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Investigation of an Adventitious Agent Test False Positive Signal in a Plant-Derived Influenza Vaccine

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Abstract

Medicago manufactures influenza vaccine virus-like particles (VLPs) in an unusual production platform consisting of *Nicotiana benthamiana* plants.

During the *in vitro* adventitious agent test (AAT) of certain Medicago B strain influenza vaccine VLP test samples, positive hemagglutination of guinea pig red blood cells was observed on day 14, but not on day 28. The positive result in the assay was surprising because the production process uses no animal-derived raw materials and contains a viral inactivation step. Plant-associated viruses would not be expected to infect the mammalian cell-based assay. No cytopathic effects or hemadsorption of red blood cells was observed in these AATs. The positive hemagglutination was observed at 2–8°C, but not at 36–38°C, and only in a few of the six detector cell lines used in the assay.

Because this is quite an unusual pattern of responses for an AAT, Medicago and the contract testing lab, Eurofins Lancaster Laboratories (ELLI) investigated the positive responses thoroughly for the presence of an adventitious agent or an alternative explanation not involving a viral contaminant. Investigation results indicated that the hemagglutinating activity associated with the vaccine test sample itself was responsible for the positive hemagglutination response. The positive hemagglutination on day 14 of these AATs was deemed an assay artifact, and preventive actions were taken to prevent recurrence of this type of false positive response.

Introduction

Medicago produces a quadrivalent influenza vaccine in the *Nicotiana benthamiana* plant. Unlike the production processes for many influenza vaccines, the Medicago production process uses no live virus at any stage in the process. In addition, *N. benthamiana* does not support replication of viruses that infect mammalian cells, nor has it been demonstrated that plant-associated viruses infect mammalian cells. However, regulatory agencies are concerned about the potential for introducing adventitious agents infectious for mammalian cells during production operations and have mandated that Medicago include a unit operation to eliminate potential contaminants. As a part of the product development lifecycle, Medicago subjected its drug substance to an *in vitro* adventitious agent test (AAT) as directed by specific guidances from the US Food and Drug Administration (FDA)[1] and the International Conference on Harmonisation (ICH).[2] During this assessment, Medicago discovered the potential for the test to return false positive signals associated with the nature of the Medicago influenza vaccine. This report details the investigation undertaken to elucidate the mechanism of the false positive AAT result.

Medicago's Vaccine

Medicago has recently completed a multi-country Phase 3 clinical trial of a quadrivalent influenza vaccine, which is composed of virus-like particles (VLP) bearing the hemagglutinin (HA) protein from influenza, produced in *N. benthamiana* plants.[3] Medicago has also conducted Phase 1 and Phase 2 clinical trials with these vaccines in the US and Canada.[4,5] The production process includes extraction of the VLP from plant leaf tissue, and clarification and purification steps that use classical unit operations such as dead-end filtration, tangential flow filtration, and chromatography.[6,7] This VLP has been extensively characterized biochemically and is morphologically similar to the influenza virus.[8,9] The production process also includes a viral inactivation step that uses ultraviolet C (UVC) light to induce nucleic acid damage to adventitious agents that could theoretically be present in the process stream.[10]

The regulatory requirement that a viral inactivation step be included in a process that uses no animal-derived raw materials and uses a plant as the host cell production system may appear, at first glance, to be unnecessary. Plant cells are not reported to be capable of allowing propagation of a virus that infects or replicates in mammalian cells. Neither have plant viruses been reported to be capable of replicating in or infecting mammalian cells.[11,12] However, regulatory agencies around the world acknowledge the possibility of adventitious agents entering a production

process as a result of raw material or environmental contamination, and therefore have good reason to require a removal or inactivation step be implemented into a plant cell production process to assure patient safety.[13-15]

In the early stages of process development, prior to the introduction of the UVC unit operation in the production process, Medicago assured product safety through the use of an *in vitro* AAT. Regulatory guidance documents such as the 1993 FDA *Points to Consider in the Characterization of Cell Lines used to Produce Biologicals*[1] and the ICH Q5A(R1) *Quality of Biotechnological Products*[2] suggest the use of an *in vitro* AAT for screening cell banks, end-of-production cells, and process intermediates as part of the safety assurance program for production of biologics. Medicago continued to test all drug substance lots made for use in clinical trials using the AAT, even after introducing the UVC unit operation. This was done to accumulate a body of data to support the safety of the *N. benthamiana* production system. During production of the investigational lots for use in the healthy adult, Phase 3 clinical efficacy study, and the clinical trial to validate the production process using consistency lots, four lots of drug substance returned a positive result, suggesting the potential presence of an adventitious agent in the AAT.

Adventitious Agent Test

The AAT used to release drug substance was performed at the Lancaster, Pennsylvania Eurofins Lancaster Laboratories (ELLI) facility according to their standard operating procedures (SOPs). Given the nature of Medicago's production process, the AAT was expanded from three to six detector cell lines for evaluating Medicago's drug substance. The AAT minimally uses a human diploid cell (such as MRC-5), a primate cell (such as Vero), and a third detector cell that is normally of the same animal species as the production cell. In this case, plant viruses are not of concern, so the additional three cell lines were selected to allow for the detection of viruses capable of infecting humans, rodent viruses (such as mouse minute virus), and insect-borne viruses (arboviruses) that theoretically could be present in the plant raw material. The cell lines selected were HeLa, a cell line with broad viral susceptibility, A9 and 324K cells for detecting the rodent parvovirus, and BHK-21 for detecting arboviruses.[16] It is important to note that Medicago plants are grown in a controlled greenhouse and an insect and rodent monitoring program is in place to minimize the risk from an insect or rodent-transmitted pathogen entering the process.

In general, the AAT involves three orthogonal measures for detecting adventitious agents. The first is the assessment of cytopathic effect (CPE) on the detector cell. The second is the presence of erythrocyte hemagglutination activity in the conditioned medium removed from the cells on days 14 and 28. The third measure is the detection of hemadsorption (HAD) of erythrocytes onto the detector cell monolayers. The AAT methodology is summarized in **Figure 1**. A drug substance test sample from each HA VLP lot is diluted 1:10 and then inoculated onto detector cells contained in 6-well culture plates. A positive and negative control are included for each detector cell line used. The cells are examined for CPE once on days 1–3 and then 2–3 times per week for the first 14 days of the assay. Cells may be re-fed if needed, typically 1–3 times in the first 14 days of the assay. On day 14, conditioned medium samples from the detector cells are used to perform a hemagglutination assay. A portion of each sample is saved for blind passage onto fresh detector cell cultures. The original detector cell monolayers are used for the HAD assay. The assay is performed in the same manner for the sub-passage (as was performed in the initial 14-day passage) with CPE being assessed periodically, and HAD and hemagglutination assays performed on day 28. The assay is considered negative if all CPE, HAD, and HA results are negative throughout the assay.

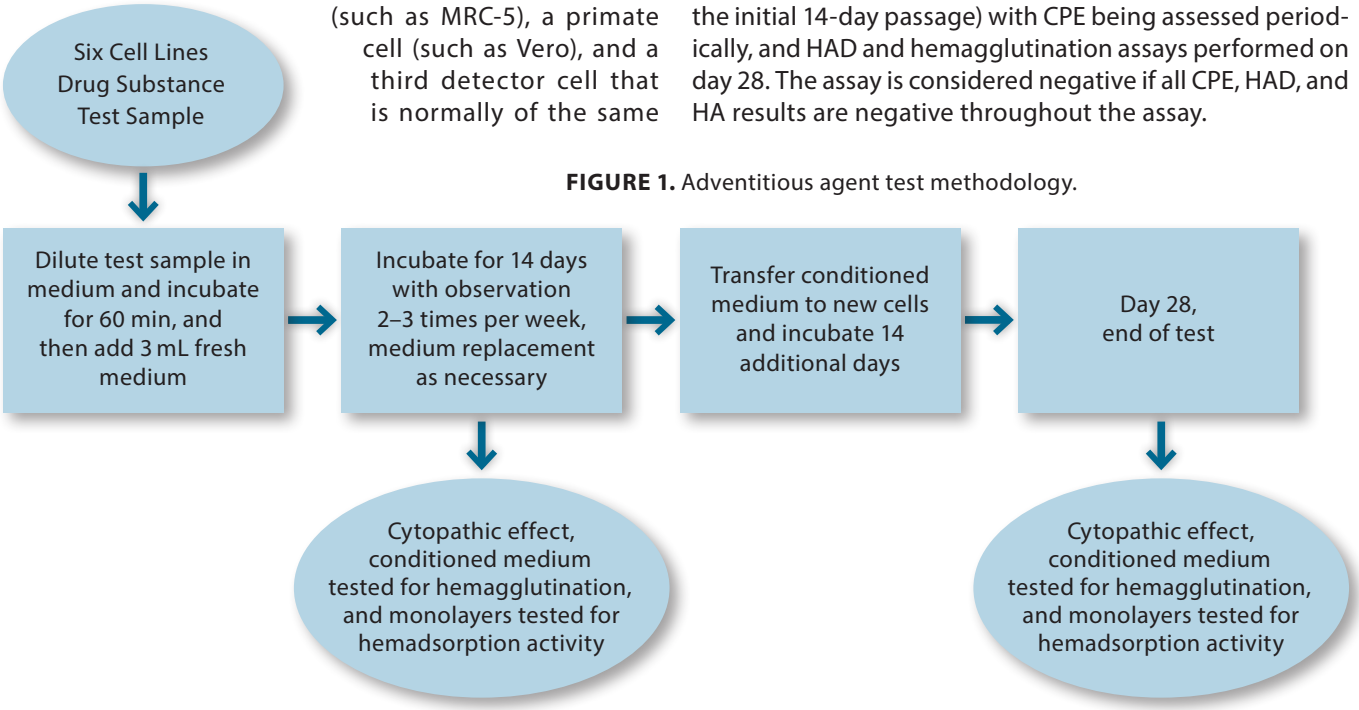


FIGURE 1. Adventitious agent test methodology.

Adventitious Agent Test Positives

AAT positives occurred during testing of three lots of B/Brisbane HA VLP drug substance and one lot of B/Phuket HA VLP drug substance produced in 2016. Up until this point in time, Medicago had never experienced a positive result for an influenza HA VLP drug substance in an AAT. The pattern of these four positive AAT results was unusual and led to an investigation of the assay, the production process, and the product. Each of the positives occurred in the hemagglutination portion of the assay only, on the day 14 test sample only, and for certain detector cell lines only, as shown in **Table 1**. The hemagglutination endpoint response was negative on day 28 in every case. The hemagglutination testing was performed according to ELLI's quality method work instruction. In the assay, red blood cells derived from three animal species (human, guinea pig, and chicken) are used, and each red blood cell type is incubated at 2–8°C and 36–38°C (separate tests). In all four cases shown in **Table 1**, hemagglutination only occurred with guinea pig red blood cells incubated at 2–8°C.

The finding of a positive hemagglutination response for a single species of red blood cells is not all that uncommon, but to have this occur at only one of the two temperatures is unusual, and more unusual is the finding of a positive hemagglutination response on day 14 that is not observed on day 28 following blind passage. An infectious adventitious agent isolated during the first 14 days of an AAT assay would be expected to amplify after blind passage onto fresh detector cells and during the ensuing 14 days of culture. These considerations led Medicago to question whether the positive hemagglutination responses represented a true indication of an adventitious contaminant, or rather an assay artifact.

After the abnormal results were obtained, Medicago enlisted the help of a virology subject matter expert (SME) to advise and assist in the oversight of an appropriately orchestrated investigation with ELLI. After examining the results from the testing, and reviewing the assay procedures and manufacturing operations, the SME provided suggestions for further assay assessment and investigatory testing. Medicago presented the planned experiments and worked with ELLI and the SME to develop the thorough investigation strategy that is described in the next section.

Investigation

In this article, we present the events and data that were derived during the investigation in chronological order, in hopes that the reader will develop an understanding of the rationale for what was done, as well as the choices made, given the desire for a rapid resolution to the problem. We ask the reader to consider that, while Medicago always kept in mind that clinical trial subject safety has highest priority, time was also critical as the company was in the midst of Phase 3 clinical study material production.

The internal investigation was performed according to Medicago SOPs, and was focused on the manufacturing processes involved in the false-positive samples and their handling prior to shipping to the contract testing facility for analysis. ELLI carried out investigations within their own quality system with Medicago present for all audits. Medicago found no indication that the manufacturing process had in any way deviated from normal operating conditions or that sample handling could have contributed to the AAT positive result. Likewise, ELLI found that the samples had been processed correctly, the analysts had been properly trained, the process version and methodology used were appropriate, the recorded data were correct, cleaning was performed properly, the correct equipment was used, and the reagents used had worked appropriately for a variety of other test samples run concurrently with the false-positive samples. ELLI also assured that no other assays performed in the same time period had returned a positive result for the AAT and that other assays performed on the Medicago samples (such as mycoplasma and spiroplasma) had returned negative results.

Special attention was given to ensure that cross-contamination of the Medicago samples with the AAT-positive virus controls had not occurred. The unusual pattern of the positive hemagglutination results was not characteristic of any of the positive control viruses employed. The continued assay investigation performed by Medicago and ELLI described in the remainder of this paper focused on the results from the two B/Brisbane HA lots 1 and 2 that tested positive, since these lots were part of the production campaigns surrounding planned clinical trials.

Given the results obtained in the original positive AAT and subsequent assay positives, along with the input from

TABLE 1. Hemagglutination-positive responses in the AAT assay.

HA Present on VLP	Detector Cell Substrate Used in AAT		
	324K Cells	HeLa Cells	BHK-21 Cells
B/Phuket Lot 1	–	–	+ day 14 only, at 2-8°C only
B/Brisbane Lot 1	+ day 14 only, at 2-8°C only	+ day 14 only, at 2-8°C only	+ day 14 only, at 2-8°C only
B/Brisbane Lot 2	–	+ day 14 only, at 2-8°C only	+ day 14 only, at 2-8°C only
B/Brisbane Lot 3	–	+ day 14 only, at 2-8°C only	–

Negative and positive agglutinations are represented by – and +, respectively.

the SME, Medicago postulated that the positive outcome may have represented an assay artifact that was due to the nature of the test sample itself. The vaccine VLP test sample is in itself comprised of empty viral capsids containing functional influenza HA on its surface. We theorized that residual hemagglutinating activity of the test sample itself might have been responsible for the positive hemagglutination responses experienced on day 14 of the assay, but not on day 28. Medicago considered the fact that the vaccine test sample was, according to procedure, allowed to remain on the detector cells in the AAT for an extended period after inoculation on the first day. In fact, the sample remains as part of the culture medium until being replaced during the first re-feed days later. This suggested that residual HA may have remained in the culture medium at some dilution up to the time of the day 14 hemagglutination test. During the re-feed procedure, which typically occurs one to three times prior to day 14 of the assay, the existing medium is removed from the culture wells with a pipette and 3 mL of fresh media are added. Some residual existing medium is always left behind in the culture wells since the removal process is not intended to be absolutely complete.

A model for the AAT assay procedure was developed in which Medicago assumed that 100 µL of existing medium remained behind in the culture wells following each re-feed. This model was used to estimate the extent of dilution of the initial VLP sample that might occur over the initial 14 days of the AAT. Medicago calculated that with the initial 1:10 dilution of the sample, a 0.5 mL volume of this dilution placed on the cells, and subsequent addition of 3 mL media, the total dilution of the test sample at the time of inoculation would have been 1:70. Once these cells had been re-fed with 3 mL of fresh medium, the total dilution would have been 1:2170 (3100 µL / 100 µL × 70 = 2170). In order to assess the accuracy of the model, 1:10, 1:70 and 1:2170 dilutions of drug substance were tested in Medicago's hemagglutination activity assay with guinea pig red blood cells. Hemagglutination was observed with the 1:10 and 1:70 dilutions, but not the 1:2170 dilution. These data indicate that there was either a smaller impact of dilution in the AAT than our model predicted or that the carryover of residual HA occurred by a mechanism other than simple liquid transfer.

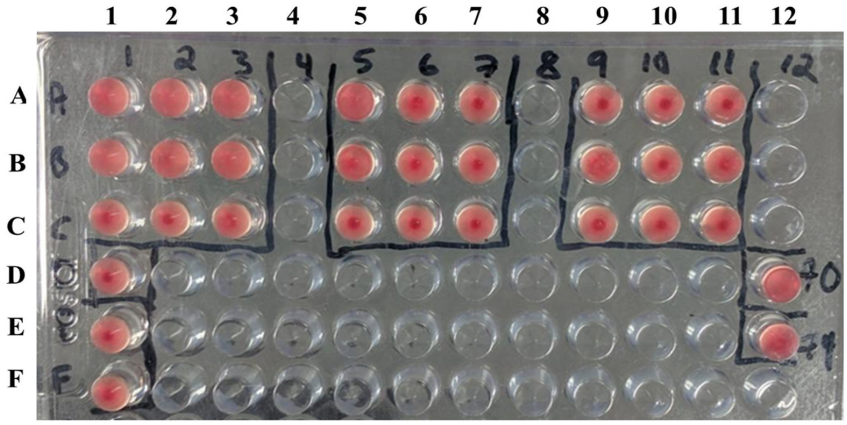
In addition to the hemagglutination activity assay, Medicago performed Western blotting using B/Brisbane-specific antiserum on the modeled dilutions to determine if the Western blot method might confirm the presence of the residual B/Brisbane HA. Western blot results indicated that B/Brisbane HA was detectable in drug substance diluted 1:10 and 1:70 but again, not at 1:2170. In order to confirm that the positive hemagglutination assay result was due to residual HA from drug substance, hemagglutination inhibition (HI) assays were performed using polyclonal sera raised against the strain of HA present in the drug substance, in this case B/Brisbane. These assays were performed by mixing a retained portion of the hemagglutination assay-positive cell supernatant with either pre-immune serum (serving as a negative control) or with B/Brisbane-specific antiserum. A positive control was prepared consisting of drug substance diluted in cell culture medium that was subsequently mixed with either pre-immune or B/Brisbane-specific antiserum. Inhibition of agglutination occurs via the binding of antibodies specific to the receptor binding domain on HA, which interferes with the ability of HA to bind to the sialic residues present on red blood cells.

The results of the HI assays performed with the supernatants from the hemagglutination-positive assays, designated as NS70 and NS74 in **Figure 2** (next page), indicated that the positive hemagglutination could have been due to B/Brisbane HA. However, subsequent results with supernatants from two additional assay-positive supernatants provided inconclusive results, due to lack of agglutination in the positive controls (data not shown). Given that the responses only occurred with undiluted supernatants, and even a 1:2 dilution did not result in hemagglutination in the assay, it appeared that the amount of HA present was at or near the level of detection for the assay. Thus, the inconsistency associated with the assay was attributed to the amount of HA present from assay-to-assay.

Although the HI assay results indicated that the positive result in the hemagglutination assay of the AAT could have been due to residual B/Brisbane HA, the HI assay results were somewhat inconsistent. Medicago was reluctant to conclude, solely on the basis of these HI results, that the test article was responsible for the positive hemagglutination responses in the AAT assays.

The investigation had taken five months by that point and a closure was desired to ensure that the planned clinical trials could occur as scheduled. Medicago had previously considered next-generation (or deep) sequencing to assess the possible presence of an unusual adventitious agent as the cause for the positive hemagglutination responses in the four impacted AAT assays. When contemplating this method, one is faced with the question: If nucleic acid is detected, is a replication-competent adventitious agent present, or just non-infectious genomic material? Medicago enlisted Eurofins Genomics Europe (Ebersberg, Germany) to analyze the positive AAT cell culture samples using next-generation sequencing (NGS).

NGS was performed on nucleic acid extracted from each of the hemagglutination-positive supernatants in order to definitively rule out the presence of an adventitious virus as a cause of the positive response. Nucleic acid extracted from control (non-inoculated) BHK-21, HeLa, and 324K cells was also tested. The Illumina MiSeq NGS system with v3 chemistry (MiSeq Control Software v2.5.0.5) was used. MiSeq RTA v1.18.54 and bcl2fastq v1.8.4 software helped in processing raw data and generating FASTQ files. Final consensus sequences were compared against the National Center for Biotechnology Information (NCBI) viral database (GenBank Release 219.0, April 2017) using BLASTn and tBLASTx to detect potential hits. In total, NGS indicated that



Well	Sample	Agglutination	Well	Sample	Agglutination	Well	Sample	Agglutination
A1	DS neat plus EMEM	+	A2	DS 1:2 plus EMEM	+	A3	DS 1:4 plus EMEM	+
B1	DS neat plus PI serum	+	B2	DS 1:2 plus PI serum	+	B3	DS 1:4 plus PI serum	+
C1	DS neat plus B/Bris serum	–	C2	DS 1:2 plus B/Bris serum	–	C3	DS 1:4 plus B/Bris serum	–
A5	NS70 neat plus EMEM	+	A6	NS70 1:2 plus EMEM	+	A7	NS70 1:4 plus EMEM	–
B5	NS70 neat plus PI serum	+	B6	NS70 1:2 plus PI serum	–	B7	NS70 1:4 plus PI serum	–
C5	NS70 neat plus B/Bris serum	–	C6	NS70 1:2 plus B/Bris serum	–	C7	NS70 1:4 plus B/Bris serum	–
A9	NS74 neat plus EMEM	–	A10	NS74 1:2 plus EMEM	–	A11	NS74 1:4 plus EMEM	–
B9	NS74 neat plus PI serum	+	B10	NS74 1:2 plus PI serum	–	B11	NS74 1:4 plus PI serum	–
C9	NS74 neat plus B/Bris serum	–	C10	NS74 1:2 plus B/Bris serum	–	C11	NS74 1:4 plus B/Bris serum	–
D1	EMEM only	–	D12	NS70 neat only	+			
E1	EMEM plus PI serum	–	E12	NS74 neat only	+			
F1	EMEM plus B/Bris serum	–						

DS: Drug substance positive control
EMEM: Medium control (Eagle's minimum essential medium)
PI: Pre-immune serum (pre-B/Brisbane vaccination)
B/Bris serum: Serum from animals inoculated with B/Brisbane VLP (antibody to B/Brisbane HA)
NS70 and NS74: Media from wells in the AAT

FIGURE 2. Inhibition of NS70 and NS74 agglutination by B/Brisbane-specific antiserum. Image of agglutination plate and well identification table. Negative and positive agglutination are represented by – and +, respectively.

there were no detectable viruses specific to the hemagglutination-positive supernatants (*i.e.*, hits that were not also detected in the DNA extracted from the non-inoculated detector cells). Extensive genomic reads for an endogenous retrovirus were detected in DNA extracted from the control BHK-21 cell extract and all test samples originating from BHK-21 cell extracts. The presence of this endogenous retrovirus in BHK-21 cells were expected, based upon previously published results.[17]

The NGS data acquisition and interpretation required approximately two months to complete. Concurrently, Medicago assessed the presence of B/Brisbane HA in the AAT by an orthogonal method to the hemagglutination test that had been performed previously. Medicago possessed in-house experience in performing immunofluorescence assays and had postulated that B/Brisbane HA might persist in the AAT assay by binding to cell surface receptors on the detector cell lines. A time-course study was designed

to test this theory. The HI assay was used in conjunction with a secondary antibody conjugated to Alexa Fluor 488 to visualize any residual HA in the detector cultures. In this study conducted at ELLI, B/Brisbane VLP drug substance was inoculated onto detector cells in 6-well plates following the usual AAT procedure. The typical AAT medium replacement schedule was followed and cells were removed from the 6-well plates at various time points up to day 14 of the assay. Two of the detector cell substrates that were shown to be positive in **Table 1**, BHK-21 and HeLa, were included in the assay along with negative controls for all time points monitored. The cells from the assay were removed from the 6-well plates, transferred to a cytoslide, and treated with primary and secondary antibodies for visualization of bound HA by fluorescent microscopy. Photographs of cells from days 0, 7, and 14 in the HeLa cell portion of the assay are shown in **Figure 3**. Similar fluorescent staining was observed in the case of the BHK-21 cells (not shown). The B/Brisbane HA-specific fluorescence in the detector cells over the course of the assay confirms the presence of residual HA at all time points for both cell types in the assay, even after medium exchange on day 7. As a complement to the immunofluorescence study, the hemagglutination activity of the supernatants from the time course was determined semi-quantitatively by endpoint dilution titration. The results in **Table 2** show that the hemagglutination activity in the detector cells was highest on day 0 and declined at each subsequent time point. The decreasing hemagglutination activity and fluorescence in these cultures over time differs from the expected amplification (*i.e.*, increasing signal with time in culture) pattern of an authentic virus infection. Had there been a replication-competent virus in the test samples, one would have expected the viral antigen to spread through the culture over time.

Assessment of the Results

Laboratory investigations by Medicago and ELLI indicated that all manufacturing, sampling, and testing operations had been performed correctly. There were no indications that the hemagglutination-positive responses were due to errors in operations at either site. Medicago then began to challenge the assay to assess what mechanism, other than an infectious adventitious agent, might be responsible for the positive result. In the first set of assays performed as a part of the investigation, Medicago determined that there was indeed residual HA associated with the test samples, as hypothesized. The dilution of the hemagglutination in the test sample during the routine AAT procedure should have prevented a positive result by day 14 of the assay, if the conditioned medium contained the residual sample HA. Of course, this model and the conclusion were based upon an assumption that 100 µL of diluted sample would have remained in the wells of the assay after a re-feed with media. While it is possible that slightly more volume might had remained, the fact that four individual assays had returned positive results in a very particular manner made this type of carry-over mechanism unlikely.

Medicago's demonstration that an antibody specific for B/Brisbane HA could prevent the hemagglutination reaction suggested that the test article might be responsible for the positive AAT result. However, the inconsistency of the hemagglutination inhibition assay prevented Medicago from confirming the hypothesis. Thus, Medicago used NGS to establish whether a complete genome for an adventitious virus might be present in assay supernatants from wells that had presented positive hemagglutination

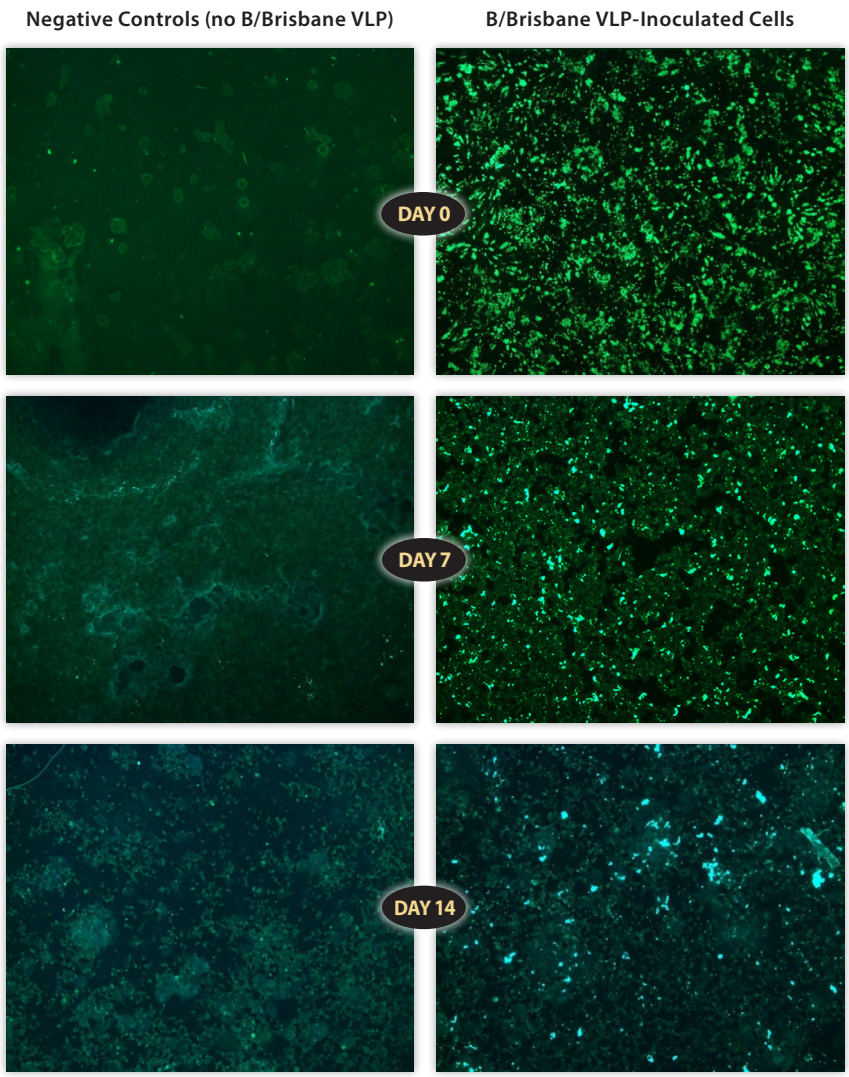


FIGURE 3. Immunofluorescence assay with HeLa cells on days 0, 7, and 14.

TABLE 2. Hemagglutination titer results (for each sample) for the conditioned medium from the time-course study.

Detector Cell Line	Pre-Inoculation (diluted 1:10)	Post-Inoculation				
	Day 0	Day 0	Day 3	Day 7	Day 10	Day 14
BHK-21	1:6144	1:1536	1:24	1:2	Neat	Neat
HeLa	1:3072	1:1152	1:16	1:16	Neat	Neat

results. After sequencing, there was no evidence of viral genomic material in the inoculated detector cell nucleic acid vs. the non-inoculated cell nucleic acid.

Medicago was able to demonstrate, using an immunofluorescence assay, that influenza HA persisted on the surface of the inoculated HeLa and BHK-21 detectors up to day 14 after inoculation, even after re-feeding. Given these results, a new model was assembled to account for the positive results observed in the hemagglutination portion of the AAT. Medicago postulated that B/Brisbane HA present on the cell surface in the assay could dissociate after the re-feed to establish a new equilibrium between cells and media. When the conditioned media was tested at the low dilution used in the hemagglutination portion of the assay, it led to a positive result due to residual vaccine HA. The amount of HA protein present could vary from assay-to-assay and could depend on the starting concentration of the HA in the sample applied to the assay. Other contributory factors might include the efficiency of media removal during re-feeding, the detector cell type, and the frequency of media replacement. With variables like these at play, an inconsistent result for hemagglutination inhibition might be expected, and an explanation of the negative result for the 1:2170 dilution in the hemagglutination assay was obtained. At that point in the investigation, based upon the data derived from multiple orthogonal assays, the assay positive was considered to represent a false positive caused by the influenza HA in the test sample.

Medicago, the SME, and ELLI reviewed the AAT methodology to ensure test sample-mediated positive results would not reoccur. Two preventive actions were implemented as a result of this investigation. First, the test sample was not allowed to remain on the detector cells for the first seven days of the assay, as in the original method. Instead, the test sample was inoculated, allowed to remain on the detector cells for 60 minutes, and then removed and replaced with fresh culture medium. This is typical practice in the industry and was not found to diminish the sensitivity of the AAT assay. After consulting with the independent SME, the second corrective action was to eliminate the hemagglutination portion of the AAT because the test sample was likely to cause future false positive hemagglutination responses in the assay. It has been observed that hemagglutination is the least sensitive of the three AAT endpoints[16], therefore removal of this endpoint is not expected to adversely impact the detection sensitivity of the method. The Medicago test methodology at ELLI was revised to capture these modifications. The revised test procedure is shown in **Figure 4**.

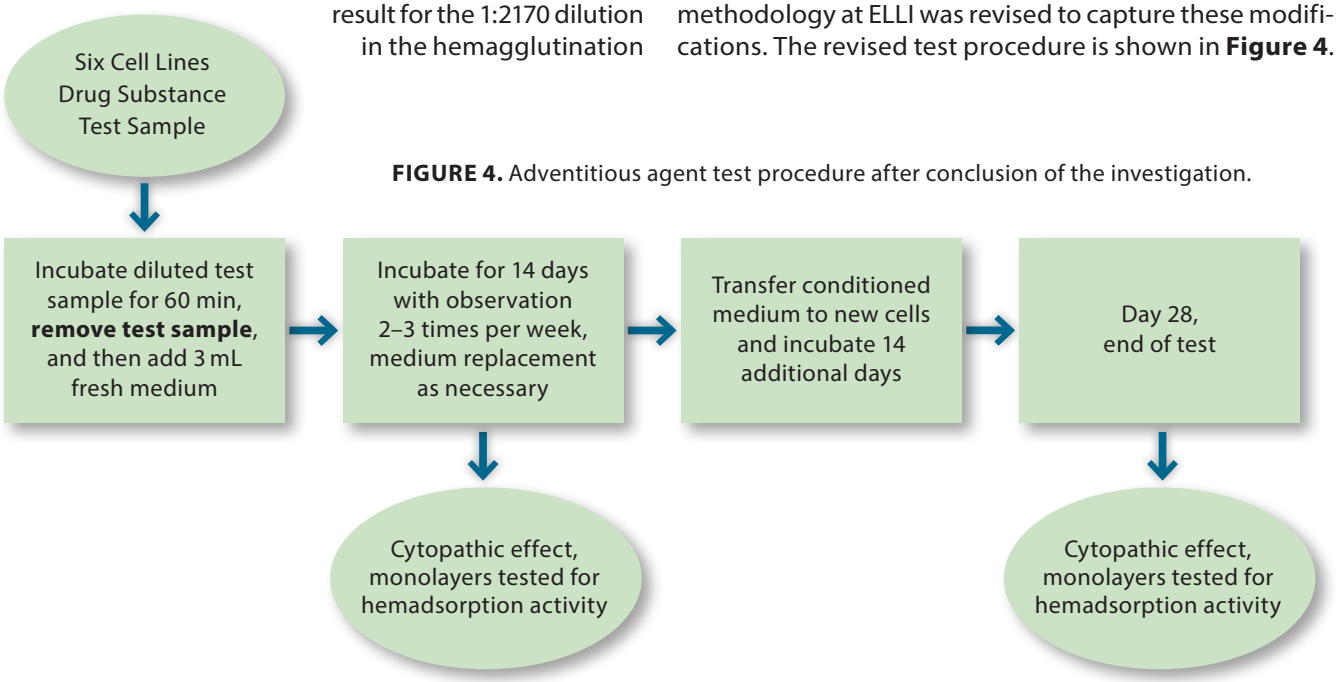


FIGURE 4. Adventitious agent test procedure after conclusion of the investigation.

Conclusion

Medicago observed AAT positives for two B strains of HA VLP during routine production. The positives occurred only in the hemagglutination portion of the AAT and presented a unique pattern that was difficult to associate with the pattern of hemagglutination expected from known viruses. In addition, Medicago's product, the test sample, was known to possess hemagglutination activity, but AAT positives had not been observed with multiple lots tested over a previous five-year period. Medicago could not demonstrate that there was sufficient test sample remaining in the AAT to cause the positive result. However, antibody specific to B/Brisbane HA was sometimes capable of inhibiting the hemagglutination assay positive, suggesting that the influenza HA was responsible for the false-positive result. Immunofluorescence assays demonstrated the presence of B/Brisbane HA protein on the surface of the cell substrates used in the AAT at the time the AAT-positive occurred. This was attributed to the HA retained on the detector cell surfaces, dissociating on day 14 of the assay, and causing the positive result in the hemagglutination portion of the assay. This explanation was also supported by the inability to detect any near complete viral genomic material in the assay fluids when subjected to NGS. Based upon the results from the body of data collected, modifications to the AAT protocol were made to prevent future false positives in the assay.

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