Efficacy of a transmissible gastroenteritis coronavirus with an altered ORF-3 gene

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Abstract

Serial passage of virulent transmissible gastroenteritis virus through cell culture reduced its virulence in 3-day-old piglets. Intramuscular inoculation of pregnant gilts with 2 doses of this modified-live virus elicited a level of lactogenic immunity that protected their nursing piglets against a lethal dose of challenge virus. Sequence analysis of a 637-bp fragment of the spike gene containing most of the aminopeptidase receptor and the 4 major antigenic sites from the original and the serially passed viruses were nearly identical. Gel analysis revealed that the fragment from the ORF-3 gene of virulent virus was smaller than the corresponding fragment from the serially passed virus. Sequence analysis of the fragment from the passed virus revealed that the sequence between nt 5310 and nt 5434 was replaced by a 636-bp fragment from the polymerase 1A gene. This replacement resulted in the loss of the CTAAACTT leader RNA-binding site and ATG start codon for the ORF-3A gene but it did not affect the ORF-3B gene.

Résumé

Le passage en série sur des cultures cellulaires d'un isolat virulent du virus de la gastro-entérite transmissible (GET) réduisit sa virulence pour des porcelets de trois jours d'âge. L'inoculation intramusculaire de deux doses de ce virus vivant modifié chez des truies en gestation entraîna l'apparition d'un niveau d'immunité d'origine lactée qui protégea les porcelets à la mamelle contre l'infection avec une dose létale du virus. Une analyse de la séquence de fragments de 637 paires de bases du gène des spicules, contenant la plupart du récepteur aminopeptidase et quatre sites antigéniques majeurs, provenant du virus original et du virus atténué, démontra qu'ils étaient pratiquement identiques. Une analyse sur gel révéla que le fragment associé au gène ORF-3 du virus virulent était de taille plus petite que le fragment correspondant provenant du virus atténué. Une analyse de la séquence du virus atténué révéla que la séquence entre nt 5310 et nt 5434 était remplacée par un fragment de 636 paires de bases provenant du gène 1A de la polymérase. Cette substitution résulta en la perte de la séquence de tête CTAAACTT du site d'attachement de l'ARN et du codon d'initiation ATG pour le gène ORF-3A mais n'affecta pas le gène ORF 3B.

(Traduit par docteur Serge Messier)

Introduction

The *Coronaviridae* family of viruses are pleomorphic enveloped viruses with a non-segmented positive-stranded RNA genome (1). The mammalian coronaviruses are divided into 2 serological groups. Members of Group I include canine coronavirus, feline enteric coronavirus, feline infectious peritonitis virus, human coronavirus 229E, porcine respiratory coronavirus (PRCV) and transmissible gastroenteritis virus (TGEV) (2). Transmissible gastroenteritis virus infects swine of all ages, producing an economically important disease. The severity of the disease is inversely related to the age of the pigs at the time of their initial exposure. Typically, when piglets less than 10 d of age are exposed to virulent TGEV, nearly 100% die within 5 to 7 d, while those 10 to 21 d of age exhibit clinical signs of a severe infection and about 50% die. Adult sows and piglets more than 3 wk of age typically have a mild response with loss of appetite and diarrhea for 1 or 2 d being common, although vomiting, elevated temperatures and death have also been observed (3).

Virulence of TGEV is associated with the infection and rapid destruction of the villous epithelial cells in the small intestine (3). Loss of these specialized absorptive cells results in a decrease in the enzymatic activity in the small intestine, thereby reducing digestion and cellular transport of nutrients and electrolytes from the intestine. This produces an acute malabsorption syndrome that is fatal in young piglets (4). To protect piglets against the disease, they must receive a continuous supply of protective antibodies via the sow's milk (lactogenic immunity) (5,6). Many viral preparations and procedures have been tested over the last 50 y in efforts to elicit this protective response in the sows (6). Sows vaccinated with commercial

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Received May 23, 2000. Accepted November 6, 2000.

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Table I. Morbidity and mortality rates of piglets exposed to serial-passaged Miller 69-7 TGEV

Virus pass(s)	No. of pigs	Number of Piglets (%)[a] Morbidity[b]		Mortality[c]	
0	11	11	(100%)	11	(100%)
15 to 30	12	12	(100%)	12	(100%)
35 to 50	12	12	(100%)	9	(75%)
55 to 65	9	6	(67%)	3	(33%)
70 to 75	6	3	(50%)	0	(0%)
Placebo	6	0	(0%)	0	(0%)

[a] Piglets exposed at 3 d of age via stomach tube to 1×10^6 pfu

[b] Number of pigs with one or more clinical sign(s) (vomiting, diarrhea, inappetence, sluggishness, dehydration)

[c] Number of piglets euthanized/total exposed

TGEV vaccines develop virus-neutralizing antibodies, but only 60 to 80% of their nursing piglets will survive a lethal dose of challenge virus (3). However, when pregnant sows are exposed to a lethal dose of virulent TGEV 3 wk before farrowing, 90 to 95% of their nursing piglets will survive a virulent TGEV challenge (7).

The purpose of this report is to evaluate the efficacy of a modified-live TGEV vaccine for pregnant sows and to identify nucleotide changes in the E2 and ORF-3 genome of the virulent and the modified-live TGEV.

Materials and methods

Cells and viruses

The McClurkin swine testicular (ST) cell line was used for virus propagation, titration, and neutralization assays. The virulent Miller TGEV was used in this study. It was originally isolated in the mid-1960s and its virulence has been maintained by serial passage through caesarian-derived, colostrum-deprived (CDCD) piglets (8,9). The Miller 69-7 (pass 0) virus was from the 3rd pig pass, had a plaque titer of 3×10^5 plaque-forming units (pfu/mL), and a pig lethal dose (PLD) titer of 1×10^5 for 3-day-old pigs (10). The Miller p439+ virus was from the 5th pig pass, had a plaque titer of 1×10^6 pfu/mL and a PLD titer of 1×10^5 pfu/mL (11). The virus isolates used in this study were free of extraneous viral, bacterial, and toxic agents as predetermined by passage in cell culture and CDCD piglets. The Miller 69-7 pass 0 virus was plaque picked 10 times and then serially passed an additional 65 times in ST cell monolayers. The Miller p439+ virus was used as a standardized challenge virus (11). All animal experiments were conducted in an laboratory certified by the Association for Assessment and Accreditation of Laboratory Animal Care.

Determination of virulence

Virulence of the serially passed virus was evaluated in 3-day-old CDCD piglets. Forty-five piglets were obtained from sows serologically negative for TGEV neutralizing antibodies from the Animal Resources Unit at the National Animal Disease Center in Ames, Iowa, USA. At 3 d of age, the piglets were randomly divided into 14 groups and each piglet was given 1×10^6 pfu of TGEV from the 15th to the 75th pass, via stomach tube; 6 piglets were held as controls. After exposure, all piglets were fed and observed 4 times a day for clinical signs of an infection. Piglets exhibiting life-threatening clinical signs of a virulent TGEV infection (vomiting, diarrhea, fever, inappetence, dehydration, and sluggishness) were euthanized and a portion of the small intestine was removed for virus isolation. Confirmation of a TGEV isolate was by virus neutralization (VN) and a reverse transcriptase polymerase chain reaction (RT-PCR) procedure (11,12).

Determination of immunizing properties

Nine sows seronegative for TGEV antibodies were obtained from the Animal Resources Unit at the National Animal Disease Center and housed in individual isolation rooms. The sows were intramuscularly vaccinated 6 wk and 3 wk before their expected farrowing date. Three of the sows were vaccinated with 2 mL of live Miller 69-7 (pass 0) virus, 3 were vaccinated with 2 mL of Miller 69-7 (pass 75) virus, and 3 were placebo controls. Three days after farrowing, their piglets were challenged with 5 mL of virulent Miller p439 TGEV containing approximately 500 PLDs via stomach tube (8). The piglets were fed and observed 4 times a day for clinical signs of an infection. Those exhibiting signs of a severe TGEV infection were euthanized and a portion of the small intestine was removed for virus isolation. Isolates of TGEV were confirmed by VN and the RT-PCR procedure.

Determination of genomic changes

The RT-PCR procedures used in this study have been reported previously (12). Sequencing of the Miller ORFs 2 to 7 of the TGEV genome indicated nucleotide changes were limited to ORF-3A-B. Primers were designed to amplify 2 independent fragments of the Miller TGEV genome. Briefly, the first set of primers amplified a 637-bp fragment of the spike gene (nt 2355 to nt 2991)(12). This sequence contains the 4 major antigenic sites and most of the aminopeptidase receptor binding site. The second set of primers (sense 5′-TTGGTATGTGTGGCTACTAATAGGC-3′ antisense 5′-GCATAGGGTCTACAAAATGC-3′) amplified a 725-bp fragment of the ORF-3A-B gene (nt 5100 to nt 5825). The amplified fragment contained the last 180 bp of the spike gene and most of the 3A-B gene. A hemi-nested set of primers (nt 5358 to nt 5825 in the 3A-B gene) was used to identify the point of insertion. Direct sequencing of the RT-PCR products was done by a commercial laboratory (Iowa State University

Table II. Transmissible gastroenteritis virus neutralizing antibody titers in serum, colostrum and milk whey of sows vaccinated with Miller 69-7 (pass 0), Miller 69-7 (pass 75) or placebo fluids after vaccination, and challenge exposure of their nursing piglets and morbidity and mortality rates of the challenge exposed piglets

		50% plaque reduction (VN titer)				Number of piglets (%)[c]	
Vaccine	Sow no.	2nd Vacc[a]	Colostrum	Challenge[b]	Day 10 PE[6]	Morbidity[d]	Mortality[e]
Miller 69-7	1	1:16	1:180	1:32	1:16	0/6 (0%)	0/6 (0%)
(pass 0)	2	1:64	1:240	1:32	1:64	5/13 (38%)	2/13 (15%)
	3	1:16	1:200	1:64	1:64	2/11 (18%)	2/11 (18%)
	GMT	1:32	1:207	1:43	1:48	7/30 (23%)	4/30 (13%)
Miller 69-7	4	1:256	1:835	1:64	1:128	7/10 (70%)	2/10 (20%)
(pass 75)	5	1:128	1:512	1:48	1:64	11/11 (100%)	3/11 (27%)
	6	1:256	1:1024	1:128	1:256	8/9 (81%)	1/9 (11%)
	GMT	1:213	1:790	1:80	1:149	26/30 (87%)	6/30 (20%)
Placebo	7	< 1:4	< 1:4	< 1:4	1:8	8/8 (100%)	8/8 (100%)
	8	< 1:4	< 1:4	< 1:4	< 1:4	11/11 (100%)	10/11 (90%)
	9	< 1:4	< 1:4	< 1:4	1:4	10/10 (100%)	9/10 (90%)
	GMT	< 1:4	< 1:4	< 1:4	1:4	29/29 (100%)	27/29 (93%)

GMT — geometric mean titer; PE — postexposure

[a] Serum titer of sow

[b] Milk whey titer

[c] Piglets exposed to 500 lethal doses of TGEV at 3 d of age via stomach tube

[d] Number of pigs with one or more clinical signs (vomiting, diarrhea, inappetence, sluggishness, dehydration)

[e] Number of piglets euthanized/total exposed

Nucleic Acid Facility, Ames, Iowa, USA) using an automated sequencer. Before sequencing, the amplified PCR products were partially purified by centrifugation through microconcentrators (Amicon Inc, Beverly, Massachusetts, USA).

Results

Viral virulence

The virus lost virulence for CDCD piglets with serial passage (Table 1). Mortality rates for the piglets given serially passed virus remained near 100% through the 50th pass. Twenty-one of 24 piglets given virus from the 15th to the 50th pass developed clinical signs of a virulent TGEV infection (vomiting, diarrhea, inappetence, gauntness, sluggishness, dehydration) and were euthanized 5 to 9 d postexposure. The surviving piglets (one each from passes 35, 40, and 45) had milder clinical signs and returned to normal by 10 d postexposure. By the 65th pass, the mortality rate was 0%. In contrast to the mortality rates, the morbidity rate varied, although piglets given virus beyond the 50th pass generally had mild clinical signs (transient diarrhea and inappetence) that lasted no more than 1 or 2 d. The piglets given placebo fluids remained free of clinical signs throughout the observation period and were serologically negative for TGEV neutralizing antibodies at the end of the experiment.

Immunizing properties

Serological results of sow vaccinations and immunity challenge of their piglets are presented in Table II. Sows vaccinated with Miller 69-7 (pass 0) and Miller 69-7 (pass 75) had high VN antibody titers in their serum and milk whey samples, while the control sows remained free of TGEV antibodies until they were exposed to the virus by their challenged piglets. The VN titers of both vaccinated groups were highest at the time of parturition. After challenge exposure of their piglets, the VN antibody response varied between the 2 vaccinated groups. The VN titer of the Miller 69-7 (pass 0) group remained constant, while the VN titer of the Miller 69-7 (pass 75) group was increased suggesting the sows had an amnestic response.

Both of the Miller 69-7 vaccinated sow groups provided a moderated level of protection for their nursing piglets (Table I). Most of the piglets that developed clinical signs recovered within 1 to 2 d postexposure. In contrast, life-threatening clinical signs were observed in the piglets nursing the control sows and their piglets were euthanized.

Sows vaccinated with the Miller 69-7 (pass 0) virus did not have any observable adverse reactions after either vaccination or exposure to their virus infected nursing piglets. The geometric mean VN titer (GMT) was 1:32 in serum, 1:207 in colostrum whey, 1:43 in milk whey collected at challenge, and 1:48 in milk whey collected 10 d postchallenge.

Sows vaccinated with Miller 69-7 (pass 75) virus did not have any observable adverse reaction after either vaccination or exposure to their virus infected nursing piglets. The GMT was 1:213 in serum, 1:790 in colostrum whey, 1:80 in milk whey collected at the time of piglet challenge and 1:149 in milk whey samples collected 10 days postexposure. All sows had higher VN antibody titers after contact with their virus infected piglets.

Virus neutralizing antibodies were not detected in any sample taken from the control sows until 10 d postexposure. Two of the 3 sows had VN titers (1:4 and 1:8), while the 3rd sow had a titer of < 1:4.

By 10 d postexposure, piglets nursing the Miller 69-7 (pass 0) vaccinated sows had a morbidity rate of 23% and a mortality rate of 13%, piglets nursing the Miller 69-7 (pass 75) vaccinated sows had a

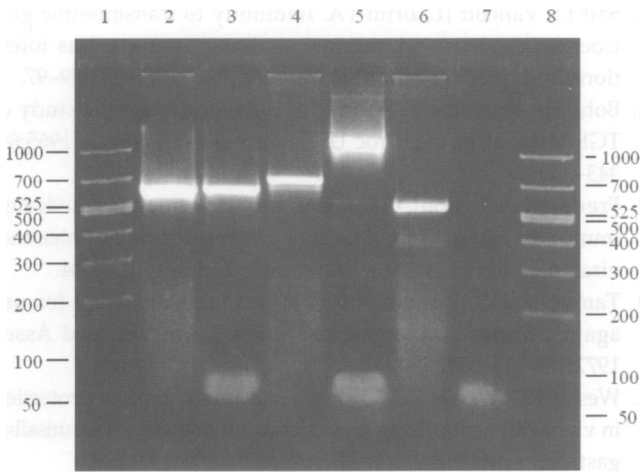

Figure 1. RT-PCR products of Miller 69-7 (pass 0) and Miller 69-7 (pass 75) viruses. Lanes 2, 4, and 6 are amplified fragments from the pass 0 virus and lanes 3, 5, and 7 are amplified fragments from the 75th pass virus. Lanes 2 and 3 spike gene primers resulting in a 637-bp fragment; lane 4 and 5 ORF-3A-B primers resulting in a 725-bp fragment in lane 3 and a 1238 bp fragment in lane 4; and lanes 6 and 7 are first half ORF-3A-B primers set producing a fragment of 547 bp in lane 6; and no product in lane 7. Lanes 1 and 8 are molecular weight standards.

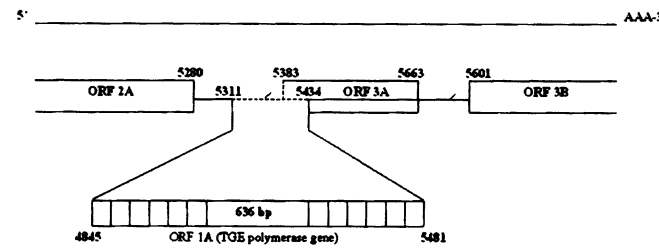

Figure 2. Location of changes in the Miller 69-7 (pass 75) 3A gene. Primer set 2 amplifies a fragment from nt 5100 to nt 5825. The CTAAACTT leader sequence indicated by "/" (nt 5354) and the start codon (nt 5383) are not present in the altered 3A gene but remain in the 3B gene.

morbidity rate of 87% and a mortality rate of 20%, and piglets nursing the control sows had a morbidity rate of 100% and a mortality rate of 93% (Table II). Diarrhea was observed in every litter of piglets challenged except for one litter in the Miller 69-7 (pass 0) group. The duration and severity of the diarrhea varied from pig to pig and from group to group. Most of the piglets nursing the control sows developed a profuse diarrhea by 36 h postexposure that persisted for the duration of the experiment, leading to dehydration and, finally, euthanasia. Piglet nursing the Miller 69-7 (pass 75) vaccinates had varied clinical signs. The litters of sows 4 and 6 had transient clinical signs (diarrhea and inappetence) that persisted for 48 to 72 h, while those nursing sow 5 had severe clinical signs (diarrhea, inappetence, dehydration, and weight loss). Piglets nursing sow 1 did not develop any observable clinical signs and most of the piglets nursing sows 2 and 3 only developed a mild diarrhea and/or inappetence that persisted for 24 to 36 h before complete recovery. Two piglets nursing sow 2 and 2 piglets nursing sow 3 were euthanized by day 9 postexposure because of dehydration and inappetence. All remaining piglets were healthy at the end of the experiment.

Genomic changes

In the RT-PCR procedure, changes in the nucleotide sequence of the Miller virus were identified (Figure 1). Three nucleotide changes were identified in the Miller 69-7 (pass 75) spike gene when compared to the Miller 69-7 (pass 0) virus. None of the changes were in the 4 antigenic sites or in the aminopeptidase receptor region: nt 2416 (C to A), nt 2621 (C to A) and nt 2955 (A to G). In contrast, with ORF-3 primers, the fragment generated from the Miller 69-7 (pass 75) virus was much larger (513 bp) than the fragment generated from the Miller 69-7 (pass 0) virus. Sequencing confirmed the fragment from the Miller 69-7 (pass 75) virus contained a 636-bp segment of the TGEV polymerase gene (ORF 1A nt 4845 to nt 5481). This fragment had replaced the sequence from nt 5311 to nt 5434 in the Miller 69-7 (pass 0) virus (Figure 2). This replacement resulted in the loss of the CTAAACTT leader RNA-binding site and ATG start codon for ORF-3A gene. The alteration in the sequence did not affect the growth of the virus in cell culture. After 30 additional serial passes, the alteration of the ORF 3 gene remained stable.

Discussion

The vaccination and challenge results in this study are similar to those in other reports concerning the virulent virus. Pregnant sows exposed to the virulent virus 3 wk before farrowing developed lactogenic antibodies that protected their piglets against a lethal dose of challenge virus. This study confirms previous reports that milk whey VN titers from sows exposed to the virulent virus are lower than milk whey VN titers from sows given the attenuated virus (Table II). None of the currently marketed commercial TGEV vaccines provide this level of protection (> 80%).

Piglets nursing sows vaccinated with 2 doses of the Miller 69-7 (pass 75) virus developed mild clinical signs of an infection after they were exposed to a lethal dose of virus. The morbidity rate of these nursing pigs (87%) was higher than the rate observed in the virulent virus group (23%), but lower than the control group (100%). The 80% protection recorded in this group was slightly lower than the 87% observed in piglets nursing sows exposed to the Miller 69-7 (pass 0) virus. This avirulent TGEV had immunogenic and protective properties similar to the Miller 69-7 (pass 0) virus, without the virulence for young piglets, suggesting its vaccine potential.

Analysis of the RT-PCR generated fragments from the spike gene of the original and 75th cell pass virus were identical in the nucleotide sequence of the 4 antigenic sites and in the aminopeptidase receptor site. Fragments generated from ORF-3 of the Miller 69-7 (pass 0) virus were smaller than fragments generated from the Miller 69-7 (pass 75) virus. Based on a blast search, the sequence of the replacement fragment had a 99% homology with a segment located in the polymerase 1A gene (13). Many coronaviruses have insertions or deletions in the 3A-B gene. Some investigators have suggested this area of the genome may be involved in tropism and pathogenicity of TGEV (14-16). In the Miller 69-7 (pass 75) virus, a replacement in the ORF-3A nucleotide sequence,

not in any other area of the genome (unreported), resulted in a virus that was avirulent for young piglets. Because there was only minor change in the spike gene sequence of the 2 viruses, the data presented suggests that ORF-3A plays a role the in virulence of TGEV.

Acknowledgments

The author thanks Julia Meimann for Figure 1, Deborah Clouser for Figure 2, and Dr. Andrew Cheung and Dr. Glenn Frank for reviewing the manuscript.

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