

## Individual and Multiplex PCR Assays for the Detection of Adventitious Bovine and Porcine Viral Genome Contaminants in the Commercial Vaccines and Animal Derived Raw Materials

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### Abstract

Animal derived raw materials such as trypsin and Fetal Bovine Serum are used in vaccine manufacturing and pose the threat of introducing animal pathogens as contaminants into the final products. Thus screening for adventitious virus/ genome is part of quality control in manufacturing of biologicals. Various *in-vitro* and *in-vivo* detection assays have been developed for the detection of potential viral contaminants in vaccines. However, these assays are expensive, time consuming, labor intensive and incomplete limiting their ability to meet the increasing demands of the biological industry. Polymerase chain reaction technology scores over the *in-vitro* and *in-vivo* assays in speed, specificity, sensitivity and robustness of detection and can replace them in regular use. In the present study, a set of multiplex and individual PCRs were developed for the detection of porcine (n=6) and bovine viral genomes (n=5). Veterinary vaccines (10), human vaccines (9), porcine tpsin lots (9), and fetal bovine serum (8) were screened for adventitious viral genomes using multiplex PCRs. It was observed that 60% of veterinary vaccines, 77.7% of trypsin, and 62.5% of fetal bovine serum were contaminated with adventitious viral genomes.

**Keywords:** Adventitious viruses; Multiplex PCR; Vaccines; Fetal bovine serum; Trypsin

### Introduction

Though infectious diseases are considered one of the major challenges for human and animal health across the world, the harmful effects of the pathogenic organisms have considerably lowered in recent times than a century ago. One of the significant factors contributing to this status is the availability of vaccines against infectious diseases coupled with vast improvements in vaccine technology and delivery systems. Eradication of smallpox from the entire world and the near elimination of wild polio virus is attributed to the availability of effective vaccines and its use. Similarly, a cattle plague, Rinderpest has been eradicated from the globe by systematic vaccination programs. Current research in vaccinology aims in developing safer and efficacious vaccines for control of infectious diseases. Vaccine safety is paramount because public fear of vaccination can dramatically reduce vaccine usage and thereby coverage. This would result in the subsequent reduction of herd immunity or in some cases even re-emergence of the disease.

One of the emerging concerns on the vaccine safety is the possibility of contamination with adventitious viruses in vaccine batches [1]. Use of animal derived raw materials in vaccine development and manufacture is the major source for such contamination events [2,3]. Bovine serum and bovine/porcine trypsin are the most common animal derived raw materials in development and manufacturing of viral vaccines [4,5]. Identification of extraneous viruses or viral genomes, such as porcine circovirus and parvovirus, in rotavirus vaccine [6-9] and nodovirus in the insect cell derived Human papilloma virus vaccine, have created greater awareness among the regulatory agencies and vaccine industry about the need for extensive screening of vaccine banks, cell substrates and vaccine batches for adventitious viruses. There are several well-documented bovine and porcine viral contamination events such as Cachevalley virus [10], Reo viruses [11], Porcine circovirus [9], BVD [12], Pestivirus [13-16] and many other animal viruses [17] in live animal and human vaccines [18,19].

Currently, both the virus banks and cell seeds in vaccine

manufacturing are tested for bovine and porcine adventitious agents in compliance with the regulations specified in the 9CFR 113 by U.S. Department of Agriculture (USDA) for veterinary products [17]. However, the 9CFR procedures are laborious, expensive and time-consuming and incorporating this method for the testing of regular vaccine batches is difficult. Moreover, the 9CFR procedure does not address the exhaustive list of viruses that are potential contaminants in animal derived raw materials [20,21]. The 9CFR procedure was essentially designed to detect the viruses that have the potential to infect bovine and porcine hosts. Since this procedure is also used to validate cell and virus banks of human vaccines, there is a possibility of overlooking the presence of zoonotic viruses [21].

Therefore, the need for additional testing procedures may be useful adjuncts to 9 CFR tests in quality control laboratories of vaccine manufacturers [3,19]. In recent times PCR assays have found wide application in genome detection in the diagnostics laboratories due to its speed, sensitivity, specificity and ease of use [22]. The present study describes a set of optimized multiplex and individual PCRs for detecting adventitious viral genome of porcine and bovine origin in the commercially available vaccines and animal derived raw materials.

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## Materials and Methods

### Study samples

Commercially available veterinary vaccines (n=10), human vaccines (n=9), trypsin (n=9) and fetal bovine serum (n=8) were procured from retail outlets. These biological products were either from different manufacturers or from different batches of the same manufacturer (Table 1).

### List of viruses and its plasmid constructs with gene of interests

Adventitious viruses that can potentially be present in trypsin and serum as contaminants were identified [18,21,23]. Conserved viral genes were chosen and the respective gene sequences were downloaded from Genbank. Primer 3 software (white head institute/MT centre for Genome Research) was used for primer designing and the primers with similar annealing temperatures were chosen for multiplex PCR. Primers were chosen such that the resulting amplicons after multiplex PCR vary in size discernably for subsequent visualization in an agarose gel as separate bands.. Primers were designed to bind the conserved regions

S No	Country of Origin	Vaccine type	Target species
1	Imported	Inactivated	Canine
2	Imported	Live	Canine
3	India	Inactivated	Canine
4	Imported	Inactivated	Canine
5	Imported	Live	Canine
6	Imported	Inactivated	Canine
7	India	Inactivated	Dogs, Cattle, Cat, Horse, Buffaloes, Sheep
8	Imported	Inactivated	Dogs, Cattle, Cat, Horse
9	India	Inactivated	Canine
10	India	Live	Sheep and Goat
11	India	Inactivated	Human
12	India	Inactivated	Human
13	Imported	Inactivated	Human
14	Imported	Inactivated	Human
15	Imported	Inactivated	Human
16	Imported	rDNA (non-living)	Human
17	Imported	rDNA (non-living)	Human
18	India	Live	Human
19	India	Inactivated	Human
20	Imported	Porcine Trypsin	
21	Imported		
22	Imported		
23	Imported		
24	Imported		
25	Imported		
26	Imported		
27	India		
28	Imported	Fetal Bovine Serum	
29	Imported		
30	Imported		
31	Imported		
32	Imported		
33	Imported		
34	Imported		
35	Imported		
36	Imported		

**Table 1:** Details of study samples used for the adventitious virus screening.

S.No	Virus	Gene	Gene Bank Accession No.	Amplicon size (bP)
1.	Porcine circo virus 2 (PCV2)	ORF1	KF695388.1	360
2.	Porcine Parvovirus (PPV)	NS1	JQ710899.1	220
3.	African swine fever virus (ASFV)	VP73	FN557520.1	215
4.	Bovine parvo virus (BPV)	ParVgp1	M14363.1	255
5.	Bovine herpes virus (BHV)	UL39	AJ 004801.1	400
6.	Porcine reproductive and respiratory syndrome virus (PRRSV)	ORF7	KC862578.1	166
7.	Classical swine fever virus (CSFV)	NS5B	HM 803932.1	300
8.	Swine vesicular disease virus (SVDV)	VP	X54521.1	550
9.	Bovine Pestivirus	5'UTR	GU395536.1	135
10.	Bovine coronavirus (BCV)	NS1	AF 239315.1	310
11.	Blue tongue virus (BTV)	NS1	JQ740775.1	121

**Table 2:** GenBank accession numbers and amplicon sizes of the PCR.

of Porcine circovirus (PCV), Porcine Parvovirus (PPV), African swine fever virus (ASFV), Porcine reproductive and respiratory syndrome virus (PRRSV), Classical swine fever virus (CSFV), Swine vesicular disease virus (SVDV), Bovine herpes virus (BHV), Bovine coronavirus, Bovine parvovirus (BPV), Bovine Pestivirus (group specific) and Blue tongue virus (BTV) genomes. The list of viruses and their respective gene targets with GenBank IDs are provided in Table 2.

Plasmid borne synthetic constructs of the target genes were obtained commercially. These plasmid constructs were used as positive control in PCRs. Trypsin, FBS, freeze drying media and algel were used as sample matrix. Trypsin, FBS and freeze drying media were treated with DNase I and then the DNase was inactivated at high temperature. Known concentration of plasmid constructs were spiked into the respective sample matrix and these samples were used for optimizing PCR conditions subsequent to the extraction of nucleic acid.

### Optimization of multiplex PCR

PCR was performed for each set of primers individually with the corresponding plasmid constructs as templates. The plasmid constructs were two fold serially diluted from 0.5 ngs to 0.029 fgs and spiked into the respective sample matrix. The analytical sensitivity (detection limit) of the PCR was determined by performing PCR from each of the dilutions individually. Multiplex PCR was optimized for the various assay parameters such as reagents, buffers and cycling conditions as described previously [24-27]. Primer concentrations were titrated for optimizing the parallel amplification of all targets simultaneously in a single tube in multiplex PCRs. Analytical sensitivity of the multiple PCR assays was also determined as mentioned previously. The specificity of the primer pairs were tested by performing PCR with unrelated combination of primers and plasmid constructs.

### Nucleic acid extraction

Total DNA and RNA were extracted from the study samples using DNA mini isolation kit (QIAGEN, USA) and RNaeasy mini kit (QIAGEN, USA), respectively, according to the manufacturer's protocol. The freeze dried vaccines were reconstituted in minimal amount of diluents and used for nucleic acid extraction. Nucleic acid was extracted individually under sterile condition in a laminar air flow to prevent contamination from the microenvironment. RNA samples were subjected to reverse transcription using cDNA synthesis kit (Invitrogen, Carlsbad, CA). The cDNA reaction mixture containing 1X RT buffer, 0.5 mM of dNTP mix, RNase inhibitor (1 unit), random primers and multiscript reverse transcriptase (1 unit) was incubated

at 25°C for 10 minutes and subsequently at 37°C for 120 minutes and 85°C for 5 minutes. PCR was performed for DNA and cDNA samples in My Cycler- thermal cycler (BIORAD, Hercules, CA) and the resulting amplicons were visualized in a 2% agarose gel.

### Sequence analysis of amplified products

The size specific PCR products obtained from various study samples were gel purified using gel extraction kit (QIAGEN, USA) as per the manufacturer's protocol. The gel purified PCR products were cycle sequenced from both directions using gene specific primers. The sequences were then compared with the respective sequences available in GenBank and the nucleotide percentage identities were determined.

## Results

### Optimization of multiplex PCR

Total of 11 sets-of-primers specific for gene targets of six porcine viruses and 5 bovine viruses were designed. These gene targets were commercially synthesized and plasmids containing the synthetic construct were used as positive controls in the PCR reactions. Porcine multiplex PCR for DNA viruses was performed using the combination of PCV, PPV and ASFV specific primers with DNA samples. Porcine multiplex PCR for RNA viruses was performed using the combination of PRRSV, CSFV and SVDV specific primers with cDNA samples. Bovine multiplex PCR for DNA viruses was performed using BHV and BPV specific primers with DNA samples and bovine multiplex PCR for RNA viruses was performed with pestivirus (includes bovine

viral diarrhea virus 1 and 2, Border disease virus) and BCV specific primers with cDNA samples. PCR for BTV was carried out separately with cDNA. Some of the viruses that have been left out in the 9CFR procedure were included in the study.

Optimization of multiplex PCR reactions was performed by varying the assay parameters such as the concentration of MgCl<sub>2</sub>, Taq DNA polymerase, primers etc. For example, the band intensity of ASFV amplicon was visibly lesser compared to the band intensities of PCV and PPV amplicons in the porcine multiplex PCR (for detecting PCV, PPV and ASFV) when all the primers were used in equal concentration (10 pmoles each in 25 µl reaction). Increasing the quantity of ASFV specific primers to 20 pmoles resulted in equal band intensities for all the three amplicons (Figure 1). The detection limit of BTV target gene reduced considerably in multiplex PCR compared to the individual PCR. Hence, the PCR for BTV was performed separately.

### Detection limits

Lower limit of detection for the individual and multiplex PCRs was determined by performing PCRs from sample matrices that were spiked with various dilutions of synthetic constructs. The PCRs could detect fg quantities of plasmid DNA and this corresponds to approx. 100 copy numbers of plasmids or lesser (Table 3). The detection limit of multiplex PCR did not vary significantly from that of individual PCRs though there was a reduction in sensitivity (Table 3). The lower limit of detection did not vary significantly when different sample matrices were used.

### Study sample screening

The multiplex PCR was used in screening commercial veterinary vaccines (N=10), human vaccines (N=9), porcine trypsin lots (N=9), and fetal bovine serum (N=8) for the detection of porcine and bovine viruses. Out of 10 veterinary vaccines, 6 were found to be positive for various porcine and bovine viral genomes (Table 4). PCV, PPV, SVDV, BPV and BCV viral genomes were identified in the veterinary vaccine batches. No adventitious viral genome was detected in the human vaccines. Many of the canine vaccines contained extraneous viral genome of porcine origin. Higher percentage of contamination was observed in trypsin with 7 out of 9 trypsin lots showing evidence for the presence of various porcine viral genomes. Genome of CSFV was identified in four of the nine trypsin batches that were tested. More than one viral genome was detected in many of the trypsin batches. Out of the 8 serum samples, 5 were positive for pestiviral genome. The results indicated that 60% of veterinary vaccines, 77.7% of trypsin batches



**Figure 1:** Multiplex PCR optimized with porcine DNA viral constructs 1. PCV ORF1 (367bp); 2. PPV NS1 (220bp); 3. ASFV VP73 (215bp); 4. PCV ORF1+PPV NS1; 5. PCV ORF1+ASFV VP73; 6. All three constructs 7. No template control 8. 100bp Marker.

S.No	Virus	Gene target	Detection limit in ≤ Copy numbers*	
			Individual PCR	Multiplex PCR
1.	Porcine circo virus 2	ORF1	1.36×10 <sup>2</sup>	5.44×10 <sup>2</sup>
2.	Porcine Parvovirus	NS1	1.41×10 <sup>2</sup>	5.62×10 <sup>2</sup>
3.	African swine fever virus	VP73	7.05×10 <sup>1</sup>	2.82×10 <sup>2</sup>
4.	Bovine parvo virus	ParVgp1	1.49×10 <sup>1</sup>	7.27×10 <sup>1</sup>
5.	Bovine herpes virus	UL39	2.66×10 <sup>1</sup>	4.44×10 <sup>2</sup>
6.	Porcine reproductive and respiratory syndrome virus	ORF7	1.38×10 <sup>2</sup>	5.84×10 <sup>2</sup>
7.	Classical swine fever virus	NS5B	1.44×10 <sup>2</sup>	5.70×10 <sup>2</sup>
8.	Swine vesicular disease virus		6.35×10 <sup>1</sup>	5.48×10 <sup>2</sup>
9.	Bovine Pestivirus	5'UTR	2.12×10 <sup>1</sup>	1.36×10 <sup>2</sup>
10.	Bovine coronavirus	NS1	1.37×10 <sup>1</sup>	5.96×10 <sup>2</sup>
11.	Blue tongue Virus	NS1	1.06×10 <sup>2</sup>	not included

**Table 3:** Lower limit of detection of synthetic plasmid constructs in individual and multiplex PCRs. \*the formula used for converting the DNA quantities into number of copies = (amount in ng× 6.022×10<sup>23</sup>) / (length in bp×1×10<sup>9</sup>ng/g ×650 g/ mole of bp).

Sample ID	Indented use of Vaccine	Adventitious Virus Screening										
		PCV	PPV	ASFV	CSFV	SVD	PRRV	BPV	BHV	BCV	Pestivirus	BTV
Veterinary Vaccines												
1	Canine distemper virus, Canine parvovirus	■										
2	Canine distemper virus, Canine adenovirus type 2, parvovirus, Canine parainfluenza virus											
3	Rabies					■						
4	Rabies											
5	Canine distemper virus, Canine adenovirus type 2, parvovirus		■									
6	Canine distemper virus, Canine parvovirus Virus, Parainfluenza and Hepatitis		■									
7	Rabies					■		■		■		
8	Rabies											
9	Canine parvovirus Virus											
10	Peste des petis Ruminants							■				
Human Vaccines												
11	Rabies											
12	Polio											
13	Rabies											
14	Hepatitis A											
15	Hepatitis A											
16	Hepatitis B											
17	Hepatitis B											
18	Rubella											
19	Japanese Encephalitis											
Trypsin												
20	Not applicable				■	■	■					
21					■							
22												
23												
24		■	■									
25		■	■									
26						■	■					
27												
28												
Serum												
29	Not applicable										■	
30											■	
31											■	
32											■	
33											■	
34											■	
35											■	
36											■	

**Table 4:** Result summary of adventitious virus screening, Positive PCR results are indicated by black boxes.

and 62.5% of bovine serum contained adventitious viral genome contamination. The amplicons from all the positive samples were sequence verified and their specificity confirmed.

### Discussion

In the present regulatory regime, vaccine safety is one of the non-negotiable attributes of vaccine quality. Revisit and refinement

of safety standards is vital part of improving the quality of vaccines. Identification of adventitious virus and viral genomes in many of the approved viral vaccines for veterinary and human use has created doubts on the robustness of the current quality testing procedures [5,21]. Considering the failure in replacement of animal derived raw materials with synthetic or non-animal derived products for vaccine manufacture, development of robust testing protocols for the detection

of extraneous agents is extremely important. The risk of introducing adventitious virus contamination exists right from the stage of virus isolation (intended for vaccine development) to the manufacture of vaccine. The potential sources include, host species of virus and cell isolation, animal derived raw materials in vaccine development and manufacture, spread from the production personnel etc. However, the most frequent source of extraneous virus contamination has been from the use of bovine serum and porcine/bovine pancreatic trypsin [4,5,21].

In the present study, commercial vaccines, trypsin and FBS were examined for the presence of adventitious viral genomes. A set of multiplex and individual PCRs were optimized for this purpose and evaluated using synthetic plasmid constructs. 9 out of the overall 12 extraneous viral genome contamination events of vaccine batches were from various porcine viruses. The potential source for porcine virus contamination is porcine derived pancreatic trypsin and 7 out of 9 trypsin batches were positive for various virus genomes with 4 of the batches positive for more than one porcine virus genome. Though CSFV genome was the frequent contaminant in the trypsin batches, CSFV and ASFV genomes were not identified in any of the vaccine batches of the present study. As per the earlier reports, PCV and PPV were the frequent contaminants in the porcine trypsin [28]. Currently, the quality guidelines for the batch-testing of trypsin is not elaborate and does not address the extraneous virus screening in detail. The European Medicines Agency (EMA) has drafted a new guideline on the use of porcine trypsin in the manufacture of human biological medicinal products. The guideline states, "specific tests for porcine viruses that are not detected by a general cell culture test should be considered on a case-by-case basis". The 9CFR procedure describes the need for detection of only PCV in the vaccine seeds. But the identification of other porcine viral genomes in vaccine and trypsin batches by various scientists reiterates the need for additional tests. It may be argued that normally viruses do not grow in the presence of trypsin and the gamma irradiation procedure during the trypsin manufacture would have inactivated the viruses. However, the risk of viral presence at low levels in trypsin batches in spite of the inactivation procedures do exist. During large-scale production of vaccines, the low level of viruses can grow multifold. Thus, it is important to include adequate extraneous virus detection protocols in vaccine batch release tests, in addition to the virus and cell banks characterization test. Moreover, trypsin and vaccine batches should be tested for the presence of all known porcine origin viruses. Usage of recombinant trypsin in place of bovine or porcine trypsin can reduce the risk of virus contamination to a great extent. Human vaccine industry should replace all the animal derived materials with serum free media, chemically defined media, recombinant trypsin, etc. Though implementing production processes without the use of raw materials from animal origin might increase the cost of production, the purity of the final product would be assured. To begin with, the characterization of cell and virus banks should include screening tests for all bovine and porcine viruses as part of the cGMP procedures for viral vaccines. The 9CFR procedure can also be modified to include the PCR based detection subsequent to three passages in permissive cell lines, until the reagents (such as antibodies) are made available to detect all the relevant viruses.

As per the earlier reports, bovine pestivirus has been the major contaminant in the FBS [5]. In the present study too, 62.5% of FBS were positive for bovine pestivirus genome. BCV genome was detected in one of the vaccine batches although the virus is not included in the 9CFR procedure. Adult bovine serum is commonly used in vaccine manufacture and this increases the potential contamination events.

The higher percentage of veterinary vaccines (60%) being positive for extraneous virus underlines the need for stringent quality control measures for the veterinary vaccine industry. Even though the number of veterinary vaccines and batches tested are small, the presence of extraneous viruses in these vaccine-batches highlights the seriousness of the problem and the lack of stringent regulation for veterinary vaccines, unlike in human vaccines. Though the 9CFR procedure cannot identify the complete range of potential adventitious viruses, the procedure is more stringently implemented in the characterization of virus and cell seeds of human vaccines. In contrast, pharmacopeial requirements of almost all the veterinary vaccines are restricted to the tests which prove the absence of haem-adsorbing and haem-agglutinating viruses from cell and virus seeds. Prevalence of veterinary pathogens varies considerably between countries and the contaminating extraneous virus can spread some of the exotic viruses into the local animal population directly through the adventitious viral contaminants in either imported vaccines or animal derived raw materials. While the human vaccines can be contaminated by animal viruses without any subsequent serious infection in humans (except for the zoonotic viruses), the contaminants in animal vaccines can cause epidemics due to the presence of viruses of animal origin.

The wide-range of adventitious viral genetic elements detected by various methodologies is an expected outcome of closer scrutiny for the nucleic acids in the vaccines. This is not necessarily a reflection of unsafe products, as the viral nucleic acid detected in the samples shall not necessarily mean live virus or infective virus [9]. At the same time, the possibility of the presence of live virus is not ruled out. Therefore, this assay could be used as a preliminary screen and positive samples for genomic contamination may subsequently be screened for viral infectivity or other confirmatory tests. Although the present study is broad based, it is not comprehensive enough to detect every potential viral contaminant. The flexibility of multiplex PCR based assays however gives enough scope to expand the detection range with the inclusion of more viruses.

## Conclusion

A set of multiplex and individual PCRs were developed for the detection of porcine and bovine viruses. This is a more convenient, reliable and adaptable method for the detection of adventitious viral genome in vaccines and animal derived raw materials covering a wider spectrum of viruses. The results reiterates the need for inclusion of adventitious virus detection as a standard test for batch release of vaccines and other animal derived raw materials used in production. The testing procedures should cover comprehensive list of animal origin viruses depending when animal derived materials are used in vaccine development and production. In the long-term, it is best for the vaccine industry to move away from the use of animal derived raw materials or at least adapt viral clearance methods to ensure the safety of the finished products.

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