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THE USE OF ARMS PCR AND RFLP ANALYSIS IN IDENTIFYING GENETIC PROFILES OF VIRULENT, ATTENUATED OR VACCINE STRAINS OF TGEV AND PRCV

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ABSTRACT

The use of ARMS (amplification refractory mutation system) PCR coupled with RFLP (restriction fragment length polymorphism) analysis has been used to identify a unique genetic marker on the Ambico oral vaccine strain. This method was also used to characterize the genetic profiles of a number of other TGEV strains. This procedure takes advantage of the nucleotide differences between the Ambico strain, and the Miller and Purdue strains. Within the S gene there are three nucleotide differences between the Ambico strain and the published Purdue sequence. There are additional nucleotide differences in the structural and non-structural gene sequences, but we have chosen to focus on the differences contained within the S gene. The Ambico strain has a closer sequence homology to the Purdue strain than to the Miller strain. The Ambico and Purdue strains contain a six nucleotide deletion at position 1122 that is not present in the Miller published sequence or the ISU-1 strain of PRCV (based on our PCR experiments). We have designed a 5' oligo whose sequence is homologous to a region located 80 nucleotides upstream of the TGEV and PRCV S gene initiation codons to be used in conjunction with either of two 3' oligos whose sequences are identical with the exception of the last six nucleotides of their 3' ends. When utilized with the appropriate PCR conditions, these oligos can differentiate between PRCV, Miller and Purdue prototype virus strains. These PCR products were then subjected to RFLP analysis using four separate restriction enzymes (*Bst*E II, *Alw*26 I, *Dra* III, or *Msp*A1 I). We have used this procedure to analyze six TGEV vaccine strains, intestinal derived virulent viruses, cell cultured viruses at different cell passage numbers, and field isolates of TGEV or PRCV.

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INTRODUCTION

TGEV was first identified in 1946 by Doyle and Hutchins [1]. TGEV causes gastroenteritis in pigs resulting in a mortality rate approaching 100% in piglets less than two weeks of age. TGEV has been shown to infect epithelial cells of the gut and respiratory tract. PRCV, a variant of TGEV with a predominate tropism for the respiratory tract, was first isolated in Europe in 1984 by Pensaert [2]. PRCV was first isolated in the United States in 1989 by Hill et. al. and designated ISU-1 [3]. Since then, four additional strains of PRCV have been isolated and characterized in the United States [4,5,6]. Based upon serology, these strains have been shown to be similar to the European strain, but based upon their nucleic acid sequence they are different. Through sequence analysis, attempts have been made to correlate characteristics of tropism and pathogenicity to genotypic changes among various TGEV and PRCV strains [7,8,9]. It is presently speculated that deletions in the 5' region of the S gene in PRCV are responsible for its altered tropism while deletions affecting the expression of mRNA 3a and 3b are responsible for altered pathogenicity [10,11,12].

MATERIALS AND METHODS

Viruses

Reference virus stocks were obtained from various sources and a description of these strains is listed in Table 1. Vaccine strains of TGEV were isolated from commercially available products from Grand Laboratories, Inc., Larchwood, Iowa; Schering-Plough Animal Health, Omaha, Nebraska; Oxford Veterinary Laboratories, Inc., Worthington, Minnesota; Diamond Laboratories, Des Moines, Iowa; Fort Dodge Laboratories, Fort Dodge, Iowa; and Ambico, Inc., Dallas Center, Iowa.

Isolation of Virus from Infected Tissue Samples.

Four TGEV seronegative piglets, two-to-three days of age were divided into two groups and housed in individual isolation boxes. Each group was inoculated either intranasally or orally with $10^{6.2}$ $TCID_{50}$ per pig of Ambico TGEV vaccine . One pig from each group was sacrificed at 24 hours and 48 hours post-inoculation. The following tissues were carefully collected using different instruments for each of the seven samples: lung; tonsils; small intestinal extract (SIE = actual tissue homogenate); nasal lavage and scrapings; mesenteric lymph nodes (MLN); small intestinal contents (SIC); and fecal samples. A sample of jejunum, ileum and duodenum was taken for staining by specific IFA of the frozen sections for TGEV. Infectious TGEV virus titers for the lung, tonsil, SIC, fecal, SIE, nasal, and MLN samples were determined by inoculation of confluent ST cell cultures and evaluation by plaque assay and TGEV specific IFA.

Isolation of Viral RNA and cDNA Synthesis

Frozen reference virus stocks consisted of either small intestinal extract (SIE) or ST cell culture passaged material. Small intestinal contents collected from infected piglets were diluted to 50% in PBS, and debris was removed by high speed centrifugation. An equal volume of 50% sucrose was added and the material was stored at -70°C as a 25% final SIE. This material was used to generate ST cell passaged stocks. Virus stocks were thawed and

clarified of particulate matter by centrifugation at 10,000 xg. The supernatant was collected and used for viral RNA isolation.

TGEV vaccine strains from Grand Laboritories, Schering-Plough, Diamond Laboratories, and Ambico, Inc. were obtained as lyophilized powdered cakes. These samples were resuspended in 10 ml of sterile purified water. The Fort Dodge material was received from Dr. Ron Wesley already rehydrated (10 dose vial rehydrated with 20 ml). The rehydrated material was clarified of particulate matter by centrifugation. The supernant was collected for viral RNA isolation. The Oxford product was received as a liquid suspension and was first diluted two-fold with sterile water and then extracted twice with toluene, once with a 1:1 ratio of toluene/phenol and one time with a 1:1 ratio of chloroform/toluene. This material was then used for viral RNA isolation.

Tissue samples were collected and a 50% homogenized extract was prepared in PBS. Samples were clarified of debris by centrifugation at 10,000 x g. The supernatants were collected, aliquoted and quick frozen at -70°C. Frozen samples were thawed and diluted two-fold in detergent containing buffer and clarified of any debris by centrifugation. The supernatant was collected and used for viral RNA isolation.

Viral RNAs were isolated by digestion in 100 mM Tris (pH 7.5),12.5 mM EDTA, 150 mM NaCl, 1% SDS and 200 μg proteinase K (Boehringer Mannheim) at 37°C for 30 minutes. After proteinase K treatment, the samples were extracted twice with phenol and once with 1:1 ratio of phenol/chloroform. RNA was precipitated using LiCl and ethanol at -70°C. The RNA was pelleted by centrifugation and the pellet was rinsed two times with 70% ethanol and resuspended in sterile RNase free water. First strand cDNA was generated using reverse transcriptase and oligo WL23 (5' TGTGTACCATTACCACAG 3'). Oligo WL23 is complimentary to the 3' ends of TGEV and PRCV S genes (corresponding to nucleotides 3591 to 3608 of the Miller sequence). Briefly, the RNAs were denatured at 94°C for 2 minutes and then incubated at 42°C for 60 minutes in a 50 μl reaction containing 50 mM Tris (pH 8.0),6 mm $MgCl_2$, 40 mM KCl, 100μM dNTPs, 5U RNAsin (Promega), 2U of AMV reverse transcriptase (Seikagaku America, Inc.) and 500 pmol of oligo WL23. Final reactions were stored at -70°C until use.

ARMS PCR and RFLP Analysis

The cDNAs were then used in PCR type-specific reactions containing 100 μM dNTPs, 2U of Taq polymerase (Boehringer Mannheim), one tenth volume of supplied 10x Taq reaction buffer, one tenth volume of the cDNA material and 250 pmol of each 5' and 3' oligos. The 5' oligo WL5 (5' GGATTACTAAGGAAGGGTAAGTTG 3') is homologous to TGEV and PRCV viral genomic sequences located at positions -80 to -56 nucleotides upstream of their S gene initiation codons and either of two 3' oligos whose sequences are identical with the exception of the last six nucleotides of their 3' ends. These oligos, utilized with the proper PCR conditions, are able to differentially amplify Purdue-like or Miller-like virus strains. This is based on the property of Taq polymerase, which will not extend a mismatched 3' end [13,14,15]. The Tms (melting temperatures) of these 3' oligos are higher than that of oligo WL23. Therefore, the annealing temperatures used are too stringent for the WL23 oligo to interfere. The 3' oligo WL29 (5' TCGAGTCACTCACTGTATCATTAT 3') is complementary to nucleotides 1121 to 1144 of the Miller S gene sequence and contains an additional six nucleotides not present in the Purdue sequence, hence this oligo is specific for Miller and PRCV strains of coronavirus. The 3' oligo WL28 (5'CGAGTCACTCACTGTATAACATG3') is complementary to nucleotides 1115 to 1137 of the Purdue strain and does not contain these additional six nucleotides, hence it is specific for the Purdue virus sequences.

Table 1. History and source of TGEV and PRCV virus strains

1	Miller MGV: virulent intestinal extract, Received from Dr Wesley, USDA, APHIS, NVSL, VS, Ames, Iowa
2	Miller PP3 · plaque purified three times on ST cells, Received from Dr Wesley, USDA, APHIS, NVSL, VS, Ames, Iowa
3	Miller TGEV APHIS 69-7· virulent intestinal extract; Received from Dr Tamogolia, Vet Biologics Div (VBD) - Animal Res Section (ARS) at USDA, Ames, Iowa.
4	Miller/ST-3. TGEV APHIS 69-7 Miller virulent strain passaged 3x in ST cells
5	Purdue TGEV Std Challenge Lot-4: virulent intestinal extract; Received from Dr. Haelterman, Purdue University, Indiana
6	Purdue/ST-4: TGEV Std Challenge Lot-4 Purdue virulent strain passaged 4x on ST cells
7	Purdue P-115 · Purdue avirulent strain; Received from Dr. Bohl, Ohio State University, Ohio
8	TGEV. Illinois Strain , Received from Dr Pat Gough, Vet Med Res Institute (V M R I), ISU, Ames, Iowa
9	PRCV ISU-I · passaged 3x in ST cells; Received from Dr Hill, Iowa State University, Ames, Iowa

We have demonstrated that oligos WL5 and WL28 amplify a PCR product of 1217 nt from the Ambico strain, whereas oligos WL5 and WL29 fail to amplify a PCR product. The reverse is true for oligos WL5 and WL29 which amplify a PCR product of 1224 nucleotides from the Miller strain and 543 nucleotides from the PRCV strain. Oligos WL5 and WL28 fail to amplify a PCR product from Miller or PRCV strains (data not shown). PCR conditions consisted of a hot start, whereby Taq was added after the tubes had reached a temperature of 94°C. The initial cycle consisted of 94°C for 3 minutes followed by 30 cycles of 94°C for 1 minute; 68°C for 1 minute; 72°C for 2 minutes; with a final cycle of 72°C for 10 minutes. One tenth volume of the PCR reactions were analyzed by gel electrophoresis. The PCR fragments were then subjected to RFLP analysis using the following restriction enzymes *Bst*E II, *Msp*A1 I, *Dra* III (New England Biolabs) and *Alw*26 I (Promega). All restriction enzyme reactions were carried out according to the manufacturer's specifications.

RESULTS AND DISCUSSION

We compared the published sequences for the Miller, Purdue-115, and PRCV (GenBank accession numbers S51223, D00118 and Z24675 respectively) with the Ambico vaccine strain, in order to choose restriction enzymes that would selectively recognize sequences within the Purdue, Miller, or Ambico generated PCR fragments. The predicted genetic profiles obtained by RFLP analysis are shown in Table 2.

Initial ARMS-PCR analysis was conducted on the reference viral material using the oligo combination of WL5-WL28 (specific for Purdue sequences) or WL5-WL29 (specific for Miller sequences). A summary of these results is presented in Table 3. All of the Miller virus strains amplified specifically with the WL5-WL29 oligos. RFLP analysis of this material showed that all of the Miller strains of virus have the predicted Miller genetic profile. Unexpectedly, the Purdue Std. Challenge SIE and the Purdue Std. Challenge ST/4 also amplified with the WL5-WL29 oligos. However, RFLP analysis of this material showed a mixed genetic profile, with a Miller profile for restriction enzyme *Dra* III and a Purdue profile for restriction enzymes *Bst*E II and *Msp*A1 I. It is unlikely that this represents a mixed viral population, because samples failed to amplify a PCR product with the WL5-WL28 combination. It more likely represents a precursor of the Purdue isolate prior to the selective

Table 2. Predicted RFLP pattern of digested ARMS-PCR amplified fragments

	Enzyme Pattern obtained with			
Virus Strain	*Bst*E II	*Alw*26 I	*Dra* III	*Msp*A1 I
Purdue-115	1000	1096	no cutting	no cutting
	217	121		
Miller	1056	1103	705	735
	110	121	519	489
Ambico	1000	615	no cutting	no cutting
	217	481		
		121		

pressures of tissue culture cell passage and plaque purification. It is interesting to note that this virus yields two plaque sizes of large and small phenotype. However, upon plaque purification of large or small plaques, the mixed phenotype of large and small plaques is still observed regardless of the size of the plaque picked (unpublished observations). We are presently attempting to isolate viral RNA from individual plaques for ARMS-PCR/RFLP analysis to determine if there are any discernible differences associated with the plaque phenotype. ARMS-PCR analysis of the Purdue-115 strain shows that it specifically amplified with the WL5-WL28 oligos and had the predicted RFLP genetic profile for the published Purdue-15 and Purdue-115 viral sequences. The Illinois strain (SIE of convalescent pigs) was the only sample that amplified a product with both sets of oligos, however it only amplified a very faint product using the WL5-WL28 oligos, whereas with the WL5-WL29 oligos the PCR product was more abundant. This most likely represents a mixed population of viruses in the Illinois sample since RFLP analysis of the WL5-WL28 PCR product with *Bst*E II and *Dra* III exhibited a Purdue profile, whereas RFLP analysis of the PCR product amplified with WL5-WL29 showed an identical genetic profile as the Purdue Std. Challenge strains. In previous studies we have shown that mixtures of Miller with Ambico virus strains will amplify PCR products from both combinations of oligos. However, RFLP analysis shows that the PCR product amplified with the WL5-WL28 oligos has the genetic profile of the Ambico strain and the product amplified with the WL5-WL29 oligos has the genetic profile of the Miller strain. This indicates that the oligos are amplifying specifically. The same six nucleotide insertion is also present in a virulent British field isolate [9].

ARMS-PCR/RFLP analysis of Grand Laboritories, Schering-Plough and Diamond Laboratories vaccine strains showed that all amplified with the WL5-WL28 oligo combination and had identical RFLP genetic profiles as the Purdue-115 strain. Only the Fort Dodge vaccine strain amplified with the WL5-WL29 oligo combination and showed a RFLP genetic profile identical to the Miller strain. The Ambico strain amplified with the WL5-WL28 oligo combination. However, it posses a unique genetic profile for RFLP analysis with the enzyme *Alw*26 I. The RFLP analysis of the Ambico strain with *Bst*E II, *Dra* III, and *Msp*A1 I correlate with the genetic profile of Purdue-115. This data is in partial agreement with the S1 nuclease mapping studies published by Register and Wesley [16] who showed that the Solvay and Fort Dodge vaccine strains exhibited a similar S1 protection pattern as the Miller PP3. They also showed that the Diamond and Ambico vaccine strains showed a similar S1 nuclease protection pattern as the Purdue-115 strain. Our data contradicts theirs in that the S1 nuclease pattern for the Ambico strain should have an additional band as compared to the Diamond and Purdue-115 strains, due to the single base change contained in the Ambico strain. In question is the ability and sensitivity of S1 nuclease to detect single base pair mismatch versus the sensitivity of ARMS-PCR/RFLP analysis. They state that they have consistently

Table 3. ARMS-PCR and RFLP analysis of reference virus and vaccine strains

	Amplification with WL5 and					
Virus Strain	WL28	WL29	*Bst*E II	*Alw*26I	*Dra* III	*Msp*A1I
Mil.MGV	–	+	M	M/P	M	M
Mil.PP3	–	+	M	M/P	M	M
Mil.APHIS 69-7	–	+	M	M/P	M	M
Mil.APHIS 69-7/ST3	–	+	M	M/P	M	M
Pur. Std. Chall/ Lot-4	–	+	P	M/P	M	P
Pur. Std. Chall./ST-4	–	+	P	M/P	M	P
Purdue P-115	+	–	P	M/P	P	P
TGEV Illinois Strain	+/–	+	P	M/P	M	P
PRCV ISU-I*	–	+	NA	NA	NA	NA
Ambico Vaccine	+	–	P	A	P	P
Grand Labs	+	–	P	M/P	P	P
Schering-Plough	+	–	P	M/P	P	P
Diamond Labs	+	–	P	M/P	P	P
Fort Dodge	–	+	M	M/P	M	M
Oxford	+/–	–	ND	ND	ND	ND

Note: M = Miller pattern; P = Purdue pattern; A = Ambico pattern; NA = Not Applicable; ND = Not done due to lack of sufficient material. (*) PRCV amplified a fragment of 543 nucleotides.

detected deletions as small as 3-6 bp but they do not indicate if they have been successful in using the S1 nuclease protection assay to detect single base pair mismatches. ARMS-PCR analysis has been used to detect allele-specific differences based on a single nucleotide difference [13,14,15,17].

To determine if the Ambico vaccine strain retains its genetic marker while passaged through piglets, we generated tissue extracts for re-isolation of the Ambico vaccine strain. The results of the PCR amplification with WL5-WL28 and the re-isolation of virus titer evaluated by plaque assay and TGEV specific IFA are shown in Table 4. None of the tissue

Table 4. Sizes of PCR fragments and virus titers isolated from tissues of piglets inoculated with the ambico vaccine TGEV

	24 Hours Post-Inoculation				48 Hours Post-Inoculation			
	Orally		Intranasally		Orally		Intranasally	
	Pig-1		Pig-2		Pig-3		Pig-4	
Sample	PCR	PFU	PCR	PFU	PCR	PFU	PCR	PFU
Fecal	Neg.	(Neg.)	1217	(Neg.)	Neg.	(Neg.)	1217	(Neg.)
Lung	1217	(Neg.)	1217	(Neg.)	Neg.	(Neg.)	1217*	(Neg.)
MLN	1217	(Neg.)	1217	(Neg.)	Neg.	(Neg.)	Neg.	(Neg.)
Nasal	1217	(Neg.)	1217	(1.9)	Neg.	(Neg.)	Neg.	(2.4)
SIE	1217	(Neg.)	1217	(Neg.)	1217*	(Neg.)	1217*	(Neg.)
SIC	Neg.	(Neg.)	Neg.	(Neg.)	Neg.	(Neg.)	Neg.	(Neg.)
Tonsil	1217	(Neg.)	1217	(Neg.)	1217	(Neg.)	Neg.	(Neg.)

samples amplified a PCR product using the oligo combination WL5-WL29 All PCR products amplified from the tissue samples exhibited the genetic profile of the Ambico strain upon RFLP analysis (data not shown) This data indicates that the genetic marker is maintained subsequent to passage in pigs Infectious TGEV was reisolated at low titers from the nasal samples of the 2 pigs that were inoculated intranasally The remaining 26 samples were negative for TGEV by IFA evaluation of plaque assays IFA specific staining for TGEV of frozen sections of jejunum, ileum or duodenum were also negative for TGEV (data not shown)

Furthermore, ARMS-PCR/RFLP analysis of field samples obtained from pigs vaccinated with the Ambico vaccine strain showed that the Ambico strain maintained its genetic marker in the field (data not shown) We have used this method to evaluate a limited number of field samples and have been successful in identification of Miller, Purdue and PRCV strains We are presently utilizing a second set of primers located downstream of the WL5-WL28/29 oligo combinations to be used for further characterization of field isolates as compared to Miller or Purdue strains of TGEV and PRCV This method represents a rapid and sensitive way to monitor virus genetic profiles, which may have application in the diagnostics field A similar system has been used to identify single nucleotide mismatches in other virus systems [15 17] ARMS-PCR analysis coupled with RFLP represents an alternative to nucleic acid sequencing in correlating nucleic acid changes and the resulting altered tropism and pathogenicity

REFERENCES

1 Doyle L P, Hutchings L M A transmissible gastroenteritis in pigs J Am Vet Med Assoc 1946,108 257-259

2 Pensaert M , Callebaut P, Vergote J Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis Vet Q 1986,8 257-261

3 Hill H T, Biwer J D , Wood R D , Wesley R D Porcine respiratory coronavirus isolated from two U S swine herds Proc Am Assoc Swine Prac 1989,333-335

4 Paul P S , Vaughn E M , Halbur P G Characterization and pathogenicity of a new porcine respiratory coronavirus strain AR310 Proc Int Pig Vet Soc Congr 1992,12 92

5 Wesley R D , Woods R D , Hill H T, Biwer J D Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus J Vet Diagn Invest 1990,2 312-317

6 Vaughn E M , Halbur P G , Paul P S , Three new isolates of porcine respiratory coronavirus with pathogenicities and spike (S) gene deletions J Clin Micro 1994,32 1809-1812

7 Britton P, Kottier S , Chen C -M , Pocock D H , Salmon H , Aynaud J M The use of PCR genome mapping for the characterization of TGEV strains Adv Exp Med and Biol 1993,342 29-34

8 Sanchez C M , Gebauer F , Sune C, Mendez A , Dopazo J , Enjuanes L Genetic evolution and tropism of transmissible gastroenteritis coronaviruses Virology 1992,190 92-105

9 Britton P, Page K W Sequence of the S gene from a virulent British field isolate of transmissible gastroenteritis virus Vir Res 1990,18 71-80

10 Laude H , Van Reeth K , Pensaert M Porcine respiratory coronavirus molecular features and virus-host interactions Vet Res 1993,24 125-150

11 Wesley R D , Wood R D , Cheung A K Genetic basis for the pathogenesis of transmissible gastroenteritis virus J Virol 1990,64 4761-4766

12 Wesley R D , Wood R D , Cheung A K Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus J Virol 1991,65 3369-3373

13 Newton C R , Graham A , Heptinstall L E , Powell S J , Summers C , Kalsheker N , Smith J C , Markham A F Analysis of any point mutation in DNA The amplification refractory mutation system (ARMS) Nuc Acids Res 1989,17 2503-2516

14 Huang M M , Arnheim N , Goodman M F Extension of base mispairs by Taq DNA polymerase implications for single nucleotide discrimination in PCR Nuc Acids Res 1992,20 4567-4573

15. Kwok S., Kellogg D.E., McKinney N., Spasic D., Goda L., Leverson C., Sninsky J.J. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nuc. Acids Res. 1990;18 999-1005.
16. Register K.B., Wesley R.D. Molecular characterization of attenuated vaccine strains of transmissible gastroenteritis virus. J. Vet. Diagn. Invest. 1994;6:16-22.
17. Ault G.S., Ryschkewitsch C.F., Stoner G.L. Type-specific amplification of viral DNA using touchdown and hot start PCR. J. Virological Meth. 1994;46:145-156.