Detection of Avian Retroviruses in Vaccines by Amplification on DF-1 Cells with Immunostaining and Fluorescent Product-Enhanced Reverse Transcriptase Endpoint Methods

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In order to ensure the safety of vaccines produced on avian cells, rigorous testing for the absence of avian retroviruses must be performed. Current methods used to detect avian retroviruses often exhibit a high invalid-test/false-positive rate, rely on hard-to-secure reagents, and/or have readouts that are difficult to standardize. Herein, we describe the development and validation of two consistent and sensitive methods for the detection of avian retroviruses in vaccines: viral amplification on DF-1 cells followed by immunostaining for the detection of avian leukosis virus (ALV) and viral amplification on DF-1 cells followed by fluorescent product-enhanced reverse transcriptase (F-PERT) for the detection of all avian retroviruses. Both assays share an infectivity stage on DF-1 cells followed by a different endpoint readout depending on the retrovirus to be detected. Validation studies demonstrated a limit of detection of one 50% cell culture infectious dose (CCID₅₀)/ml for retrovirus in a 30-ml test inoculum volume for both methods, which was as sensitive as a classical method used in the vaccine industry, namely, viral amplification on primary chicken embryo fibroblasts followed by the complement fixation test for avian leukosis virus (COFAL). Furthermore, viral amplification on DF-1 cells followed by either immunostaining or F-PERT demonstrated a sensitivity that exceeds the regulatory requirements for detection of ALV strains. A head-to-head comparison of the two endpoint methods showed that viral amplification on DF-1 cells followed by F-PERT is a suitable method to be used as a stand-alone test to ensure that vaccine preparations are free from infectious avian retroviruses.

Vaccines are very effective at reducing death and suffering caused by viral diseases. Many vaccines are generated on primary avian cells, such as the vaccines for yellow fever, measles, and mumps. International health authorities require vaccines produced on avian cells to be tested for and demonstrated to be free of infectious avian retroviruses such as avian leukosis virus (ALV) and reticuloendotheliosis virus (REV) (1, 2, 3, 4). For example, detailed regulatory requirements are outlined specifically for the detection of ALV and REV in the European Pharmacopoeia (Ph. Eur.) (2). Furthermore, the detection of reverse transcriptase (RT) activity, which is indicative of the presence of retroviruses, by highly sensitive PCR-based methods is recommended by several international regulatory authorities (3, 4, 5).

The detection of RT activity in yellow fever, measles, and mumps vaccines (6, 7) clearly demonstrated a need for effective methods to test for the presence of replication-competent retroviral contaminants. It was discovered upon further investigation that nonreplicative endogenous avian retroviral elements not associated with infectious retroviruses were responsible for the positive RT signals observed in these vaccines (8, 9, 10, 11, 12). As a consequence, it is now understood that it is necessary to test for the presence of infectious retroviral properties in addition to the presence of RT activity. Regulatory guidelines for RT assays recommend the use of an amplification strategy (i.e., infectivity) to determine whether a positive RT result can be attributed to the presence of an infectious retroviral agent (3, 4).

Current test methods used to detect infectious retroviruses in vaccine lots include an infectivity culture method followed by either a complement fixation test for avian leukosis (COFAL) or an

enzyme-linked immunosorbent assay (ELISA) readout specific for ALV (2) or infectivity with a reverse transcriptase PCR endpoint for the detection of amplified RT activity (3). Unfortunately, these tests often exhibit high invalid test rates, may use primary chicken embryo fibroblast (CEF) cells that can result in inconsistent results, rely on the use of detection reagents (i.e., antibodies) that are difficult to secure in sufficient quantities for routine lot release testing, and have readout systems that are time-consuming and difficult to standardize. For example, it is difficult to consistently determine the appropriate cutoff point between positive and negative results for the ELISA method, and the consistency of the COFAL readout is directly related to the quality of the complement and erythrocytes used. Therefore, the vaccine industry is very interested in developing alternative methods that are more reliable and standardized than those mentioned above for the detection of avian retroviruses in vaccines.

DF-1 cells are an immortalized avian cell line spontaneously

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derived from *ev-0* fibroblasts of 10-day-old chicken embryos (13). *ev-0* cells do not contain endogenous ALV sequences, and thus these cells can be used as the substrates for the detection of exogenous ALV and other avian retroviruses (2, 14). Maas et al. (15) demonstrated that DF-1 cells are a suitable replacement for primary CEFs for the detection of ALV retroviruses. As such, the use of this cell line was added to Ph. Eur. 2.6.24 (in July 2009, version 6.5) and is considered appropriate for infectivity-based assays for retroviral safety testing of vaccines.

The fluorescent product-enhanced reverse transcriptase method (F-PERT) is a technique that was developed as an improvement upon reverse transcriptase PCR-based assays (16). A specific RNA (i.e., bacteriophage MS2 RNA) is added to the test sample to serve as a template for any RT activity present. If RT activity is present in the sample, a specific cDNA is produced that is then used as a target for fluorescent quantitative PCR. The presence of a fluorescent signal above a background threshold indicates the presence of RT activity in the original sample. F-PERT is a very sensitive assay and an acknowledged advancement in the detection of retroviral contaminants. However, precautions are required to limit the occurrence of false positives with this method, such as limiting the amount of RNA template available in the reaction, including activated DNA in the testing protocol to reduce the detection of nonspecific RT activity produced by cellular DNA polymerases, and performing infectivity assays to confirm whether positive signals are associated with infectious retrovirus (4, 16).

Herein, we describe the development of alternative and improved methods for the detection of retroviruses in avian cell culture supernatants. These methods rely on the use of the DF-1 cell line to amplify any infectious retroviruses that may be present in a test sample. The presence of any infectious retrovirus is then detected by either immunostaining that is specific for ALV or F-PERT, which is able to detect RT produced by any retrovirus. In this study, these methods were validated for use as safety tests for the detection of potential retroviral contaminants in vaccines.

MATERIALS AND METHODS

Virus strains. ALV strains Rous-associated virus (RAV)-1 (subgroup A), RAV-2 (subgroup B), RAV-49 (subgroup C), and RAV-50 (subgroup D) and REV (at a titer of 7 log 50% cell culture infectious dose [CCID₅₀]/ml) were obtained from ATCC (Manassas, VA). ALV strain RAV-J (subgroup J) was obtained from Charles River (Wilmington, MA). Working banks of each virus were created, titrated as described below, and stored at -60°C or lower. Murine leukemia virus (MuLV) was obtained from the ATCC. HIV-1 strain Bru, HIV-2, and human T-cell lymphotropic virus 1 were obtained from the Pasteur Institute in Paris, France. Human adenovirus type 5, cytomegalovirus, and herpes simplex virus 1 were provided by P. Boulanger (Rockefeller University, Lyon, France).

Titration of ALV strains. Titration of the ALV strains was performed as described previously (17). Briefly, DF-1 cells were seeded in 96-well microplates and incubated at 36°C for 48 h. The viral suspension was then serially diluted and inoculated onto the cells, and the plates were incubated for a further 11 to 12 days at 36°C . After this incubation period, the plates were fixed with acetone and stained for the ALV p27 protein (Charles River SPAFAS, Wilmington, MA). A secondary antibody (goat anti-rabbit IgG antibody; Clinisciences, Montrouge, France) linked to alkaline phosphatase was used as the detector antibody, followed by the addition of the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium (BCIP-NBT) (Sigma-Fast; Sigma-Aldrich, Saint-Quentin Fallavier, France). Enumeration of the wells showing black coloration (positive wells) was performed, and the infec-

tious titer, expressed as the 50% infectious dose (log CCID₅₀/ml), was calculated using the least-squares method.

CEF matrix generation. The chicken embryo fibroblast (CEF) matrix used for the validation of the CEF amplification plus COFAL method was obtained during the manufacture of vaccine lots by Sanofi Pasteur (France). The CEF matrix used for the validation of the DF-1 amplification methods was generated by harvesting supernatants from CEF cultures. Briefly, CEFs (Charles River SPAFAS) were seeded in roller bottles at 2×10^8 cells/roller bottle and maintained for 2 days in Dulbecco modified Eagle medium (DMEM)-F-12 medium containing 5% fetal bovine serum (FBS) and 50 $\mu\text{g}/\text{ml}$ neomycin. The spent medium was then replaced with fresh medium containing 1% FBS and 50 $\mu\text{g}/\text{ml}$ neomycin, and the cells were allowed to grow for a further 3 to 4 days. The culture supernatants were harvested, clarified at $2,000 \times g$ for 10 min, aliquoted, and stored at -60°C or lower.

Viral amplification on CEFs followed by COFAL. Primary cells derived from specific-pathogen-free flocks were used for retroviral amplification. On day 0, CEFs were seeded in DMEM-F-12 medium containing 5% FBS and 0.2% penicillin-streptomycin (Eurobio, Les Ulis, France). The test virus was thawed and diluted in the CEF matrix to the desired test concentration(s). For each concentration of virus, the required number of replicates (i.e., flasks freshly seeded with CEFs) was inoculated with 30 ml of diluted virus and incubated at $36 \pm 1^{\circ}\text{C}$, in $5\% \pm 2\% \text{CO}_2$. Each replicate was amplified on CEFs as described below for amplification on DF-1 cells. After the amplification period, the flasks were washed with dextrose-gelatin-Veronal (DGV) buffer (TechGEN, France) and cell lysates were prepared by freeze-thawing. The cell lysates were then clarified by centrifugation and the supernatants were stored at -60°C or lower for COFAL analysis. Cell lysates from uninoculated cells were prepared in parallel as negative controls. To prepare the hemolytic mixture, rabbit anti-sheep hemolytic serum (bioMérieux, Craponne, France) was added to an equal volume of 2% sheep red blood cells (bioMérieux) and incubated at $37 \pm 1^{\circ}\text{C}$ for 1 h. While the hemolytic mix was incubating, guinea pig complement (bioMérieux) was titrated as follows. Serial dilutions of complement in DGV buffer were prepared; 50 $\mu\text{l}/\text{well}$ DGV buffer, 25 $\mu\text{l}/\text{well}$ of each complement dilution, and 50 $\mu\text{l}/\text{well}$ hemolytic mix were then added to a 96-well plate. The plate was incubated at $37 \pm 1^{\circ}\text{C}$ with shaking for 30 min and then centrifuged at $220 \times g$ for 5 min at $5 \pm 3^{\circ}\text{C}$. The last complement dilution that resulted in total hemolysis was designated 1 U of complement. The cell lysates prepared as described above were then thawed and analyzed by COFAL in 96-well plates as follows. Diluted rabbit anti-p27 serum (Charles River) or serum nonreactive to ALV (as a negative control) and the cell lysate were added in a 1:1 ratio to the plates. Then, 2 U of complement (per the complement titer determined above) was added. The plates were incubated for 2 h at $37 \pm 1^{\circ}\text{C}$ with shaking. After the incubation, a 50- $\mu\text{l}/\text{well}$ hemolytic mix was added to the plates and another incubation was performed for 30 min. The plates were then centrifuged at $220 \times g$ for 5 min at $5 \pm 3^{\circ}\text{C}$. Total hemolysis indicated a negative result. The lack of hemolysis (i.e., the presence of a pellet of red blood cells) indicated a positive result.

DF-1 cell line maintenance. DF-1 cells (ATCC) were passaged twice a week and maintained in DMEM-F-12 plus GlutaMax medium (Invitrogen, Carlsbad, CA) containing 5% FBS. For the DF-1 amplification procedure, the cells were not passaged beyond passage 168.

Viral amplification on DF-1 cells. A schematic of the DF-1 amplification procedure is shown in Fig. 1. Three or 4 days before inoculation with virus, DF-1 cells were seeded in T175 flasks at a concentration of 5×10^6 cells/flask in DMEM-F-12 medium containing 5% FBS. On day 0, the DF-1 cells were passaged and seeded into new T175 flasks at a concentration of 5×10^6 cells/flask in 70 ml of medium. Immediately after DF-1 cell passaging, the test virus was thawed and diluted in the CEF matrix to the desired test concentration(s). For each concentration of virus, the required number of replicates (i.e., T175 flasks freshly seeded with DF-1 cells) was inoculated with 30 ml of the diluted virus and incubated at $36 \pm 1^{\circ}\text{C}$, in $5\% \pm 2\% \text{CO}_2$, for 3 days. The following steps were performed to

FIG 1 Schematic diagram of viral amplification on DF-1 cells followed by immunostaining and F-PERT endpoints. Thirty milliliters of sample was inoculated into a T175 flask freshly seeded with DF-1 cells. The inoculated cells were then passaged 4 times to allow for amplification of any infectious retroviruses potentially present in the initial sample. On day 17 of the assay, aliquots of the culture supernatants were taken for F-PERT analysis. (A) The flasks were then fixed and immunostained for the p27 protein to detect ALV, or (B) the supernatant aliquots were analyzed by F-PERT to detect RT activity associated with any retrovirus present at the end of the assay.

amplify the virus for each replicate (i.e., for each T175 flask). On day 3, spent medium was discarded. The inoculated cells were passaged by resuspending the trypsinized cells in 10 ml/flask of DMEM-F-12 plus 5% FBS and dividing the cell suspension equally into 2 new T75 flasks (therefore, each T175 flask was passaged into 2 new T75 flasks). The medium volume was then brought up to a total of 40 ml/flask. On day 4, the spent media on the cell cultures were exchanged for fresh media according to the confluence of the cell monolayer, as follows. For a confluence of >80%, the medium was exchanged with DMEM-F-12; for a confluence of 50% to

80%, it was exchanged with DMEM-F-12 plus 1% FBS; and for a confluence of <50%, it was exchanged with DMEM-F-12 plus 5% FBS. On day 6, spent media were discarded. The cells were passaged 1:2 by resuspending the trypsinized cells in 10 ml/flask of DMEM-F-12 plus 5% FBS and pooling the cell suspensions from the 2 T75 flasks together (20 ml total). Five milliliters of the pooled cell suspension was then aliquoted into each of 2 new T75 flasks, and the medium volume was brought up to a total of 40 ml/flask. On day 7, the spent media on the cell cultures were exchanged as described for day 4. On day 10, cells were passaged 1:2 per the procedure described for day 6. On day 11, the spent media on the cell cultures were exchanged as described for day 4. On day 13, cells were passaged 1:2 per the procedure described for day 6. On day 14, the spent media on the cell cultures were exchanged as described for day 4. On day 17, the cell culture supernatants from the 2 T75 flasks were pooled, and 1-ml aliquots of the pooled supernatant were sampled and stored at -60°C or lower for subsequent F-PERT analysis. The remainder of the supernatant was discarded, and the flasks were fixed and immunostained with anti-p27 as described below.

Fixation and immunostaining with anti-p27. After DF-1 amplification, flasks were gently washed with 10 ml/flask chilled 85% acetone and then placed at -30°C to -20°C for at least 15 min in 85% acetone. The acetone was discarded and the flasks were allowed to dry for at least 1 h prior to staining. If necessary, fixed flasks were stored at -30°C to -20°C for up to 1 week. Prior to immunostaining, the fixed flasks were warmed to room temperature and then placed in blocking buffer (2.5% skim milk [VWR, Radnor, PA], 0.05% Tween 20 [Sigma, St. Louis, MO], and 0.5% Triton X-100 [Sigma] in phosphate-buffered saline [PBS]) for approximately 30 min at 36 ± 1°C. Rabbit anti-p27 serum (Charles River) was diluted in blocking buffer at a dilution of 1/1,200 and then added to the flasks. Incubation with the primary antiserum was allowed to proceed for approximately 45 min at 36 ± 1°C. Flasks were washed with 0.05% Tween 20 in PBS before addition of the secondary goat anti-rabbit antibody coupled to alkaline phosphatase (Southern Biotech, Birmingham, AL) (diluted in blocking buffer at a dilution of 1/1,000) for approximately 45 min at 36 ± 1°C. For the specificity experiment, chicken anti-REV (Charles River) was used as the primary antibody, and alkaline phosphatase-AffiniPure F(ab)2 donkey anti-chicken antibody (Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody. After further washes, the substrate BCIP-NBT (Sigma-fast SAFC, Arklow, Ireland) prepared in water containing 0.1% levamisole (Sigma) was added to each flask, and the reaction was allowed to proceed for at least 1 h at ambient temperature.

F-PERT. The 1-ml cell culture supernatants that were stored frozen from day 17 of the DF-1 amplification procedure were thawed and clarified at 17,000 × g for 10 min, and then the supernatants were transferred to fresh tubes. Clarified samples were pretreated with 0.2% Triton X-100 (Sigma), to release RT activity associated with retrovirus particles, for a minimum of 1 h on ice. The samples were then filtered through a 0.22-μm filter and aliquoted. At this stage, the pretreated samples were stored at -60°C or lower, if necessary. The Qiagen QuantiTect PCR kit (Qiagen, Valencia, CA) was used to perform F-PERT with the following primers and minor groove binding (MGB) probe: primer qPERT1 (5'-AAC ATG CTC GAG GGC CTT A-3'), primer qPERT2 (5'-GCC TTA GCA GTG CCC TGT CT-3'), qPERT MGB probe (5'-6-carboxyfluorescein-TGG GAT GCT CCT ACA TG-MGB-3'). The following controls were included in every experiment: a no-template control (consisting of master mix and 0.3× buffer A with no template added), a negative extraction control (consisting of nuclease-free water processed in parallel with the samples), and a positive control (consisting of Moloney murine leukemia virus [M-MLV] RT standard curve serial dilutions). For the RT stage of the assay, pretreated samples were tested neat or diluted 1/10 or 1/100 in 0.3× buffer A (1× buffer A: 50 mM Tris [pH 8.0], 0.25 mM EDTA [pH 8.0], 50 mM KCl, 0.025% Triton X-100, 50% glycerol, and 5 mM dithiothreitol [DTT]). The standard curve (M-MLV RT; Invitrogen) was prepared in 0.3× buffer A to cover a range of 10⁻⁸ to 10⁻³ U/10 μl and was kept on

ice. The RT master mix was prepared to contain 2× RT buffer (10× RT buffer: 328 mM KCl, 66 mM Tris [pH 8.0], 36 mM MgCl₂, 0.66% Nonidet P40 [Roche, Basel, Switzerland], and 72 mM DTT), 0.4 mM deoxynucleoside triphosphate (dNTP) mix (Roche), 0.8 μM qPERT2 primer, 5 ng/μl bacteriophage MS2 RNA (Roche), 0.8 U/μl RNasin Plus RNase inhibitor (Promega, Fitchburg, WI), and 0.15 μg/μl activated DNA from calf thymus (Sigma) in nuclease-free water, and added 1:1 with each template in 96-well plates. Reverse transcriptase PCR was performed on a Roche LightCycler 480 as follows. Reverse transcription was performed at 37°C for 1 h, followed by denaturation at 95°C for 5 min and cooling at 40°C for 2 min. Immediately after the cooling step, the plate was cold shocked for 5 min on ice. For the fluorescent PCR stage, the PCR master mix was prepared to contain 1× QuantiTect probe master mix, 0.9 μM each primer qPERT1 and qPERT2, 1 ng/μl RNase A, and 0.18 μM qPERT MGB probe in nuclease-free water. The PCR master mix was added to the 96-well plates following the RT stage, and the fluorescent PCR amplification was performed on the Roche LightCycler 480 as follows. RNase A treatment at 37°C for 30 min, *Taq* polymerase activation at 95°C for 10 min, 55 cycles of 95°C for 15 s and 60°C for 1.5 min, and then cooling at 40°C for 2 min. Data were analyzed on the Roche LightCycler 480.

RESULTS

Validation of viral amplification on CEFs followed by COFAL.

The term validation refers to the demonstration that the performance of a particular test method is reliable and reproducible for its intended use with a given biological matrix (18). As a basis for this study, viral amplification on CEFs followed by COFAL (hereafter referred to as the CEF amplification plus COFAL method), a classical method used for the detection of ALV in vaccine preparations, was validated to determine the level of sensitivity exhibited by a method commonly used in the vaccine industry. Primary CEF cells were chosen for the infectivity portion of the method since this cell type was used almost exclusively for these kinds of tests until the acceptance of DF-1 cells by the Ph. Eur. in 2009 (2). The COFAL endpoint is one of the two readout options listed in Ph. Eur. 2.6.24 (2) and is based on the detection of complement-mediated lysis of erythrocytes. In the absence of ALV retroviruses in the test sample, free complement is able to lyse erythrocytes in the hemolytic serum to indicate a negative result. In the presence of retroviruses in the test sample, the complement is “fixed” into an immune complex with anti-p27 antiserum (p27 is a capsid protein of the virion core that is common to all ALV strains [19]) and thus cannot cause lysis, resulting in intact erythrocytes sedimenting out of solution to indicate a positive result.

The CEF amplification plus COFAL method was validated for the detection of ALV in the supernatant of CEF cells. This sample matrix was chosen for use in the validation because testing for the presence of avian retroviruses is routinely performed on the supernatant of CEF control cells during vaccine development and production. As the CEF amplification plus COFAL method represents a limit test (i.e., it detects the presence of a desired analyte in a test sample down to a certain limit), it was validated for limit of detection (LOD) and specificity as recommended by International Conference on Harmonisation (ICH) guidelines (18).

The LOD refers to the smallest amount of analyte which can be reliably detected by the method in a given test sample matrix (18). This is generally determined by the analysis of multiple replicates of matrix spiked with known concentrations of the analyte (i.e., avian retrovirus). Therefore, the LOD for the CEF amplification plus COFAL method was determined by testing 4 different concentrations, 6 replicates per concentration, of multiple ALV strains. Three different ALV strains representing different sub-

TABLE 1 Validation results for determining the LOD of the CEF amplification plus COFAL method to detect ALV in CEF culture supernatants^d

ALV strain	ALV subgroup	No. of positive replicates/total no. of replicates at concn (CCID ₅₀ /ml) of ^a :				
		7–19 ^b	1–4 ^c	0.1–0.4 ^e	0.01–0.04 ^f	0.004
RAV-1	A	6/6	6/6	5/6	1/6	ND ^g
RAV-2	B	6/6	6/6	2/6	0/6	ND
RAV-49	C	ND	6/6	4/6	0/6	0/6

^a Concentration of virus in the 30-ml inoculum used to infect the cell culture.

^b Concentrations of 7 and 19 CCID₅₀/ml were used for RAV-1 and RAV-2, respectively.

^c Concentrations of 1, 2, and 4 CCID₅₀/ml were used for RAV-1, RAV-2, and RAV-49, respectively.

^d The LOD for each ALV virus strain, defined as the lowest concentration at which all replicates tested positive at the end of the assay, is shown in boldface type.

^e Concentrations of 0.1, 0.2, and 0.4 CCID₅₀/ml were used for RAV-1, RAV-2, and RAV-49, respectively.

^f Concentrations of 0.01, 0.02, and 0.04 CCID₅₀/ml were used for RAV-1, RAV-2, and RAV-49, respectively.

^g ND, not determined.

groups known to be infectious to chickens were tested, RAV-1 (subgroup A), RAV-2 (subgroup B), and RAV-49 (subgroup C) (20). A negative control (medium alone), a matrix control (supernatant from CEF cultures), and a positive control (RAV-1) were included in each replicate. The LOD for each strain was defined as the lowest concentration of virus that resulted in a positive result for all replicates at the end of the assay. The overall LOD of the method was conservatively defined as the highest LOD determined for any of the strains tested.

The LOD results for the CEF amplification plus COFAL method are summarized in Table 1. The method exhibited an LOD of 1, 2, and 4 CCID₅₀/ml for RAV-1, RAV-2, and RAV-49, respectively. Therefore, the overall LOD for the method was determined to be a 30-ml inoculum volume containing 4 CCID₅₀/ml of ALV virus.

Specificity refers to the ability of the method to unequivocally assess the analyte in question (18). The CEF amplification plus COFAL method should not be able to detect nonavian retroviruses, as the COFAL readout is specific to ALV strains. Therefore, in order to validate the specificity of the method, the nonavian retroviruses human immunodeficiency virus type 1 (HIV-1) and murine leukemia virus (MuLV) were tested. As expected, the CEF amplification plus COFAL method was unable to detect HIV-1 or MuLV. Full hemolysis in the COFAL readout was observed in both cases (data not shown), thus confirming the specificity of the p27 antiserum. Therefore, the CEF amplification plus COFAL method as described in this study was validated to be specific for ALV retroviruses (i.e., RAV-1, RAV-2, and RAV-49).

Development of viral amplification on DF-1 cells followed by immunostaining. In order to avoid the use of primary CEF cells, which are difficult to standardize and often lead to inconsistent test results, we investigated the use of the DF-1 cell line for the infectivity portion of the test method. Optimal ALV amplification conditions on DF-1 cells were determined in order to achieve the best sensitivity level possible for the method. Instead of the COFAL endpoint, a simple immunostaining procedure was developed to observe successful amplification of the ALV retrovirus. At the end of the amplification procedure, each flask was fixed and stained with an antiserum to the ALV virion core protein p27 (19).

FIG 2 Optimization of ALV amplification on DF-1 cells. (A) Examples of positive (left) and negative (right) immunostaining results after DF-1 amplification of test samples. The positive flasks were derived from a 30-ml inoculation with ALV strain RAV-1 at a concentration of 0.1 CCID₅₀/ml. The negative flasks were derived from an inoculation of medium alone. Flasks were fixed at day 17 of the assay and immunostained with anti-p27 antibody. (B) Flasks were derived from a 30-ml inoculation of ALV strain RAV-49 at a concentration of 10 or 1 CCID₅₀/ml. At days 6 and 10, one flask of the pair for each concentration was passaged per the protocol, and the other was fixed and immunostained with anti-p27 antibody. Day 6 staining of the 1-CCID₅₀/ml inoculum exhibits the rocket staining pattern observed before the entire monolayer became infected due to amplification (as visible on day 10).

This anti-p27 serum was the same as that used for the CEF amplification plus COFAL method described above. A secondary antibody coupled to alkaline phosphatase followed by the addition of the BCIP-NBT substrate was used to visualize positively stained ALV-infected cells. The BCIP-NBT substrate is hydrolyzed/reduced by alkaline phosphatase to form a deep-purple/black precipitate which is readily detectable by eye.

We assessed a number of different DF-1 amplification parameters, such as infection time, number of amplification stages, duration of amplification stages, and the passaging of cells versus cell culture supernatant. For a summary of the amplification models investigated, see Table S1 in the supplemental material. The optimal results were achieved using 5 amplification stages of 3 to 4 days each, with passaging of half of the cells after each stage (thus 4 passaging events) (Fig. 1A). This amplification scheme was the same as that used for the CEF amplification plus COFAL method described above and resulted in a strong positive detection signal

(Fig. 2A). In fact, a positive signal could be observed as early as day 6, in the form of a “rocket” staining pattern. Successful amplification of infectious virus could be visualized by the incomplete staining seen at day 6, progressing to complete staining of the entire flask by day 10 (Fig. 2B). The protocol was extended to 17 days total to allow for even further enhancement of the signal and for the amplification of retroviruses with potentially reduced replication rates, thus increasing method interpretation and sensitivity. Hereafter, this method is referred to as the DF-1 amplification plus immunostaining method.

Validation of viral amplification on DF-1 cells followed by immunostaining. The DF-1 amplification plus immunostaining method was validated for detection of ALV in the supernatant of CEF cells, similarly to what was performed for the CEF amplification plus COFAL method, as described above. The LOD for the DF-1 amplification plus immunostaining method was determined by testing at least 4 different virus concentrations, with at least 6 replicates for each concentration, of multiple ALV strains. Five different ALV strains representing 1 of each of the 5 subgroups known to be infectious to chickens were tested, RAV-1 (subgroup A), RAV-2 (subgroup B), RAV-49 (subgroup C), RAV-50 (subgroup D), and RAV-J (subgroup J) (20). Three replicates of each of a negative control (medium alone), a matrix control (supernatant from CEF cultures), and a positive control (RAV-1 at a concentration of 50 CCID₅₀/ml) were included for each series of tests. The LOD for each strain was defined as the lowest concentration of virus that resulted in all replicates staining positive at the end of the assay. The overall LOD of the method was conservatively defined as the highest LOD determined for any of the strains tested.

The LOD results for the DF-1 amplification plus immunostaining method are summarized in Table 2. The method exhibited an LOD of 1 CCID₅₀/ml for all the strains tested, except for RAV-49, which exhibited an LOD of 0.1 CCID₅₀/ml. Therefore, the overall LOD for the method was determined to be a 30-ml inoculum volume containing 1 CCID₅₀/ml of ALV virus.

Similar to the CEF amplification plus COFAL method, the DF-1 amplification plus immunostaining method should not be able to detect nonavian or non-ALV retroviruses because the immunostaining readout is specific to ALV. Therefore, in order to validate the specificity of the method, the nonavian retroviruses HIV and MuLV, as well as REV (a non-ALV avian retrovirus), were tested. RAV-1 was included as a positive control.

As expected, the DF-1 amplification plus immunostaining

TABLE 2 Validation results for determining the LOD of the DF-1 amplification plus immunostaining method to detect ALV in CEF culture supernatants^b

		No. of positive replicates/total no. of replicates								
		At concn (CCID ₅₀ /ml) of ^a :						Control		
ALV strain	ALV subgroup	50	10	1	0.1	0.01	0.001	Negative	Matrix	Positive ^c
RAV-1	A	6/6	6/6	6/6	8/9	0/6	0/6	0/6	0/3	NA
RAV-2	B	ND	ND	6/6	3/6	1/6	0/6	0/3	0/3	3/3
RAV-49	C	6/6	6/6	6/6	3/6	ND	ND	0/3	0/3	3/3
RAV-50	D	ND	ND	ND	6/6	3/6	1/6	0/3	0/3	3/3
RAV-J	J	ND	6/6	6/6	5/6	2/6	ND	0/3	0/3	3/3

^a Concentration of virus in the 30-ml inoculum volume used to infect the cell culture. ND, not determined.

^b The LOD for each ALV virus strain, defined as the lowest concentration at which all replicates stained positive at the end of the assay, is shown in boldface type.

^c The positive control was RAV-1 at a concentration of 50 CCID₅₀/ml. NA, not applicable.

TABLE 3 Validation results for specificity of the DF-1 amplification plus immunostaining method for the detection of ALV in CEF culture supernatants

Inoculum or control	Antibody used to stain the flasks	No. of positive replicates/total no.
REV (50 CCID ₅₀ /ml)	Anti-p27	0/6
	Anti-REV	6/6
Control		
Negative	Anti-p27	0/3
	Anti-REV	0/3
Matrix	Anti-p27	0/3
	Anti-REV	0/3
Positive ^a	Anti-p27	3/3
	Anti-REV	0/3

^a The positive control was RAV-1 at a concentration of 50 CCID₅₀/ml.

method was unable to detect HIV or MuLV (data not shown). The method was also unable to detect the non-ALV avian retrovirus REV, even though REV was capable of amplifying on DF-1 cells (as confirmed by staining with an antibody to REV at the end of the assay; Table 3). However, the RAV-1 positive control was detected in all cases. Therefore, the DF-1 amplification plus immunostaining method was validated to be specific for ALV retroviruses. It should be noted that this method can be modified to be specific for other viral strains, depending on the protein detected by the immunostaining endpoint. However, if the endpoint of the method is altered, the validation would have to be repeated to determine the specificity and LOD for the virus in question.

Development of viral amplification on DF-1 cells followed by F-PERT. As the immunostaining endpoint developed as described above is specific only for ALV retroviruses, we pursued the development of an alternative molecular biology-based endpoint to allow for the detection of all retroviruses capable of replication in DF-1 cells. After the completion of the 17-day amplification period, the culture supernatants were pooled and aliquots prepared for F-PERT analysis to detect RT activity (Fig. 1B). F-PERT is a quantitative reverse transcriptase PCR-based test that is known to be extremely sensitive and thus may exhibit a high rate of false positives. Therefore, activated DNA was included in the F-PERT protocol to reduce the detection of nonspecific RT activity produced by cellular DNA polymerases (16). A standard curve obtained by using known amounts of M-MLV RT was included in every experiment (see Materials and Methods). The test sample was considered positive if its crossing point (Cp) value was less than that of the 10⁻⁷ U/10 µl standard curve point and negative if its Cp value was greater than or equal to that of the 10⁻⁷ U/10 µl standard curve point. Hereafter, this method is referred to as the DF-1 amplification plus F-PERT method.

Although DF-1 cells do not contain endogenous ALV retroviral sequences (13), they do contain endogenous avian retrovirus (EAV) sequences, which are noninfectious but may still produce functional RT (9, 11, 12). Therefore, developmental studies were performed to evaluate whether background levels of RT activity in supernatants from DF-1 cell cultures would yield RT activity in negative controls at levels that could invalidate the test (i.e., negative-control Cp < 10⁻⁷ U/10 µl standard curve point Cp). Multiple replicates of negative controls (medium alone) and CEF matrix controls were amplified on DF-1 cells and tested by F-PERT. As a positive control, RAV-49 at 1 CCID₅₀/ml was also included in

TABLE 4 No detectable RT activity by F-PERT was observed in the supernatant of negative and matrix control samples after amplification on DF-1 cells

Supernatant sample	No. of positive replicates/total no. of replicates ^{a,b} at a supernatant sample dilution of:		
	Neat	1/10	1/100
Negative control 1	0/3	0/5	0/5
Negative control 2	0/3	0/5	0/5
Negative control 3	0/3	0/5	0/5
Matrix control 1	0/3	0/5	0/5
Matrix control 2	0/3	0/5	0/5
RAV-49 positive control ^b	3/3	3/3	1/1

^a The numbers of replicates that tested positive for RT activity out of the total number of replicates tested by F-PERT for that condition are shown. Replicates refers to the number of times the indicated sample was tested by F-PERT.

^b Sample generated using 1-CCID₅₀/ml RAV-49 virus.

the test. No detectable background RT activity was observed in DF-1 cell culture supernatants at day 17 by the F-PERT assay (Table 4; Fig. 3). Therefore, DF-1 cells do not contribute detectable RT activity to the supernatants of the amplification samples when the F-PERT readout is performed as described in this study. Notably, it was not necessary to clarify the amplification supernatant samples prior to F-PERT analysis as has been done in other studies to reduce background signal from RT-like activity of endogenous cellular enzymes (16).

Validation of viral amplification on DF-1 cells followed by F-PERT. The DF-1 amplification plus F-PERT method was validated for detection of avian retroviruses (i.e., ALV and REV) in CEF supernatants. Similar to the DF-1 amplification plus immunostaining method described above, specificity and LOD were determined for validation (18).

To evaluate the LOD of the DF-1 amplification plus F-PERT method, the supernatants of the amplified samples from the DF-1 amplification plus immunostaining method validation were used. Prior to fixation of the flasks for immunostaining on day 17, the supernatants of the two flasks were pooled, and aliquots were taken and stored at -60°C or lower for F-PERT analysis (Fig. 1B). RAV-1, RAV-2, RAV-J, and REV amplification sample supernatants were tested by F-PERT, with the inclusion of at least 2 virus concentrations per strain (4 concentrations for REV) and 6 replicates per concentration. One replicate of each of the negative and matrix controls was also included in each series of tests. The appropriate F-PERT negative controls and standard curve (see Materials and Methods) were included on each plate tested by F-PERT. The LOD was defined similarly to that for the DF-1 amplification plus immunostaining method (see above).

The validation results for the LOD of the DF-1 amplification plus F-PERT method are shown in Table 5. The DF-1 amplification plus F-PERT method demonstrated an LOD of 1 CCID₅₀/ml for all strains tested and therefore an overall method LOD of 1 CCID₅₀/ml of avian retrovirus in the 30-ml inoculum volume.

F-PERT should be capable of detecting all retroviruses but unable to detect nonretroviral strains that do not produce the RT enzyme. In this study, specificity was validated for the F-PERT endpoint alone by testing 3 different retroviruses and 3 different nonretroviral strains by F-PERT. As expected, the F-PERT method was able to detect the retroviruses HIV-2, human T-cell

FIG 3 F-PERT amplification curves of negative and matrix control DF-1 amplification sample supernatants. Shown are the F-PERT amplification curves as generated by the Roche Light Cycler 480. The M-MLV standard curve dilutions are indicated in red, the positive control samples in black, and the negative and matrix control samples in blue. The test sample was recorded as positive if its Cp value was less than that of the 10⁻⁷ U/10 µl standard curve point (i.e., the amplification curve was detected at a cycle before that of the 10⁻⁷ U/10 µl standard) and negative if its Cp value was greater than or equal to that of the 10⁻⁷ U/10 µl standard curve point (i.e., the amplification curve was detected at a cycle after that of the 10⁻⁷ U/10 µl standard).

lymphotropic virus 1, and RAV-1 but not the nonretroviruses human adenovirus 5, cytomegalovirus, and herpes simplex virus 1 (data not shown). Determining the specificity of the F-PERT endpoint alone supports the validation of the DF-1 amplification plus F-PERT method. However, it would be expected that the DF-1 amplification plus F-PERT method would be able to detect only avian retroviruses capable of replicating on DF-1 cells.

Comparison of the different methods to detect ALV in CEF culture supernatants. Table 6 shows a comparison of the validated LODs for the various methods described in this study. Although different amplification samples were used for the COFAL endpoint validation, the strains used for spiking the initial test sample inocula were from the same virus stocks. All of the methods exhibited comparable detection limits.

The DF-1 amplification plus immunostaining and DF-1 amplification plus F-PERT methods were validated using the exact same DF-1 amplification samples. Therefore, it was possible to carry out a direct comparison of the two different endpoint methods (immunostaining versus F-PERT). A replicate-by-replicate comparison of DF-1 amplification followed by the immunostain-

ing versus F-PERT endpoints is detailed in Table S2 in the supplemental material and demonstrates that both endpoints resulted in positive/negative determinations that were identical for each sample replicate. These results are consistent with the positive/negative signal present in the original sample being enhanced by amplification of the retrovirus in the DF-1 cells.

DISCUSSION

Many vaccines are produced using primary avian cells. Although these cells are derived from specific-pathogen-free avian species, the vaccine products must be tested for the possible presence of avian retroviruses as part of the safety-testing regimen. In this study, two distinct methods to detect avian retroviruses in avian cell culture supernatants (as would be performed during control cell testing of vaccine products) were developed, validated, and compared to a method classically used in the vaccine industry for this application. Both of the new methods use amplification of the test sample on the DF-1 avian cell line but differ in the endpoint method used to detect any potential retroviruses present in the original sample. The DF-1 amplification plus immunostaining

TABLE 5 Validation results for determination of the LOD of the DF-1 amplification plus F-PERT method to detect avian retroviruses in CEF culture supernatants^c

Retrovirus strain	ALV subgroup	No. of positive replicates/total no. of replicates ^a					
		At a concn (CCID ₅₀ /ml) ^b of:				Control	
		50	10	1	0.1	Negative	Matrix
RAV-1	A	ND	ND	6/6	8/9	0/2	0/1
RAV-2	B	ND	ND	6/6	3/6	0/1	0/1
RAV-J	J	ND	ND	6/6	5/6	0/1	0/1
REV	Non-ALV	6/6	6/6	6/6	0/6	0/1	0/1

^a The numbers of replicates that tested positive for RT activity by F-PERT out of the total replicates tested are shown.
^b Concentration of virus in the 30-ml inoculum volume used to infect the cell culture. ND, not determined.
^c The LOD for each virus strain, defined as the lowest concentration that resulted in all replicates testing positive for RT activity, is shown in boldface type.

TABLE 6 Comparison of LODs for the CEF amplification plus COFAL, the DF-1 amplification plus immunostaining, and the DF-1 amplification plus F-PERT methods^a

Strain	ALV subgroup	LOD (CCID ₅₀ /ml)		
		CEF amplification, COFAL endpoint	DF-1 amplification	
			Immunostaining endpoint	F-PERT endpoint
RAV-1	A	1	1	1
RAV-2	B	2	1	1
RAV-49	C	4	1	ND ^b
RAV-J	J	ND	1	1

^a The LODs for each ALV strain tested are shown. The same DF-1 amplification samples were used to evaluate the immunostaining and F-PERT endpoints. The CEF amplification samples for validation of the COFAL method were prepared separately using the same virus stocks as used with the other methods. For all methods, 30 ml of test article was initially inoculated per flask.
^b ND, not determined.

method was validated to an LOD of 1 CCID₅₀/ml in a 30-ml inoculum volume specifically for the detection of ALV retroviral strains. Likewise, the DF-1 amplification plus F-PERT method was validated to an LOD of 1 CCID₅₀/ml in a 30-ml inoculum volume for the detection of all avian retroviral strains.

The current regulatory requirements for an ALV-specific test outline an infectivity method followed by a COFAL or an ELISA readout (2). Classically, CEF cells were used for the infectivity amplification portion of the procedure, although DF-1 cells are now an accepted alternative (2). Comparison of the DF-1 amplification plus immunostaining method versus the more traditional CEF amplification plus COFAL method demonstrated that the two methods exhibit similar sensitivities for the detection of ALV (Table 6). However, the COFAL readout is complicated, time-consuming to perform, and reliant on a number of difficult to control parameters. For example, this readout method requires standardization and titration of complement and fresh erythrocytes for every assay run. As such, controls must be routinely included in the assay to ensure that the erythrocytes and complement are suitable, and a consistent supply of fresh reagents must be ensured. Therefore, the DF-1 amplification plus immunostaining method is just as sensitive as the CEF amplification plus COFAL method for the detection of ALV but provides a much simpler and more consistent readout.

The DF-1 amplification method we investigated in this study utilizes a passaging regime similar to that described in Ph. Eur. 2.6.24, i.e., multiple amplification stages of 3 to 4 days each (2). The DF-1 amplification plus immunostaining method includes 5 amplification stages (4 passaging events) (see Fig. 1) and takes a minimum of 17 days, whereas the method outlined in Ph. Eur. 2.6.24 requires an infectivity portion of a minimum of 9 days followed by a COFAL or ELISA readout (2). With the DF-1 amplification plus immunostaining method, it is possible to detect ALV at early time points (i.e., 6 days after inoculation; refer to Fig. 2B); however, the method was extended to 17 days to allow for enhanced sensitivity and amplification of any retroviruses with potentially reduced replication rates. Indeed, the benefit of extending the length of the amplification stage was demonstrated during validation of the method, as one replicate of RAV-50 at 0.01 CCID₅₀/ml exhibited a rocket staining pattern, which is typical of low levels of ALV, only at the 17-day time point (data not shown). Excitingly, the DF-1 amplification plus immunostaining method provides a greater level of sensitivity than that which is required by regulatory authorities. Validation of the DF-1 amplification plus immunostaining method demonstrated an LOD of 1 CCID₅₀/ml, which exceeds the LOD of 100 CCID₅₀/ml required by Ph. Eur. 2.6.24 (Table 7) (2).

The second method developed and validated in this study, the DF-1 amplification plus F-PERT method, provides additional benefits to the DF-1 amplification plus immunostaining method. The F-PERT endpoint can detect the RT activity of any amplified retrovirus originally present in the test sample. A head-to-head comparison between the immunostaining and F-PERT endpoints demonstrated that the DF-1 amplification plus F-PERT method is capable not only of detecting ALV strains to the same LOD as the DF-1 amplification plus immunostaining method (Table 6; also see Table S2 in the supplemental material) but also of detecting other avian retroviruses outside of the ALV family (i.e., REV) to the same LOD (Table 5). Therefore, it is possible to use the DF-1 amplification plus F-PERT method to meet all of the regulatory

TABLE 7 Comparison of the DF-1 amplification plus immunostaining method to detect ALV in CEF culture supernatants with current requirements^a

Method	Total amount of sample tested	Amount of virus detected per flask (CCID ₅₀) ^b	Concn of virus detected (CCID ₅₀ /ml)
DF-1 amplification	30 ml (30 ml in 1 replicate)	30	1
Ph. Eur. 2.6.24	0.5 ml (0.1 ml in 5 replicates)	10 ^c	100 ^d

^a As outlined in Ph. Eur. 2.6.24 (2).

^b One flask is equal to 1 replicate.

^c Detection of at least 10 CCID₅₀ of the positive controls in 5 out of 6 replicates is required (2).

^d Calculated from 10 CCID₅₀/0.1 ml sample per flask. This detection level is required in 5 out of 6 positive control replicates (2).

requirements for avian retroviral contaminant safety testing during vaccine development and production. Importantly, the immunostaining and COFAL endpoint methods both rely on the use of the same p27 antiserum, a critical reagent that is difficult to obtain in large and secure quantities. The F-PERT endpoint thus provides an additional advantage in terms of reagent supply, which is particularly important in the context of routine testing during vaccine lot manufacturing.

In addition to the benefits mentioned above, the DF-1 amplification plus F-PERT method represents an advancement compared to a precursor reverse transcriptase PCR-based amplification method previously used in the industry, known as the combined reverse transcriptase (CoRT) test (Sanofi Pasteur, unpublished data). The CoRT test used amplification of the test article on primary CEFs followed by a readout based on reverse transcriptase PCR and agarose gel electrophoresis. For each batch of primary cells used for the amplification portion of the procedure, a panel of positive control avian retroviruses was inoculated in parallel with the test article. The number of PCR cycles used for the reverse transcriptase PCR readout was then modified to control for the background signal exhibited by the primary CEF cells. A direct comparison of the DF-1 amplification plus F-PERT and CoRT methods was not carried out in this study. However, the DF-1 amplification plus F-PERT method provides a significant advantage compared to this precursor assay as it does not require the number of PERT cycles to be modified to account for the background signal, potentially providing a more sensitive assay.

In this study, we developed and validated two novel methods for the detection of ALV or any avian retrovirus in the control cell supernatants of vaccines produced on avian cells. The DF-1 amplification plus immunostaining and the DF-1 amplification plus F-PERT methods demonstrated identical LODs that exceed the current sensitivity levels required by health authorities for the detection of ALV strains (2). Furthermore, the methods are simpler and more streamlined than, and as sensitive as, other currently used techniques (i.e., the CEF amplification plus COFAL method), making them ideal for use in vaccine development and safety testing.

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