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SHORT COMMUNICATIONS

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Isolation of Encephalomyocarditis Virus from Dormice (*Myoxus glis*) in Italy

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ABSTRACT: Two isolates of encephalomyocarditis (EMC) virus (ZRC 276RA/90 and ZRC 292RA/90) were isolated from two dormice (*Myoxus glis*) in Tuscany, Italy. The two isolates were lethal for laboratory mice and caused a rapid cytopathic effect characterized by rounded and wrinkled cells in both baby hamster kidney cells (BHK21) and African green monkey kidney cells (Vero). We found neutralizing antibodies against EMC virus in 408 (77%) of 529 domestic pigs (*Sus scrofa scrofa*) and in 165 (49%) of 338 wild boars (*S. scrofa ferus majori*) in Tuscany.

Key words: Encephalomyocarditis virus, dormouse, isolation, characterization.

Encephalomyocarditis (EMC) virus is an RNA-virus of the *Cardiovirus* genus, within the Picornaviridae family. The members of this genus have been classified for many years among murine enteroviruses, as their effects range from inapparent infections to death in rodents (Andrewes et al., 1978). Many other animal species, both domestic and wild, are susceptible to infection (Gainer, 1967; Acland and Littlejohns, 1986). However the rat is considered the main natural reservoir (Sanford et al., 1985), because it can have a continuous and lifelong virus excretion from its gut (Acland and Littlejohns, 1986). Encephalomyocarditis virus also has been associated with several epizootics among swine (*Sus scrofa scrofa*) (Cardeti, 1989) and with an influenza-like syndrome in humans (Warren, 1965).

In Europe the virus was isolated from swine in Greece (Paschaleri-Papadopoulou et al., 1990), from a red squirrel (*Sciurus vulgaris leucourus*) in Great Britain (Vizoso et al., 1964) and from a pheasant (*Phasianus colchicus*) in Czechoslovakia (Gresìkovà et al., 1978). Epizootics of EMC in swine recently have been reported in Italy (Sidoli et al., 1989). We report the isolation and identification of two isolates of EMC virus (ZRC 276RA/90 and ZRC 292RA/90) from the brains of two dormice (*Myoxus glis*) found clinically ill in two different areas of Tuscany: Lucca Province (43°50′N, 10°27′E) and Livorno Province (43°32′N, 10°20′E).

Samples of brain were ground in Eagle's minimal essential medium (MEM) (Whitaker Bioproducts, Inc., Baltimore, Maryland, USA) containing antibiotics (1,000 IU/ml penicillin, 100 mg/ml streptomycin and 2 mg/ml amphotericin B) (Sigma Chemical Company, St. Louis, Missouri, USA); the resulting homogenates were centrifuged at 5,000 rpm for 30 min and the supernatants were inoculated by adsorption onto baby hamster kidney (BHK21) (CCL 10, American Type Culture Collection, Rockville, Maryland, USA), and African green monkey kidney (Vero) (CCL 81, American Type Culture Collection) cell monolayers (Sidoli et al., 1989).

The viral suspensions were observed by electron microscopy transmission in negative staining using 2% phosphotungstic acid (Poli et al., 1991). Infected cell cultures slides after inoculation were fixed at 12-hr intervals in Bouins's solution before staining with hematoxylin and eosin (Castrucci et al., 1968). The type of nucleic acid was determined by titration of the virus diluted in MEM containing 50 μg/

ml of 5 bromo-2′ deoxyuridine 5′ monophosphate sodium salt (BUDR) (Sigma Chemical Company) (Castrucci et al., 1970). The viral isolates also were evaluated for their survival at pH 3.0 and in the presence of ether (20%) and chloroform (10%), as described by Castrucci et al. (1968). Viral infectivity was tested after incubation at pH 6.2 and at a sodium chloride concentration of 0.14 M for 30 min (Mak et al., 1970).

For the heat stability study, three ampules, each containing 2 ml of the undiluted viruses, were placed in a waterbath at 50 C, 56 C and 70 C, respectively. After 30 min the content of each ampule was evaluated (Sidoli et al., 1989) for virus by titration (Reed and Muench, 1933). The stability at high temperatures and in the presence of a divalent cation was tested after diluting the viruses in distilled water and in 2 M $MgCl_2$ solution. Dilutions were placed in a waterbath either at 50 C for 30 min or at 70 C for 15 min, and finally their titers were determined on BHK21 cell cultures (Wallis and Melmick, 1961).

The ability of the isolates to agglutinate erythrocytes of guinea pig, rabbit, sheep, chicken, pigeon, rat and mouse was tested according to the procedure described by Craighead and Shelokov (1961). Identification procedures were performed by a serum neutralization test (Hoskins, 1975) using rabbit antiserum produced against the Pirbright strain of swine EMC virus (Central Veterinary Laboratory, Weybridge and Institute for Animal Health, Pirbright) (Sangar et al., 1977). Serial tenfold dilutions of the viruses were mixed with an equal volume of antiserum containing 20 neutralizing units.

Serological comparisons among the isolates and the Pirbright strain were evaluated using serial two-fold dilutions of the same immune-serum mixed with 100 median tissue culture infectious doses (TCID50) of virus. The serum-virus mixtures first were incubated at 37 C for 60 min and then inoculated onto a monolayer of Vero cells. Moreover, the same reference serum was used in immunoelectro-microscopy (Poli et al., 1991).

Three monoclonal antibodies (Mabs) produced at Istituto Zooprofilattico Sperimentale, Brescia, Italy, against a swine strain of EMC virus were used in a sandwich immunoenzymatic assay (ELISA) as described by Brocchi et al. (1993).

The dormice's brain suspensions (0.3 ml) were inoculated intracerebrally (IC) and subcutaneously (SC) into 20-day-old mice. These were observed daily for clinical signs or death. The brains of the mice dying from inoculation were aseptically removed and their suspensions were again inoculated IC into mice.

To evaluate the diffusion of the infection among other susceptible species, 529 and 338 blood samples from domestic pigs and wild boars (*S. scrofa ferus majori*), respectively, were collected from several areas of Tuscany (between 42° and 45°N, 10° and 12°E) during hog cholera investigations in 1991 and 1992. Serum samples were from healthy pigs at abattoirs and from wild boars killed according to the control plan. Antibody detection was conducted by a neutralization test (Sidoli et al., 1989) using the ZRC 276RA/90 isolate as the antigen.

From the first passage on BHK21 and Vero cells, a cytopathic agent (ZRC 276RA/90 and ZRC 292RA/90) was isolated from each brain homogenate. The cytopathic effect on both tissue cultures was identical for the two isolates, and was represented by scattered, rounded cells of minor size with nuclear pyknosis which appeared between the first and second day after inoculation. Gradually the entire monolayer was affected and destroyed. Based on electron microscopy, the virus particles were about 27 nm in diameter with an icosahedral structure typical of picornavirus morphology; no envelope or spikes were observed (Fig. 1a).

We observed intracellular inclusions with hematoxylin-eosin staining. The cells appeared wrinkled, with nuclear pyknosis. The isolates were determined to be RNA

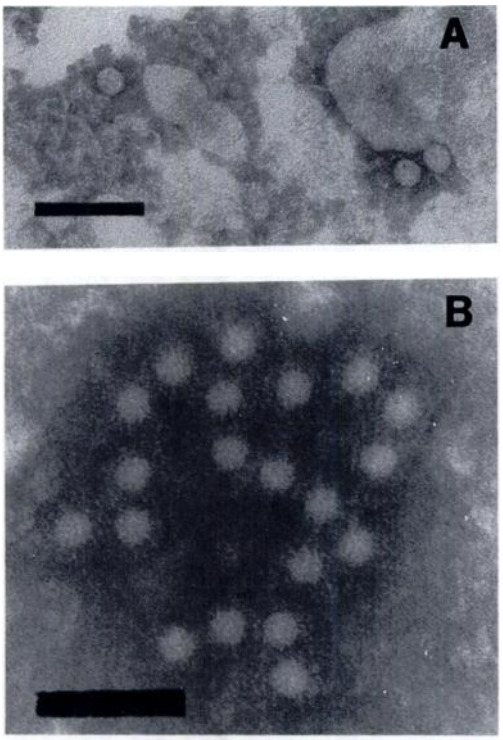

FIGURE 1. Electron micrographs of single and aggregated viral particles of encephalomyocarditis virus: A) Single viral particles (27 nm in diameter) in negative staining. B) Immunoelectromicroscopy: Aggregate of viral particles surrounded by a dense halo of antibodies. Each bar = 100 nm.

types since their multiplication was not inhibited by BUDR. The viruses were not destroyed by pH 3.0, but were inactivated by pH 6.5 in a solution 0.14 M of chlorine ions and were resistent to ether (20%) and chloroform (10%). Further, they were not inactivated at 50 C for 30 min but had a significant drop in titer when held at 56 C for 30 min and at 72 C for 15 min; on the other hand they were heat stabilized by 2 M $MgCl_2$. Strains ZRC 276RA/90 and ZRC 292RA/90, at their fifth passage level in Vero cells, had hemagglutination endpoints of 1:16 and 1:8/50-μl, respectively, for guinea pig erythrocytes.

The strains in this study closely resembled the Pirbright strain of swine EMC virus. The titer of the reference serum with the two viruses was identical to that obtained for the homologous Pirbright virus (1:2,560).

Based on immunoelectro-microscopy, we observed the presence of large aggregates of viral particles surrounded by a dense halo of antibodies (Fig. 1b). The isolates were positive in the ELISA test performed with anti-swine EMC virus monoclonal antibodies.

Three mice were found dead 3 days post inoculation; the remaining two showed ruffled coats, hunched postures and progressive flaccid posterior paralyses. When passed to other mice, inoculated either IC or SC with the brain filtrate material, these mice showed nervous signs and death in 24 hr. The viruses were reisolated from the brains of these mice.

Of the 529 serum samples taken from swine, 408 (77%) were positive for neutralizing antibodies. Among the 338 sera from wild boars, 77 were cytotoxic and of the remaining 261 samples, 131 (50%) had neutralizing antibodies. The neutralizing titers of the two species ranged from 1:6 to 1:768.

The viral isolates (ZRC 276RA/90 and ZRC 292RA/90) had dimensions and morphology, together with an RNA nucleic acid, as described for the Picornaviridae family. Moreover, their resistance to ether and chloroform confirmed the absence of an envelope as observed in electron microscopy. In contrast to isolates from *Aphtovirus* spp. and the *Rhinovirus* spp., these isolates were resistant to pH 3. However they were similar to *Enterovirus* spp. due to their heat stability in a magnesium solution, but distinct from them for their sensitivity to pH 6.2 in a 0.14 M sodium chloride solution. This would classify them within the *Cardiovirus* genus. Antigen correlation tests, seroneutralization and ELISA results, were further evidence that the isolates were cardioviruses due to their close antigenic relationship with the reference strain of swine EMC virus. Until now, no isolations of EMC virus from dormice have been reported in literature; thus this rep-

resents the first viral isolation in this species.

In the past, seroepizootological surveys have been conducted among pigs in Italy (Gualandi et al., 1989) and in other countries (Sangar et al., 1977; Tesh, 1978; Sanford et al., 1985); a high prevalence for EMC virus antibodies was found. Based on our serological survey, in Tuscany, EMC virus presents a wide distribution not only in pigs (77%) but also in wild boars (50%), classifying the latter as another species that may be susceptible to the same virus.

The high antibody prevalence in swine together with the strict homology of these isolates with the Pirbright strain of EMC virus and the pathogenicity of such strains for the dormouse, is evidence that these dormice were infected by swine. We found no information in literature about EMC virus infection (neither isolation nor antibodies) in wild boars. We do not know if the antibodies in this species derive from a contact with infected pigs, dormice, or other rodents. The isolation of two isolates within a 2-wk period in nearby sites is evidence that both domestic and wild animals of the same region might have been infected.

Further characterization of the two isolates as well as serological surveys in dormice and other wild rodents in Tuscany should be conducted to better understand if these cases represent accidental spill-over of Pirbright EMC virus from swine or another rodent species into dormice or whether this is truly an unknown dormouse strain of EMC virus.

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