band with the tuf gene primer set but sequencing was unsuccessful, and five were depleted in volume and unable to be tested further.

Twenty-eight preparations with positive results by either the Lonza or Sigma kits but negative results by the ATCC and ABM kits were selected for sequencing. Of these 28 preparations, 17 were most recently passaged in SMB. None were confirmed as mycoplasma positive and were therefore considered to be false positive results. A detectable PCR product of the correct size was not visualized or the PCR product was sequenced and identified as bacterial contaminants. Non-specific bands (product not the correct size) were observed and sequenced but resulted in identifying host DNA (data not shown).

4. Discussion

If managed correctly, historical virus repositories can provide valuable resources to researchers. Maintenance of the viruses in the

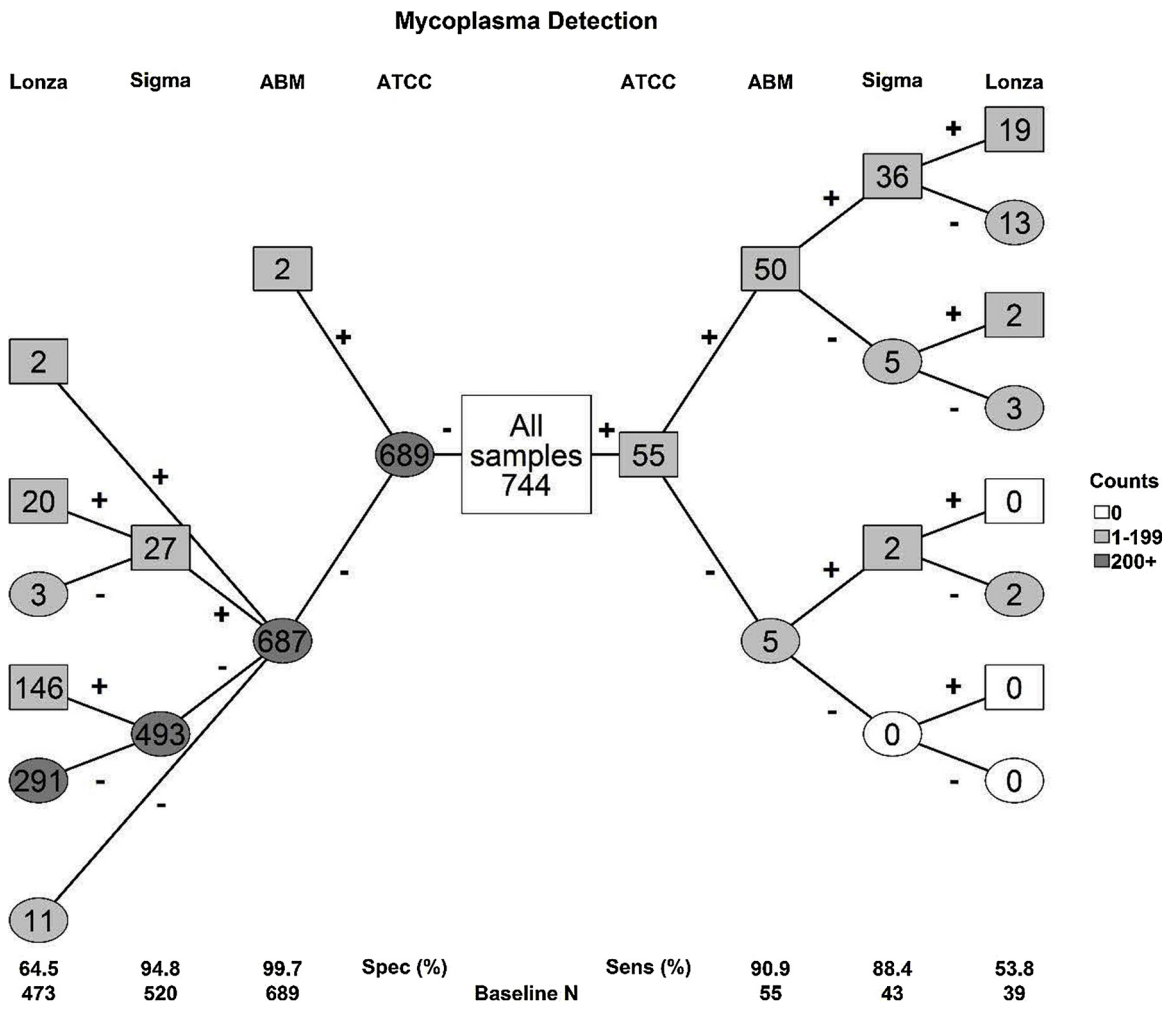

Fig. 1. Summary of results, including sensitivity and specificity of the Lonza, Sigma, and ABM kits compared to the ATCC kit. Virus preparations were tested by the 4 kits to determine which kit to incorporate in the QA program. Not all samples were tested by all 4 assays. Based on results, the ABM kit was selected for additional side-by-side testing with the ATCC kit. The baseline number of samples (N) for each assay is listed in the figure.

repository should be performed in a way that minimizes the passage number and maintains the quality of the virus preparation. Mycoplasma detection has become an important part of quality management of historical virus repositories due to the impact on research and challenges associated with treatment of virus preparations. Implementing a QA program for a large repository can be expensive and time consuming. We performed this comparison of commercial kits to determine if a more cost-effective and/or time-saving alternative was available for mycoplasma detection.

The ATCC kit was used as the reference assay for two reasons: it is the kit produced by ATCC for QC testing of cells and it is the kit used by our in-house cell culture lab for QC testing of cells. The ATCC kit is known to produce reliable results, but it can be cost prohibitive especially for QC testing of a large virus repository. The ATCC kit is unique compared to the other molecular detection kits in that it detects spiroplasma contaminants. Spiroplasma are mollicutes that have been found in adult female mosquitoes (Clements, 2012). This is of particular importance for the ARC as many of the viruses were originally isolated from mosquitoes and are subsequently cultured in mosquito cells. Although spiroplasma contaminants were not identified in the preparations tested in this evaluation, the possibility of spiroplasma contaminants existing in the ARC cannot be ruled out. The preparations that were tested have been passaged multiple times in the lab since isolation, so a mosquito-specific mollicute, such as spiroplasma, could have been lost through passaging over time. This can occur if the virus has been maintained in mammalian cells or live animals, such as SMB. In the future, the ATCC kit could be useful for specific screening of recent mosquito isolates to rule out contamination of spiroplasma before introduction to the ARC, after which a more cost-effective kit could be used for routine testing. In 2016, ATCC provided guidance to users, recommending a reduction in the number of amplification cycles to 12 cycles to reduce the potential for false positive results. Three preparations tested prior to 2016 had positive results based on our use of 15 cycles by the ATCC kit but negative by both the tuf gene and 16S primers. These samples will be retested in the future following the recommended changes to confirm the ATCC results.

The ABM kit provides a viable alternative to the ATCC kit, as it had the highest sensitivity and specificity, and has the added benefit of a lower cost per test. When this work was performed the cost was almost 6 times less expensive per sample, compared to the ATCC kit. The ABM kit detected two of four preparations contaminated with *A. laidlawii*, although this species is not listed as detectable by the ABM kit. This data helps to further support the use of this kit as a cost-effective alternative to the ATCC kit. We have adopted this kit in the ARC as our primary mycoplasma detection kit.

The Sigma kit includes an internal control to ensure functionality, but had a lower sensitivity and specificity compared to the ABM kit. In addition, at the time this work was performed the Sigma kit was more expensive per sample than the ATCC kit. In this application, routine QC testing of a large historical repository, this is not a viable option as a

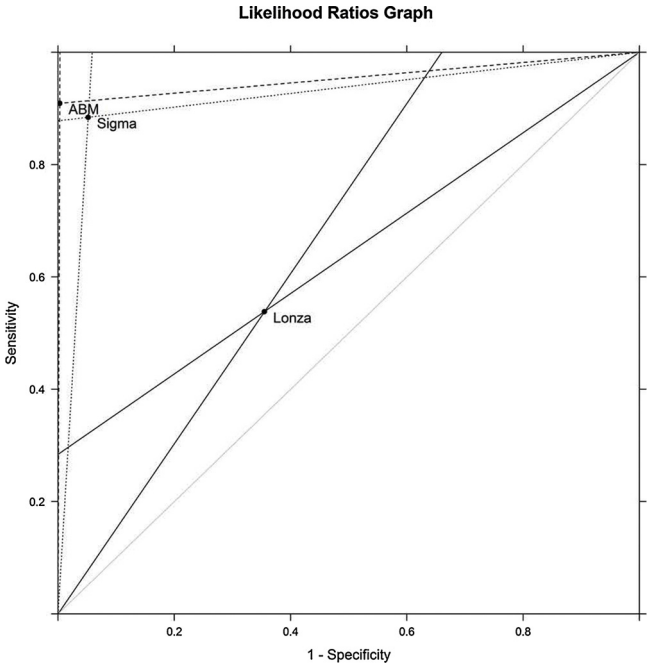

Fig. 2. Plotting the true positive rate (sensitivity) against the false positive rate (1-specificity) for the ABM, Sigma, and Lonza kits. This analysis is used for a visual measurement of the overall performance of the kit. The closer the kit performs to 100% true positives and 0% true negatives, the better the overall performance of the kit. If a kit falls below the diagonal line, it is not a suitable kit because the false positive rate is higher than the true positive rate. As shown in this figure, the ABM kit has the best overall performance (lowest proportion of false positives and highest proportion of true positives) using the ATCC kit as the standard.

mycoplasma detection assay.

The Lonza kit had the lowest sensitivity and specificity compared to the ABM and Sigma kits. The results were impacted based on the cell type, specifically virus produced in SMB or invertebrate cells. The Lonza kit showed a significant increase in false positive results for virus produced in SMB, suggesting non-specific reactivity to components in the mouse brain (Table 3). In addition, the invertebrate C6/36 cells are maintained at a temperature of 28 °C, which is not the ideal temperature for mycoplasma growth (35 °C–37 °C), potentially providing a false negative result in the Lonza kit, which detects viable mycoplasma, not genomic DNA. If the mycoplasma is not actively replicating due to the low temperature, the kit will not detect the contaminant. Virus preparations inoculated in both Vero and C6/36 cells showed conflicting results with the Lonza kit, further supporting the potential for false negative results in invertebrate cells. The ideal sample type for the Lonza kit is fresh culture, which contraindicates its use for QC testing of viruses frozen in storage. Downstream manipulation of live virus with the Lonza kit also introduces safety considerations that are not required with the molecular kits, which detect a RNA/DNA mixture that if extracted correctly should be non-infectious. Additionally, manipulation of live virus prevents testing from being completed outside of the appropriate biocontainment. In this comparison, we did not attempt to confirm infectivity status of the samples after addition of the buffers. It is possible the buffers are inactivating the virus. If a lab would choose to implement this kit, infectivity status is something that would need to be confirmed prior to moving downstream sample manipulation outside of containment. Alternatively, the work could be carried out in containment, similar to this comparison. In its favor, the Lonza kit required the least hands-on time to produce results, an obvious benefit for routine QC testing of a large repository. However, for our laboratory, we feel that the increased safety requirements, additional sample volume, and decreased sensitivity and specificity make this a poor choice for this application.

The application of the kits for detection of mycoplasma contaminants in virus preparations may have influenced the results. It is important to note that the kits used in this comparison are marketed for QC testing for mycoplasma contaminants in clean cell cultures, not in virus preparations. For this reason, the kits are designed to detect common cell culture contaminants, which are introduced by laboratory staff or media components. These kits are not marketed for the detection of all mycoplasma species, which means mycoplasma introduced from other sources may be missed. However, the confirmatory sequencing of the 28 viruses, 17 of which were replicated in SMB, were negative for mycoplasma. If a non-specific assay, such as the Lonza kit, was detecting mycoplasma that the molecular kits were not, this should have been confirmed with the more broadly reactive tuf gene primers. As this comparison has shown the virus preparation (replication host) can influence results depending on the kit. The confirmatory sequencing results from the 28 false positives further support that the Sigma and Lonza kits are not suited for this specific application.

Direct culture of the mycoplasma contaminants for confirmatory testing was not performed due to the additional safety issues of working

Table 2
Mycoplasma results by virus family and kit.

Family	Mycoplasma Kits										
	Negative by all kits	Positive by:									Total
		ATCC	ABM	ABM ATCC	Sigma	Sigma ATCC	Sigma ABM ATCC	Lonza	Lonza ABM ATCC	Lonza Sigma	All kits	
Arenaviridae	1	0	0	0	0	0	0	2	0	1	0	4
Flaviviridae	164	0	1	5	4	2	12	50	1	8	9	256
Hantaviridae	1	0	0	0	0	0	0	0	0	1	0	2
Nairoviridae	18	0	0	3	1	0	0	2	0	0	0	24
Orthomyxoviridae	6	0	0	0	0	0	0	0	0	0	0	6
Paramyxoviridae	1	0	0	0	0	0	0	0	0	0	0	1
Peribunyaviridae	133	3	1	2	1	0	2	53	0	1	6	202
Phenuiviridae	32	0	0	0	1	0	0	6	0	1	0	40
Pircornaviridae	1	0	0	0	0	0	0	0	0	0	0	1
Reoviridae	32	0	0	0	0	0	0	2	0	0	1	35
Rhabdoviridae	58	0	0	0	0	0	3	12	1	2	1	77
Togaviridae	56	0	0	2	0	0	0	16	0	4	2	80
Unclassified	0	0	0	0	0	0	0	0	0	1	0	1
Ungrouped Order Bunyavirales	9	0	0	0	0	0	0	5	0	1	0	15
Total	512	3	2	12	7	2	17	148	2	20	19	744

Table 3
Mycoplasma results by culture type and kit.

Culture Type	Mycoplasma Kits											Total
	Negative by all kits	Positive by:										
		ATCC	ABM	ABM ATCC	Sigma	Sigma ATCC	Sigma ABM ATCC	Lonza	Lonza ABM ATCC	Lonza Sigma	All kits	
AP61 (*Aedes* pseudoscutellaris)	0	0	0	0	0	0	0	1	0	0	0	1
BHK-21 (hamster kidney)	34	2	1	4	1	0	1	1	0	1	5	50
C6/36 (*Aedes albopictus*)	54	0	1	3	2	2	6	3	0	1	1	73
C7/10 (*Aedes albopictus*)	2	0	0	0	0	0	0	0	0	0	0	2
CER (hamster kidney)	0	0	0	0	0	0	1	0	0	0	0	1
DE (duck embryo)	1	0	0	0	0	0	0	0	0	0	0	1
LLC-Mk2 (rhesus monkey kidney)	4	1	0	0	0	0	0	0	0	0	0	5
Monkey	1	0	0	0	0	0	0	0	0	0	0	1
Mouse	170	0	0	0	4	0	0	139	0	18	0	331
MRC-5 (human lung)	0	0	0	0	0	0	0	1	0	0	0	1
PK-15 (pig kidney)	2	0	0	0	0	0	1	0	0	0	0	3
PS (pig kidney)	0	0	0	0	0	0	0	0	0	0	1	1
Vero (African green monkey kidney)	236	0	0	4	0	0	8	3	2	0	12	265
Vero E6 (African green monkey kidney)	5	0	0	0	0	0	0	0	0	0	0	5
HuH-7 (hepato cellular carcinoma)	2	0	0	0	0	0	0	0	0	0	0	2
RML-12 (*Aedes albopictus*)	1	0	0	0	0	0	0	0	0	0	0	1
Unknown cell culture passage history	0	0	0	1	0	0	0	0	0	0	0	1
Total	512	3	2	12	7	2	17	148	2	20	19	744

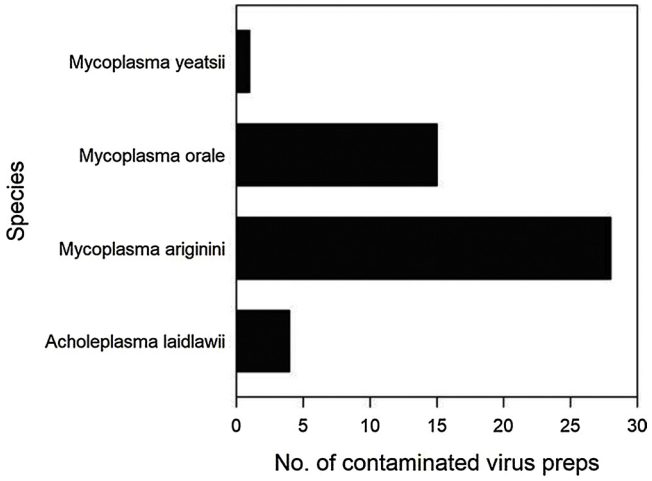

Fig. 3. Mycoplasma species identification was successful on 48 of the 57 virus preparations positive by either the ATCC or ABM kits. The amplification and sequencing for species identification was used as a confirmatory assay in this comparison. The primers used for the amplification and sequencing reactions are directed to the tuf gene of *Mollicutes* (Schnee et al., 2012).

with live virus in streaked plates. In addition, the potential to spread the mycoplasma contamination by performing direct culture influenced the decision. Sending samples for outside testing was considered, but was cost prohibitive due to the fact that the samples contained BSL2 and BSL3 viruses. For this reason, sequencing and species identification was used for additional confirmatory testing. It is interesting to note that in a recombinant virus preparation we detected a species not common to cell culture, *M. yeatsii,* which is found in goats (Calcutt et al., 2015).

Overall, there was a mycoplasma contamination detection rate of 7.7% in virus preparations tested from the ARC. Based on the historical nature of the ARC and the poor QC of media components in the early years of cell culture, this number is lower than expected. This may be due to viruses historically being maintained in SMB, not in cell culture. Although there are mycoplasma species that infect mice, such as *M. pulmonis*, all SMB preparations tested in the ARC to date had no detectable mycoplasma contamination. In addition, screening for *M. pulmonis* in research animal labs is common, although one study in Taiwan showed a 40% positive rate in rat colonies from 2004 to 2007 (Liang et al., 2009). Passage in mice can also be used for treatment of mycoplasma contamination in cells (Weng et al., 2017; Nikfarjam and Farzaneh, 2012). Based on improved cell culture media, use of aseptic techniques, and the mycoplasma detection methods we employed, we were surprised to find contamination in more recent isolates including those submitted to the ARC within the last 10–15 years. A possible explanation for this is a previously undetected contaminated cell line used for virus isolation. This highlights the gaps that still exist regarding QA in laboratories using cell culture for isolation.

Over time, the same species of mycoplasma contaminant is often consistently detected in a laboratory (Drexler and Uphoff, 2002). Our results support this finding as well. The isolates in the ARC that are contaminated with *M. orale* represent the majority of the historical contaminants maintained in the ARC over the years. This indicates that poor technique contributed to either contamination of the host cells used for virus replication combined with a lack of a QA program or poor

technique during the manipulation of the virus preparations in the laboratory. The isolates contaminated with *M. arginini* were all received in recent years from one outside source. This could indicate that the submitting laboratory has an issue with their FBS source, a mycoplasma contaminant was introduced into the cells used for the virus replication or isolation, and/or a lack of a QA program has allowed the contaminant to persist.

Although Table 3 only reflects the most recent host in which the virus was passaged, it is important to consider the complete passage history when analyzing the results. If a virus has only been passaged in SMB according to laboratory records, but a bovine mycoplasma species is detected, it could indicate a laboratory contaminant was introduced during testing or that the laboratory records are incomplete. If a virus has historically been passaged only in mammalian cells, but the current host is invertebrate cells and a negative mycoplasma result is obtained, it could be a false negative due to the mycoplasma growth being suppressed. We observed this phenomena with the Lonza kit, which detects only actively replicating mycoplasma.

It is also important to note if the virus preparation has been previously treated for mycoplasma. In this comparison, one virus had been treated for mycoplasma contamination by serial filtration. This sample was positive by both ATCC and ABM prior to treatment and negative by both assays after treatment. This sample produced a faint band with the tuf gene primers, but sequencing was unsuccessful. We have seen other samples that have been treated with serial filtration still have a positive result. However, after an additional passage in mammalian cells the virus preparation had a negative result when retested. As these are molecular assays detecting DNA, not necessarily viable organisms, the additional passage was needed to clear the residual DNA from the sample.

Once a virus culture becomes contaminated with mycoplasma, treatment can be difficult without altering the integrity of the virus and, in our experience, of limited success. Commercial products for treating cell lines, such as Plasmocin, require the virus to be passaged in the presence of the agent, which may destroy the virus viability, impose selective pressure, alter the quasispecies population, and/or increase the passage number of the virus. Serial filtration to remove mycoplasma contaminants has shown limited success. RNA extraction of the virus and electroporation in cells for recovery of uncontaminated virus is currently being explored. Both serial filtration and RNA electroporation help to keep the passage number low, but any method that alters the virus population and imposes additional selective pressures on the overall virus population needs to be examined thoroughly. Additional studies are needed on mycoplasma treatment methods to determine which method imposes the least selective pressure on the virus population and if the treatment imposes more selective pressure on the virus than the mycoplasma contaminant.

When maintaining cell lines in the laboratory, if a contaminant is introduced into a cell line the recommended practice is to autoclave the cells and replace the cell line from a reputable cell repository. It is not recommended to attempt treatment and recovery unless the cell line is valuable or unique and does not exist in other repositories. If treatment and recovery is required, then isolation of the contaminated cell line is recommended while treatment is pursued (Freshney, 2010). Implementing a QA program for cells involving routine QC mycoplasma testing and replacement of a potential contaminated cell line used for isolation or maintenance of viruses is much easier and cheaper than treating a mycoplasma-contaminated virus repository.

These results highlight the importance of a QA program that includes QC testing for mycoplasma contamination of historical virus isolates, new isolates submitted to the ARC or other repositories, and the cell lines used to maintain the viruses with sensitive and specific mycoplasma assays. The ABM kit is suitable for routine QC testing of viruses. New mosquito isolates should be QC tested with the ATCC kit, which includes spiroplasma detection. The Lonza kit should be avoided when QC testing virus replicated in SMB, due to its propensity to produce false positives in this case. The Sigma kit has low sensitivity and is cost prohibitive for routine QC testing. To ensure the quality of the viruses available to researchers, QA programs are needed at both levels of virus maintenance (initial isolation and repositories). These results can help inform decisions to implement a new QA program or modify an existing QA program for improved virus maintenance.

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Declarations of interest

None.

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