J. Vet. Med. B. 41, 249–256 (1994)

ISSN 0931–1793

From the Dpto. Patalogia Animal I, Faculted de Veterinaria, Universidad Compultense, Spain

Canine-coronavirus (CCV) Characterization in Spain Epidemiological Aspects

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With 7 figures

(Received for publication September 29, 1993)

Summary

In this paper the characterization of a canine-coronavirus (CCV) strain isolated in Spain is reported. The CCV cellular-infection cycle on A-72 cells was studied using electron-microscopy techniques. The isolate was found to have similar electrophoretic profile and antigenic properties to an American reference strain. Sera samples were obtained from several canine populations and tested for viral antibodies using two immunoenzymatic methods. Sera specificity was confirmed using immunoblot analysis. The agreement between both ELISA tests produced adequate results (kappa value = 0.64).

Introduction

Canine coronavirus (CCV) has been identified as one of the causal agents of viral enteritides in members of the Canidae and has a worldwide distribution. CCV infection is a highly contagious disease affecting mainly young pups in closed populations (HELFER-BAKER et al., 1980; RIMMELZWAAN et al., 1991).

The CCV epidemiological situation in Spain has not yet been reported on, so virus characterization and comparison with an American reference strain was one of the main objectives of this study. In this way, an experimental ELISA was evaluated by comparing it both with another immunoenzymatic method and with immunoblot analysis.

In this report, a serologic survey has been conducted to determine the incidence of exposure to CCV in open and closed canine populations.

Materials and Methods

Feces and sera specimens

A total of 51 sera samples were analysed. They had been obtained from an open population (25 sera) and from the kennel where the CCV was isolated for the first time in Spain (26 sera) (MARTIN-CALVO et al., 1992).

Viruses and cells

Two CCV strains were used: a Spanish isolate (P-Madrid/90) and an American strain (s3786/6). The latter strain, and canine A-72 cell line (CRL 1542), were received from the American Type-Culture Collection (Rockville, Maryland).

Control sera

Two control sera were used: hyperimmune anti-CCV serum and SPF dog serum, received from Fort Dodge Laboratories, Iowa.

Electronic microscopy

Ultra-thin sections were made from infected cultures collected at 14, 24 and 48 h post-infection (hpi). The cultures were fixed with a 1.5 % glutaraldehyde solution and 1 % osmium tetroxide solution. They were then washed, dehydrated and embedded in Epon for thin sectioning. Sections were stained with uranyl acetate and lead citrate, and examined using a Jeol 100B.

Polyacrylamide gel electrophoresis and immunoblot assay

CCV strains were grown on A-72 cells and purified in sucrose gradient as described previously (BRIAN et al., 1980). The proteins of the purified virus were separated in slab gels containing 12.5 % acrylamide, 0.1 % bisacrylamide, 375 mM Tris-hydrochloride (pH 8.8) and 0.1 % SDS (SDS-PAGE) (LAEMMLI, 1970). Molecular weights of structural polypeptides were estimated by comparing them with those of the reference strain (s3786/6) and a commercial marker (Pharmacia).

Immunoblot analysis was designed to complete identification of strain P-Madrid/90 and to verify reaction specificity of analysed sera. The two purified CCV strains had been used in previous electrophoresis and their proteins were electroblotted onto nitrocellulose sheets using a Bio-Rad Trans-Blot Cell (TOWBIN et al., 1979). Filters were washed with blocking buffer and then stained with diluted sera, followed by staining with peroxidase-conjugated A-protein for 1 h. Filters were then developed by the substrate 4-chloronaphtol.

Immunoenzymatic methods

Commercial ELISA

A commercial kit was used for anti-CCV antibody detection (Clark KVC-100). Optical density was measured at 490 nm with a Titertek Multiskan (Flow).

Experimental ELISA

Purified virus antigen (P-Madrid/90 strain) was diluted to an appropriate concentration with carbonate buffer and 100 μl of the dilution was dispensed into the wells of 96-well flat-bottomed immunoplates (Nunc). Sera were diluted with PBS-Tween 20 (0.05 %). Peroxidase-conjugated A-protein and DMAB-MBTH substrate were used. Optical density was measured at 620 nm.

Statistical methods

An episcope program was used to evaluate the diagnostic tests and to calculate the test agreement. The agreement between both tests is expressed by the value of kappa. The program was developed by K. FRANKENA and J. O. GOELEMA from the Agricultural University (Wageningen, The Netherlands).

Results

Electronic microscopy (ultrathin sections)

In the early stage of infection (14 hpi), virus particles were located singly or in small clumps enclosed in smooth vesicles (vacuoles in either the Golgi region or in the cisternae of the smooth endoplasmic reticulum). The virus particles were generally

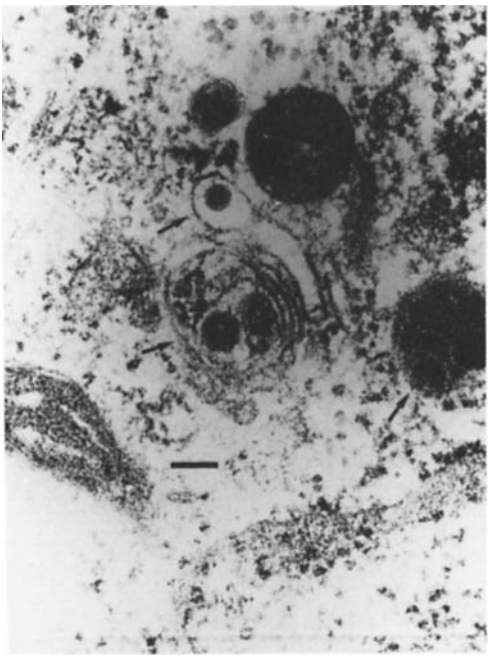

Fig. 1. Virus particles (arrows) enclosed in small clumps in smooth citoplasmic vesicles of an infected cell (14 hpi). Ultra-thin section. Bar represents 100 nm. (×128 000).

circular in shape, displaying some pleomorphism, and their diameter was in the range of 60–100 nm (mean 75 nm). They were doughnut-shaped, with an electron-lucent core surrounded by an electron-dense nucleocapsid (Fig. 1).

In latter stages of infection, virus particles were found in larger clumps enclosed in bigger vesicles. They were almost completely electron-dense (Fig. 2).

Great numbers of extracellular particles were present at 48 hpi. These particles were seen in large clusters on or near the surface of the cell, or lining the surface of the cell (Fig. 3). Budding structures could not be found on any part of the intracellular membrane-bound structures in this study.

Polyacrylamide gel electrophoresis and immunoblot assay

No differences in the electrophoretic behaviour of the major structural polypeptides from the two CCV strains were detected. Estimated molecular weights were similar to those previously described for CCV: 204 kD (S glycoprotein), 50 kD (N protein) and 32 kD (M glycoprotein) (GARWES and REYNOLDS, 1981). The structural polypeptide of 22 kD described by GARWES and REYNOLDS (1981) could not be found in any of the analysed gels (Fig. 4).

Specific serologic response to structural polypeptides of both CCV strains were identical (Fig. 5). Furthermore, specificity of the positive sera reaction was clearly confirmed by immunoblot assay (Fig. 6).

ELISA

As a first step, an assay was performed with two pools of field sera using both ELISA methods. Mean optical densities are shown in Figure 7. Results revealed a lower sensibility, but a higher specificity for the experimental ELISA. A high interspecific

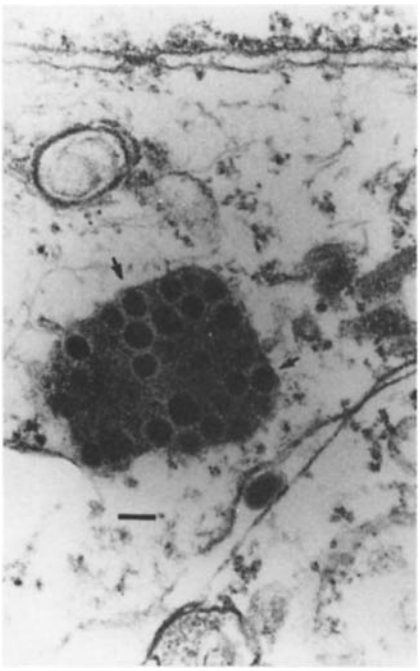

Fig. 2. Virus particles (arrows) in large clumps in citoplasmic vesicles (24 hpi). Ultra-thin section. Bar represents 100 nm. (×128 000).

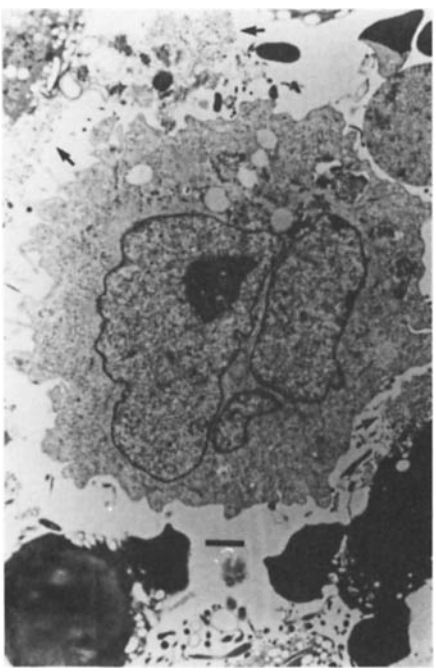

Fig. 3. Great number of extracellular virus particles (arrows) (48 hpi). Ultra-thin section. Bar represents 1000 nm. (×10 000).

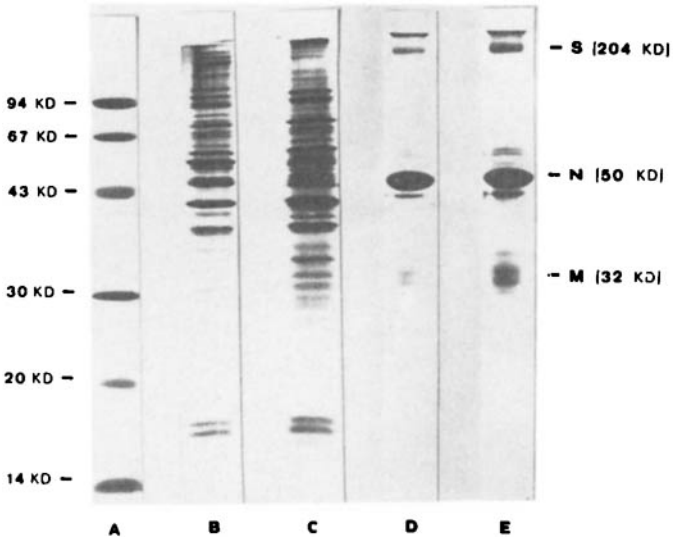

Fig. 4. CCV structural polypeptide analysis. SDS-PAGE: Commercial marker (lane A), A-72 cells (lane B), A-72 cells infected with P-Madrid/90 strain (lane C), purified CCV reference strain s3786/6 (lane D), purified CCV strain P-Madrid/90 (lane E).

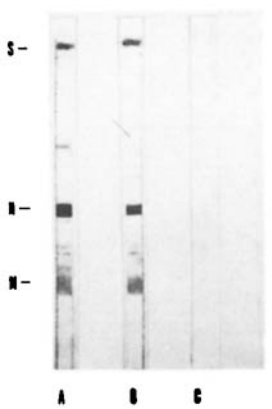

Fig. 5. Isolated strain identification. Immunoblot analysis: hyperimmune control serum reaction to structural polypeptides of CCV (P-Madrid/90 strain; lane A) and to structural polypeptides of reference CCV strain s3786/6 (lane B). Negative control serum reaction to structural polypeptides of strain s3786/6 (lane C).

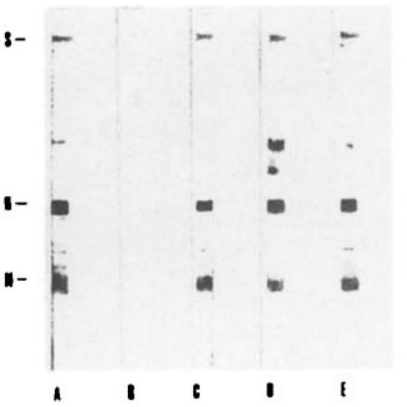

Fig. 6. Reaction specificity of some field sera to structural polypeptides of the isolated strain P-Madrid/90. Immunoblot analysis: hyperimmune control serum (lane A), negative control serum (lane B), field positive sera (lanes C–E).

background in low sera dilutions appeared with the commercial method. The ELISA developed in the authors' laboratory revealed an appropriate agreement with the commercial ELISA (kappa value = 0.64).

Using the commercial assay, 54 % of the closed dog population and 40 % of the open population were seropositive. With the experimental ELISA, a 54 % and 32 %

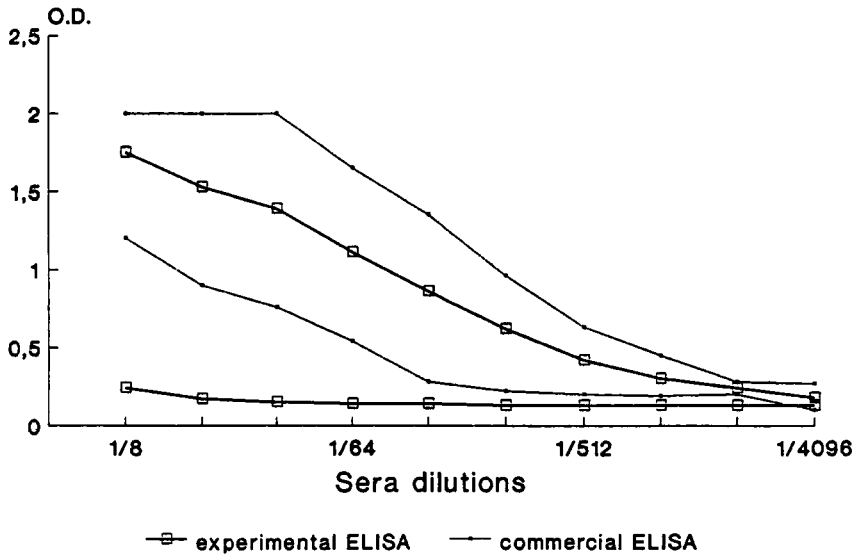

Fig. 7. Mean optical densities obtained by the two immunoenzymatic methods using two pools of field sera (positive and negative).

prevalence values were obtained for both the closed and the open population, respectively. These results show the presence of a higher number of seropositive dogs in the closed population (kennel) using both techniques.

Discussion

Ultra-thin sections were obtained from cultures infected with a Spanish CCV isolate (P-Madrid/90) to study the infectious cycle (MARTIN-CALVO et al., 1992). Size differences between negatively stained virions (105 nm) and virions in ultra-thin sections of cells (75 nm) were attributed to the collapse of the spherical structure, with the consequent diameter increase caused by procedures that are necessary to obtain negatively stained preparations. Furthermore, as described by MEBUS et al. (1973), the diameter of virions in section is probably decreased slightly by fixation.

Electron-microscopic observations revealed that virus particles were located in vacuoles in the Golgi region and in the cisternae of the smooth endoplasmic reticulum. Some of these vesicles were formed by the pinching-off from the end of the Golgi sacs. However, budding structures were not found in any part of the intracellular membrane-bound structures in this study. These observations not only differ markedly from one coronavirus to the other, but are also dependent on the host or cell type (KOJIMA et al., 1986).

Electrophoretic profiles and specific serologic response to structural polypeptides of both CCV strains were very similar. They correlate with those previously described for CCV (GARWES and REYNOLDS, 1981). However, the structural polypeptide of 22 kD described by GARWES and REYNOLDS (1981) could not be found in any of the analysed samples. To find this polypeptides, it would be necessary to try another kind of assay using radioactively marked virus.

Serum antibody titers in CCV infections are not generally used because of the localized site of the infection and the time it takes to detect measurable serum-antibody titers (14–21 days). However, measurement of serum-antibody titers to enteric viruses

has proven useful to determine the prevalence of infection at a particular time (EVERMANN et al., 1989).

In this study, an immunoenzymatic method using the Spanish CCV isolate P-Madrid/90 was developed (MARTIN-CALVO et al., 1992). This technique was compared to a commercial ELISA and contrasted by immunoblotting. Sensibility and specificity values were high and the agreement between both ELISAs was satisfactory (kappa value = 0.64).

Systematic use of the commercial ELISA (slightly higher sensibility) may lead to overestimation of seropositives. In contrast, the experimental ELISA would not detect some low-antibody titers. This higher specificity would, however, undoubtedly increase diagnostic accuracy.

These results indicate that the developed immunoassay is adequate for diagnostic purposes in canine populations. Test sensibility must be adjusted (corrected) by obtaining paired sera samples. It may be desirable to enlarge the serologic survey in order to contrast results more effectively.

The immunoblot assay clearly verified the specificity of sera reaction in the dogs that were analysed. This technique produces a better agreement with the experimental ELISA than with the commercial ELISA. Seropositivity prevalences obtained using both ELISAs were always higher in the closed population (>50 %). Usually, the number of seropositive animals in kennels (closed populations) is double that in open populations. The authors' results agree with those described by other European and American authors (HELFER-BAKER et al., 1980; RIMMELZWAAN. et al., 1991). In Spain, during the study period (1990–1991), there was no registered vaccine for CCV, so the authors' sera results can be used to establish the prevalence of CCV in at least two different canine populations.

Acknowledgements

The authors wish to thank Dr L. E. CARMICHAEL of James A. Baker Institute, Cornell University, NY., USA, and COFAL SA Laboratories, for kindly providing A-72 cells and control sera respectively. The authors also thank the Electronic Microscopy Service and the Animal Health Department of INIA for their help and cooperation in this study.

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