Chapter 2

PCR Methods for the Detection of Turkey Hemorrhagic Enteritis Virus DNA

2.1 Abstract

A standard and nested PCR assay was developed for the specific detection of Turkey Hemorrhagic Enteritis Virus (THEV) DNA. This assay is used as one of the basic diagnostic tools in suspect cases of hemorrhagic enteritis (HE) in turkeys. Two quantitative PCR methods were developed based on the standard PCR: competitive PCR (cPCR) and real-time quantitative PCR (qPCR). The plasmid HEV2 (pHEV2) was engineered for use as a competitor molecule in the cPCR.

The ability of the cPCR to quantify an unknown sample was tested and validated against the qPCR method. The quantity of viral genomes present in each of twelve HE vaccines was determined by qPCR and cPCR. In addition, the relative amount of antigen in each vaccine was determined by agar gel immunodiffusion (AGID). The results were compared against the number of labeled vaccine doses.

The cPCR was found to be an effective tool for the estimation of DNA copies. The results of each sample tested were within one log of the value determined by qPCR. Interestingly, the cPCR estimate was consistently larger than the qPCR value in every vaccine tested. This is indicative of slight variations in PCR efficiency inherent to both PCR methods.

The limit of detection of the standard PCR was found to be 439 template copies or less as determined by qPCR. There were 43,000 to 181 million genome copies per dose detected in each vaccine product. This great variation suggests that the number of infectious virions per dose varies widely depending on the vaccine product used. However, there was no direct correlation between the number of genomes per dose and the AGID titer. The infectious dose 50% (ID_{50}) values of each product are not known. Knowing the ID_{50} values would allow for a more accurate correlation of the number of

genome copies and number of infectious virus. Once this ratio has been determined for each vaccine, both quantitative PCR methods would be useful tools for titration of HE vaccine products.

2.2 Introduction

Polymerase chain reaction (PCR) is a method of specific DNA amplification. The use of PCR in diagnostic and research applications is widespread, as it allows for fast detection of specific DNA populations. Standard PCR is not inherently quantitative, so special techniques must be used to adapt PCR protocols to be used for the quantification of DNA in a sample (Reddy *et al*., 2000; Mackay *et al*., 2002).

Diagnostic tools for the detection of THEV in turkeys have changed greatly in recent years, as the progression of science and technology has extended the sensitivity and specificity of testing. There has only been one diagnostic PCR protocol published to date (Hess, 1999). This PCR protocol is now seven years old, and an update using more modern techniques was needed. Analysis of all sequence data available for the different strains of THEV revealed a very high level of sequence homology in the structural genes (Pitcovski *et al*., 1998; Jucker *et al*., 1996; Suresh *et al*., 1995). PCR assays amplifying the hexon region would allow for specific detection of a broad range of THEV strains.

The lack of an adherent cell line for propagation of THEV *in vitro* prevents accurate titration of infectious virus by use of viral plaque assays. Commercial HE vaccines that are produced in MDTC RP-19 cells are titrated based on their ability to cause cytopathic effect (CPE). Infected cell cultures must be visually examined for ballooning of cells and other CPE, which can be mistaken for cells undergoing necrosis. This causes the potential for large variability and poor precision in the estimated titer. Alternatively, THEV may be titrated in live birds using AGID to detect antigen in the spleens (Domermuth *et al*., 1972, 1973). AGID is a relatively crude technique, and has a relatively low sensitivity. This method also requires the sacrifice of many turkeys.

The purpose of this research was to develop quantitative PCR assays in order to determine the number of viral genome copies present in DNA extracts. These assays will provide alternatives to the *in vitro* and *in vivo* titration methods for HE vaccines. The assays were developed for experimental and diagnostic use, and are the basis for subsequent sequencing and persistent infection studies.

2.3 Materials and Methods

Virus:

Genomic DNA from the Virginia Avirulent Strain (VAS) of THEV was used to develop these PCR protocols. The VAS was originally isolated in 1977 from enlarged spleens of pheasants suspected of having marble spleen disease (Domermuth *et al*., 1977). It has been maintained as a low passage splenic vaccine for prevention of HE in turkeys for over 20 years, with 5-6 live-bird passages since 1985.

Turkeys:

Large white turkey poults were provided by British United Turkeys of America (BUTA, Lewisburg, WV) and raised in isolation to six weeks-of-age. At six weeks-of-age, turkeys were orally or intravenously inoculated with 100 turkey infectious dose 50% (TID_{50}) VAS and euthanized by cervical dislocation 3-5 days post-inoculation. Spleens were collected from each bird and stored at -20°C until used.

DNA Isolation:

Spleens collected from infected birds were homogenized 50:50 in phosphate buffered saline (PBS). This crude splenic homogenate was tested for viral antigen by AGID to verify the presence of a large quantity of virus (Domermuth *et al*., 1972, 1973). Splenic homogenate was added to an equal volume of InstaGene Matrix (BioRad) and mixed thoroughly using a vortex. Mixture was then incubated for 30 minutes in a 56°C water bath, vortexed thoroughly, and incubated in a 100°C heat block for 8 minutes. The

mixture was then vortexed thoroughly a third time, and centrifuged at 15,000 rpm for 10 minutes. The supernatant was collected and used as a positive DNA control for each PCR method developed.

DNA was extracted from HE vaccine products for the cPCR validation study using the blood and body fluids protocol of the QiaAmp DNA mini-kit (Qiagen). Lyophilized vaccines were reconstituted in sterile water. DNA was extracted from 200 uL of each vaccine DNA, eluted in 50 ul of sterile DNase/RNase free water and stored at -20°C until used.

Standard PCR

DNA polymerase, $MgCl_{2,}$ deoxynucleotide triphosphates (dNTPs), oligonucleotide primers, and sterile de-ionized water, and sample DNA were all combined in a total reaction volume of 25-50 uL. PCR methods were developed using automatic hot-start DNA polymerases, eliminating the need for preparation of reactions on ice. The two types of polymerase used during the development of the methods were Platinum Taq Readymix (Invitrogen) or Hotmastermix (Eppendorf), both of which contain thermostable DNA polymerase, $MgCl_2$ and dNTPs.

Three sets of primers were designed for the specific detection of THEV DNA (Table 1). Set C is nested within set B, which is nested within set A. PCR primers were stored in 5 mM aliquots, with a final reaction concentration of 200 uM for each primer. All PCR reactions were performed in an iCycler thermocycler (BioRad). The same basic thermocycler program was used for every round of PCR amplification (Table 2). Thermocycler times and temperatures sometimes varied depending on the PCR reagents used. After completion of the thermocycler program, reactions were electrophoresed on a 1% agarose gel containing ethidium bromide at 100 V for about 1 hour. Positive reactions were identified as bright bands that were of expected size as compared to a kb ladder standard.

Table 2-1: PCR Primer Sequences

Name	Position+	Sequence (5′ to 3′)	Amplicon Size	Amplicon ID
HEV14521	14521	ctg tag cca aga acc act a	399 bp	A
HEV14122	14122	gtt cct tca cct aat act gg		
nHEVL	14138	gtg gtt cag cag aaa gtt ctt	270 bp	B
nHEVR	14410	cag tag act cat aag caa cta t		
HEVrtL	14160	cct aca aca gag gga gct agt tg	160 bp	C
HEVrtR	14329	cac tgc cag att tac cag ca		

+Nucleotide position relative to VAS sequence

Table 2-2: Basic Thermocycler Program

Temperature	Time	Cycles
95°C	3 minutes	1x
95°C	30 seconds	35x
58°C	30 seconds	
65-72°C*	45 seconds+	
4°C	infinite	hold

*Optimal extension temperature varies by polymerase

+Extension time may vary based on amplicon length (bp)

For the most sensitive detection of viral DNA, nested PCR was used. Nested PCR involves the use of two consecutive PCR reactions. The first reaction was a standard PCR, and the second used a set of primers “nested” within the first set.

Competitive PCR:

Although nested PCR is very sensitive and useful for detection of viral DNA, it is only semi-quantitative. In cPCR, molecules of template DNA compete with each other for a limited supply of primers in the reaction tube. Competitor molecules are engineered such that primers react equally to both sample and competitor templates. Amplification of the competitor template results in a product with a larger size than is produced by the sample template (Reddy *et al*., 2000). In order to quantify unknown samples, serial ten-fold dilutions of the competitor were made. A series of PCR reactions were set up, each containing 2 uL of the unknown sample, and one dilution of the competitor. Reactions were run in the thermocycler and visualized on 1% agarose gels with ethidium bromide. Results were analyzed based on the relative intensity of the resultant bands. An example agarose gel from a cPCR test is shown in Figure 1. Reactions that contained a larger quantity of competitor than genomic DNA predominately contained the larger band. Conversely, if the starting quantity of viral DNA was significantly larger than the competitor molecule, the amplicon of the sample template was more intense. In reactions where the two populations were roughly equal, the intensity of the resultant bands were approximately equivalent.

Competitor Molecule Design:

A molecule of DNA, plasmid HEV2 (pHEV2) was engineered specifically for use in a competitive PCR for quantification of THEV DNA (Figure 2). Using standard PCR, the 399 bp amplicon A was amplified from THEV DNA extracted from VAS infected turkey spleens. The amplicon was then inserted into the 3.9 kb TA-cloning vector pCR 2.1 (Invitrogen) according to manufacturer’s instructions. Briefly, the PCR product was ligated with linear vector pCR 2.1 using T4 DNA ligase incubated at 14°C overnight. The resultant circular plasmid (pHEV1) was used to transform chemically competent *E. coli*

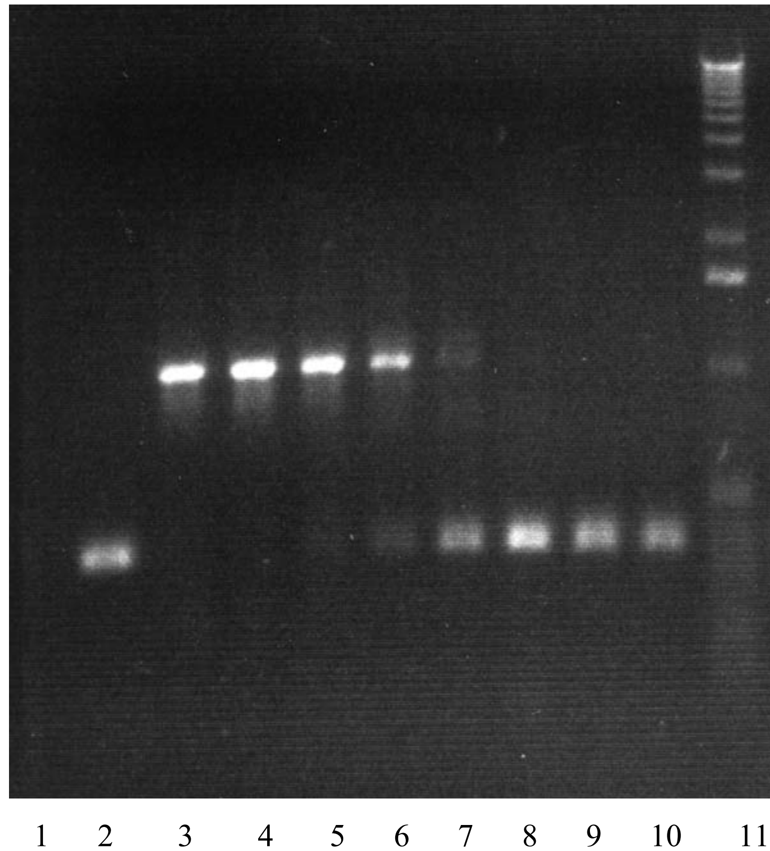

Figure 2-1: Agarose Gel from a Competitive PCR

Example of a competitive PCR gel, with a single unknown sample tested against 8 serial ten-fold dilutions of pHEV2. Lanes 1 and 2: negative and positive controls; Lanes 3-10: reactions containing the sample template and a decreasing dilution of pHEV2. Lane 11: kilobase ladder standard. The 400 bp amplicon begins to be visible in lane 5, and increases in intensity through lane 8. The equivalence dilution of the sample and competitor template is estimated to be between 10^{-4} and 10^{-5}.

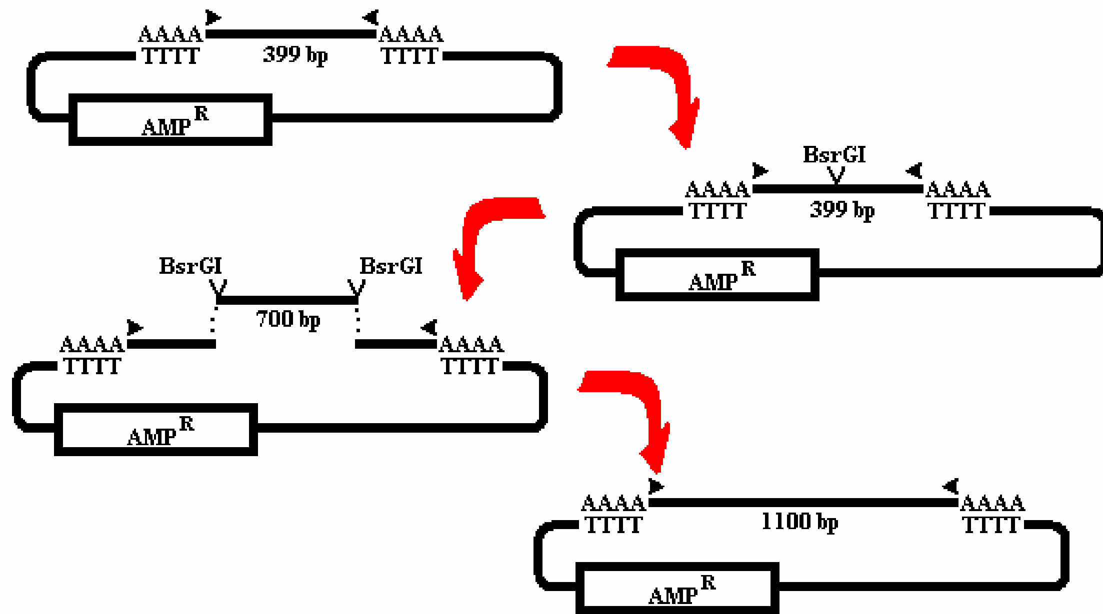

Figure 2-2: Competitor Molecule Creation

Amplicon A was amplified from VAS DNA, inserted into pCR 2.1 (Invitrogen). A 700 bp restriction fragment from the THEV genome was then inserted within amplicon A. The resultant plasmid results in an 1100 bp product when PCR primer set A is used.

INVaF′. Blue/white screening on LB amp$^+$ agar was used to isolate a transformant containing the insert. Successful insertion was verified by restriction endonuclease digestion and PCR. The molecular weight of pHEV1, as calculated based on its sequence, is 2.632x10^6 daltons.

Filler DNA was prepared by amplification of an 850 bp region of the THEV genome spanning the 52K and pIIIa genes (precise location of the primers on the THEV genome is: nt 9201-9221, 5'- GGCAGCTATAAACACTAGTC -3'; and nt 10042-10063, 5'- ATCATCTTCAGATCGCACAAC -3'). This region was chosen because it contains two BsrGI restriction sites 703 bp apart (nt 9283 and nt 9986). BsrGI is a Type II restriction enzyme that cleaves at the recognition palindrome T/GTACA. The PCR product was visualized on an agarose gel with ethidium bromide. The 850 bp band was excised from the gel and DNA was recovered using the QiaQuick gel extraction kit (Qiagen). The filler DNA and pHEV1 were each digested using BsrGI, combined and ligated overnight at room temperature with T4 DNA ligase. OneShot competent *E. coli* (Invitrogen) cells were transformed and plated on LB amp$^+$ agar. Transformants were screened for the expected insert using BsrGI digestion and the Clone-Checker System (Gibco) and verified using PCR. The resulting plasmid (pHEV2) contains an 1102 bp insert that can be PCR amplified with primer sets A and B. The molecular weight of pHEV2 is 3.057x10^6 daltons.

Real-Time Quantitative PCR Technique

qPCR was adapted directly from the standard PCR protocol using the same reaction conditions, except that iTaq qPCR ready-mix (BioRad) was used in all qPCR reactions. The iTaq mix contains optimal concentrations of SYBR green I dye, a non-specific intercalating dye that fluoresces only in the presence of double-stranded DNA. qPCR reactions were run in the iCycler real-time PCR thermocycler (BioRad).

Validation of PCR efficiency

qPCR was used to validate the efficiency of a standard PCR reaction. The DNA concentration of the stock of purified pHEV1 was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The concentration of undiluted pHEV1 was 96 ng/uL, which is the equivalent of 2.196×10^{10} copies/uL. A set of serial tenfold dilutions of pHEV1 was prepared, containing 220 to 2.196×10^{7} copies/uL. qPCR was performed on duplicate samples containing 2 uL of each dilution. Standard qPCR was run with primer set B according to the established protocol (Table 2).

Validation of cPCR

Several commercial tissue culture propagated HE vaccines from 1990-2006 were collected. The Virginia Avirulent Strain (VAS) splenic vaccine was also tested (lot VA113004, $10^{4.25}$ TID_{50}/mL). Reconstituted vaccines were tested for viral antigen by AGID according to established protocol (Domermuth *et al*., 1972, 1973). Serial two-fold dilutions of each vaccine were tested, ranging from 1:2 to 1:32, and the highest positive dilutions were determined. qPCR was performed on DNA from each vaccine, according to standard procedure. cPCR was performed first with serial ten-fold dilutions of pHEV2 in order to determine the range of genome equivalents. cPCR was then repeated using serial two-fold dilutions of pHEV2 in order to more accurately determine the copy number.

2.4 Results

The calculated efficiency of the qPCR was 101.0%, with a correlation coefficient of 0.999 (Figure 3). The results indicate that the PCR is almost perfectly efficient over a six-log range of template concentration. The detection threshold was confirmed to be approximately 439 genome equivalents.

The results of the comparison of quantitative PCR methods are listed in Table 3. The VAS lot VA113004 contained 33 to 181 million genome copies per dose. This is

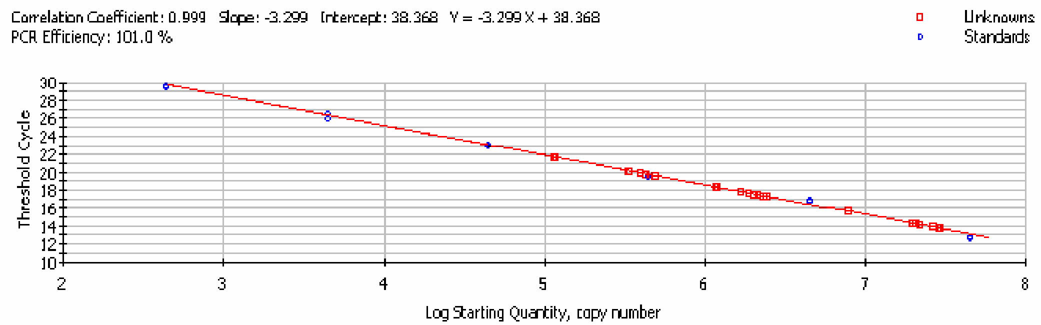

Figure 2-3: Standard PCR Efficiency Calculation

Standard curve calculated based on qPCR of serial dilutions of pHEV1. Experimental fluorescence was compared with theoretical fluorescence in order to determine reaction efficiency.

Table 2-3: Quantification of Viral Genomes in HE Vaccines

Vaccine ID	Year	AGID		Genome copies per dose		
		Doses	Titer[+]	cPCR*	qPCR	Log difference
TC-A	1998	5	3:8	1.814E+08	1.725E+07	1.021908
TC-B	1991	5	1:4	3.629E+06	8.775E+05	0.61648
TC-C	2004	10	3:16	2.267E+07	1.084E+07	0.320498
TC-D	1989	3	1:4	3.024E+06	2.288E+06	0.121185
TC-E	1991	10	3:16	5.805E+07	7.875E+06	0.867552
TC-F	1991	10	3:16	4.534E+05	4.350E+04	1.017968
VAS	2004	20	1:32	1.814E+08	3.338E+07	0.735231
TC-H	1991	6	3:8	1.512E+06	2.850E+05	0.724671
TC-I	1990	3	1:2	1.511E+06	5.100E+05	0.471766
TC-J	1991	3	1:8	6.048E+06	2.600E+06	0.366603
TC-K	1991	12	1:4	3.023E+06	2.416E+06	0.097471
TC-M	2006	6	N/D	3.024E+06	2.506E+06	0.081521

*Results based on cPCR using two-fold serial dilutions.

[+]Titer is the highest dilution that tested positive, and the average of two AGID tests.

N/D: Not determined

equivalent to 3.75 to 20.4 million genome copies per TID_{50}. It is not known for certain exactly how many infectious virions are present for each genome copy. In theory, the numbers would be expected to be equivalent, but in reality there are likely far more copies of DNA than infectious virus. The TID_{50} rating of the commercial tissue culture vaccines are unknown, though it is likely that the labeled dose of each vaccine is calculated based on a similar tissue culture infectious dose 50% ($TCID_{50}$) rating. Based on the qPCR results, a single labeled dose of TC vaccine contained 44,000-17 million genome copies, depending on the product. Based on the cPCR using serial twofold dilutions, a single dose of TC vaccine contained 453,000-181 million genome copies. While there is a great deal of variation between each vaccine product, the cPCR and qPCR values for each were consistently within one log of each other. The VAS had the highest AGID titer, although a greater number of doses were present in the sample.

2.5 Discussion

qPCR is a method that measures fluorescence in each reaction tube and monitors the progress of a PCR reaction in real time. There are several different techniques in use, but all result in an increase in fluorescence as the PCR product is created. This fluorescence change directly or indirectly correlates with the initial amount of template DNA in the reaction. qPCR is the fastest, most accurate method for determining the amount of viral DNA present in a sample. Unfortunately, it requires the use of very expensive equipment that is not available in every laboratory setting (Mackay *et al*., 2002).

One way to introduce fluorescence to a PCR reaction is to use an intercalating dye that fluoresces only in the presence of double-stranded DNA. At the start of the reaction, the only DNA present in the tube is the relatively small quantity of template. The amount of DNA increases exponentially as product accumulates during amplification. A measurement of fluorescence during the extension phase of amplification is taken and compared to the background level of fluorescence.

Because the fluorescence is non-specific, its increase does not correlate directly with the amount of template in the starting sample. In order to quantify the sample, the change in fluorescence must be compared to that of a standard of known quantity. A serial dilution of a known quantity of template DNA is amplified, and a standard curve is generated based on the fluorescence of the standard dilutions. Any sample run with the standards may then be matched to that curve, allowing for a quantity to be determined. Use of a standard curve also allows for the determination of PCR efficiency. At 100% efficiency, PCR theoretically results in the doubling of the template every cycle. In reality, many PCR reactions proceed less efficiently. This may be due to DNA secondary structure, non-specific binding of the primers, or reaction conditions that are out of the optimal range of the DNA polymerase being used. Comparison of the experimental results from a standard dilution set with the theoretical "optimal" results allows for the calculation of PCR efficiency. Having a high PCR efficiency is essential in order to accurately quantify an unknown (Mackay *et al*., 2002).

There are advantages to the use of a non-specific intercalating dye for qPCR. The dye is relatively inexpensive and may be used in corporation with standard PCR protocols already in use. Use of a non-specific intercalator allows for the generation of a melt-curve profile for each sample reaction. This permits the quick differentiation of product populations based on the melting temperature (T_m) of each amplicon (Mackay *et al*., 2002).

There are disadvantages to using a dye as well. Fluorescence is non-specific, so accurate quantification depends on the consistency of the standard dilutions and 100% optimal PCR reaction efficiency. PCR protocols must be optimized to minimize non-specific product formation during amplification. Non-specific product includes primer-dimers and any amplified DNA that is not considered to be the intended product of the reaction. Any production of such non-specific DNA molecules increases the fluorescence in the reaction tube and may lead to an over-estimation of template quantity (Mackay *et al*., 2002).

The cPCR assay was developed as an alternative means to quantify THEV in viral stocks without the need for live birds, cell culture, or expensive real-time fluorescent PCR thermocyclers. In order to validate the cPCR as a quantitative technique, THEV DNA in several commercial vaccines was extracted and quantified by qPCR and cPCR. The results were compared with AGID titers and the labeled dose for each vaccine. The average for all vaccines tested was 2.2 to 7.4 million copies per labeled dose. The highest copy:dose ratio was found in the splenic VAS vaccine. This indicates that there was a greater amount of THEV in each dose of the splenic vaccine than the tissue culture products. This was not surprising, as splenic vaccine products have been shown to cause a greater degree of splenomegaly in infected turkeys than tissue culture products (Sharma, 1994). There was no correlation between age of the product and number of copies per dose, however three products from 1998-2005 (including the VAS) had some of the highest copy per dose values. The AGID results did not correlate with the number of copies detected in each labeled dose. TC-J had the largest AGID titer relative to the number of doses tested. It is not known if AGID titers correlate directly with the number of infectious virions present in each dose, as precipitating antigen may be present in the form of incomplete capsids and individual viral proteins.

The copy-dose ratios determined by qPCR were different than those determined by cPCR, though this difference was relatively consistent (Figure 4). In every sample, the cPCR estimated 0.2-10 times the number of copies than the qPCR. This could have been caused by the difference in amplification efficiency of the pHEV2 molecule compared to the viral genome. During amplification of competing molecules, slightly fewer pHEV2 regions may have been produced, leading to an overestimation of the number of viral genomes in each sample. Alternatively, the difference could be associated with a decrease in qPCR efficiency when amplifying a genomic DNA template compared to the pHEV1 standards. A slight decrease in amplification of the genomic template would lead to the under-estimation of genome copies in each sample.

The cPCR assay was capable of detecting and quantifying a large range of genome copy numbers, and the results of the assay correlated with the qPCR assay. The

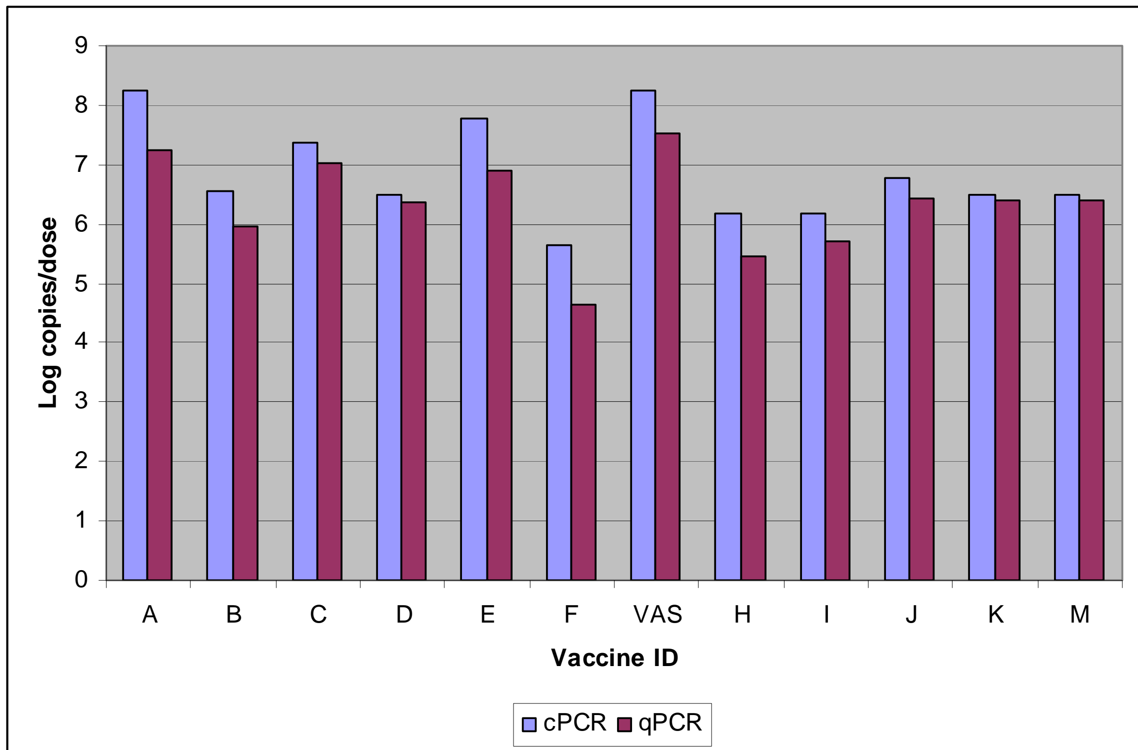

Figure 2-4: Comparison of cPCR and qPCR Estimates of Genome Copy Number

Quantities of viral genome copies present in 12 HE vaccines were determined by cPCR and qPCR. The estimate by cPCR was consistently higher than that of qPCR. The quantity of viral genome copies varied greatly between vaccines.

usefulness of this competitive PCR technique in a high-throughput diagnostic laboratory is limited because of the number of reactions that must be run in order to quantify one unknown. However, competitive PCR allowed for estimation of viral copy number without the need for an expensive real-time PCR thermocycler. It is useful for applications that do not require large numbers of samples, such as vaccine titration. The cPCR assay is not a true replacement for titration of the number of infectious virions present in each product. The results must be correlated with the experimental TID_{50} or $TCID_{50}$ before cPCR can be used to estimate numbers infectious virus, and even then the true number of infectious particles cannot be calculated with certainty.

2.6 References

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