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VIRAL INFECTIONS OF THE CENTRAL NERVOUS SYSTEM IN SPAIN: A PROSPECTIVE STUDY

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Running head: Viral infections of the central nervous system in Spain

ABSTRACT

Objective. To determine the incidence of viruses causing aseptic meningitis, meningoencephalitis and encephalitis in Spain.

Methods. Prospective study, in collaboration with 17 Spanish hospitals, including 581 cases (CSF from all and sera from 280): meningitis (340), meningoencephalitis (91), encephalitis (76), febrile syndrome (7) and other neurological disorders (32), and 35 cases without clinical information. CSF were assayed by PCR for enterovirus (EV), herpesvirus (herpes simplex [HSV], varicella-zoster [VZV], cytomegalovirus [CMV], Epstein-Barr [EBV], and human herpes virus-6 [HHV-6]), mumps (MV), Toscana virus (TOSV), adenovirus (HAdV), lymphocytic choriomeningitis virus (LCMV), West Nile virus (WNV), and rabies. Serology was undertaken when methodology was available.

Results. Amongst meningitis cases, 57.1% were characterised; EV was the most frequent (76.8%), followed by VZV (10.3%) and HSV (3.1%) (HSV-1: 1.6%; HSV-2: 1.0%, HSV non typed: 0.5%). Cases due to CMV, EBV, HHV-6, MV, TOSV, HAdV and LCMV were also detected. For meningoencephalitis, 40.7% of cases were diagnosed, HSV-1 (43.2%) and VZV (27.0%) being the most frequent agents, while cases associated with HSV-2, EV, CMV, MV and LCMV were also detected. Turning to encephalitis, 27.6% of cases were caused by HSV-1 (71.4%), VZV (19.1%) or EV (9.5%). Other positive neurological syndromes included cerebellitis (EV and HAdV), seizures (HSV), demyelinating disease (HSV-1 and HHV-6), myelopathy (VZV) and polyradiculoneuritis (HSV). No rabies or WNV cases were identified.

Conclusion. EV are the most frequent cause for meningitis, as is HSV for meningoencephalitis and encephalitis. A significant number of cases (42.9%

meningitis, 59.3% meningoencephalitis and 72.4% encephalitis) still have no etiological diagnosis.

KEY WORDS

Aseptic meningitis, encephalitis, meningoencephalitis, enterovirus, herpes simplex virus, varicella-zoster virus, polymerase chain reaction, serology, viral infections, central nervous system, incidence.

INTRODUCTION

The aseptic forms of meningitis, encephalitis and meningoencephalitis are non-purulent inflammatory processes of the central nervous system (CNS), where initial assessment using the usual staining and cultures of cerebrospinal fluid (CSF) is negative. Epidemiological studies indicate that viruses are the main identified cause of acute meningitis and encephalitis (Koskiniemi et al, 2001; Glaser et al, 2003; Kupila et al, 2006). Of major importance are the enteroviruses (EV), herpes simplex virus (HSV)-2 and varicella zoster virus (VZV) in meningitis, and HSV-1 in encephalitis. Human immunodeficiency virus (HIV) has been identified as an important agent causing meningoencephalitis (del Saz et al, 2008).

In Spain there have been some studies based on the detection of etiological agents, and on studies of antibodies which examine the involvement of viral agents in the production of meningitis and encephalitis. In the case of meningitis, the most frequent agents are EV, particularly in children, followed by VZV and HSV, more frequently in adults (Téllez et al, 1989; Echevarría et al, 1997a; de la Loma et al, 2002). Other pathogens detected in prior studies were mumps (MV) (Téllez et al,

1989), Toscana virus (TOSV) (Navarro et al, 2004; De Ory et al, 2009), West Nile virus (WNV) (Kaptoul et al, 2007), and lymphocytic choriomeningitis virus (LCMV) (De Ory et al, 2009). As regards encephalitis, the information available in Spain is more limited. A previous report found that HSV and VZV were the most frequent agents while Epstein Barr virus (EBV), cytomegalovirus (CMV), human herpes virus-6 (HHV-6) and EV were the cause of a minor number of cases (Echevarría et al, 1997b).

Other neurological disease-producing agents are human adenovirus (HAdV) (Lema et al, 2005). With regard to rabies, the pathognomonic hallmarks are not always apparent and it may present with general symptoms of encephalitis. Although the rabies virus (RV) was eradicated from the Spanish mainland and the islands in 1978, imported cases of canine rabies are still being reported on a regular basis in the Spanish North-African cities of Ceuta and Melilla. In 2004 one imported human case was declared in Ceuta (Strauss et al, 2005). In addition, the European bat lyssavirus type 1 is endemic in some Spanish bat species and can cause rabies in man, not always accompanied by the characteristic symptoms (Vázquez-Morón et al, 2008).

Recently the situation of encephalitis and meningoencephalitis surveillance in Europe, which highlighted the advisability of running studies on the etiological characterisation of neurological syndromes in European countries, was analysed. This would reveal the incidence of pathogens of concern for public health, including emerging viral infections (Donoso-Mantke et al, 2008). The aim of this study was to identify the viruses responsible for CNS infections, by analysing EV, HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, MV, TOSV, HAdV, LCMV, Flavivirus (WNV and tick borne encephalitis virus [TBEV]) and Rhabdovirus.

MATERIALS AND METHODS

Design. Prospective observational study of acute viral CNS infections involving diagnostic virology labs at 17 hospitals from the Spanish Health National System. These hospitals serve a population of 7,572,771 inhabitants (6,762 million adults over 14 years of age and 810,771 children under 14), accounting for 17.17% of the Spanish population (17.9% of those over 14 and 12.9% under 14), according to data from the 2005 municipal census (http://www.ine.es).

Patients. A total of 581 cases classified at admission as meningitis, meningoencephalitis or encephalitis was studied. At discharge the cases were definitively classified as acute cases of meningitis (340 cases), meningoencephalitis (91), encephalitis (76), febrile infant syndromes (7), and other conditions (32) (headache [2], cerebellitis [2], seizures [6], confusion [1], diplopia [1], demyelinating disease [3], hypotension [1], leukoencephalopathy [1], cerebral lymphoma [1], meningeal lymphomatosis [1], myelitis [3], myelopathy [4] multineuritis [1], optic neuritis [2], paralysis [1] and polyradiculoneuritis [2]), diagnosed by Internal Medicine or Neurology or Paediatrics Services in the hospitals participating from March 1, 2008 to February 28, 2009. In 35 of the cases no clinical information was available. Informed consent was obtained from all patients (or guardians in the case of children under 14 years). For inclusion, all patients had to be immunocompetent, their CSF sample had to present lymphocytic pleocytosis >5 cells/mm^3 and the sample had to be collected during the first week of onset of symptoms. Meningitis cases coursed with fever, headache, and neck rigidity. For diagnosis of encephalitis, cases needed to present one or more clinical signs of focal cerebral involvement, an altered level of consciousness, neuropsychiatric clinical symptoms, or compatible

radiological signs. Meningoencephalitis included signs or symptoms from both encephalitis and meningitis.

Clinical and epidemiological data. A form specifically designed for this purpose was completed in each case, recording demographic data (age, sex, seasonality of episode, length of hospitalisation) and clinical data (presence of fever ≥38°C and its duration in days, neurological symptoms and association of systemic symptoms such as diarrhoea, arthralgia, myalgia, lymphadenopathy, respiratory symptoms).

Samples. In all cases CSF sample from the first week of onset of symptoms was available (minimum of 400 μl). Three hundred and twenty one serum samples from 280 cases were studied: 2 cases with three samples, 37 cases of acute and convalescent serum, acute serum only from 236 cases, and only convalescent serum in 5 cases.

Virological study. This was run on the CSF samples, preparing two aliquots of 200 μl each. With the first one, the presence of at least HSV-1 and -2, VZV and EV by polymerase chain reaction (PCR) was determined in each hospital, using each laboratory's own procedures. The second aliquot was mixed with a lysis buffer based on guanidinium thiocyanate (300 μl) (MagnaPure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics), kept at -20ºC for subsequent submission to the National Microbiology Centre (CNM), where the nucleic acids were automatically extracted (MagnaPure LC, Roche Diagnostics) and processed in a multiplex PCR assay for EV, HSV, VZV, CMV, EBV and HHV-6 (Casas et al, 1997). Where the result was negative, samples were processed by PCR for HAdV (Avellón et al, 2001), MV (Palacios Poggio et al, 2000), RV (Echevarría et al, 2001), TOSV (Sánchez-Seco et al, 2003; Pérez-Ruiz et al, 2007), for the identification of as yet undescribed members of the

Herpesviridae family (using a generic PCR [Pozo F et al, in preparation]); *Flavivirus* (Sánchez-Seco et al, 2005); *Arenavirus* (or LCMV) (Ledesma et al, 2009); *Lyssavirus* (Vazquez-Morón et al, 2006); RV, and *Dimarhabdovirus* (Aznar C, in preparation). In samples which tested positive for HSV, a typing test based on the polymerase gene was used (Echevarría JE et al, in preparation).

Cases which showed positive results at the sender laboratory and/or in the CNM were considered positive.

Samples positive for EV, HAdV or MV were genotyped by previously described assays (Casas et al, 2001; Casas et al, 2005; Palacios et al, 2005; Cabrerizo et al, 2008).

Bacterial and fungal aetiology was discarded in all cases.

Serological Study. The following tests were performed depending on sample availability: antibodies to HSV and VZV by complement fixation (CF), IgM antibodies to HSV and VZV, and IgG and IgM antibodies to MV and TBEV by ELISA (Enzygnost, Siemens, Germany), IgG antibodies to HSV-1 and HSV-2 by immunoblot (Virotech, Germany), IgG and IgM antibodies to TOSV, by ELISA (Diesse, Italy); IgG and IgM antibodies to WNV by ELISA (Focus, USA) and IgG and IgM antibodies to LCMV by indirect immunofluorescence as described (De Ory et al, 2009).

In one case serum/CSF paired samples with 26 days of evolution were available. The intrathecal synthesis of IgG antibodies to HSV and VZV was examined, establishing the ratio of antibody to albumin (Echevarría et al, 1990).

RESULTS

DIRECT VIRAL DETECTION BY PCR. Result were obtained for 566 of the 581 samples studied, with positive results in 250 cases using PCR (44.2%): this was 161 for EV

(66.4% of positives; 28.4% of the total); 31 for HSV-1 (12.4% and 5.5%); 28 for VZV (11.2% and 4.9%); 3 for HSV-2 (1.2% and 0.5%); 1 HSV-non typed (nt) (0.4% and 0.2%); 4 CMV (1.6% and 0.7%); 3 EBV (1.2% and 0.5%); 2 HHV-6 (0.8% and 0.4%); 2 TOSV (0.8% and 0.4%); 2 HAdV (0.8% and 0.4%); 1 MV (0.4% and 0.2%); 1 LCMV (0.4% and 0.2%); and mixed infections in 11 cases (4.4% and 1.9%): 5 EV, 1 HSV-1, 1 VZV , and 4 EV plus HSV-nt. Thus, including mixed infections, 170 EV, 32 HSV-1, 5 HSV-nt and 29 VZV were identified. No cases due to RV, WNV or herpes virus other than those already known were identified.

GENOTYPE CHARACTERISATION. The samples which tested positive for EV, HAdV and MV were genotyped. As regards EV, 132 were positive in the CNM, of which 70 were genotyped (53%) (Cabrerizo et al, in preparation). Furthermore, the only sample which identified MV positively by PCR was characterised as genotype G1. The two cases of HAdV belonged genetically to serotype HAdV-5.

SEROLOGY. For HSV and VZV 315 samples were studied from 277 cases: one case with three samples; 36 cases of acute and convalescent sera; 235 cases of acute serum only, and 5 cases of convalescent only. Fourteen cases of HSV-nt infection (5.1% of those studied) were serologically diagnosed; 4 cases by seroconversion, 2 cases by presence of IgM, and 8 cases by high titres in CF. Two cases were positive for HSV infection but negative in PCR. With regard to specific response to HSV-1, the result was positive in 11 cases, 5 of them simultaneously with detection of the virus by PCR. Finally, one case of infection with HSV-2, simultaneously with identification of the virus by PCR was diagnosed.

Seventeen cases (6.1%) turned out to be VZV-positive: 9 with a simultaneously positive PCR, 7 were positive only in serology, and one last case was diagnosed by

specific intrathecal IgG production and by serology. In addition, three cases with positive direct detection of other viruses (2 EV and 1 HSV-1) showed positive VZV serology.

MV IgM was studied in 321 samples from 280 cases, with positive results in 4 cases (1.4%): one with G1 genotype identification by PCR, and 3 with negative PCR. Three other cases with positive direct detection of EV (2 cases) and VZV showed positive results. In addition, IgG-MV was studied in 221 cases, obtaining positive results in 187 cases (84.6%). High titres (≥3,500) were identified in 49 cases (22.2% of the total i.e. 26.2% of the positives).

TOSV IgG and IgM were analysed to in 318 serum samples from 278 patients, diagnosing one case with reactivity to both isotypes (0.4%) and positive PCR. The presence of specific IgG was detected in 18 cases out of the 277 remaining cases (6.5%).

LCMV IgG and IgM antibodies were studied to in 270 samples from 35 cases, obtaining positive IgM in three cases (1.3%), one with a positive PCR in CSF for LCMV, a second with negative PCR, and a third from a mixed infection with direct identification of EV. When analysing IgG, 10 positive cases were obtained from those not revealing recent infection (4.3%). IgG and IgM antibodies to WNV were determined in 303 serum samples from 267 patients. No cases of recent infection with the virus were diagnosed, however 5 positive IgG samples were identified (1.9%). Finally, with regard to TBEV, 138 samples were studied from 121 cases. No cases indicative of recent infection were identified but IgG was detected in 3 cases (2.5%).

OVERALL RESULTS. Overall results, including both the results for PCR, such as serology in serum and CSF, are presented in Table I. A total of 270 cases were finally characterised (46.5% of the 581 cases received).

ETIOLOGICAL CHARACTERISATION OF CASES OF MENINGITIS, MENINGOENCEPHALITIS AND ENCEPHALITIS. Both final results obtained by clinical picture and those obtained by both clinical characteristics and age group are shown in Tables II and III.

For cases of meningitis, 194 cases gave positive results (57.1% of those studied); for meningoencephalitis, 37 (40.7%), and for encephalitis, 21 (27.6%). An etiological diagnosis was most often reached in cases of meningitis (χ^2 = 20.868, p<0.00001), and less frequently than in the rest in the cases of encephalitis (χ^2 = 16.671, p<0.0001).

Among the cases of meningitis, EV were the most frequent cause, both overall (149 cases, 43.8% of the total, 76.8% of positives) (Table II) and in all age groups (Table III). Interestingly, 65% of meningitis in children was positive for EV. Next in importance came VZV (20 cases, 5.9% and 10.3%); HSV (6 cases, 1.8% and 3.1%) (3 HSV-1: 1.6%; 2 HSV-2: 1.0%, and 1 HSV-nt: 0.5%); EBV (3 cases, 0.9%, 1.6%), CMV (2 cases, 0.6% 1.0%); HHV-6 (1 case, 0.3% and 0.5%); MV (3 cases, 0.9%, 1.6%); TOSV (2 cases, 0.6%, 1.0%) and HAdV and LCMV (1 case, 0.5%, 0.3% each). Double positives were found in 6 cases (1.8% and 3.1%), as listed in Table IV.

Among the cases of meningoencephalitis, HSV-1 was the most frequent cause, both overall (16 cases, 17.6% of the total, 43.2% of positives) (Table II) and in all age groups, followed by VZV (10 cases, 11.0% and 27.0%) (Table III). Importantly, in

16-60 and >60 age groups both HSV and VZV caused respectively 35% (14/40) and 36% (9/25) of cases studied. EV caused 3 cases (3.3% and 8.1%); CMV, 2 cases (2.2% and 5.4%); and HSV-2, MV, and LCMV 1 case each (1.1% and 2.7%). Double positives were found in 3 cases (3.3% and 8.1%), as listed in Table IV.

Among the cases of encephalitis, the most common cause was HSV-1 (15 cases, 19.7% of the total, 71.4% of positives), followed by VZV (4 cases, 5.3% and 19.1%) and EV (2.6% and 9.5%). All positive cases in patients older than 16 years were due to HSV-1 and VZV (Table III). No multiple infections were detected.

In both meningoencephalitis and encephalitis etiological diagnosis was not achieved in younger age groups.

Six cases of febrile syndrome in infants revealed infection by EV.

The results obtained in cases with other clinical pictures are shown in Table V. Positive results were obtained in 12/32 (37.5%). Of the cases with etiological identification, one case of conjunctivitis associated with cerebellitis was characterised, caused by HAdV-5; three cases with seizure associated with HSV (two cases with HSV-1 and one with HSV-nt) out of the six studied; two cases of demyelinating disease (one associated with HSV-1 and another with HHV-6); out of three analysed, one case of VZV myelopathy, out of four studied, and one case of polyradiculoneuritis due to HSV-nt, from two analysed.

DISCUSSION

A total of 270 positive cases were identified (46.5% of those studied). Among the cases of meningitis, the most frequent were the EV, while among the cases of meningoencephalitis and encephalitis the most frequent cause were the HSV and

VZV. These results basically concur with studies run in Spain on both meningitis (Téllez et al, 1989; Echevarría et al, 1997a; de la Loma et al, 2002) and encephalitis (Fernandez Viladrich et al, 2007).

In children the most frequent cause for meningitis was EV, as has been described in Spain (Téllez et al, 1989). The relative participation of both HSV and VZV in causing meningitis increases with age. The findings in older people are similar to those recently described (Delerme et al, 2009). EV was the most frequent cause, being HSV and VZV also important ones.

As regards encephalitis, the information available is more limited. A study conducted from 1989 to 1995 in Spain found that HSV and VZV were responsible for more than 75% of cases with an etiological diagnosis of acute and subacute encephalitis; and EBV, CMV, and HHV-6, as well as EV were the cause of a number of cases (Echevarría et al, 1997b). Prospective studies run in France (Mailles et al, 2009), Sweden (Studahl et al, 1998) and England (Ambrose et al, 2011) found that HSV and VZV are the main agents of encephalitis in adults.

Although HIV has been identified as an important agent causing meningoencephalitis (del Saz et al, 2008), this virus was not tested since immunodepression was a cause of exclusion from the study.

Human parechoviruses (HPeV) are recognised as a relatively important cause of neurological disease, especially in neonates (Harvala and Simmonds, 2009). HPeV1 and HPeV2 were formerly classified as Echovirus 22 and 23. Unfortunately, the assay used to detect EV (Casas et al, 1997) was unable for detecting them, and no data on

other HPeV more recently described are available. Anyway, the importance of HPeV as neurological pathogens cannot be neglected.

Apart from the identification of EV as the most common cause of meningitis and HSV-1 and VZV amongst meningoencephalitis and encephalitis, the use of broad spectrum tools and specific serological tests have provided us the identification of other agents as cause of CNS infection in Spain.

Before widespread use of the measles-mumps-rubella vaccine, infection with MV was a major cause of aseptic meningitis, particularly in children (Tellez et al, 1989), its importance decreasing with increasing vaccine coverage (Echevarría et al, 1997a). Mumps re-emerged when the use of a vaccine with poor immunogenicity became widespread, with an increase in cases of meningitis in 1996 (Echevarría et al, 1999). In the present study four cases were identified, figure coinciding with low circulation of the virus at the time when the cases were collected (in 2008 there were reported 2,044 cases in Spain, and 3,741 in 2009) (Boletín Epidemiológico Semanal, 2009). Furthermore, only one case of MV by direct detection has been identified, characterised as G1, the most prevalent genotype in previous seasons with the largest circulation of MV (Echevarría et al, 2010). Furthermore, quantification of specific IgG in serum has been suggested as a marker of recent infection in periods of viral circulation (Sanz et al, 2006). On this point, high titres (≥1/3,500) were seen in about 25% of cases. However, considering the conventional indicators of recent infection (PCR and IgM), the high titres obtained in this study may reflect infections in previous seasons, rather than current infection.

LCMV has been identified in rodents in Spain (Ledesma et al, 2009), being responsible for 1.5% of cases negative for frequent aseptic meningitis agents (de Ory et al, 2009). In this study two cases of meningitis (one with simultaneous detection of EV), and another of meningoencephalitis, have been identified. The three cases were located in the south-east of the Spanish mainland, one in Granada and the other two in Murcia. The first, which was diagnosed by both PCR and serology, was in a deprived urban area, where another case caused by the virus was diagnosed one year later (Perez-Ruiz et al, 2012), thus identifying an area of risk for this virus. On the other hand, the global LCMV IgG seropositivity obtained in the cases studied is equivalent to what has been previously documented in a similar sample (de Ory et al, 2009). The current study shows the circulation of the virus in Spain, with the implications that may arise from the seriousness of infection in patients with transplants from apparently healthy donors (Fischer et al, 2006).

With respect to TOSV, two cases were diagnosed, both in south-east Spain, the number of cases being lower than obtained in a previous report (Navarro et al, 2004). A study on sera from cases which were negative for the common agents which cause meningitis, confirmed that TOSV causes 6% of cases, with major year-on-year variations (from 1.2% to 12.5%) (de Ory et al, 2009). Possible causes of the low incidence obtained may be the climatic differences in terms of temperature and humidity in different years, which may affect the circulation of the vector (*Phlebotomus perniciossus*).

Flaviviruses are known to cause neurological syndromes. TBEV and WNV have been identified in Europe, in central Europe (TBEV) and in the Mediterranean basin (WNV). To date there have been no known cases of infection by TBEV in Spain, and only

sporadic cases of neurological disease caused by WNV (Kaptoul et al, 2007; Jesús de la Calle et al, 2012) have been documented. In this study, no cases due to these viruses were identified, although some individuals were found to be IgG positive. Specifically, WNV seroprevalence obtained in Catalonia (4.4%, results not shown) was higher than documented in a previous study (Bofill et al, 2006). WNV and TBEV belong to the genus *Flavivirus*, and show a high degree of cross-reaction between each other and other members of the genus (dengue, yellow fever and others) (Domingo C et al, in preparation). Thus, the reactivity detected in some of these cases may be due to infection by another or other flaviviruses circulating in Spain.

Of the 51 known HAdV serotypes, types -2, -3, -7, -26 and -31 and -49 (Schnurr et al, 1995; Ohtsuki et al, 2000; Dubberke et al, 2006; Nagasawa et al, 2006; Vincentelli et al, 2010) have been linked to neurological infection**.** Meningitis by HAdV has been identified as a complication of pneumonia as a result of disseminated infection in immunocompromised patients; the infection rarely presents primarily as a CNS disease (Studahl et al, 1998). HAdV-5, identified in this study as the cause of one case of cerebellitis with a history of conjunctivitis, and another of meningitis, is usually recognised as a major cause of respiratory infection in infants (bronchiolitis) (Belsy et al, 2009).

Apart from this HAdV-5 cerebellitis case, other medical conditions of interest have been diagnosed (Table V). Three cases of seizures associated with HSV (two cases with HSV-1, and one with HSV-nt), out of six studied were identified. This relationship has previously been described, confirming HSV in 40% of epilepsy cases (Sanders et al, 1997). Otherwise, the findings of HSV-1 and HHV-6 in cases of demyelinating disease can be considered nonspecific, as previously reported (Gordon

et al, 1996). The relationship of VZV with cases of myelopathy has been described previously (Morita et al, 2003). Finally, infection with HSV in cases of polyradiculoneuritis is considered a rare finding (Cosson et al, 2002).

Serological tests for EV for the diagnosis of neurological infection are influenced by cross-reactions (Miao et al, 2009), and the large number of EV which can give rise to these diseases. As genomic amplification assays perform adequately, diagnosis is based almost exclusively on PCR. By contrast, in infections by other viruses, whose serological diagnosis has been more decisive than in the case of EV, the two diagnostic approaches have been seen to be complementary, improving the total number of cases diagnosed, as in the case of HSV, VZV, TOSV, LCMV and MV in this study. However intrathecal production of antibodies was not properly explored, since no paired serum/CSF samples taken more than 10 days after onset were available. This approach showed to be an excellent complementary tool to direct diagnosis for encephalitis (Echevarría et al, 1990; Ambrose et al, 2011).

Dual infection in the central nervous system is a not frequent issue, and seems to be mainly related to herpesviruses. HSV-1 and VZV were simultaneously diagnosed in encephalitis patients by PCR and serology (in CSF and serum) (Casas et al, 1996), and CMV and HSV and CMV and EBV dual infections were described in two aids patients showing respectively encephalitis and polyradiculomielitis (Casas et al, 1999). It has been difficult to find an explanation to mixed infections showed in table iv. In one case acute and convalescent serum sample was available, allowing the confirmation of both HSV-1 and VZV infections. Serum sample from the four patients with double PCR result of EV and HSV-nt was not available to confirm at least the HSV infection; additionally, in the cases showing IgM reactivity (LCMV [1 case], MV

[3 cases] or VZV [2 cases]) no further analysis were done, since no follow up serum sample was available.

The current study has the strong point of being a prospective study, conducted in Spanish Health National System public hospitals, serving a major proportion of the Spanish population; thus the results obtained are a reflection of the true incidence of these microorganisms in the production of neurological syndromes in Spain. A potential limitation, however, is that the cases included come from clinics' requests made to the laboratory; consequently the real circulation of these agents in Spain may actually be underestimated.

As conclusion, this study has shown which are the most frequent viruses related with neurological disorders in Spain (EV, HSV-1 and -2, VZV, MV), allowing to detect some other causing sporadic cases (TOSV, LCMV, HAdV), and ruling out other neurotropic viruses as relevant cause of neurological disease in Spain (as WNV or RV).

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REFERENCES

1. Ambrose HE, Granerod J, Clewley JP, Davies NW, Keir G, Cunningham R, Cunningham R, Zuckerman M, Mutton KJ, Ward KN, Ijaz S, Crowcroft NS, Brown DW, UK Aetiology of Encephalitis Study Group. 2011. Diagnostic strategy used to establish etiologies of encephalitis in a prospective cohort of patients in England. J Clin Microbiol 49:3576-3583.

2. Avellón A, Pérez P, Aguilar JC, Lejarazu R, Echevarría JE. 2001. Rapid and sensitive diagnosis of human adenovirus infections by a generic polymerase chain reaction. J Virol Methods 92:113-120.

3. Belsy A, Odalys V, Alexander P, Clara S, Angel G, Grehete G, Guelsys G, Luis S, Pedro M, Guadalupe GM, Alina L, Pilar PB, Inmaculada C. 2009. Molecular characterization of adenoviral infections in Cuba: report of an unusual association of species D adenoviruses with different clinical syndromes. Arch Virol 154:619-627.

4. Bofill D, Domingo C, Cardeñosa N. Zaragoza J, De Ory F, Minguell S, Sánchez-Seco MP, Domínguez A, Tenorio A. 2006. Human West Nile virus infection in the Ebro delta, Catalonia, Spain. Emerg Infect Dis 12:1163-1164.

5. Boletín Epidemiológico Semanal. 2009. 17:277-288.

6. Cabrerizo M, Echevarria JE, González I, de Miguel T, Trallero G. 2008. Molecular epidemiological study of HEV-B enteroviruses involved in the increase in meningitis cases occurred in Spain during 2006. J Med Virol 80:1018-1024.

7. Casas I, Tenorio A, de Ory F, Lozano A, Echevarría JM. 1996. Detection of both herpes simplex and varicella-zoster viruses in cerebrospinal fluid from patients with encephalitis. J Med Virol 50:82-92.

8. Casas I, Tenorio A, Echevarría JM, Klapper PE, Cleator GM. 1997. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. J Virol Methods 66:39-50.

9. Casas I, Pozo F, Trallero G, Echevarría JM, Tenorio A. 1999. Viral diagnosis of neurological infection by RT multiplex PCR: a search for entero- and herpesviruses in a prospective study. J Med Virol 57:145-151.

10. Casas I, Palacios GF, Trallero G, Cisterna D, Freire MC, Tenorio A. 2001. Molecular characterization of human enteroviruses in clinical samples: comparison between VP2, PV1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. J Med Virol 65:138-148.

11. Casas I, Avellón A, Mosquera M, Jabado O, Echevarría JE, Campos RH, Rewers M, Perez-Breña P, Lipkin WI, Palacios G. 2005. Molecular identification of adenovirus in clinical samples by analyzing a partial hexon genomic region. J Clin Microbiol 43:6176-6182.

12. Cosson A, Tatu L, Decavel P, Parratte B, Rumbach L, Monnier G. 2002. Polyradiculoneuropathie aiguë au cours d'une primo-infection à virus Herpès simplex. Rev Neurol (Paris) 158:833-835.

13. De la Loma A, Trallero G, De Ory F, Tenorio A, Sanz M, Echevarría JM. 2002. Meningitis linfocitaria en España. Posible situación epidémica en el año 2000. Med Clin (Barc) 118:694-695.

14. De Ory F, Gegúndez MI, Fedele CG, Sánchez-Seco MP. 2009. Los virus Toscana, West Nile y de la coriomeningitis linfocitaria como causantes de meningitis en España. Med Clin (Barc) 132:587-590.

15. del Saz SV, Sued O, Falcó V, Agüero F, Crespo M, Pumarola T, Curran A, Gatell JM, Pahissa A, Miró JM, Ribera E. 2008. Acute meningoencephalitis due to human immunodeficiency virus type 1 infection in 13 patients: clinical description and follow-up. J Neurovirol 14:474-479.

16. Delerme S, Castro S, Viallon A, Boutoille D, Bendahou M, Riou B, Ray P. 2009. Meningitis in elderly patients. Eur J Emerg Med 16:273-276.

17. Donoso Mantke O, Vaheri A, Ambrose H, Koopmans M, De Ory F, Zeller H, Beyrer K, Windorfer A, Niedrig M. 2008. Analysis of the surveillance situation for viral encephalitis and meningitis in Europe. Euro Surveill 13:1-10.

18. Dubberke ER, Tu B, Rivet DJ, Storch GA, Apisarnthanarak A, Schmidt RE, Weiss S, Polish LB. 2006. Acute meningoencephalitis caused by adenovirus serotype 26. J Neurovirol 12:235-240.

19. Echevarría JE, Avellón A, Juste J, Vera M, Ibáñez C. 2001. Screening of active lyssavirus infection in wild bat populations by viral RNA detection on oropharyngeal swabs. J Clin Microbiol 39:3678-3683

20. Echevarría JE, Castellanos A, Sanz JC, Pérez C, Palacios G, Martínez de Aragón MV, De Ory F, Royuela E. 2010. Circulation of mumps virus genotypes in Spain from 1996 to 2007. J Clin Microbiol 48:1245-1254.

21. Echevarría JM, De Ory F, León P, Téllez A, Martínez-Martín P, Martín-Fontelos P. 1990. Comparative sensitivity of different criteria for interpreting antibody tests in cerebroespinal fluid from patients with acute viral meningitis. Serodiagn Immunother Infect Disease 4:193-199.

22. Echevarría JM, De Ory F, Echevarría C, Lozano A, Tenorio A, Casas I. 1997a. Participación del virus de la parotiditis en la etiología de las meningitis linfocitarias

agudas diagnosticadas durante el período 1989-1995. Enferm Infecc Microbiol Clin 15:113-114.

23. Echevarría JM, Casas I, de Ory F, Tenorio A, Echevarría C, Lozano A. 1997b. Diagnóstico de laboratorio en casos de encefalitis aguda y subaguda de probable etiología vírica: experiencia en 7 años. Neurología 12:381-383.

24. Echevarría JM, De Ory F, Echevarría C, Lozano A, Tenorio A. 1999. Reemergencia de la meningitis linfocitaria aguda por virus de la parotiditis en España. Enferm Infecc Microbiol Clin 17:373-374.

25. Fernández-Viladrich P, Kaptoul D, García-Vidal C, Domingo C, Niubó J, Martinez-Yélamos S, De Ory F, Tenorio A. 2007. Emergencia de arbovirus como causa de meningitis en pacientes adultos inmunocompetentes en nuestro país. Comunicación a la XII Reunión de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. A Coruña, 2007. Enferm Infecc Microbiol Clin 25 (Especial Congreso):32.

26. Fischer SA, Graham MB, Kuehnert MJ, Kotton, CN, Srinivasan A, Marty FM, Comer JA, Guarner J, Paddock CD, DeMeo DL, Shieh WJ, Erickson BR, Bandy U, DeMaria A Jr, Davis JP, Delmonico FL, Pavlin B, Likos A, Vincent MJ, Sealy TK, Goldsmith CS, Jernigan DB, Rollin PE, Packard MM, Patel M, Rowland C, Helfand RF, Nichol ST, Fishman JA, Ksiazek T, Zaki SR; LCMV in Transplant Recipients Investigation Team. 2006. Transmission of lymphocytic choriomeningitis virus by organ transplantation. N Engl J Med 354:2235-2249.

27. Glaser CA, Gilliam S, Schnurr D, Forghani B, Honarmand S, Khetsuriani N, Fischer M, Cossen CK, Anderson LJ. 2003. In search of encephalitis etiologies: diagnostic challenges in the California Encephalitis Project, 1998-2000. Clin Infect Dis 36:731-742.

28. Gordon L, McQuaid S, Cosby SL. 1996. Detection of herpes simplex virus (types 1 and 2) and human herpesvirus 6 DNA in human brain tissue by polymerase chain reaction. Clin Diagn Virol 6:33-40.

29. Harvala H, Simmonds P. 2009. Human parechoviruses: biology, epidemiology and clinical significance. J Clin Virol 45:1-9.

30. http://www.ine.es

31. Jesús-De La Calle I, Espinosa-García MJ, Pérez-Ramos S, Cruz-Rosales E. Primeros casos confirmados de meningoencefalitis humanas debida a virus West Nile en Andalucía, España. 2012. Enfer Infecc Microbiol Clin 30:426-427.

32. Kaptoul D, Viladrich P, Domingo C, Niubó J, Martínez Yélamos S, De Ory F, Tenorio A. 2007. West Nile virus in Spain. Report of the first diagnosed case in a human with aseptic meningitis. Scand J Infect Dis 39:70-71.

33. Koskiniemi M, Rantalaiho T, Piiparinen H, von Bonsdorff CH, Färkkilä M, Järvinen A, Kinnunen E, Koskiniemi S, Mannonen L, Muttilainen M, Linnavuori K, Porras J, Puolakkainen M, Räihä K, Salonen EM, Ukkonen P, Vaheri A, Valtonen V, Study Group. 2001. Infections of the central nervous system of suspected viral origin: a collaborative study from Finland. J Neurovirol 7:400-408.

34. Kupila L, Vuorinen T, Vainonpää R, Hukkanen V, Marttila RJ, Kotilainen P. 2006. Etiology of aseptic meningitis and encephalitis in an adult population. Neurology 66:75-80.

35. Ledesma J, Fedele CG, Carro F, Lledó L, Sánchez-Seco MP, Tenorio A, Soriguer RC, Saz JV, Domínguez G, Rosas MF, Barandika JF, Gegúndez MI. 2009. Independent lineage of lymphocytic choriomeningitis virus in wood mice (Apodemus sylvaticus), Spain. Emerg Infect Dis 15: 1677-1680.

36. Lema CL, Cisterna DM, Freire MC. 2005. Enfermedad neurológica por adenovirus. Medicina (B. Aires) 65:196-200.

37. Mailles A, Stahl JP; Steering Committee and Investigators Group. 2009. Infectious encephalitis in France in 2007: a national prospective study. Clin Infect Dis 49:1838-1847.

38. Miao LY, Pierce C, Gray-Johnson, DeLotell J, Shaw C, Chapman N, Yeh E, Schnurr D, Huang YT. 2009. Monoclonal antibodies to PV1 recognize a broad range of enteroviruses. J Clin Microbiol 47: 3108-3113.

39. Morita Y, Osaki Y, Doi Y, Forghani B, Gilden DH. 2003. Chronic active VZV infection manifesting as zoster sine herpete, zoster paresis and myelopathy. J Neurol Sci 212:7-9.

40. Nagasawa H, Wada M, Kurita K, Iseki C, Katagiri T, Kato T. 2006. A case of non-herpetic acute limbic encephalitis associated with a type-2 adenovirus infection. Rinsho Shinkeigaku 46:322–327.

41. Navarro JM, Fernández-Roldán C, Pérez-Ruiz M, Sanbonmatsu S, de la Rosa M, Sánchez-Seco MP. 2004. Meningitis por el virus Toscana en España: descripción de 17 casos. Med Clin (Barc) 122:420-422.

42. Ohtsuki N, Kimura S, Nezu A. 2000. Three cases with acute encephalopathy with adenovirus type 7 infection. No To Hattatsu 32:68–72.

43. Palacios Poggio G, Rodriguez C, Cisterna D, Freire MC, Cello J. 2000. Nested PCR for rapid detection of mumps virus in cerebrospinal fluid from patients with neurological diseases. J Clin Microbiol 38:274-289.

44. Palacios G, Jabado O, Cisterna D, De Ory F, Renwick N, Echevarría JE, Castellanos A, Mosquera M, Freire MC, Campos RH, Lipkin WI. 2005. Molecular

identification of mumps virus genotypes from clinical samples: standardised method for analysis. J Clin Microbiol 43:1869-1878.

45. Pérez-Ruiz M, Collao X, Navarro-Marí JM, Tenorio A. 2007. Reverse transcription, real-time PCR assay for detection of Toscana virus. J Clin Virol 39:276-281.

46. Pérez-Ruiz M, Navarro-Marí JM, Sánchez-Seco MP, Gegúndez MI, Palacios G, Savji N, Lipkin WI, Fedele G, De Ory-Manchón F. 2012. Lymphocytic choriomeningitis virus infection within the same geographical area (Southern Spain) during a two-year period. . Emerg Infect Dis 18:855-858.

47. Sánchez-Seco MP, Echevarría JM, Hernández L, Estévez D, Navarro-Marí JM, Tenorio A. 2003. Detection and identification of Toscana and other phleboviruses by RT-nested-PCR assays with degenerated primers. J Med Virol 71:140-149.

48. Sánchez-Seco MP, Rosario D, Domingo C, Hernández L, Valdés K, Guzmán MG, Tenorio A. 2005. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. J Virol Methods 126:101-109.

49. Sanders VJ, Felisan SL, Waddell AE, Conrad AJ, Schmid P, Swartz BE, Kaufman M, Walsh GO, De Salles AA, Tourtellotte WW. 1997. Presence of herpes simplex DNA in surgical tissue from human epileptic seizure foci detected by polymerase chain reaction: preliminary study. Arch Neurol 54: 954-960.

50. Sanz JC, Mosquera MM, Echevarria JE, Fernández M, N. Herranz, Palacios G, De Ory F. 2006. Sensitivity and specificity of Immunoglobulin G titer for diagnosis of mumps virus in infected patients depending on vaccination status. APMIS 114:788-794.

51. Schnurr D, Bollen A, Crawford-Miksza L, Dondero ME, Yagi S. 1995. Adenovirus mixture isolated from brain of an AIDS patient with encephalitis. J Med Virol 47:168-171.

52. Strauss R, Granz A, Wassermann-Neuhold M, Krause R, Bago Z, Revilla-Fernandez S, Simón-Soria FS, Echevarría JE, Popow-Kraupp T, Allerberger F, Schönbauer M, Hrabcik H. 2005. A human case of travel-related rabies in Austria, September 2004. Euro Surveill 10:225-226.

53. Studahl M, Bergström T, Hagberg L. 1998. Acute viral encephalitis in adults. A prospective study. Scand J Infect Dis 1998 30: 215-220.

54. Téllez A, Bernal A, De Ory F, Estévez E, Martínez Martín P, Barreiro G, Martín FP, Echevarría JM. 1989. Meningitis linfocitaria. Estudio virológico. Análisis del estudio realizado entre 1984-1986 (632 casos). Enferm Infecc Microbiol Clin 7:77-82.

55. Vázquez-Morón S, Avellón Calvo A, Echevarría JE. 2006. RT-PCR for detection of all seven genotypes of lyssavirus genus. J Virol Methods 135:281-287.

56. Vázquez-Morón S, Juste J, Ibáñez C, Ruiz-Villamor R, Avellón A, Vera M, Echevarría JE. 2008. Endemic circulation of European bat lyssavirus type 1 in serotine bats, Spain. Emerg Infect Dis 14:1263-1266.

57. Vincentelli C, Schniederjan MJ, Brat DJ. 2010. 35-year-old HIV-positive woman with basal forebrain mass. Brain Pathol 20:265-268.