Received: 14 November 2017 | Revised: 11 May 2018 | Accepted: 10 June 2018

DOI: 10.1111/zph.12501

ORIGINAL ARTICLE

WILEY

Detection of Japanese Encephalitis Virus in bone marrow of healthy young wild birds collected in 1997–2000 in Central Italy

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Abstract

Japanese Encephalitis Virus (JEV) is a flavivirus responsible for an important zoonotic, vector-borne disease included in the OIE list. JEV is endemic in a large area of Asia. In Italy, JEV has been found in dead birds collected in 1997–2000 and in a pool of *Culex pipiens* mosquitoes collected in 2010. Viral ecology in the inter-epidemic periods is not known. The aim of this study was to investigate JEV in FFPE archival samples of healthy birds collected in 1997–2000 in Tuscany (Italy) in the same area and a few months after collection of birds resulted infected by JEV. Different samples from 37 young birds and 83 adults were available. Immunohistochemistry detected JEV antigen only in bone marrow samples from 12 young healthy birds. Positive cells were morphologically referable to monocyte–macrophages lineage and were positive for anti-CD11b in serial sections. Real-time PCR detected JEV RNA in four of these samples. These results suggest that healthy birds can harbour JEV in bone marrow cells, while no other organs resulted infected. The role of healthy birds in JEV ecology should be better investigated. Surveillance programmes should include sampling of the most appropriate target organs.

KEYWORDS

bone marrow, immunohistochemistry, Japanese encephalitis virus, real-time PCR, reservoir, wild birds

1 | INTRODUCTION

Japanese Encephalitis Virus (JEV) is a flavivirus closely related to West Nile Virus (WNV) and is responsible for an important zoonotic, vector-borne disease included in the OIE list. JEV is endemic in a large area of Asia, with cases being reported from Japan, China, India, the Philippines and Pakistan (Erlanger, Weiss, Keiser, Utzinger, & Wiedenmayer, 2009). Encephalitis is the most severe disease observed in humans and horses, which are considered as dead end hosts. More than 60,000 cases occur in humans annually, despite established or developing vaccination programmes, and children are more affected than adults. Mortality of encephalitis can reach 30%, and approximately a half of the surviving patients suffer from neuropsychiatric sequelae (Campbell et al., 2011).

Japanese Encephalitis Virus is maintained in an enzootic transmission cycle among mosquitoes and sylvatic avian reservoirs; domestic and feral pigs act as amplifying hosts (Mansfield, Hernández-Triana, Banyard, Fooks, & Johnson, 2017; Ricklin et al., 2016). WNV is proved to circulate in Europe from decades (Hubálek & Halouzka, 1999) and is actually considered as an emerging pathogen in Italy, whereas JEV has been found only sporadically in Italy in mosquito and bird samples (Mani et al., 2009; Platonov et al., 2012; Ravanini et al., 2012). Formalin-fixed tissue samples from birds collected in Italy (Padule di Fucecchio, Tuscany) during episodes of bird mortality between 1997 and 2000 resulted positive for JEV group antigens by immunohistochemistry. RT-PCR assays detected JEV but not WNV sequences in six birds collected in 2000 and in one bird collected in 1997 (Platonov et

ZPH | Journal Name
12501 | Manuscript No.
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Dispatch: 23-6-2018 | No. of pages: 7
CE: Murugan S | PE: Chitra R.

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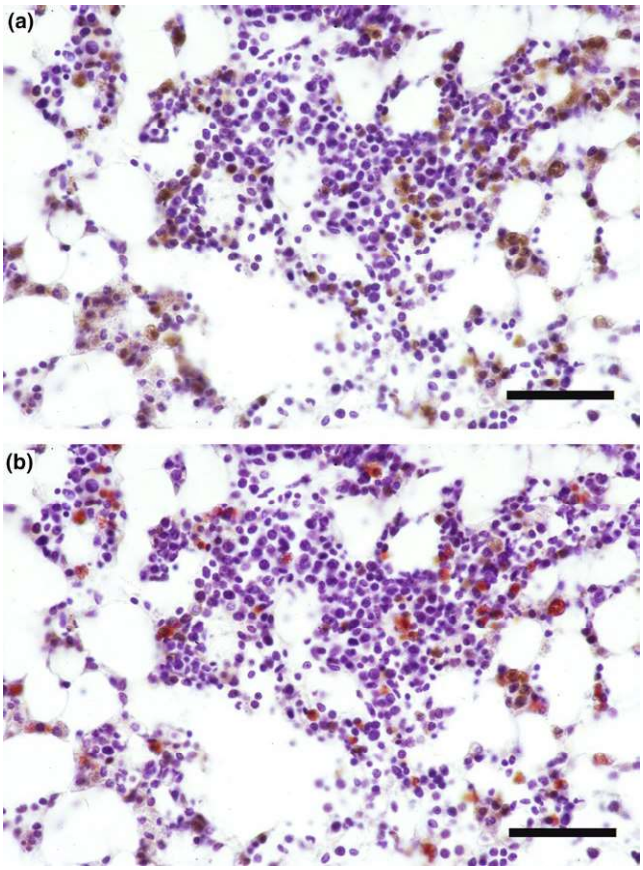


FIGURE 1 (a) Blackbird (*Turdus merula*); bone marrow with many cells of myeloid lineage that showed a strong brown stain (DAB stain), indicative of a positivity for Cd11b antibody, interspersed between other not unstained erythroid lineage cells and adipocytes (Nuclear counterstain with Harris's Hematoxylin. Scale bar = 100 μm). (b) The consequent section of bone marrow belonging to the same bird, stained with JEV specific antibody: note the lower number of positive—orange-red stained cells (Vector Red stain) that correspond to the same cells stained also for Cd11b in section A. (Nuclear counterstain with Harris's Hematoxylin. Scale bar = 100 μm)

al., 2012). Subsequently, a partial sequence of the JEV NS5 gene was found in a pool of *Culex pipiens* collected in summer 2010 in Sasso Marconi (Italy), which is distant about 100 km from Padule di Fucecchio (Ravanini et al., 2012). Subsequent investigations on sera and cerebrospinal fluid samples from 38 human subjects with

Impacts

- Japanese encephalitis virus (JEV) was detected in archival samples (1997–2000) of young healthy birds collected in Italy a few months later and in the same area where birds resulted infected by JEV.
- JEV antigen and partial genome sequences were detected only in bone marrow samples but not in other organs.
- Bone marrow of young healthy birds could be a reservoir of JEV, which can be transported by asymptomatic migratory birds to other geographic areas. These findings should be considered to collect the proper samples for detecting JEV during surveillance programmes

clinical symptoms of acute meningoencephalitis collected in the province of Bologna in 2011 did not reveal IgM or IgG anti-JEV antibodies (Gaibani et al., 2011). The ecology of JEV in Italy is not well investigated and, in particular, the epidemiology of JEV during the inter-epidemic periods is not clear.

The aim of this study was to investigate the JEV presence in archival samples of healthy birds collected in Tuscany (Italy) a few months after collection of birds resulted infected by JEV.

2 | MATERIALS AND METHODS

2.1 | Samples

Archival formalin-fixed and paraffin-embedded (FFPE) samples collected from 120 healthy birds (*Turdus merula*, *Turdus pilaris*, *Turdus philomelos* and *Turdus iliacus*) were available for this study. In particular, 37 (30.8%) were young birds (less than 2 months old) and 83 (69.2%) were adults. The samples had been collected during the hunting seasons 1997–2000 (late September–January) in the same geographic areas and more than one month after episodes of mortality of birds subsequently resulted infected by JEV (Platonov et al., 2012).

Three serial sections were obtained from FFPE blocks of kidney, spleen, liver, lung, brain, intestine and bone marrow and were placed

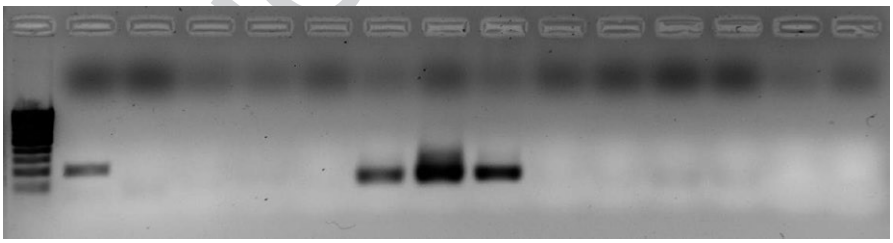
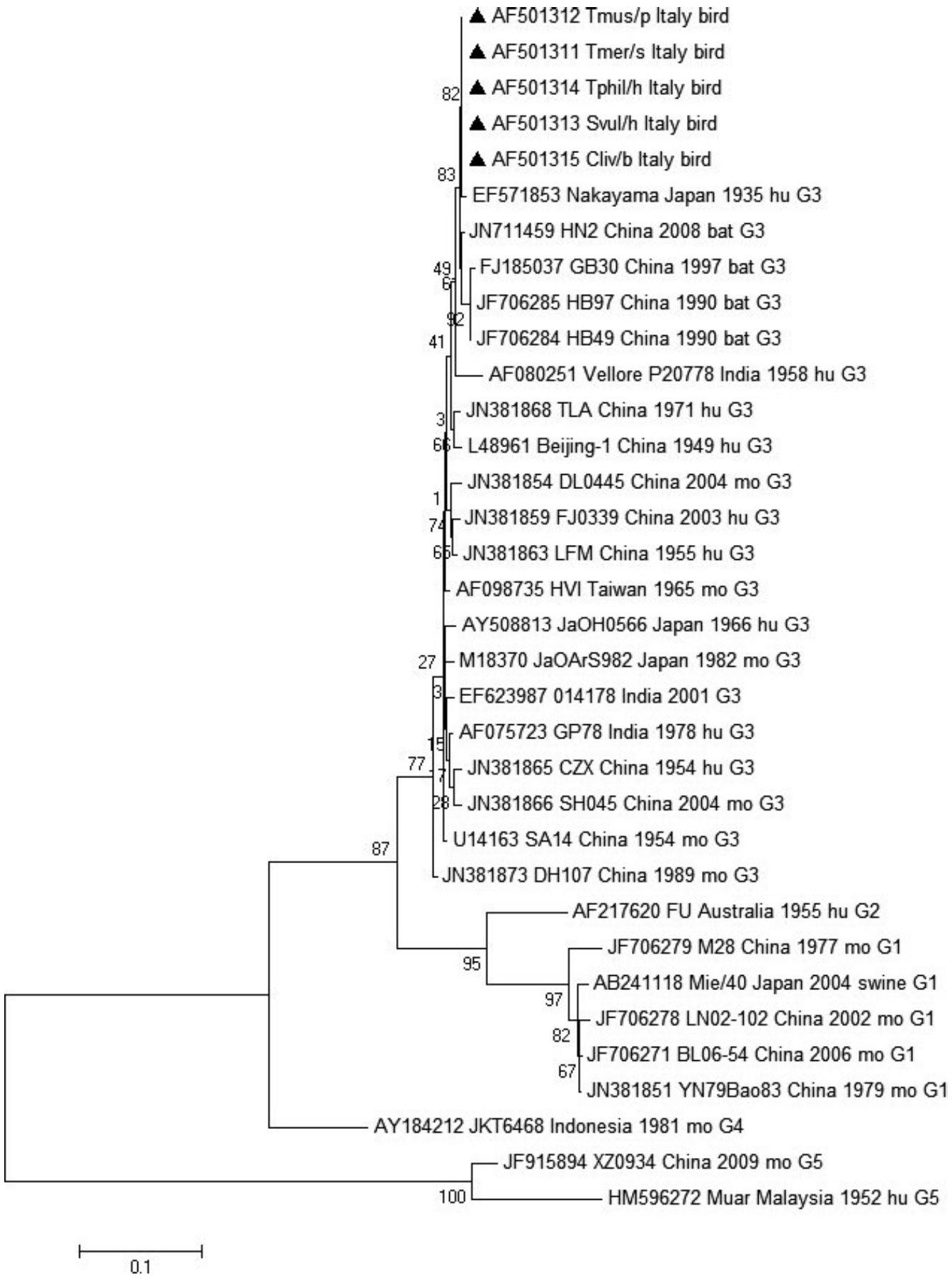


FIGURE 2 1.5% agarose gel electrophoresis of Sybr green real time PCR products. Line 1:100 bp ladder; lines 2, 7, 8, 9: positive samples; lines 3, 4, 5, 6, 10, 11, 12, 13: negative samples; lines 14 and 15: negative controls

FIGURE 3 Molecular Phylogenetic analysis by maximum likelihood method based on the Kimura 2-parameter model. The analysis was based on sequence information derived from a 156 bp region spanning the NS5 gene of JEV. The sequences of JEV previously detected in Italy in birds (Platonov et al., 2012) have been included in the data set (▲)

LOW RESOLUTION FIG



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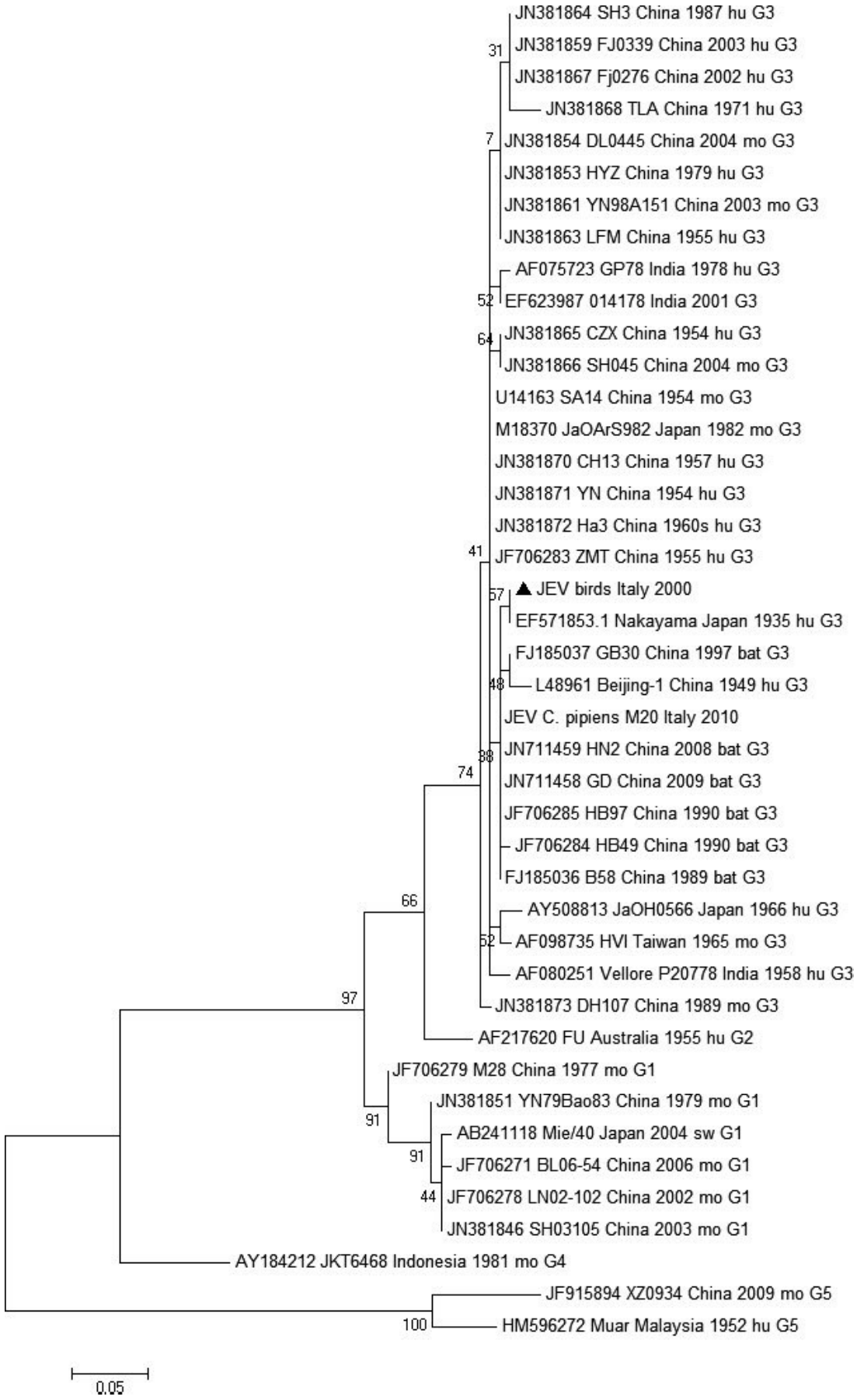

FIGURE 4 Molecular Phylogenetic analysis by maximum likelihood method based on the Kimura 2-parameter model. The analysis was based on sequence information derived from a 551 bp region spanning the gE gene of JEV. The sequences of JEV previously detected in Italy in birds (Platonov et al., 2012) have been included in the data set (▲)

on positively charged slides (Superfrost plus; Histoline, Milan, Italy) for histopathology (haematoxylin and eosin staining) and immunohistochemistry (IHC).

On the basis of IHC results obtained, additional four serial sections 10 μm thin were cut from bone marrow blocks and were placed in a 2.0 ml tube for RNA extraction, paying special attention to avoid cross-contamination. Negative control FFPE samples were also used.

2.2 | Immunohistochemistry

Immunohistochemical staining was carried out using a rabbit JEV-specific antibody at a dilution of 1:250 (pAb; PG 10004; Genesis Biotech Inc.). The consequent sections were stained using a rabbit CD11b-specific antibody, diluted 1:100 (ab75476, ABCAM, pAb). After incubation with biotin-labelled secondary antibodies, the binding of the antibody was detected with the Elite kit (Vector Laboratories) and the immunoreaction was developed using a brown chromogen (DAB, Vector).

2.3 | RNA extraction and PCR

RNA was extracted by RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion, USA). After deparaffinization, slides were incubated with protease at 50°C for 15 min and at 80°C for 15 min. Subsequent RNA isolation steps have been performed using the Filter Cartridge following the procedure reported in the kit. RNA was eluted in 60 μl Elution Solution. Two microlitres of RNA were immediately retrotranscribed to cDNA in 20 μl final volume following the kit instructions (PrimeScript RT reagent Kit - Perfect Real Time, Takara, Japan). Real-time PCR was carried out in a total reaction volume of 20 μl, containing 10 μl 2× SYBR Premix Ex Taq (TIi RNase H Plus; Takara, Japan), 0.4 Rox Reference Dye 50× (Takara, Japan), 10 pmol F3 forward primer (ACGAGACCGATCAATCGCT), 10 pmol B3 reverse primer (CTTTGTGGACGATCTTCGCT) and about 100 ng of cDNA. The primers amplify a 220 bp sequence of NS1 gene of JEV and have been recently designed for standardization of real-time PCR (Dhanze et al., 2015). Reactions were carried out in a Step One instrument (Applied Biosystem) with the following programme: 95°C for 30 s followed by 45 cycles of PCR at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Following amplification, a melting curve analysis (Tm) was performed to verify the authenticity of the amplified products. On the basis of previous studies, a Tm of about 83.5°C was expected (Dhanze et al., 2015). As a further control, real-time PCR products were visualized by agarose gel electrophoresis.

Conventional PCR was carried out on positive samples to obtain products for sequencing. The PCR mix consisted of Taq PCR Master Mix 1X (Qiagen, Germany), each of the primers F3 and B3 0.4 mM, 5 μl of cDNA and water up to 100 μl final volume. PCR was conducted as previously reported (Dhanze et al., 2015).

2.3.1 | Phylogenesis

Model: Jukes-Cantor.

For phylogenetic analysis, JEV gE and NS5 gene sequences obtained from Italian birds (Platonov et al., 2012) and mosquitoes (Ravanini et al., 2012), GenBank sequences from strains with higher identity with our sequences by BLAST analysis and sequences belonging to different JEV genotypes (Gao, Liu, Li, Fu, & Liang, 2015) were used.

Sequences were aligned using Clustal X (Larkin et al., 2007) and manual editing was performed with BioEdit version 7.2.5 (Hall, 1999). MEGA version 7.0 was used to select the simplest evolutionary model that adequately fitted the sequence data (Kumar, Stecher, & Tamura, 2016). The model with the lowest Bayesian Information Criterion (BIC) was considered to describe the substitution pattern the best. On the basis of the BIC values obtained, the model Kimura-2 parameter with a discrete Gamma distribution (+G) with five parameters was used. Phylogeny was estimated by the neighbour-joining algorithm (NJ) and the maximum likelihood (ML) method. In both analyses, 1,000 bootstrap replicates were used.

3 | RESULTS

Japanese Encephalitis Virus antigen was detected only in bone marrow of 12 young birds by IHC. The staining was detectable only in cells morphologically referable to monocyte–macrophages lineage, which were positive for anti-CD11b in serial sections (Figure 1a,b).

PCR products of 220 bp with a Tm of about 83.5 ± 0.3°C were obtained from four of these 12 bone marrow samples by Real-Time PCR (Figure 2) but not by conventional PCR. All other samples from adults and young birds were negative for JEV. As new sequences were not obtained from these samples, phylogenetic analysis was performed with JEV sequences obtained previously (Platonov et al., 2012) form birds dead a few months earlier in the same areas. The results confirmed that JEV found in Italian birds belongs to genotype III and NS5 sequences had the higher homology with JEV strains Nakayama, obtained from human brain in Japan in 1935 (Figures 3 and 4).

4 | DISCUSSION

Japanese Encephalitis Virus is primarily a cause of human encephalitis, but a range of domestic animals are susceptible to disease. Despite infection in livestock is frequently asymptomatic, severe

encephalitis in horses and stillbirth and congenital deformity are reported in pigs (Mansfield et al., 2017). JEV is endemic in many areas of Asia, and for long time, it has been never reported in Europe. However, JEV has been found in dead birds collected between 1997 and 2000 and in a pool of *C. pipiens* mosquitoes collected in 2010 in Italy (Platonov et al., 2012; Ravanini et al., 2012), suggesting a potentially underestimated circulation of JEV outside of Asia. In this study, we investigated archival samples collected from birds in the same areas where JEV-infected birds had been collected in Tuscany (Italy). Healthy birds were killed during the hunting seasons immediately subsequent to the sampling of dead birds resulted infected by JEV, about one-two months later, to evaluate if healthy migratory birds can be reservoir of the virus and which organs are mostly infected. A total of 12 samples resulted positive by IHC for JEV antigen; positive cells were found only in bone marrow and were belonging to the monocyte/macrophage lineage cells. Primer specific for the NS1 gene of JEV gave expected products in four of 12 positive samples only by real-time PCR but not by conventional PCR, probably because the low amount of virus. All JEV-positive samples were from young bird, while all adults were negative in all organs tested.

Experimental subcutaneous infection of ducklings and chicken with JEV has shown that youngest animals were more susceptible to the infection than older animals and that the mean peak viremia in birds of both species decreased as the age at infection increased from 2 to 42 days, suggesting that young poultry may be amplifying hosts of importance in disease-endemic regions (Cleton, Bosco-Lauth, Page, & Bowen, 2014). In our study, young birds resulted infected by JEV were younger than 2 months and thus they were born probably when episodes of bird mortality occurred in Tuscany. They could become infected during the epidemic period some months earlier, or by mosquitoes survived in the cold season a few days earlier. These findings suggest that asymptomatic young birds can be subclinically infected and could play a role in JEV epidemiology, serving as a possible source of virus.

Because the actual epidemiological situation, the compulsory surveillance programme for West Nile Virus in Italy includes sampling of hearth, brain, kidney and spleen of *Pica pica*, *Corvus corone cornix* and *Garrulus glandarius* for RT-PCR investigations. This sampling programme could be not optimal for JEV detection because, to our knowledge, no data are available about the susceptibility of these species to the virus. Furthermore, our data show that healthy birds can harbour JEV only in bone marrow, which is not included in the sampling programme. Although more investigations are required, we suggest Real-Time PCR tests on bone marrow samples from healthy young birds for detecting JEV circulation.

Unfortunately, new genetic sequences were not obtained from these samples; thus, phylogenetic studies were carried out on sequences obtained from bird dead in the same geographic areas 1–2 months earlier (Platonov et al., 2012). Previous analysis by BLAST revealed that these sequences had the highest identity with the genotype III strain Kakayama. Our data confirm that Italian birds were infected by a genotype III JEV. Unfortunately, only FFPE samples were available and longer sequences are not obtainable. Aligning of NS5 sequences of JEV found in Italian birds collected in 1997–2000 (Platonov et al., 2012) with those obtained from *Culex* in Italy in 2010 (Ravanini et al., 2012) demonstrated only a single mutation G/A at position 96 in the nucleotidic sequence, which is silent because the amino acidic sequences are identical. Although the sequences available from Italian samples are too short to obtain definitive data, we speculate that the same strain found in 1997–2000 circulated subclinically in that area with low levels of viraemia in wild animals and it was found in *Culex* during entomological surveillance activity in 2010. Otherwise, migratory birds could periodically reintroduce genotype III JEV from endemic regions. JEV genotypes GI and GIII are the most diffuse genotypes in the world. While genotype I strains are mainly present in tropical areas, genotype III strains are mainly found in temperate countries such as Central Asia (Schuh, Ward, Leigh Brown, & Barrett, 2013), which could be the origin of the strains found in Italy.

In conclusion, young healthy birds are suspected reservoir of JEV. Their role in maintaining the virus in the inter-epidemic periods in a specific area or in transporting it for long distance should be better investigated. Bone marrow could be a reservoir organ and it should be evaluated whether monocyte/macrophages lineage cells are persistently infected by JEV. These findings can be helpful for designing specific surveillance programmes to detect JEV circulation in Europe.

CONFLICT OF INTEREST

None.

AUTHORS' CONTRIBUTIONS

SP and GR designed the study and wrote the article, all authors carried out experiments, participated in the results interpretation and approved the final manuscript.

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How to cite this article: Preziuso S, Mari S, Mariotti F, Rossi G. Detection of Japanese Encephalitis Virus in bone marrow of healthy young wild birds collected in 1997–2000 in Central Italy. *Zoonoses Public Health*. 2018;00:1–7. https://doi.org/10.1111/zph.12501