

biologic results. Since both strains were identified as *L. pneumophila* serogroup 1, we performed sequence-based typing (SBT) (3) of 6 genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA*) by using the protocol and database of EWGLINET. Both isolates showed identical SBT patterns (2,3,18,15,2,1).

Isolates from 4 patients in the Zaragoza outbreak were identified at the Spanish Reference Laboratory as *L. pneumophila* serogroup 1 (Philadelphia monoclonal antibody type) and had identical SBT patterns (3,4,1,1,14,9). Collaboration between public health authorities in France and Spain enabled us to eliminate the association of patient 2 with the Zaragoza outbreak and establish an association of both patients with the same site in France. Control measures were taken at the hotel, but we could not obtain environmental cultures for comparison with those of the patients. Lack of environmental data prevented investigation of the relationship with the other accommodation sites visited.

The SBT method provides robust genotyping with high discriminatory power (index of discrimination >0.94) (3). This method is less effective at discriminating between strains than pulsed-field gel electrophoresis (4), but it shows excellent reproducibility and may be useful in epidemiologic investigation of outbreaks caused by *L. pneumophila*. The availability of an online database with accessible information is key for sharing results and determining the geographic distribution of isolates that cause Legionnaires' disease (4,5).

This study demonstrates the critical role of sharing results between countries that participate in a network. Agreement is essential on a standardized questionnaire that includes more information on the patient's exposure to a disease. Moreover, despite the performance of the urine antigen test, cultures of clinical samples should be encouraged by clinicians and microbi-

ologists. This step would permit use of techniques, such as SBT, in reference laboratories and sharing of results. Our investigation would have been more difficult without this technique in identifying the site where the infection potentially originated.

**Rosa Cano,* Sophie Jarraud,†
Joana Pardos,‡
Christine Campese,§
and Carmen Pelaz***

*Instituto de Salud Carlos III, Madrid, Spain; †Institut National de la Santé et de la Recherche Médicale E0230, Lyon, France; ‡Public Health Laboratory of Girona, Catalonia, Spain; and §Institut de Veille Sanitaire, Saint-Maurice, France

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Address for correspondence: Carmen Pelaz, Laboratorio de Legionella, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda 28220 Madrid, Spain; email: cpelaz@isciii.es

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Influenza A Virus PB1-F2 Gene

To the Editor: Recently, Chen and co-workers described the expression of an 11th influenza A virus protein, designated PB1-F2 because this protein is encoded in the +1 open reading frame of the segment-2 RNA (1). Later, Chen et al. presented a preliminary analysis of 336 PB1 sequences from GenBank (2). We have extended the work on PB1-F2 and analyzed 1,864 partial and complete segment-2 sequences deposited in GenBank; these sequences belong to 79 influenza A virus subtypes. In summary, the following 8 observations should receive attention:

First, the size of PB1-F2 polypeptides ranges from 79 to 101 amino acids (aa); most isolates encode versions of either 87 or 90 aa. Because polypeptides of 79 aa are located within mitochondria, their truncation has no effect on the protein function. The frequency of the 79-aa PB1-F2 is ≈5%.

Second, a functional PB1-F2 is expressed by 92% of all segment-2 sequences, i.e., a polypeptide >78 aa. The proportion of intact PB1-F2 varies according to host (humans 90%, swine 76%, other mammals 100%, birds 95%).

Third, the H1N1 subtype comprises 3 genetic lineages. One clade has 2 branches: 1 branch includes the human viruses, with the pandemic 1918 virus at its root; the other branch includes the classic swine viruses. The third clade represents the European porcine isolates. Although all classic swine sequences have a truncated PB1-F2 (in-frame stop codons after 11, 24, and 35 codons), the early human isolates (H1N1 sequences from 1918 through 1947) have an intact PB1-F2. After 1956, however, a mutation became prevalent such that the recent sequences starting from A/Beijing/1/56 terminate after 57 codons. An exception to

this rule is A/Taiwan/3355/97. Two human H1N1 isolates with an intact PB1-F2 coding sequence cluster in the H3N2 clade (A/Kiev/59/79, A/Wisconsin/10/98). The PB1 sequences of European porcine influenza A virus isolates cluster with European porcine H3N2 and H1N2.

Fourth, all H2N2 sequences are monophyletic and encode an intact PB1-F2. Fifth, the main sequence cluster of the H3N2 subtype comprises 3 branches: 1) porcine H3N2 and porcine H1N2 sequences from the United States, 2) porcine H3N2 isolates from Hong Kong and human H1N2, and 3) recent human H3N2 and some Japanese H3N2 isolates. Most of these sequences encode an intact PB1-F2.

Sixth, the cluster of European porcine influenza A virus isolates comprises the subtypes H1N1, H1N2, and H3N2. The lack of distinct clades for each subtype indicates frequent reassortment in the evolution of these viruses. Of the segment-2 sequences, 56% encode an intact PB1-F2.

Seventh, other porcine isolates of various subtypes represent trans-species infections or single reassortment events. And eighth, the segment-2 sequences of many avian influenza A virus isolates encode intact PB1-F2. Considerable proportions of truncated PB1-F2 genes were found in the H5N2, H6N6, H9N2, and H13N2 subtypes. However, because of the small number of sequences available, this observation may not be important.

In conclusion, PB1-F2 is expressed in most avian and many porcine influenza A virus isolates. This finding contrasts with those in the initial publication, which stated that PB1-F2 is not expressed in many animal isolates, particularly those of porcine origin (1). Because PB1-F2 was described as a proapoptotic protein probably counteracting the host immune response, why numerous

human and porcine isolates lack this protein without selective disadvantage remains unclear.

**Roland Zell,* Andi Krumbholz,*
and Peter Wutzler***

*Friedrich Schiller University, Jena, Germany

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Address for correspondence: Roland Zell, Institute of Virology and Antiviral Therapy, Medical Centre at the Friedrich Schiller University, Hans Knoell Str 2, D-07745 Jena, Germany; email: Roland.Zell@med.uni-jena.de

In response: Zell et al. (1) performed an extensive genetic investigation of PB1-F2, based on up-to-date GenBank sequences. Their sample size (1,864) greatly outnumbered ours (336) in a previous study (2) and thus definitely better portrays the genetic characteristics of PB1-F2. We appreciate their analyzing these samples by subdividing nonhuman strains into different species, which we did not do (2). Their analysis is especially meaningful for the global pandemic threat from avian influenza viruses, which increases the need to study interspecies adaptation and transmission.

Zell et al. found that 92% of PB1 RNA encodes a functional PB1-F2, compared with our 79% (264/334), which supports the increasingly crucial role of PB1-F2 in influenza virology. They found the proportion of intact human PB1-F2 to be 90%, a

substantial boost from our 68% (67/99), which was based on data from late 2003 (2). This increase is apparently caused by the increasing number of human H3N2 sequences (mostly encoding an intact PB1-F2 compared with H1N1) deposited in the past 2 years.

Human H1N1 from 1918 through 1947 contains full-length PB1-F2, whereas human H1N1 beginning in 1956 has a truncated PB1-F2 after codon 57. As reported by Zell et al., only 3 human H1N1 strains contain full-length PB1-F2: A/Kiev/59/79, A/Taiwan/3355/97, and A/Wisconsin/10/98. The PB1 genes of A/Kiev/59/79 and A/Wisconsin/10/98 were found clustered with human H3N2 as a result of natural reassortment between human H1N1 and H3N2 strains. On the other hand, the asynonymous mutation found on A/Taiwan/3355/97 enabled the translation to get past the usual stop codon at position 58, which other H1N1 strains exhibit. A/Taiwan/3355/97 (H1N1) was isolated from a patient with severe pneumonia. Animal study has demonstrated that the existence of full-length PB1-F2 contributed to pathogenesis in mice (3). We speculate that the expression of a full-length PB1-F2 may contribute to disease severity in humans.

The C-terminal domain of PB1-F2 contains the mitochondrial signal and can trigger apoptosis in specific immune-related cells. Our recent work (4) comparing avian and human influenza A viruses also found that many species-associated amino acid signatures are located on the C terminal of PB1-F2. This finding highlights the importance of further investigating the role of PB1-F2 on interspecies infection.

**Guang-Wu Chen*
and Shin-Ru Shih***

*Chang Gung University, Taoyuan, Taiwan

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Address for correspondence: Guang-Wu Chen, Chang Gung University, Department of Computer Science and Information Engineering, 259 Wen-Hua 1st Rd, Kwei-shan, Taoyuan, Taiwan 333; email: gwchen@mail.cgu.edu.tw

Enterovirus 75 and Aseptic Meningitis, Spain, 2005

To the Editor: Although most human enterovirus (EV) (genus *Enterovirus*, family *Picornaviridae*) infections are asymptomatic, they can cause upper respiratory illness, febrile rash, aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis, and neonatal sepsislike disease (1). Most EVs have been implicated in aseptic meningitis, most notably echovirus (E) 30, 9, 6, and 11 and coxsackie B virus (CBV) type 5 (2); other serotypes are less frequently associated with neurologic disease.

New EV serotypes have come to light, chiefly as a result of molecular typing methods (3-6). EV75 was proposed as a new serotype of the EV genus in 2004 (5). Retrospective analysis showed that it had circulated sporadically in Asia, the United

States, and Africa since at least 1974. Only 8 isolates of this serotype have been reported worldwide, in 1974, 1985, 1986, 1987 (n = 2), 1998, and 2000 (n = 2). Infection in those cases was associated with respiratory disease, acute flaccid paralysis, neonatal jaundice, failure to thrive, or unspecified neurologic disease or was asymptomatic. At the time of writing this manuscript, EV75 had not been linked to aseptic meningitis.

From May 2005 through January 2006, 106 EVs were received for typing from Spanish hospital laboratories; 46 of them were from patients with aseptic meningitis, 10 from patients or contacts of patients with acute flaccid paralysis, 27 from patients with fever, 7 from patients with respiratory diseases, and 16 from other patients. Twenty EVs could not be typed by serum neutralization (7); however, 3' terminus VP1 gene sequence analysis (8) showed that they were E18 (n = 7), CBV3 (n = 1), and E16 (n = 2); 2 could not be typed with serologic or molecular methods because the 3' terminus of VP1 gene amplification was negative. The analysis of the 3' terminus of VP1 gene of the remaining 5 cerebrospinal fluid (CSF) and 3 nasopharyngeal isolates showed that they were similar to the recently proposed EV75 serotype (5). These 8 isolates were obtained from samples from children in Bilbao (n = 3), Granada (n = 3), Barcelona (n = 1), and the Canary Islands (n = 1). In 4 patients with aseptic meningitis, EV75 was isolated from CSF. EV75 was isolated from CSF of a fifth patient who had symptoms of fever and irritability. The remaining 3 EV75 isolates were from nasopharyngeal swabs of children who had fever, respiratory disease, or gastroenteritis. All isolates were grown in cell lines (rhabdomyosarcoma, lung adenocarcinoma, and human fetal lung fibroblast) and identified as EV by immunofluorescence with pan-EV antibody assays (Pan Entero Blend Chemicon,

Temecula, CA, USA, and Monoclonal Mouse Anti-Enterovirus, Dako, Glostrup, Denmark).

Phylogenetic analysis of the isolates from 2005 was performed on the basis of complete VP1 gene sequence (GenBank accession nos. DQ468137-DQ468142). The 5' terminal domain was obtained by reverse transcriptase-PCR with specific primers EV75_sense: 5'-GAAAGCTTYTTC-CAAGGGGA-3' and EV75_anti: 5'-GAGAAGTGKGCACCAWCCATC-3'. Phylogenetic analysis of the Spanish isolates and representatives of all other species B EVs showed that the Spanish isolates clustered (bootstrap value 100, Figure) with strains USA/OK85-10362, ETH74-1341, USA/VA86-10363, USA/CT87-10364-5, OMA98-10366, and BAN00-10367-8 (accession nos. AY556063-AY556070), corresponding to the proposed EV75. The Spanish isolates constitute a subgroup (bootstrap value 100, Figure). The similarity between the Spanish cluster and other EV75 isolates was 82.8%-85.4% at the nucleic acid level. Although the entire VP1 sequence was not available for the isolates from 2006, the VP1 3' terminal analysis showed the strains belonged to the same cluster.

To our knowledge, this is the first isolation of EV75 in Spain. Indeed, isolation of EV75 has not been reported in Europe. Given that the European EV75 isolate grows easily in a variety of cell lines, is detected by common EV genus-specific antibodies, and that EV surveillance and typing were performed in Spain since 1988 (2), EV75 might have begun to circulate in Spain recently. However, because isolates are not obtained from all aseptic meningitis patients and many EVs are detected by PCR but never typed, we cannot rule out the possibility of previous asymptomatic circulation.

The European strains of EV75 appear to represent a different evolutionary lineage than those previously