

Characterisation of ORF3, M, N and E gene sequences of porcine epidemic diarrhoea virus from domestic pigs in Poland

Monika Olech^{1,2✉}, Marta Antas², Anna Szczotka-Bochniarz²

¹Department of Pathology, ² Department of Swine Diseases,
 National Veterinary Research Institute, 24-100 Puławy, Poland
 monika.olech@piwet.pulawy.pl

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Abstract

Introduction: Porcine epidemic diarrhoea virus (PEDV) is an enteric pathogen causing porcine epidemic diarrhoea and acute gastroenteritis in pigs of all ages. Previous analysis of the viral genome of PEDV in Poland was only based on the spike protein (S) gene sequences and no analysis of other genes has been performed. The aim of this study was to analyse the envelope (E), membrane (M) and nucleocapsid (N) protein and open reading frame 3 (ORF3) gene sequences. **Material and Methods:** Viral RNA from 18 Polish pig faecal samples that were quantitative reverse transcription PCR-positive for PEDV was analysed in four genomic regions (E, M, N and ORF3). **Results:** Phylogenetic analysis based on these regions' sequences revealed that Polish PEDV isolates were highly related and were clustered into group G2a across the four genes compared. Moreover, the Polish strains were located in distinct subclusters on the phylogenetic trees, which suggests the presence of at least three independently evolving PEDV genetic lines circulating in Poland. The occurrence of unique mutations in the sequences of Polish PEDV strains suggests that PEDV continues to undergo evolutionary processes, accumulating the mutations necessary for viral fitness in its natural hosts. The Polish PEDV strains differed genetically from the CV777 vaccine strain, suggesting the risk of relatively low vaccine efficacy if this strain is used. **Conclusion:** Our results promote a better understanding of the genetic diversity of PEDV field isolates in Poland and highlight the importance of molecular characterisation of PEDV field strains for the development of an effective vaccine against PEDV.

Keywords: porcine epidemic diarrhoea virus, E gene, M gene, N gene, ORF3, phylogenetic analysis.

Introduction

Porcine epidemic diarrhoea virus (PEDV) is an enteric pathogen causing porcine epidemic diarrhoea, an acute enteric disease of pigs, the severity of which decreases with age. Neonatal piglets are affected the most, with a mortality rate of up to 100%. Clinical signs principally include vomiting, anorexia and diarrhoea, which may lead to dehydration, loss of body weight and death. Transmission occurs predominantly through faeces, *via* the airborne route and fomites (14, 26, 30). This virus was first reported in the early 1970s in Europe and has subsequently been detected in different areas throughout the world, causing outbreaks in many countries in Europe and Asia despite the use of vaccines (20, 26).

The disease agent is an enveloped, positive single-stranded RNA virus in the *Alphacoronavirus* genus, *Coronavirinae* subfamily, and *Coronaviridae* family.

The genome of PEDV is approximately 28 kb in length and is composed of seven open reading frames (ORFs) named ORF1a, ORF1b, ORF3, spike (S), envelope (E), membrane (M) and nucleocapsid (N) (4, 30). The ORFs 1a, 1b and 3 encode non-structural polyproteins, whereas the remaining genes encode structural proteins. The S and ORF3 proteins play important roles in the pathogenesis of the virus. The S protein is involved in the binding of viral particles to the receptors of host cells, cell membrane fusion and virus entry. It is also the main glycoprotein which induces the production of neutralising antibodies (13). In addition, the S gene exhibits high genetic diversity and is commonly used to investigate PEDV evolution. The ORF3 gene encodes an ion channel protein and regulates virus production (28). It is also an important determinant for PEDV biological properties. A 49-bp deletion observed in the ORF3 genes of attenuated PEDV strains resulting from the process of

adaptation to serial passages in cell culture may be an important site for the pathogenicity of PEDV and can be used for differentiation of wild and attenuated PEDV strains, providing a valuable tool to study the molecular epidemiology of PEDV infections (19). The E and M proteins are involved in the formation and budding of the coronavirus envelope (7, 9). The N protein is a highly conserved phosphoprotein which interacts with viral genomic RNA, providing the structural basis for the helical nucleocapsid during viral assembly. It has multiple functions in viral replication, assembly and pathogenesis (18, 21). Furthermore, this protein is abundantly expressed during the early stages of PEDV infection and is therefore a preferred target for PEDV diagnosis.

In a previous study, we showed that Polish PEDV strains belonged to the G1b (S gene insertion/deletion – S-INDEL) subgroup and were closely related to other European PEDV strains (2). It was the first report on the molecular characterisation of PEDV strains in Poland. The genetic analysis in that study was based on S gene sequences only, and analysis of other genes has never been performed. Analysis of a single gene may skew epidemiological studies and elucidation of the virus' evolution because it reflects only a portion of the virus genome. Moreover, PEDV diagnostic tests such as PCR targeted at the S protein might have misidentified swine enteric coronavirus (SeCoV) as PEDV, while most of the genome of this new chimaeric virus is derived from TGEV, the S gene is derived from PEDV. Hence the aim of this study was to extend the molecular characterisation of previously identified PEDV strains by analysis of the E, M, N and ORF3 gene sequences. This study will provide a better understanding of the molecular epidemiology and genetic diversity of PEDV field strains in Poland.

Material and Methods

The 18 faecal samples analysed in this study were collected from pigs with clinical signs suggestive of PEDV infection. The faeces were quantitative reverse transcription (RT-q) PCR-positive for PEDV (2). All of the samples were non-invasively collected by veterinarians, therefore ethical approval was not required. Samples originated from four herds located in three voivodeships of Poland (Wielkopolskie, Dolnośląskie and Kujawsko-Pomorskie). The samples were diluted 1:10 (v/v) with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄ and 1.8 mM KH₂PO₄), homogenised and centrifuged for 8 min at 6,000 × g at 4°C. Ribonucleic acid was extracted from 140 µL of the collected supernatant using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). An RT-PCR was carried out using a OneStep RT-PCR Kit (Qiagen, Hilden, Germany). A pair of primers (E-F 5' ATTCAACTAGACGAGTATGCTACAA 3' and E-R 5' CACCTCATCAACGGGAATAGAA 3') was designed to amplify the E gene. The M, N and ORF3 genes were amplified by specific primers reported previously (8, 12, 23).

RT was performed at 50°C for 30 min. The PCR cycling parameters were initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 1 min; annealing at 55°C for 1 min for the ORF3, M and N genes, and 56°C for 1 min for the E gene; extension at 72°C for 2 min; and a final extension at 72°C for 10 min.

The amplified PCR products were subjected to gel electrophoresis, and after purification using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Hamburg, Germany) were sequenced in both directions on a 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA) using a Big Dye Terminator v3.1 Cycle Sequencing kit. The obtained sequences were edited and analysed using Geneious Pro 5.3 software (Biomatters, Auckland, New Zealand). Sequences were assembled into multiple sequence alignment using ClustalW. Unrooted phylogenetic trees were constructed using the neighbor-joining (NJ) method with the maximum composite likelihood model. Alignment and NJ tree building were performed using MEGA software version 6.06 (24). Sequence percentage identity (the percentage of bases/residues which are identical) was derived using Geneious software. All of the novel sequences reported in this study were submitted to the GenBank database under accession numbers MZ216018–MZ216031, MZ268115, MZ313556, MZ313557 and MZ325484–MZ325487. Sequences from seven samples for which whole genome sequences were obtained earlier (2) were also included in the phylogenetic analysis. The Recombination Detection Program version 4 (RDP4) (17) was used to detect possible recombination events.

Results

The fragment encoding protein E was successfully amplified and sequenced from all 18 tested samples, while the M, N and ORF3 sequences were obtained from 16, 14 and 15 samples, respectively. Polish PEDV sequences shared 99.1–100%, 99.6–100%, 99.6–100% and 99.3–100% nucleotide identity with each other and 97.4%, 97.9–98.2%, 95.0–95.3% and 96.1–96.7% nucleotide identity with the vaccine strain CV777 on the basis of the E, M, N and ORF sequences, respectively. The ORF3 of Polish PEDV isolates is 675 nucleotides in length and encodes a protein of 225 amino acids. A phylogenetic analysis based on the ORF3 gene revealed that PEDV isolates from all submitting locations were divided into two groups, G1 and G2, each of which was further subdivided into G1a and G1b and G2a and G2b subgroups (Fig. 1). All PEDV isolates from Poland were allocated to group G2a and were distributed into two potential subclusters. Sixteen Polish PEDV sequences formed the first subcluster together with the Hungarian S236, French FR2019001 and Spanish H3 and 1931-1-1 strains (99.0–99.8% nucleotide identity), while the remaining six Polish sequences formed the second subcluster together with other PEDV strains from Spain, France, Hungary, Italy, Slovenia, Germany, Romania,

Belgium and Austria (99.8–100% nucleotide identity). All PEDV sequences belonging to the first subcluster possessed unique ^{T114^C} and ^{A517^G} nucleotide substitutions, while only Polish sequences belonging to this subcluster had a unique ^{A426^G} substitution. All sequences from this subcluster had a unique ^{I173^V} amino acid substitution when compared to other groups/clusters and, noteworthy, to the CV777 vaccine strain. Moreover, all Polish PEDV strains had nine amino acid substitutions compared to the ORF sequence of the CV777 vaccine strain (^{V21^A}, ^{V54^I}, ^{V79^I}, ^{F80^V}, ^{L85^I}, ^{L92^F}, ^{A101^T}, ^{N166^S} and ^{H182^Q}). Polish PEDV sequences belonging to the first subcluster had one more aa substitution (^{I173^V}) compared to strain CV777. None of the Polish PEDV strains had the 49-bp deletion at positions 245–294 in the ORF3 gene that is specific to attenuated-type PEDV strains.

The E genes of PEDV isolates from Poland contained a single ORF of 231 nucleotides which encoded a 77-amino-acid-long peptide. Phylogenetic analysis of the E gene showed that all of the PEDV isolates could be divided into two main clusters made up of clearly distinguished G1a, G1b, G2a and G2b subgroups (Fig. 2). All PEDV isolates from Poland belonged to subgroup G2a and were allocated to three potential subclusters which had also been identified on the basis of S gene sequences (2). Twenty sequences of Polish PEDV isolates and the sequences of the Hungarian S236, French FR2019001 and Spanish H3 and 1931-1-1 strains formed the first subcluster (99.6–100% nucleotide identity) and had a substitution of thymine by cytosine at position 90, which was also noted for sequences in subgroup G1a. Three sequences were allocated to the second subcluster, along with European PEDV isolates and isolates from the United States of America, South Korea, Japan, Canada and Colombia, while two PEDV Polish sequences together with sequences of the Hungarian HUN/5031/2016 and Slovenian SLOreBAS-1/2015 and SLOreBAS-2/2015 strains (100% nucleotide identity) formed the third subcluster and had a unique ^{C96^T} substitution. The Polish PEDV strains had one amino acid substitution (^{R65^Q}) compared to the E sequence of the CV777 vaccine strain and shared 99.9% amino acid identity.

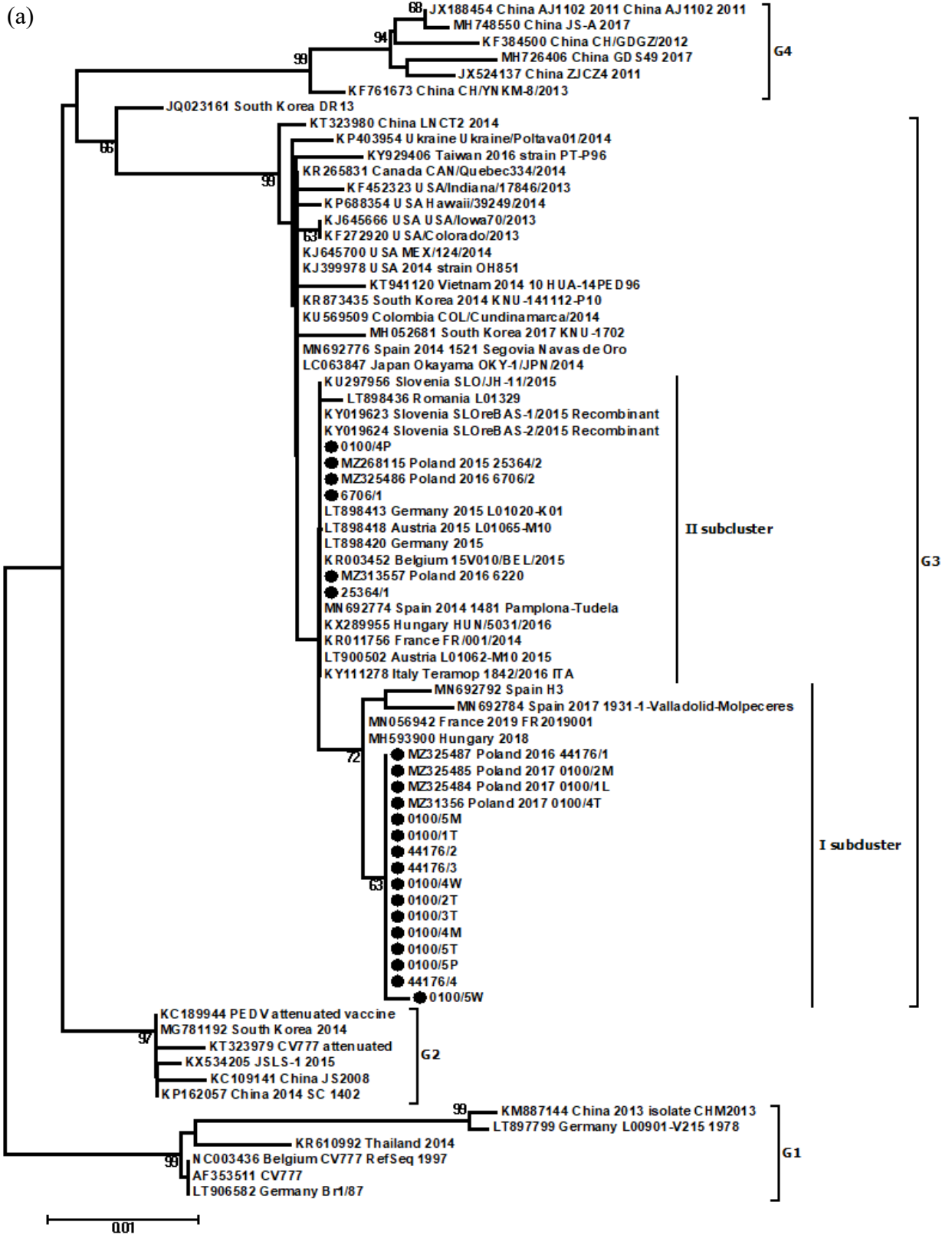
The M gene of all Polish PEDV isolates was 681 nucleotides in length and encoded a protein of 226 amino acids. No insertions or deletions were found in the M gene of these isolates except point mutations. Phylogenetic analysis indicated that all PEDV strains were divided into two main clusters, G1 and G2. Strains in the G1 cluster were clearly distinguished into G1a and G1b subgroups, while G2 strains were divided into G2a and G2b subgroups. Sequences of the Polish PEDV isolates were clustered in subgroup G2a and, similarly to the phylogenetic assignment inferred by the E gene sequences, were allocated to three subclusters (Fig. 3). Isolates belonging to the first subcluster had a ^{C591^T} substitution, and isolates belonging to the third subcluster had a ^{C336^T} substitution compared to isolates

from the other groups/subclusters. Compared to the M sequence of the CV777 vaccine strain, three amino acid substitutions were detected (^{F13^Q}, ^{V42^A} and ^{A214^S}).

Our phylogenetic analysis of the nearly complete 1,068-bp-long N gene showed that, as occurred for the other genes, all PEDV isolates were separated into two main groups, G1 and G2, and divided into G1a, G1b, G2a and G2b subgroups (Fig. 4). All PEDV isolates from Poland were allocated to group G2a. Out of 19 analysed Polish PEDV strains, 16 had a unique ^{A287^C} substitution and all Polish PEDV strains had unique ^{A802^G}, ^{A802^T} and ^{G976^{CT}} substitutions. These substitutions subsequently resulted in three amino acid substitutions (^{Q96^P}, ^{K268^V} and ^{A326^P}) when compared to the other groups. The sequences of the N gene of the Polish PEDV strains had 15 amino acid substitutions compared to the CV777 vaccine strain (^{K37^N}, ^{A56^T}, ^{N119^K}, ^{R155^K}, ^{H156^L}, ^{K166^R}, ^{N169^S}, ^{K268^V}, ^{L295^P}, ^{L309^Q}, ^{Q311^L}, ^{H312^N}, ^{F314^D}, ^{A322^L} and ^{V326^{SP}}). Our results revealed that the 3F12 linear epitope was conserved in all PEDV strains, while in the sequences of the NEP-D4 and NEP-D6 epitopes, some variations between PEDV strains belonging to subgroups G2b, G1b and G1a and PEDV strains belonging to subgroup G2a were observed. No evidence of recombination was found for any of the four analysed fragments based on RDP analysis.

Discussion

Based on spike gene phylogenetic analysis, PEDV is mainly divided into two genogroups, G1 (including the G1a and G1b subgroups) and G2 (with the G2a and G2b subgroups). The G1a subgroup includes the prototype PEDV strains, vaccine strains and other cell culture-adapted strains, whereas G1b comprises S-INDEL strains which shared the same nucleotide insertion/deletion event in the S gene as the classical PEDV strains belonging to subgroup G1a. Group G2 contains non-S-INDEL virulent field isolates that cause pandemics in North America and Asia (G2a strains) and Chinese non-S-INDEL strains (G2b strains). In this study, a phylogenetic analysis performed on the basis of the E, M, N and ORF3 genes revealed that all of the PEDV isolates could also be divided into two main groups (G1 and G2). However, some differences in the classification of subgroups occurred. The G1a subgroup includes the prototype PEDV strains, while G1b includes most cell culture-adapted vaccine strains. Global S-INDEL strains, which on the basis of S gene sequences belonged to subgroup G1b, belonged to group G2 on the basis of the E, M, N and ORF3 gene sequences together with American, Asian and Chinese non-S-INDEL pandemic strains. Within group G2, most of the Chinese pandemic strains formed a separate cluster (the G2b subgroup), while the S-INDEL and American and Asian non-S-INDEL strains clustered together (the G2a subgroup).



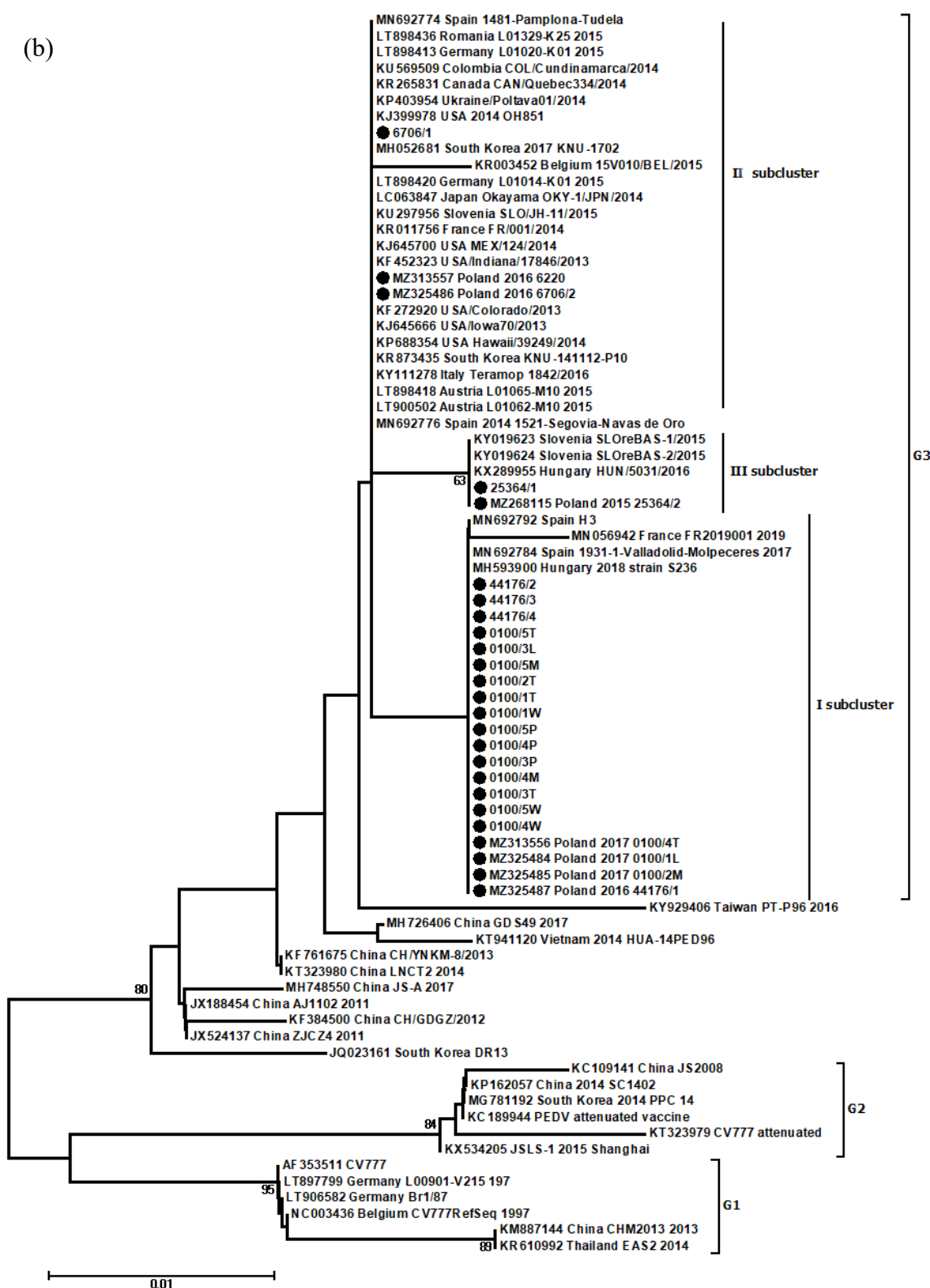


Fig. 1. Phylogenetic relationship between the sequences of PEDV Polish strains and sequences of reference strains obtained from GenBank. The phylogenetic trees were constructed on the basis of the ORF3 (a) and E (b) nucleotide sequences with MEGA6 software using the neighbour-joining method. Bootstrap values >70 are shown. The numbers of each branch represent the bootstrap value calculated using 1,000 replicates. The scale bars indicate nucleotide substitutions per site. PEDV isolates identified in this study are indicated with solid black circles

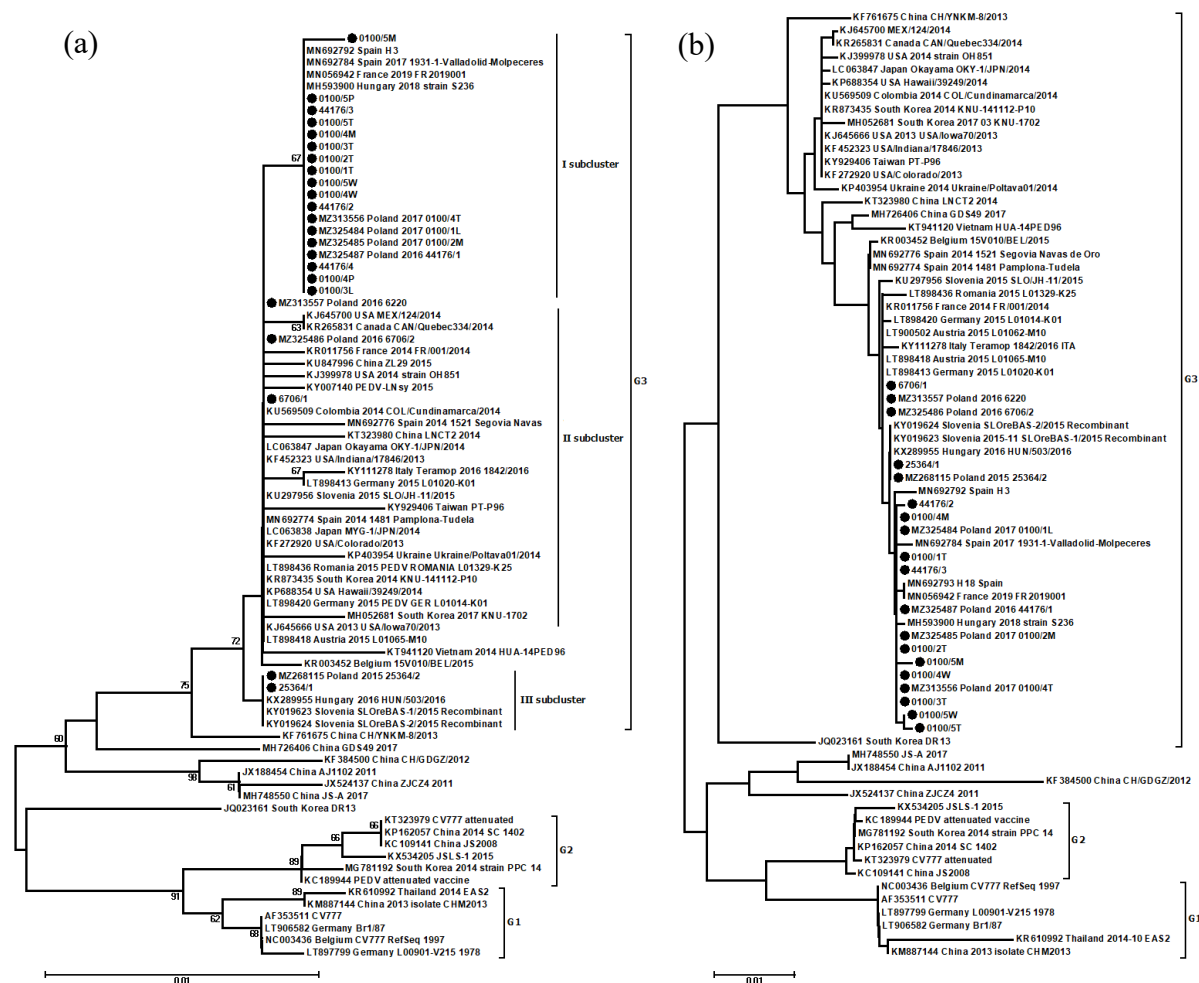


Fig. 2. Phylogenetic relationship between the sequences of PEDV Polish strains and sequences of reference strains obtained from GenBank. The phylogenetic trees were constructed on the basis of the M (a) and N (b) nucleotide sequences with the MEGA6 software using the neighbour-joining method. Bootstrap values >70 are shown. The numbers of each branch represent the bootstrap value calculated using 1,000 replicates. The scale bars indicate nucleotide substitutions per site. PEDV isolates identified in this study are indicated with solid black circles

Phylogenetic analysis based on the investigated regions revealed that Polish PEDV isolates were highly related and belonged to group G2 and subgroup G2a. Polish PEDV strains were phylogenetically distant from the vaccine and classical PEDV strains of group G1. The Polish strains were also observed to be located in distinct subclusters on the phylogenetic trees, suggesting the presence of at least three independently evolving PEDV genetic lines circulating in Poland. Our findings also supported previous research which indicated that Polish PEDV strains were closely related to European PEDV strains (2). This is consistent with the potential transmission of PEDV from farm to farm through animal transportation and trade.

None of the Polish PEDV strains had the 49-bp deletion in the ORF3 gene that is specific to attenuated-type PEDVs. Because most cell culture-adapted strains present this deletion at positions 245–294, it is considered a possible marker of cellular adaptation. This type of deletion occurs during extensive passaging of the virus in cell culture and is correlated with viral attenuation (25). Therefore, ORF3 can be used to discriminate the vaccine strains from the wild-type

PEDV strains (15). This suggests that ORF3 may be a multifunctional protein involved in growth *in vitro*; however, its biological function needs to be further investigated. All Polish PEDV strains had nine amino acid substitutions compared to the ORF3 sequence of the CV777 vaccine strain ($^V21^A$, $^V54^I$, $^V79^I$, $^F80^V$, $^L85^I$, $^L92^F$, $^A101^T$, $^N166^S$ and $^H182^Q$). Seven of these substitutions occurred in transmembrane domains (TMDs): one ($^V54^I$) in TMD1, four ($^V79^I$, $^F80^V$, $^L85^I$ and $^L92^F$) in TMD2 and two ($^N166^S$ and $^H182^Q$) in TMD4. It has been reported that the ORF3 protein of the CV777 strain contains four TMDs: TMD1 (Gln-40 to Ser-63), TMD2 (Arg-75 to Ile-97), TMD3 (Tyr-116 to Tyr-139) and TMD4 (Gly-150 to Ile-173) (28). These domains were described as forming a tetrameric assembly which is important for potassium channel activity and being associated with the virulence of PEDV (28). The mutations detected in the Polish PEDV strains may presumably have an impact on the virulence of these strains, and warrant further investigations.

The N protein is commonly used as a target for diagnosis and vaccine development (10, 22). Originally, the N protein was thought to be highly conserved, but

Kim *et al.* (11) recently revealed that the N gene has an evolutionary rate similar to the S gene and thus is likely to have high genetic diversity. This genetic diversity may lead to changes in the antigenicity of the N protein and may pose a problem with regard to PEDV testing and diagnosis. Three epitopes have been identified in the N protein: 3F12 (VAAVKDALKSLGI) (31) and NEP-D4 and NEP-D6, these latter two located within residues 18–133 and 252–262, respectively (29). Our results revealed that the 3F12 linear epitope was conserved in all PEDV strains, while in the sequences of NEP-D4 and NEP-D6 some differences between classical PEDV strains belonging to groups G2b, G1b and G1a and PEDV strains belonging to group G2a were observed, which may have an impact on the antigenicity of the N protein. It was shown that the antibodies induced by the G2b PEDV strains reacted poorly with a commercial N-based PEDV ELISA kit, this kit showing a sensitivity of only 37% (6). This could be due to PEDV strain differences; therefore, molecular analysis of the N gene is very important and should be conducted constantly to update knowledge of its epitopicity and ensure the reliability of diagnostic tests.

Interestingly, a ^{C591T} substitution was observed in the B cell epitope (WAFYVR) (32) of the M protein of some Polish strains; however, this mutation did not result in an amino acid change. The B cell epitope was conserved in all PEDV strains. Compared to the M sequence of the CV777 vaccine strain, three amino acid substitutions were detected (^{E13Q}, ^{V42A} and ^{A214S}). It was noted that the ^{E13Q} mutation altered the hydrophobicity of the N terminus of the M protein and therefore had an impact on its antigenic activity, while the other mutations did not influence the hydrophobicity of the M protein (23).

Generally, compared with the CV777 prototype strain, Polish PEDV strains had a number of novel common mutations which may have an effect on the virus' antigenicity and pathogenicity and may be contributory factors to vaccine failure. Further research is needed to investigate the biological significance of these mutations.

Recombination plays a crucial role in the diversity and evolution of coronaviruses by creating new strains. Recently, recombination between PEDV and TGEV has generated a new swine chimaeric (recombinant) coronavirus – SeCoV. This new coronavirus has been reported in several European countries and causes the same clinical signs as PEDV and TGEV (1, 3, 5, 16, 27). Notably, SeCoV may have been misidentified as PEDV using S-protein or S-gene assays since most of the genome of this new chimaeric virus is derived from TGEV while the S gene is derived from PEDV (3). However, SeCoV sequences were not detected in our study; nor was evidence of recombination found for any of the four analysed fragments based on RDP analysis.

In conclusion, phylogenetic analysis based on the E, M and N and ORF3 sequences revealed that Polish PEDV isolates were highly related to each other and

were clustered into group G2a across the four genes compared. The Polish strains were located in distinct subclusters on the phylogenetic trees, which suggests the presence of at least three independently evolving PEDV genetic lines circulating in Poland. No sequences of SeCoV were found. The occurrence of unique mutations only in sequences of Polish PEDV strains suggests that PEDV continues to undergo evolutionary processes, accumulating the mutations necessary for viral fitness in its natural hosts. Furthermore, our study revealed that all Polish PEDV strains differed genetically from the CV777 vaccine strain, suggesting the relatively low vaccine efficacy of preparations using this strain in Poland.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: None required.

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