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Sequence analysis and cDNA probe hybridization of the nucleocapsid(N) protein gene of transmissible gastroenteritis virus(TGEV) and porcine epidemic diarrhea virus(PEDV)

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Transmissible gastroenteritis virus(TGEV)와 porcine epidemic diarrhea virus(PEDV)의 nucleocapsid(N) 단백질 유전자에 대한 염기서열 분석과 cDNA probe hybridization

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초록 : Coronaviridae에 속하는 transmissible gastroenteritis virus(TGEV)와 porcine epidemic diarrhea virus (PEDV)를 specific하게 detection할 수 있는 방법을 개발하고자 본 연구를 수행하였다.

두 바이러스 모두 RNA 바이러스이기 때문에 reverse transcription-polymerase chain reaction(RT-PCR)으로 nucleocapsid(N) protein gene의 cDNA를 증폭시켰다. *Sma*I으로 처리한 pTZ19R에 ligation시킨 후 염기서열을 밝히고자 sequencing하였다. 각각의 prototype virus와 비교하여 상동성을 밝혔다. 두 바이러스에 대한 cDNA probe를 제작하여 Southern blot hybridization을 실시하였다.

TGEV의 경우 백신주인 P45와 병독주인 Miller strain을 사용하였다. cDNA를 증폭시키기 위해 N1/N1R과 N2/N2R 두 가지 primer를 이용한 결과, N1/N1R primer의 경우 586bp 크기의 PCR product를 얻을 수 있었고, N2/N2R primer로 582bp의 cDNA를 증폭시킬 수 있었다.

PEDV 실험을 위하여 PED 임상 증상을 나타내는 분변을 이용하여 RT-PCR을 실시하였다. P2/P2R primer로 753bp의 PCR product를 얻을 수 있었다.

TGEV의 두 가지 strain의 N protein gene을 sequencing하여 prototype인 Purdue strain과 염기서열 상동성을 조

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사한 결과, 97% 이상의 높은 homology를 나타내었다. PED-V 역시 N protein gene을 sequencing하여 CV777과 염기서열 상동성을 조사한 결과 97% 이상의 homology로 PEDV임을 알 수 있었다.

TGEV와 PEDV의 염기서열을 비교한 결과 29%의 낮은 homology를 관찰할 수 있었다.

두 가지 바이러스의 N protein gene에 대한 cDNA probe를 제작하여 Southern blot hybridization을 한 결과, 각 바이러스에 매우 특이적 반응을 나타내었다.

Key words : TGEV, PEDV, RT-PCR, Southern hybridization

Introduction

The coronaviruses are a monogeneric family of single-stranded RNA viruses. The enveloped particles are pleomorphic but roughly spherical, with diameters ranging from 60 to 220nm(average 100nm).

The genomic RNA of coronavirus is a linear, single stranded molecule that is capped, polyadenylated and infectious. The genomic RNA of coronavirus is 27-30 kb in size. Structurally virions are composed of three major proteins; a phosphorylated nucleocapsid(N) protein of 50 to 60×10^3 daltons, a glycosylated peplomer(spike, S) protein associated with glycopolypeptides of 90 to 180×10^3 daltons and a transmembrane matrix(M) protein associated with polypeptides of 20 to 35×10^3daltons, with varying degrees of glycosylation. The genome is organized into seven regions, containing one or more open reading frames(ORFs) which are separated by junctions[6]. These sequences contain the signal(s) for the transcription of multiple subgenomic mRNAs. The ORFs encoding the structural viral proteins have been identified from their location and a consensus gene order 5' S-M-N 3' can be inferred[18].

Coronaviruses are important pathogens in human and animals. Several antigenic groups are categorized on the basis of results obtained by serology[2]. Transmissible gastroenteritis virus(TGEV), feline infectious peritonitis virus(FIPV), feline enteric coronavirus(FECV), canine coronavirus(CCV) and human respiratory coronavirus(HCV-229E) are classified into one antigenic group[10,17]. The other antigenic group contains murine hepatitis coronavirus(MHV), bovine coronavirus (BCV), hemagglutinating encephalomyelitis coronavirus of swine, human coronavirus(OC43) and rat coronavirus.

Transmissible Gastroenteritis(TGE) is one of the highly contagious enteric disease of swine characterized by vomiting, severe diarrhea and a high mortality(often 100%) in piglets under 2 weeks of age[4]. Although swine of all ages are susceptible to this viral infection, the mortality in swine over 5 weeks of age is very low[12].

In recent years, porcine epidemic diarrhea virus(PEDV) antigenically distinct from TGEV and hemagglutinating encephalomyelitis virus has been incriminated in epizootics of porcine diarrhea in Europe, Canada, and Japan[11,12]. Clinical signs may not differentiate TGE from porcine epidemic diarrhea.

The sequences of the nucleotides nearest to the 3' end of the genome comprising the N gene of coronavirus 777(CV 777), first identified by Pensaert and DeBouck in Belgium, were determined[1]. This showed significant homology to the N genes of coronaviruses and thus the status of PEDV as a member of family the Coronaviridae was formally established.

The purposes of this paper are to determine the nucleotide sequences of N protein gene of TGEV and PEDV isolated from Korea and to develope a specific detection and differentiation system of these two coronaviruses. We have performed the cloning and sequencing of nucleocapsid(N) protein gene and developed cDNA probe for the specific detection and differentiation of TGEV and PEDV.

Materials and Methods

Cells and viruses : Swine testicular(ST) cells were grown in modified Eagle's medium(Gibco) supplemented with fetal bovine serum(10%).

The P45, avirulent vaccine strain and the virulent Miller strain of swine TGEV were obtained from the

Veterinary Research Institute, Anyang, Korea. The virus was plaque-picked four times and passaged twice on ST cells at the moi less than 0.1. Stock virus(titers ranged from 0.6×10^7 to 5×10^7 pfu/ml) was prepared from infected cell supernatants of the third and fourth passages.

The PEDV samples were obtained from the diarrhea stool from the pigs manifesting clinical signs of PED.

RNA extraction : RNA was extracted by acid guanidium thiocyanate phenol-chloroform(AGPC) method of Chomczynski and Sacchi[3]. 100μl of purified TGEV or clarified stool sample for PEDV was mixed with same volume of 2× solution D(Sol D = 4M guanidium thiocyanate, 25mM sodium citrate, 0.5% Sarcosyl, 0.1M 2-mercaptoethanol, pH 7.0). The mixture was treated with 400μl of phenol/chloroform(P/C 1:1) by vortexing for 10 min vigorously. After centrifugation for 10 min at 15,000×g and the supernatent was transferred to a new eppendorf tube. And then 350μl of isopropanol was added, stored at -80℃ for 2hrs. RNAs were obtained from centrifugation for 20min at 15,000×g and resuspended in 20μl of Sol D and 300ml of diethyl pyrocarbonate(DEPC) water. The mixture was centrifuged for 10min at 15,000×g and the supernatant was transferred to a new eppendorf tube and then 20μl of 5M NaCl and 200μl P/C was added. The mixture was vortexed vigorously for 10 min, centrifuged for 5 min at 15,000×g and the supernatant was transferred to a new eppendorf tube. 500μl of chloroform/isoamylalcohol(C/I 24:1) was added, vortexed for 1min, centrifuged for 2min at 15,000×g and the supernatant was transferred to a new eppendorf tube. 350μl of isopropanol was added and stored at -80℃ for 2hrs. The mixture was centrifuged for 20min at 15,000×g and then resuspended in 10μl of DEPC water.

Reverse-Transcription Polymerization Chain Reaction(RT-PCR) : cDNA was synthesized from purified total RNA from TGEV P45, Miller strain, and PEDV using antisense primer of nucleoprotein(N protein) gene and reverse transcriptase(Superscript II, Gibco BRL). RNA sample was added to 2μl of 5× first strand synthesis reaction buffer(1M Tris-Cl 0.5ml, 1M KCl 0.7ml, 25mM $MgSO_4$ 0.4ml, 0.1 M dithiothreitol 2μl), 2μl of 2.5mM dNTP(Perkin Elmer), 1ml of reverse primer, and 3μl of DEPC water. The mixture was incubated at 70℃ for 10 min and slowly cooled down to 37℃. The 200 units of reverse transcriptase was added to the mixture and incubated at 37℃ for 60min.

The 1st strand cDNAs were used for PCR amplification. The sequences of primers for TGEV and PEDV were derived from the published sequences of Purdue strain and Belgian CV777 strain[1], respectively. N1 and N1R primer were used to amplify from 5' end of N protein gene. The N2 primer set was applied to the amplified 582bp from 3' end of N protein gene. The P2 primer set of PEDV was obtained from the N protein gene sequence of 1700 nucleotides nearest to the 3' end of genome. All the sequence primers were listed in Table 1. Fig 1 shows the positions of the oligonucleotide primers on the TGEV and PEDV genome.

The PCR was carried out in a 50μl volume containing 10mM Tris-Cl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001%(w/v) gelatin, 0.3mM dATP, dCTP, dGTP and dTTP, 1μl of each oligonucleotide primer, 5 units of Taq polymerase(Perkin-Elmer), and 10 to 100ng of the cDNA template. The reaction mixture was overlaid with mineral oil(Sigma) and amplified in a DNA thermal cycler(Perkin-Elmer). The template of TGEV was denatured for 10min at 98℃, then put through 35 cycles consisting of 1min at 94℃, 2min at 55℃ for annealing and 2min at 72℃ for extension. In case of the cDNA of PEDV, the amplification was performed through 40 cycles of 94℃ for 1min, 47℃ for 2min and 72℃ for 3min with final elongation step of 72℃ for 8min in a thermal cycler.

Cloning of the cDNAs : The PCR-amplified cDNAs of N protein genes generated with the primer sets, N1/N1R and N2/N2R for TGEV and P2/P2R for PEDV were electrophoresed on 1.2% agarose gels (Seakem, FMC Bioproducts), excised and purified using Geneclean II Kit(Bio 101, USA).

One ml of PCR products of TGEV or PEDV was ligated to 0.5μl of SmaI- digested pTZ19R with vector DNA ligase at 16℃ for 2hrs. Ligated plasmid was

Table 1. The position of the oligonucleoticdes used for RT-PCR

Oligonucleotides	Sequence	Nucleotide Position
OLIGO N1	5' CTAAACTTCTAAATGGCCAA 3'	5822-5841[a]
OLIGO N1R	5' GTTCTACACTATCATCCTTC 3'	6403-6422[a]
OLIGO N2	5' AGAACAAGCTGTTCTTGCCG 3'	6436-6455[a]
OLIGO N2R	5' GTTTAGTTCGTTACCTCGTC 3'	6978-6997[a]
OLIGO P2	5' GAGAATTCCCAAGGGCGAAA 3'	881- 900[b]
OLIGO P2R	5' GGCACGCTCTTCCATATAGG 3'	1616-1635[b]
OLIGO P35	5' CAGTGTACTTGAGATTGTT 3'	423- 442[b]
OLIGO P32	5' ATCTTTAATTACTCGTGCAA 3'	1584-1603[b]

[a] derived from N protein gene of TGEV strain, Purdue and [b] derived from PEDV strain, CV777.

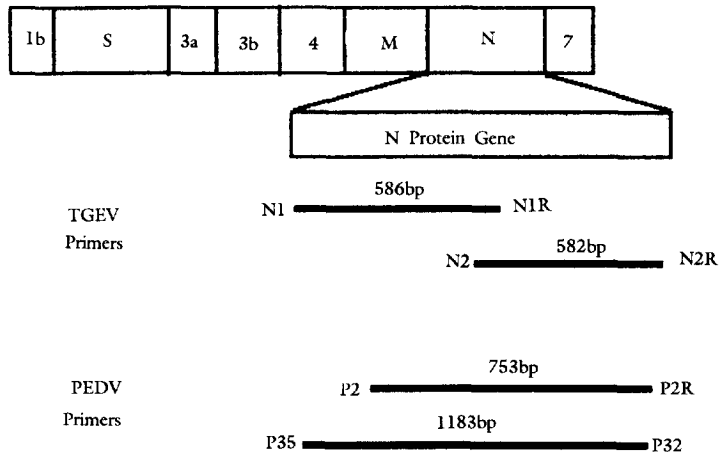

Fig 1. Schematic diagram of the TGEV and PEDV genome showing the position of the oligonucleotides used for RT-RCR. The boxes show the positions of the TGEV and PEDV genes. The lines show the sizes of the PCR-amplified fragments expected from the TGEV and PEDV genome.

transformed into *Escherichia coli* JM 105[7].

White colonies were selected from the selective plate, and cultured in 5ml LB broth containing ampicillin(50μg/ml) for 18hrs at 37℃. Cells were pelleted by centrifugation for 3min. Plasmid DNA was isolated by alkaline lysis method[14]. The cells were resuspended in 100μl of solution I(50mM glucose, 10mM EDTA, 25mM Tris-Cl, pH 8.0), stored at room temperature for 5min and 200μl of solution II (0.2 N NaOH, 1% SDS) was added and mixed. 150μl of solution III(3M potassium acetate, pH 4.8) was added to the mixture, vigorously mixed and stored on

ice for 10min. The mixture was added by 450μl of 5M LiCl, left on ice for 5min and centrifuged for 5min at 12000×g to yield a clear supernatant. 900μl of aqueous phase was transferred to a new eppendorf tube and precipitated with 600μl of isopropanol on ice for 10min. The DNA was precipitated by centrifugation at 12,000×g for 10min.

The purified DNA was treated with restriction endonucleases and examined the cloning efficiency of DNA. The mixture contained 1mg of DNA, 2 units of XbaI, 2 units of EcoRI and 10×reaction buffer was incubated at 37℃ for 2hrs. The digested samples were electrophoresed on 1.2% agarose gel and examined the presence of the inserted DNA under the ultra-violet(UV) illuminator.

DNA sequencing : Analysis of nucleotide sequence was performed by the dideoxynucleotide chain termination method of Sanger et al[15].

1μg of the template DNA was mixed with 2ml of 2N NaOH at 37℃ for 5min to denature the double stranded DNA. 8μl of 5M ammonium acetate(pH 4. 5), and 100μl of 100% ethanol were added and the template DNA was precipitated for 15min in a dry-ice bath. The pelleted DNA was washed with 70% ethanol, and dried under vacuum.

1μg of the denatured template DNA, 2μl of 5× sequencing buffer(40mM Tris-Cl, pH 7.5, 20 mM MgCl2, 50 mM NaCl) and 1μl of pTZ19R universal (U)(5' CACTTAAGCTCGAGCCATGG 3') or reverse(R)(5' ATGCTGAGTGATATCC 3' sequencing primer was mixed.

In this DNA mixture, 1μl of 0.1M DTT, 0.4μl of labelling mix, 1.75μl of enzyme dilution buffer, 1μl of ^{35}S-dATP, 1.6μl of distilled water and 0.25μl of DNA polymerase(Sequenase, USB) were added and incubated at room temperature for 4min. 3.5μl of the labelled mixture were transferred to each tube containing ddGTP, ddATP, ddTTP and ddCTP, respectively. They were incubated continuously for the termination reaction at 37℃ for 5min. The reactions were stopped by adding 4μl of stop solution(95% formamide, 20mM EDTA, 0.25% bromophenol blue, 0. 25% xylene cyanol). Samples were treacted at 75℃ for 2min immediately before loading onto sequencing gel. Each 2.5μl of aliquots of four reactions was loaded on the 6% polyacrylamide sequencing gel containing 8M urea in TBE(90mM Tris-borate, 1mM EDTA, pH 8. 0) buffer.

Southern hybridization

Gel electrophoresis : DNA samples were applied to 1.2% agarose gel containing ethidium bromide, and allowed to run until bromophenol blue migrates to the two thirds of the way down the gel. Fresh depurination solution(250mM HCl) was added to cover the gel and agitated. Treatment should be stopped when the bromophenol blue dye has turned completely yellow. The gel was rinsed with distilled water, and treated with denaturation solution(1.5M NaCl, 0. 5M NaOH) until bromophenol blue dye returned to its blue color.

Capillary blotting : The agarose gel was placed on a supporting platform and a dish filled partly with 20× SSC(3M Na_3 citrate, 3M NaCl, pH 7.0). On the top of the gel, the nylon membrane(Hybond-N+, Amersham) was placed and three sheets of 3MM filter paper(3MM) wetted with 10× SSC were placed. The stack of absorbent paper towels were put on the filter paper with a weight and left overnight for capillary blotting.

Preparation of labelled probe : The DNA to be labelled was diluted to a concentration of 10ng/ml. The 100ng of the DNA sample(10ml) was denatured by heating for 5min in a boiling water bath, and then cooled on ice for 5min. An equivalent volume of DNA was added to 10ml of labelling reagent and 10ml of glutaraldehyde solution. The mixture was incubated for 10min at 37℃.

Hybridization : The blotting membrane in hybridization buffer(0.5M NaCl, 5%(w/v) blocking agent) was pre-heated to 42℃ for 15min with gentle agitation. The labelled probe was added to the pre-hybridization buffer and incubated overnight at 42℃ with gentle agitation. The primary washing buffer(0. 4% SDS, 0.5× SSC) was prepared and preheated to 42℃. The blots were transferred carefully to this solution and washed twice for 20min with gentle agitation. The blots were placed in a clean container and secondary washing buffer(20× SSC) was added at

room temperature for 5min.

Detection : An equal volume of detection reagent 1 mixed with detection reagent 2, was added to the blot on the side carrying the DNA and incubated for 1min at room temperature. The blot was covered in wrap and placed in the film cassette. After 1min exposure, the film was developed.

Results

cDNA synthesis and amplification of the N protein gene of TGEV and PEDV : The cDNAs of P45 and-Miller strains of TGEV were synthesized and amplified using the four primer sets designed from conserved sequences in the coronaviral N genes(N1/N 1R and N2/N2R). Two cDNA fragments of 586bp (N1/N1R) and 582bp(N2/N2R) were amplified as we expected from the TGEV sequence data. Fig. 2 shows RT-PCR positive fragments of TGEV. Lanes A and D are positive controls for S protein gene. Lanes B, C and E show the amplifications of N protein gene of TGEV. The smaller bands could be the amplified DNAs by non-specific binding of primers to the templates.

The cDNA from the 3' end of PEDV N gene was amplified using P2/P2R primer directly from the stool samples. We have tried to detect the PEDV N protein gene from at least 20 different stool samples collected from the pigs manifesting clinically signs of PED. However, only 4 samples showed positive cDNA fragment by RT-PCR. The size of cDNA was 753bp as we expected from the CV777 sequence data (Fig 3).

Fig 4. shows the amplified entire segments of N gene from TGEV and PEDV. The cDNA fragments in lanes A and B were originated from TGEV P45 and Miller strains, respectively. The sizes of cDNAs were 1162bp as we expected. The DNA fragments in lane C represent the cDNAs originated from PEDV N gene. The size of cDNA was 1183bp.

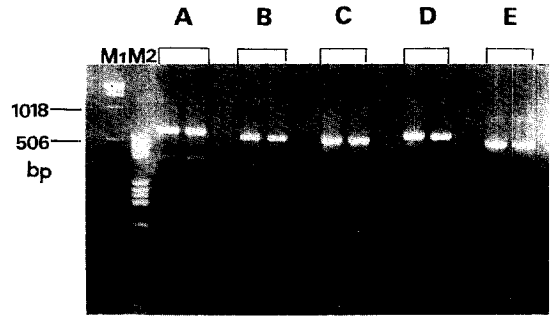

Fig 2. Electrophoresis of TGEV PCR products. Lane M1: 1Kb DNA ladder, lane M2: Marker V, lane A: S2 of P45, lane B: of P45, lane C: N2 of P45, lane D: S2 of Miller, and lane E: N2 of Miller

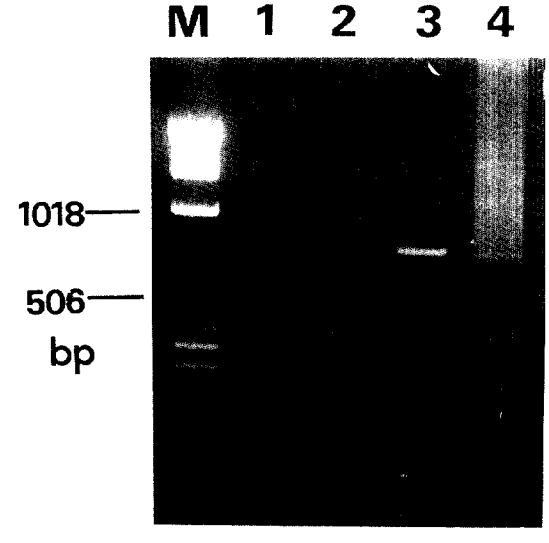

Fig 3. PCR products of PEDV stool samples. Lane M: 1Kb DNA ladder, lane 1: stool 9, lane 2: stool 12, lane 3: stool C, and lane 4: stool 5

Cloning into plasmid vector : After the PCR products were treated with Klenow fragment and T4 polynucleotide kinase, and they were ligated into the linearized pTZ19R. The ligated clones(white colony)

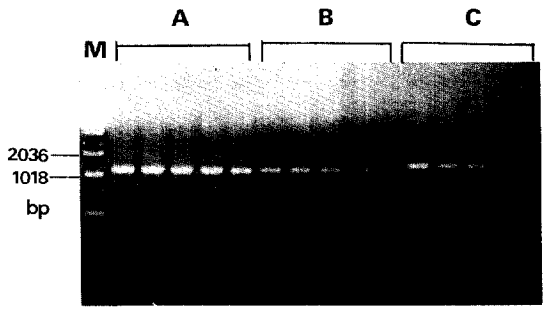

Fig 4. Amplified cDNA of N gene of TGEV and PEDV by PCR Lane M: 1Kb DNA ladder, lane A: 1162bp of P45 N gene, lane B: 1162bp of Miller N gene, and lane C: 1183bp of PEDV N gene.

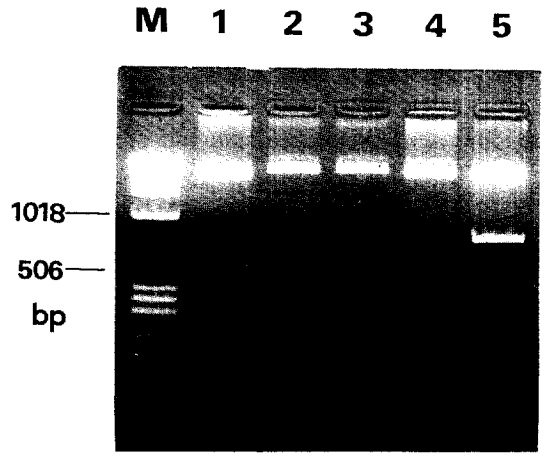

Fig 5. Cleavage patterns of TGEV and PEDV recombinant DNA with *Eco*RI and *Xba*I. Lane M: 1Kb DNA ladder, lane 1: pTZN1, lane 2: pTZN2, lane 3: pTZN1m, lane 4: pTZN2m, and lane pTZP2

on the LB containing ampicillin(50mg/ml), X-Gal (20mg/ml) and 1M IPTG were selected and used for plasmid DNA preparation.

The recombinant positive clones were confirmed by restriction endonucleases, *Eco*RI and XbaI, as the presence of inserts(Fig 5). The selected clones were named as pTZN1 and pTZN2 from P45 and pTZN1m and pTZN2m from Miller strain, respectively. The selected clone from PEDV was named as pTZP2.

However, pTZN1 and pTZN1m inserts were smaller in size than those of expected size(586bp). That was thought that these inserts included a recognition site of EcoRI or XbaI. After the sequencing of these cDNA clones, We have confirmed that they have EcoRI site at the position of 6287. After digestion with EcoRI and XbaI, the inserts from pTZN2, pTZN2m and pTZP2 showed the expected size. The

Table 2. Nucleotide and amino acid sequence homology among three strains of TGEV

	P45/Purdue	Miller/Purdue	P45/Miller
Nucleotide Sequence	1131/1162	1135/1162	1144/1162
Homology	97.3%	97.6%	98.4%
Amino Aicd Sequence	368/382	370/382	372/382
Homology	96.3%	96.8%	97.6%

Table 3. Nucleotide sequence homology of N protein gene between PEDV and TGEV

	Miller/CV777	P45/CV777	PED-C/CV777
Nucleotide Sequence	140/438	142/483	302/311
Homology	29.2%	29.4%	97.8%

size of pTZN2/pTZN2m and pTZP2 were 582bp and 753bp, respectively.

Nucleotide and amino acid sequence analysis of N gene from TGEV : The nucleotides and amino acids of N genes from TGEV P45 and Miller strain were sequenced and shown in Fig 6 and 7, respectively. There were compared to the sequences of Purdue strain as a prototype. The repeated sequence 5' CTAAAC 3' which assumed to act as a signal for the transcription of subgenomic RNA in coronaviruses

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Purdue  CTAAACTCTAAATGGCCAACCAGGGACAACGTGTCAGTTGGGGGGATGA  5884
P45     ----------------------------------------------A-----
Miller  ----------------------------------------------A-----

Purdue  ATCCACCAAAATACGTGGTCGCTCCAATTCCCGTGGTCGGAAGATTAATA  5934
P45     ---T-------C-------------------------------A-----
Miller  -----------C-------------------------------G-----

Purdue  ACATACCTCTTTCATTCTTCAACCCCATAACCCTCCAGCAAGGTGCAAAA  5984
P45     ----------------------------------------------T-----
Miller  ----------------------------------------------T-----

Purdue  TTTTGGAACTCATGTCCGAGAGATTTTGTACCCAAAGGAATAGGTAATAG  6034
P45     ----------T------------C----------------------C--
Miller  ----------T------------C----------------------C--

Purdue  GGATCAACAGATTGGTTATTGGAATAGACAAACTCGCTATCGCATGGTGA  6084
P45     --------------------------------------------------
Miller  ------G-------------------------------------------

Purdue  AGGGCCAACGTAAAGAGCTTCCTGAAAGGTGGTTCTTTTACTACTTAGGC  6134
P45     ------------------------------------C-----------T
Miller  ------------------------------------C-----------T

Purdue  ACTGGACCTCATGCAGATGCCAAATTTAAAGATAAATTAGATGGAGTTGT  6184
P45     --------------------------------------------------
Miller  --------------------------------------------------

Purdue  CTGGGTTGCCAAGGATGGTGCCATGAACAAACCAACCACGCTTGGTAGTC  6234
P45     --------------------------------------------------
Miller  --------------------------------------------------
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Purdue   GTGGTGCTAATAATGAATCCAAAGCTTTGAAATTCGATGGTAAAGTGCCA   6284
P45      --------------------------------------------------
Miller   --------------------------------------------------

Purdue   GGCGAATTTCAACTTGAAGTTAACCAGTCTAGGGACAACTCAAGGTCACG   6334
P45      -----G----------------------A--A--------T-------T---
Miller   -----A--------------------GG-TA--------------------

Purdue   CTCTCAATCTAGATCGCGGTCTAGAAACAGATCTCAATCTAGAGGTAGGC   6384
P45      ---------------T-----------T-----------------C----
Miller   ---------------T-----------------------------C----

Purdue   AACAATCCAATAACAAGAAGGATGACAGTGT[redacted]   6434
P45      --------------------------------------------------
Miller   --------------------------------------------------

Purdue   CACTTAAAAAGTTAGGTGTTTACACAGAAAAACAACAGCAACGCTCTCG   6484
P45      --------------------------------------------------
Miller   ----------------------G---------------------------

Purdue   TTCTAAATCTAAAGAACGTAGTAACTCTAAAACAAGAGATACTACGCCTA   6534
P45      ------------------------------G-T-----------------
Miller   --------------------------------------------------

Purdue   AGAATGAAAACAAACACACCTGGAAGAGAACTGCAGGTAAAGGTGATGTG   6584
P45      --------------------------------------------------
Miller   --------------------------------------------------

Purdue   ACAAGATTTTATGGAGCTAGAAGCAGCTCAGCCAATTTTGGTGACAGTGA   6634
P45      ---------------A----------T----------------------
Miller   --------------------------T----------------------
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Purdue   CCTCGTTGCCAATGGGAGCAGTGCCAAGCATTACCCACAATTGGCTGAAT   6884
P45      --------------------------------------------------
Miller   --------------------------------------------------

Purdue   GTGTTCCATCTGTGTCTAGCATTTTGTTTGGAAGCTATTGGACTTCAAAG   6934
P45      --------------------------------------------------
Miller   --------------------------------------------------

Purdue   GAAGATGGCGACCAGATAGAAGTCACGTTCACACACAAATACCACTTGCC   6984
P45      --------T-----------------------------------------
Miller   --------------------------------------------------

Purdue   AAAGGATCATCCTAAAACTGAACAATTCCTTCAGCAGATTAATGCCTATG   7034
P45      -------G-----------G-------------------------------
Miller   -------GC----------------------------------------

Purdue   CTAGCCCATCAGAATTGGCAAAAGAACAGAGAAAAAGAAAGTCTCGTTCT   7084
P45      --C-T---------G------------------------A---------
Miller   --C-----------G------------------------A---------

Purdue   AAATCTGCAGAAAGGTCAGAGCAAGAGGTGGTACCTGATTCATTAATAGA   7134
P45      ----------------------------------------G---------
Miller   ----------------------------------------G---------

Purdue   AAACTATACAGATGTGTTTGATGACACACAGGTTGAGATGATTGACGAGG   7184
P45      ---T-------------------------------------A---------
Miller   ---T-------------------------------------A---------

Purdue   TAACGAACTAAAC                                       7197
P45      -------------
Miller   -------------
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Fig 6. Sequence analysis of N protein of TGEV three strains. The dark filled boxes show sense primers of N gene, and the light filled boxes show antisense primers. The dot lines display identical bases.

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Purdue  MANQGQRVSWGDESTKIRGRSNSRGRKINNIPLSFFNPITLQQGAKFWNS   50
P45     ----------------T----------N----------------S----L
Miller  ----------------T----------S----------------S----L

Purdue  CPRDFVPKGIGNRDQQIGYWNRQTRYRMVKGQRKELPERWFFYYLGTGPH  100
P45     --------------------------------------------------
Miller  --------------------------------------------------

Purdue  ADAKFKDKLDGVVWVAKDGAMNKPTTLGSRGANNESKALKFDGKVPGEFQ  150
P45     --------------------------------------------------
Miller  --------------------------------------------------

Purdue  LEVNQSRDNSRSRSQSRSRSRNRSQSRGRQQSNNKKDDSVEQAVLAALKK  200
P45     ----Q------L-------------------------------------
Miller  ----RL------------------------------------------

Purdue  LGVYTEKQQQRSRSKSKERSNSKTRDTTPKNENKHTWKRTAGKGDVTRFY  250
P45     ----------------------I---------------------------
Miller  ---D------------------------------------------

Purdue  GARSSSANFGDSDLVANGSSAKHYPQLAECVPSVSSILFGSYWTSKEDGD  300
P45     -T------------------------------------------------
Miller  --------------------------------------------------

Purdue  QIEVTFTHKYHLPKDHPKTEQFLQQINAYASPSELAKEQRKRKSRSKSAE  350
P45     ---------------D---G----------R---V---------------
Miller  ---------------A--------------R---V---------------

Purdue  RSEQEVVPDSLIENYTDVFDDTQVEMIDEVTN                    382
P45     ----------A---------------I------
Miller  ---------A---------------I------
```

Fig 7. The amino acid sequences corresponding to the N gene of TGEV. The dot lines displayed identification of amino acid among the strains.

```
CV777  GAGAATTCCCAAGGGCGAAAATAGCGTAGCAGCTTGCTTCGGACCCAGAG   932
PED-C  ----------------------------------------------------

CV777  GGGGCTTCAAAAACTTTGGAGATGCGGAATTTGTCGAAAAAGGTGTTGAT   982
PED-C  ------------T---------------------------------------

CV777  GCGTCAGGCTATGCTCAGATCGCCAGTTTAGCACCAAATGTTGCAGCATT  1032
PED-C  ---C------------------------------------------------

CV777  GCTCTTTGGTGGTAATGTGGCTGTTCGTGAGCTAGCGGACTCTTACGAGA  1082
PED-C  ----------------------------------T-------------

CV777  TTACATACAACTATAAAATGACTGTGCCAAAGTCAGATCCAAATGTTGAG  1132
PED-C  ----G-----------------------------------------------

CV777  CTTCTTGTTTCACAGGTGGATGCATTTAAAACTGGGATGCAAAACTCCAG  1182
PED-C  ----------------------------------------------------

CV777  AGAAAGAAGGAAAAGAAGAACAAGCGTGAAACCACGCTGCAGCAGCATGA  1232
PED-C  ----G-----------------------------A-------------

CV777  AGAGGCCATCTACGATGATGTGGGTGCGCCATCTGATGTGACCCATGCCA  1282
PED-C  ----T--------------------T-----------------------

CV777  ATCTGGAATGGGACACAGCTGTTGATGGTGGTGATACGGCCGTTGAAATT  1332
PED-C  ---------------------------------C-----A---------

CV777  ATCAACGGATCTTCGATACAGGAAATTAAACAATGTTAGACCGGCTTATC  1382
PED-C  ----------------C--------C---------------T---C----

CV777  CTGGCTATGTTCCAGGGTAGTGCCATTACACTGTTATTACTGAGTGTTTT  1432
PED-C  --------------------C-----------------------------

CV777  TCTAGCGACTTGGCTGCTGGGCTATGGCTTTGCCCTCTAACCAGCGGTCT  1482
PED-C  ----------------------------C----------T--------

CV777  TGGTCTTGCACACAACGGTAAGCCAGTAATGTCAGTGCAAGAAGGATATT  1532
PED-C  ----------------------------------------------------

CV777  ACCATAGCACTGTCACGAGGGGAACGCAGTACCTTTTCATCTAAACCTTT  1582
PED-C  ------------------------A-------------------------

CV777 GCACGAGTAATTAAAGATCCGCTTGACGAGCCTATATGGAAGAGCGTGCC   1632
PED-C ----------C-------T-----------------------------
```

Fig 8. Comparision of nucleotide sequence of 3' end of N protein gene of PEDV. The dot lines display identical bases.

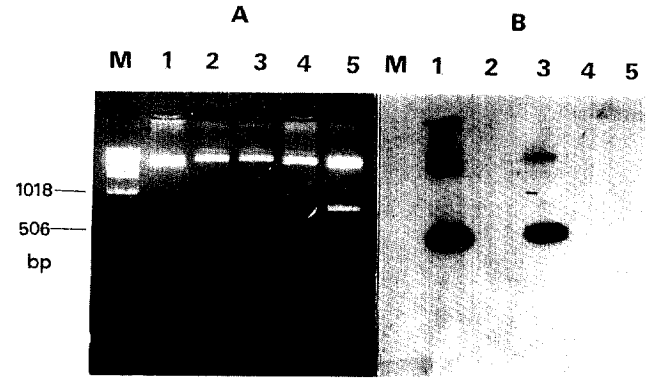

Fig 9. Cleavage patterns of TGEV and PEDV recombinant DNA with *Eco*RI and *Xba*I(A) and Southern blot hybridization using a N1 probe(B). Lane M: 1Kb DNA ladder, lane 1: pTZN1, lane 2: pTZN2, lane 3: pTZN1m, lane 4: pTZN2m, and lane 5: pTZP2.

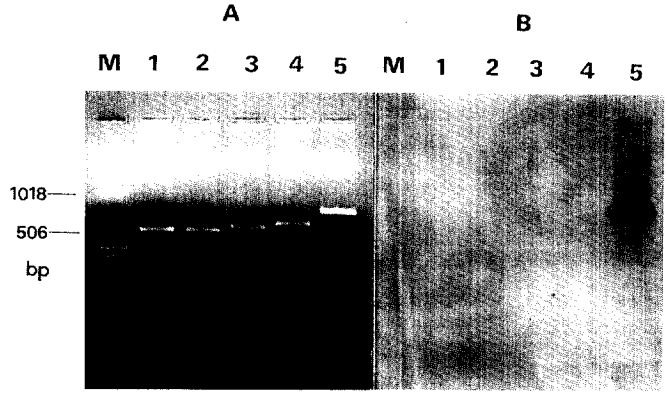

Fig 10. Cleavage patterns of TGEV and PEDV recombinant DNA with *Eco*RI and *Xba*I(A) and Southern blot hybridization using a P2 probe(B). Lane M: 1Kb DNA ladder, lane 1: pTZN1, lane 2: pTZN1m, lane 3: pTZN2, lane 4: pTZN2m, and lane 5: pTZP2.

was found at upstream of open reading frame(ORF)[8]. The differences among the three strains were displayed by letters.

The nucleotide and amino acid sequence homology among three strains of TGEV were summarized in Table 2. The sequences between P45 and Purdue strain were very similar with over 97.3% homology in nucleotides and 96.3% in amino acids. The nucleotide sequence homology between Miller and Purdue strain was 97.6% and the amino acid homology was 96.8%.

The nucleotide and amino acid sequences between P45 and Miller strain were 98.4% and 97.6% homology respectively(Table 2). The variation of two strains were observed at identical position.

Nucleotide sequence analysis of N gene of PEDV : The nucleotide sequence of pTZP2 clone was compared to that of CV777 published data(Fig 8). The nucleotide sequence homology between two strains were over 97%. The comparison of the nucleotide sequence of PEDV N gene to that of TGEV showed less than 30% homology(Table 3).

cDNA probe Southern hybridization : The purified

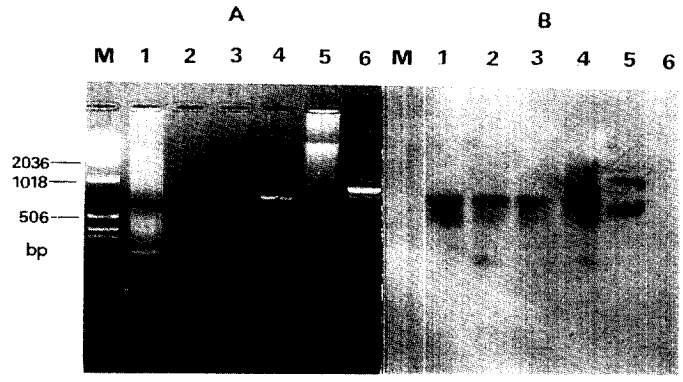

Fig 11. PCR products of PEDV and TGEV(A) and Southern blot hybridization using a P2 probe(B). Lane M: 1Kb DNA ladder, lane 1: stool 5, lane 2: stool 9, lane 3: stool 12, lane 4: stool C, lane 5: stool P32, and lane 6: P45 of TGEV.

insert DNAs from pTZN1 and pTZP2 were used as probes. Fig 9 shows the Southern hybridization pattern with pTZN1 insert probe. This probe showed the specific reaction with only pTZN1(lane 1) and pTZN1m(lane 3) DNA. In Fig 10, the probe from pTZP2 shows the specific reaction with only pTZP2. These probes were recognized as specific to detect TGEV and PEDV N gene.

Fig 11 shows the specific detection of RT-PCR products from PEDV positive stool samples with pTZP2 probe by Southern hybridization. These probes could be used for specific detection and differentiation of TGEV and PEDV genome.

Discussion

The cDNAs of the complete nucleoprotein(N) gene of TGEV, from a Korean field isolate(P45) and Miller strain, were synthesized and amplified with reverse transcription-polymerase chain reaction(RT-PCR). cDNAs fragments were cloned and sequenced. In PEDV, RNAs were isolated from Korean field stool samples and cDNAs were amplified with 3' end primers of N protein genes of PEDV. 20 stool samples from the piglets with clinical signs of PED were used in this study. About 25% of samples showed positive reaction with P2/P2R primer.

cDNAs were cloned into pTZ19R and nucleotide and amino acid sequences were determined. Overall 97% homology was found between Purdue and P45 strain of TGEV N gene at both nucleotide and amino acid level. Also the nucleotide and amino acid sequence homology between P45 and Miller strain were more than 97%.

Fig 6 showed some variations on identical site between P45 and Miller strain. Although P45 strain was avirulent, vaccine strain, we could assume that P45 strain isolated from Korea was more closely related to Miller strain than to Purdue strain. And nucleotide sequence of N gene of PEDV isolated from Korea showed 97% homology compared to that of CV777. This work has shown that the nucleotide sequence of PEDV N gene was 29% homology to that of TGEV.

More than 10 coronavirus N genes have now been sequenced, including those of human respiratory coronavirus(HCV) 229E, porcine respiratory coronavirus(PRCV), feline infectious peritonitis virus (FIPV), canine coronavirus(CCV), murine hepatitis virus(MHV), human coronavirus(HCV) OC 43, bovine coronavirus(BCV) and infectious bronchitis virus (IBV)[1,5]. The nucleotide homology of the PEDV N protein gene with these coronaviruses was ranged from 12 to 19% with MHV, IBV, HCV OC 43 and

BCV and ranged from 32 to 37% with FIPV, CCV, PRCV, TGEV and HCV 229E[9]. It indicates the comparatively weak identity between the PEDV N protein and those of other coronaviruses[1]. However, recently reported PEDV is more closely related to the human respiratory virus 229E than to other porcine enteric virus, TGEV[1].

The initiation codon of open reading frame was preceded by the heptameric sequence ACTAAAC, which is similar to the sequences preceding the nucleoprotein(N), matrix(M), spike(S or E2) genes and other open reading frames in IBV, MHV and PEDV. The heptameric sequence contains the hexameric sequence, CTAAAC[5], for the Purdue strain of TGEV. It has been suggested that this sequence is involved in the initiation of mRNA synthesis for other coronaviruses[16]. In our sequence data, we could find their conserved hexameric sequence, CTAAAC, similar to the previous findings.

The N protein contains the largest open reading frame extended from base 5847 to 7032 and predicts a 382-amino acid protein. This protein is phosphorylated and 46-50kb by SDS-polyacrylamide electrophoresis. The amino acid analysis of our sequence data has shown that serine residues are riched as 42(10.9%) of the residues that is the most abundant amino acid. Assuming this protein was phosphorylated at serine residues and the protein was basic and the property expected to be nucleic acid-binding proteins[5].

Nowadays, PEDV has been identified to cause severe enteric disease in swine industry in Korea. It is urgent to develop the detection and differentiation system for TGEV and PEDV infection. The five cloned cDNAs were used to make the probe for specific detection between TGEV and PEDV. The PCR products from supernatent of tissue culture or intestinal samples of pigs were used for hybridization. Fig 9 and 10 showed that TGEV(pTZN1) probe reacted with only TGEV cDNA and PEDV(pTZP2) probe showed signals with only PEDV cDNA strongly in Southern hybridization because of the low nucleotide sequence homology(29%) between TGEV and PEDV.

Conclusion

The purposes of study were to make specific detection systems for transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus(PEDV) of Coronaviridae. Two viruses have shown the similarities of pathogenesis in swine.

Two strains of TGEV were used in this examination. One was provided from domestic strain(P 45), the other was appeared as a virulent strain(Miller). PEDV was found from the stool or intestinal samples of pigs. Two sets of oligonucleotide primer(N1 and N 2 for TGEV and P2/P2R for PEDV) were synthesized. Each N protein gene was amplified with these primer sets using the reverse transcription-polymerase chain reaction(RT-PCR). The expected size of PCR products as 1162bp for N protein gene of TGEV and as 753bp for that of PEDV were amplified. Amplified cDNAs were used for cloning, sequencing and Southern blotting. The DNA probe derived from TGEV and PEDV displayed highly specific reaction with PCR productions of all tested samples.

In conclusion, the prepared DNA probes could be used for the specific diagnostic methods for TGEV and PEDV infection.

References

1. Bridgen A, Duarte M, Tobler K. Sequence detection of the nucleocapsid protein gene of the porcine epidemic diarrhoea virus confirms that this virus is a coronavirus related to human coronavirus 229E and porcine transmissible gastroenteritis virus. *J Gen Virol* 1993; 74: 1795-1804.
2. Calleqaut P, Pensaert MB, Hooyberghs J. A competitive inhibition ELISA for the differentiation of seroantibodies from pigs infected with transmissible gastroenteritis virus(TGEV) or with TGEV-related porcine respiratory coronavirus. *Vet-Microbiol* 1989; 20: 9-19.
3. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-

phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156-159.

4. Cox E, Pensaert MB, Callebaut P. Intestinal protection against challenge with transmissible gastroenteritis virus of pigs immune after infection with the porcine respiratory coronavirus. *Vaccine* 1993; 11(2): 267-272.
5. Kapke PA, Brian DA. Sequence analysis of the porcine transmissible gastroenteritis coronavirus nucleo-capsid protein gene. *Virol* 1986; 151: 41-49.
6. Kapke PA, Tung FYT, Brian DA. Nucleotide sequence between the peplomer and matrix protein genes of the porcine transmissible gastroenteritis coronavirus identifies three large open reading frames. *Virus Gene* 1988; 2(3): 293-294.
7. Nishimura A, Morita M, Nishimura Y, et al. A rapid and highly efficient method for preparation of competent Escherichia coli cells. *Nucleic Acids Res* 1990; 18(20): 6169.
8. Page KW, Mawditt KL, Britton P. Sequence comparison of the 5'end of mRNA 3 from transmissible gastroenteritis virus and respiratory coronavirus. *J Gen Virol* 1991; 72: 579-587.
9. Page KW, Britton P, Boursnell MEG. Sequence analysis of the Leader RNA of two porcine coronavirus: transmissible gastroenteritis virus and porcine respiratory coronavirus. *Virus Gene* 1990; 4(4): 289-301.
10. Penderson NC, Ward J, Mengeling WL. Antigenic relationship of the feline infectious peritonitis virus to coronaviruses of other species. *Arch Virol* 1978; 58: 45-53.
11. Pensaert MB. Porcine epidemic diarrhea. In " Diseases of swine", Dunne HW, Leman AD ed. Ames: Iowa state press, 1986; 402-406.
12. Saif LJ, Bohl EH. Transmissible gastroenteritis. In "Disease of swine", Dunne HW, Leman AD ed. Ames: Iowa state press, 1986; 255-274.
13. Saif LJ, Bohl EH. Passive immunity transmissible gastroenteritis of swine: immunoglobulin classes of milk antibodies after oral-intranasal inoculation of sows with a live low cell culture-passaged virus. *Am J Vet Res* 1979; 40(1): 115-117.
14. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory. NY: Cold spring Harbor, 1989.
15. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci* 1977; 74: 5463.
16. Shieh CK, Soe LH, Makino S, et al. The 5'-end sequence of the murine coronavirus genome: implications for multiple fusion sites in leader-primed transcription. *Virol* 1987; 156: 321-330.
17. Siddell S, Wege H, Meulen VY. Review article: The biology of coronaviruses. *J Gen Virol* 1983; 64: 761-776.
18. Spaan W, Cavanagh D, Horzinek MC. Coronaviruses: structure and genome expression. *J Gen Virol* 1988; 69: 2939-2952.