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Molecular Characterization Confirms the Presence of a Divergent Strain of Canine Coronavirus (UWSMN-1) in Australia

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Canine coronavirus (CCV) UWSMN-1 was originally identified from an outbreak of fatal gastroenteritis in breeding colonies. In this report, we examined whether UWSMN-1 represents a novel divergent strain or is the result of recombination events between canine and feline coronavirus strains. Sequencing of various regions of the spike and polymerase genes confirms that UWSMN-1 is widely divergent from other CCV and feline coronavirus strains. These data raise the possibility that this strain is the first member of a novel third subtype of CCV.

Canine coronavirus (CCV) is a common pathogen of dogs that generally produces symptoms of mild to moderate gastroenteritis (1–3, 14). In combination with other pathogens such as canine parvovirus, or in young or stressed animals, CCV infection causes more severe symptoms or can be fatal (1). It has recently been demonstrated that CCV is prevalent in dog populations throughout Australia, and persistent reinfection is common in breeding colonies (11).

The spike (S) gene of the coronavirus family is reported to be important in virus-host attachment and is involved in antigenic differences between strains (for a review, see reference 12). Coronaviruses are known to undergo frequent recombination events in vitro (7, 8), and an increasing body of evidence suggests that recombination of coronaviruses occurs in the field (5, 6, 15, 16). Several canine and feline coronaviruses (FCoVs) have been identified that have an S gene originally derived from other coronavirus species (5), and CCVs can be separated into two subtypes (feline- or canine-like) on the basis of S gene origin (5, 6, 15, 16). The frequent recombination events of coronaviruses have been suggested to be an important means of avoiding host immunity and may occur due to the close proximity of domesticated cats and dogs (5).

We have previously reported the identification of a novel Australian strain of CCV (UWSMN-1) from a fatal case of gastroenteritis in pups from breeding colonies by using an S gene nested PCR assay (10). Initial sequencing of the variable 5′ region of the S gene of UWSMN-1 showed that UWSMN-1 formed an intermediate group between FCoVs and CCVs (10). These data raised two possibilities: (i) UWSMN-1 is a divergent coronavirus strain, or (ii) the differences observed in the 5′ region of the S gene are not reflective of divergence across the whole UWSMN-1 genome but may reflect recombination events between FCoVs and CCVs in the S gene only. To address these competing scenarios, we sequenced the conserved 3′ region of the S gene as well as part of the polymerase (Pol) gene from UWSMN-1 and compared them to various coronavirus strains on the basis that these usually conserved regions would give good discrimination of the phylogenetic relationships between strains.

A 751-bp region from the 3′ end of the S gene and a 409-bp conserved region of the Pol gene were amplified by using the primers CCVSF1 (forward primer, 5′-AGCACTT TTCCTATTGATTG-3′) and CCVSR1 (reverse primer, 5′-GTTAGTTTGTCTAATAATACCAACACC-3′) for the S gene and CCVPF1 (forward primer, 5′-ATGGGATGGGA CTATCCTAAGTGTGA-3′) and CCVPR1 (reverse primer, 5′-ATCTTTGTTGTAGCACACAACTCCATCATC-3′) for the Pol gene, with PCR conditions as previously described (10). The PCR products were purified by using a Bresa-Clean DNA purification kit (Geneworks, Adelaide, Australia), and DNA was cloned by using pGEM-T Easy Vector system II (Promega, Madison, Wis.). Nucleotide sequencing was performed in both orientations by automated sequencing at the Australian Genome Research Facility (University of Queensland, St. Lucia, Australia [http://www.agrf.org.au]).

Alignments of the DNA sequences from different coronaviruses and CCV strains were made by using the PILEUP program of the Genetics Computer Group package through the Australian National Genomic Information Service (Sydney University, Sydney, Australia [www.angis.org.au]). The calculation of percentages of identity, construction of DNA parsimony phylogenetic trees, and bootstrap analysis were

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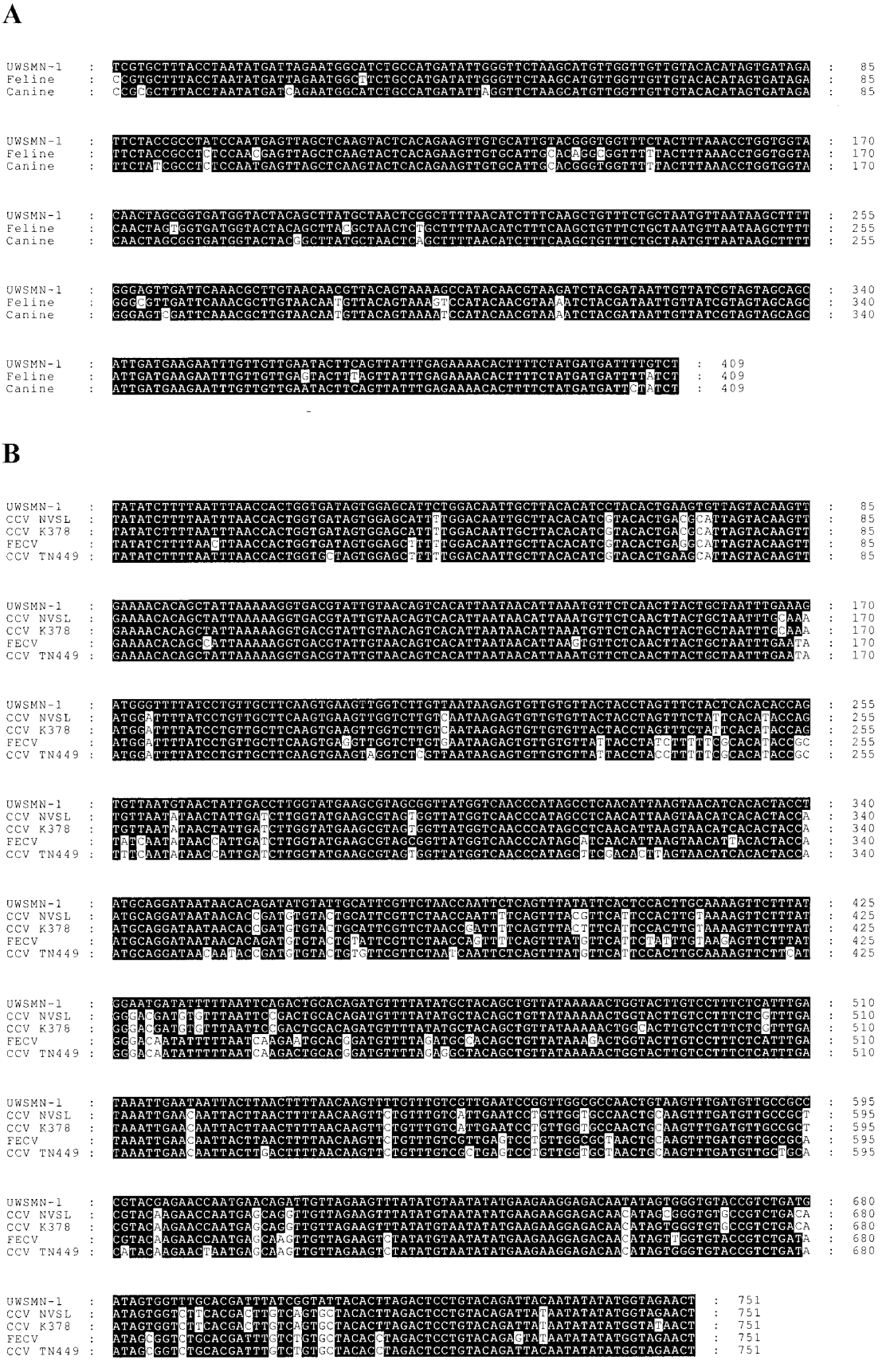

FIG. 1. (A) DNA alignment of Pol genes of UWSMN-1, feline infectious peritonitis virus (AF124987), and CCV 1-71 (AF124986). (B) DNA alignment of S genes of UWSMN-1, CCV TN449 (AF116245), feline enteric coronavirus (FECV) (X80799), CCV NSVL (AF116244), and CCV K378 (X77047). GenBank accession numbers are noted in parentheses. Shaded regions indicate conserved amino acid residues among the different CCV and FoCV strains. Variable regions of the consensus sequence are indicated by white boxing.

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A

	TURKEY	RAT	HUMAN	BOVINE	CCV 1-71	CCV UWSMN-1	TGEV
RAT	57.4						
HUMAN	58.0	83.9					
BOVINE	56.7	84.1	96.1				
CCV 1-71	55.7	60.2	58.0	58.9			
CCV UWSMN-1	56.0	60.4	58.0	58.9	96.1		
TGEV	56.7	60.4	57.7	58.0	95.6	95.4	
FoCV	56.7	60.6	57.7	58.4	95.6	95.4	96.8

B

	TGEV	CCV TN449	FoCV	CCV UCD-2	CCV UCD-1	CCV INSVAC-1	CCV UWSMN-1	CCV 5821	CCV 6	CCV K378	CCV NVSL
CCV TN449	90.9										
FoCV	90.7	94.4									
CCV UCD-2	92.3	94.7	93.9								
CCV UCD-1	92.3	93.9	92.6	95.8							
CCV INSVAC-1	89.3	89.8	89.7	91.1	91.2						
CCV UWSMN-1	89.0	89.8	89.9	90.7	90.6	91.9					
CCV 5821	90.7	91.9	91.8	92.7	92.7	94.1	93.4				
CCV 6	90.6	91.3	91.6	92.2	92.0	93.1	92.7	94.4			
CCV K378	90.6	91.3	91.6	92.2	92.0	92.3	92.8	94.4	98.9		
CCV NVSL	91.0	91.6	92.0	92.6	92.4	92.7	93.1	94.8	99.6	99.3	
CCV 1-71	91.1	91.8	92.2	92.7	92.6	92.8	93.2	95.0	99.5	99.5	99.9

FIG. 2. Percentages of nucleotide identity between the Pol (A) and S (B) gene sequences of CCV strain UWSMN-1 and other coronaviruses, including the CCV and FoCV strains denoted in Fig. 1. TGEV, porcine transmissible gastroenteritis virus.

performed as previously described (10) by using the HOMOLOGIES (Genetics Computer Group), ESEQBOOT, EDNAPARS, and ECONSENSE programs of the PHYLIP package through the website of the Australian National Genomic Information Service.

A BLAST search (http://www.ncbi.nlm.nih.gov) and subsequent DNA parsimony analysis of the sequenced region of the Pol gene from UWSMN-1 showed that this strain is most closely related to group I coronaviruses (CCV, porcine transmissible gastroenteritis virus, and FoCV) (Fig. 1). Calculation of the percentages of identity between coronavirus strains demonstrated that the Pol gene of USWMN-1 shares the highest homology (96.1%) with Pol genes of other CCVs, followed closely by 95.4% identity with the Pol genes of both FoCV and porcine transmissible gastroenteritis virus (Fig. 2A). As previously reported, these group I coronaviruses share relatively poor homology with coronaviruses of other species (Fig. 2). Phylogenetic analysis confirmed this relationship, with an unrooted parsimony tree based on the Pol sequences forming two main groups corresponding to the group I (including UWSMN-1) and group II coronavirus serogroups (Fig. 3). These data indicate that, within the Pol gene, UWSMN-1 is most closely related to the group I coronaviruses, particularly CCV.

The sequence of the 3′ end of the S gene of UWSMN-1 also showed only a small apparent bias for other CCVs, with relatively similar average identities of 90.3% to comparable sequences of feline-related coronaviruses and of 93.0% to those of other CCVs (Fig. 2B). In comparison to FoCVs and other CCVs, the Australian isolate (UWSMN-1) had the least homology with the other strains for the 3′ region of the S gene (Fig. 2B). Phylogenetic analysis confirmed the data on percentages of identity, with UWSMN-1 forming a distinct branch on the 3′ S gene nucleotide parsimony tree between different CCV and FoCV strains (Fig. 3B). The finding that the conserved 3′ end of UWSMN-1 forms an independent subgroup when compared to FoCVs and other CCVs mirrors our previous findings with the highly variable 5′ end of the S gene (Fig. 3C). The distinctness of UWSMN-1 among CCVs is therefore confirmed here to be a feature that is not confined only to the variable 5′ region of the S gene but is likely to be a general feature of this strain.

Sequencing of the various regions of the coronavirus genome has been previously used to investigate the phylogenetic relationship of various coronavirus strains (5, 6, 15, 16). In these studies, recombination events between different CCV, FoCV, and porcine coronavirus strains were identified (5, 6, 15, 16). However, in the present study, we demonstrate that UWSMN-1 forms an independent group between CCV and FoCV strains even with regard to the conserved regions of the S and Pol genes. Moreover, in comparing the 751 nucleotides in the 3′ region of the S gene, it was found that UWSMN-1 had 21 unique sites and that there were 112 sites where UWSMN-1 was different from at least one of the other strains analyzed (Fig. 1B). There was no obvious pattern to the locations of these differences with respect to the other CCVs or FoCVs, as they appeared to be randomly interspersed (Fig. 1). These data demonstrate that our original finding of a divergent 5′ region of the S gene in UWSMN-1 is probably not the result of recombination events between FoCVs and CCVs, as would be indicated if the S gene shared blocks of homology with either FoCV or CCV S genes. Rather, UWSMN-1 appears to be generally divergent, indicating an earlier break from a common ancestor and the gradual accumulation of mutations throughout its genome, which may be reflective of its isolated evolution in Australia. To date, no "typical CCVs" have been identified in Australia by using electron microscopy and reverse transcription-PCR as detection methods (4, 9, 10, 13). These data indicate that CCV UWSMN-1 forms a novel third CCV subgroup that is distinct from the two feline- and canine-like subtypes.

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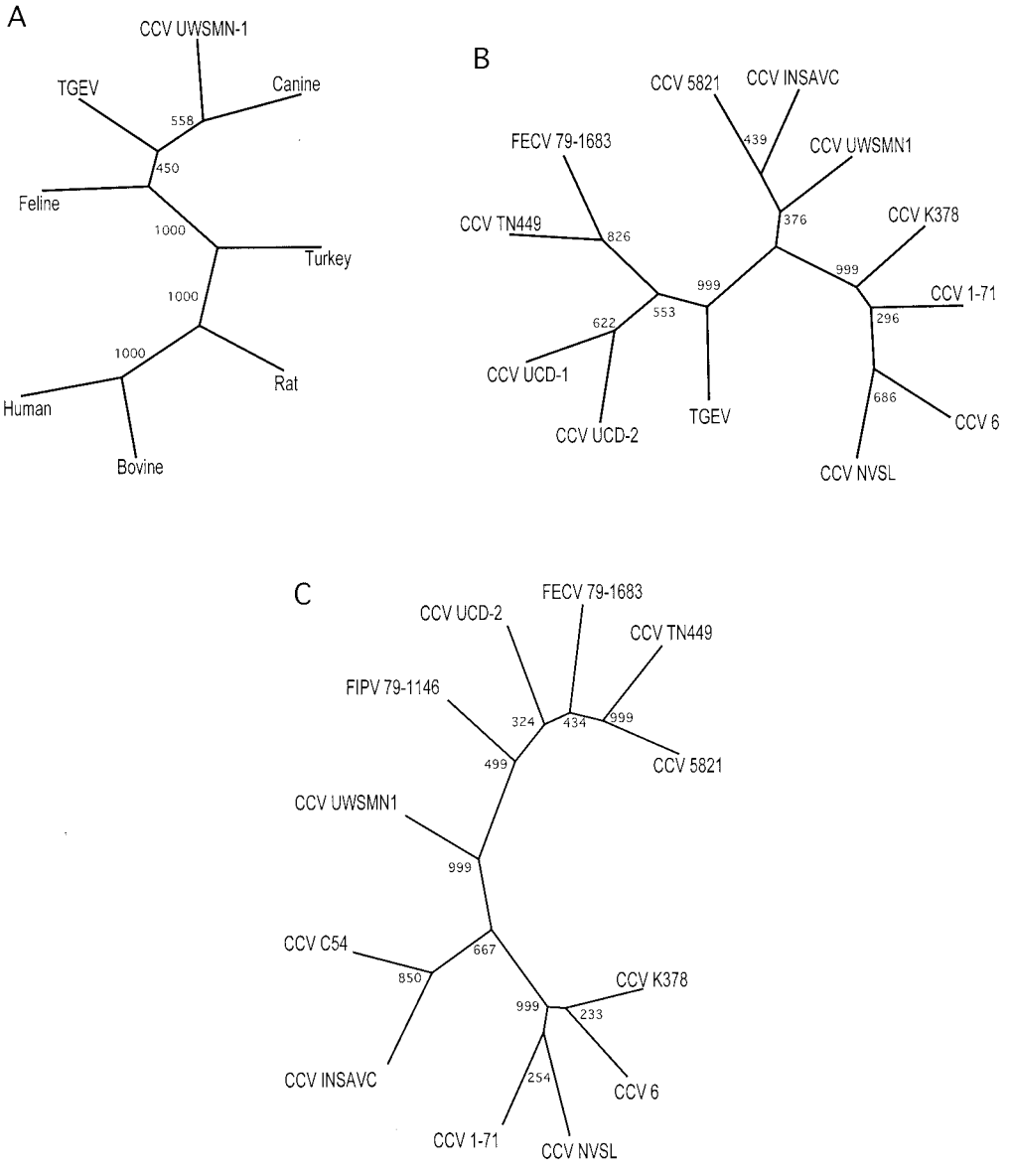

FIG. 3. Phylogenetic trees of coronaviruses, CCV, and FoCV. (A) Pol gene; (B) 3′ region of S gene; (C) 5′ region of S gene (reproduced from reference 10 with permission). The sequences are based on DNA parsimony analysis using the PHYLIP package as described in Materials and Methods. Bootstrap values indicate the number of times out of 1,000 iterations that a branch point was identified.

Nucleotide sequence accession numbers. The sequences of the S and Pol genes for CCV UWSMN-1 were deposited in GenBank and assigned accession no. AF516906 and AF516907, respectively.

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