Marek's disease in genetically susceptible Cochin chickens in Italy: a case report

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Paralysis.

Summary
The present study investigates an outbreak of classical Marek's disease (MD) in backyard Cochin chickens reared for hobby in Italy. Examined chickens showed spastic paralysis of the legs and at necropsy, enlargement and discoloration of the peripheral nerves and plexuses that matched microscopic A- and B- type MD lesions. Molecular analysis of the *meq* gene of the detected *Gallid alphaherpesvirus* 2 (GaHV-2) strain, showed typical markers of low virulence and the strain shared the entire *meq* gene sequence with strains circulating in Italian backyard chickens. Furthermore, the haplotype B[19] of the major histocompatibility complex (MHC) was defined in the affected chickens, showing that the birds possessed a genetic profile of high susceptibility to MD, allowing the appearance of a classical nervous clinical form after infection with an apparently low pathogenicity GaHV-2 strain. Trade of live ornamental purebred chickens occurs frequently between hobby farmers and biosecurity practices, such as quarantine periods, should be applied to avoid the introduction of infected animals. Veterinarians should raise awareness of this issue and promote the use of vaccines against MD.

Introduction

Marek's disease (MD) is a disease of chickens caused by a lymphotropic and ubiquitous oncogenic virus, *Gallid alphaherpesvirus* 2 (GaHV-2), belonging to the genus *Mardivirus* of the *Alphaherpesvirinae* subfamily. Four GaHV-2 pathotypes are currently recognized: mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) (Witter 1997).

GaHV-2 infection may induce neoplastic transformation of T cells resulting in development of lymphomas in the visceral organs, peripheral nerves or muscles and skin (Schat and Nair 2013). Birds may experience different clinical signs according to the virulence of the strains. Weakly virulent strains are responsible for the classical form of the disease characterized by spastic paralysis of the extremities, while infection with highly virulent strains results in the development of multiple visceral lymphomas or paralysis (Witter 1997).

Within the GaHV-2 genome, the Marek's *Eco* RI-Q (*meq*) gene has been recognized as playing a crucial role in GaHV-2-induced neoplastic transformation, in association with other auxiliary genes. The *meq* gene is generally 1,020 base pairs (bp)-long and encodes for Meq, a 339-amino-acid basic leucine zipper (bZIP) protein (Chang *et al.* 2002). The *meq* gene sequence analysis is frequently used for the prediction of GaHV-2 strain virulence thanks to the polymorphic nature of the gene and to the presence of molecular markers of virulence (Shamblin *et al.* 2004, Renz *et al.* 2012, Padhi and Parcells 2016), such as the number of stretches of four proline molecules (PPPP) within the transactivation domain of the Meq protein.

The well-known genetic resistance to MD in chickens is associated with several chicken genes, in particular, the B locus of the chicken major histocompatibility complex (MHC) seems to play a significant role in conferring resistance to MD

(Bumstead and Kaufman 2004). The existence of a hierarchy of resistance determined by B haplotypes has been revealed, with B^{21} haplotype conferring the strongest resistance to MD and B^{19} conferring the greatest susceptibility (Briles *et al.* 1977, Blankert *et al.* 1990).

Rearing backyard chickens is a common practice worldwide, especially in rural areas. Although infectious disease surveillance in backyard flocks is limited (Felice *et al.* 2019) and mostly undocumented, MD is recognized as one of the leading causes of mortality in these birds in several countries. MD in backyard flocks has been reported in Mexico (Rodriguez *et al.* 1997), the United Kingdom (Whitehead and Roberts 2014), Finland (Pohjola *et al.* 2015), Ethiopia (Demeke *et al.* 2017), the United States (Mete *et al.* 2013, Crespo and Senties-Cue 2015, Mete *et al.* 2016, Bell *et al.* 2019, Cadmus *et al.* 2019, Vaught *et al.* 2019), Canada (Brochu *et al.* 2019), Italy (Mescolini *et al.* 2019), Brazil (Chacón *et al.* 2019) and Israel (Davidson, unpublished data).

The present study investigates an outbreak of MD in backyard Cochin chickens reared for hobby. Clinical signs as well as macroscopic and microscopic findings are described and molecular analysis of the *meq* gene of the detected GaHV-2 is performed. Finally, the MHC haplotype of the affected chickens is molecularly established.

Materials and methods

Birds and sample collection

In 2014, two MD unvaccinated Frizzle Cochin Bantam chickens from a multi-species backyard farm in Italy (Sarsina, FC) were presented to the Avian Pathology Service of the Department of Medical Veterinary Sciences of the University of Bologna for diagnostic investigation. The flock consisted of approximately thirty chickens of various breeds (Brahma, Cochin, Silkie, Leghorn). Peacocks and pigeons were raised on the same farm but in different fences. Three birds showed clinical signs suggestive of classical MD at two months of age, two of them were presented for examination. In addition, another young bird died but was not conferred for post-mortem examination. Both birds were clinically examined and necropsied after euthanasia, and liver, lung, kidney, proventriculus, bursa of Fabricius, brachial plexus, sciatic plexus and sciatic nerve were collected for microscopic examination. Feather tips were also collected for PCR analysis.

Histopathology

Portions of the organs listed above were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Sections (4 µm thick) from paraffin embedded tissues were stained with haematoxylin and eosin following standard histological techniques (Dalle Zotte *et al.* 2017).

DNA extraction and PCR amplification of the *meq* gene of GaHV-2

DNA was extracted from pools of five feather tips per bird using the commercial kit High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), with a subtle adjustment to the manufacturer's instructions as previously described (Mescolini *et al.* 2019). Total DNA was then subjected to the PCR protocol described by Mescolini and colleagues (Mescolini *et al.* 2019), capable of amplifying the full-length *meq* gene.

Sequencing and phylogenetic analysis

The amplification products of the *meq* gene were purified using ExoSAP-IT™ Express PCR Product Cleanup (Thermo Fisher Scientific, Massachusetts, USA) and sequenced using PCR primers *Eco*R-Q for 5'-GGT GAT ATA AAG ACG ATA GTC ATG-3' and *Eco*R-Q rev 5'-CTC ATA CTT CGG AAC TCC TGG AG-3' (Shamblin *et al.* 2004) plus an additional internal primer, *meq*-F, 5'-ATG TCT CAG GAG CCA GAG CCG-3' (Hassanin *et al.* 2013), by a commercial sequencing service (Macrogen Europe, Amsterdam). The obtained nucleotide and deduced amino acid (aa) sequences were edited with BioEdit Sequence Alignment Editor Version 7.0.5.3 (Tom Hall, Ibis Therapeutics, Carlsbad, California, USA), aligned and compared using Clustal W software with the *meq* gene sequences of reference GaHV-2 strains, available in the GenBank database, and with 33 GaHV-2 Italian strains recently detected during MD outbreaks in backyard and commercial flocks (Mescolini *et al.* 2019, Mescolini *et al.* 2020a) and in a turkey flock (Mescolini *et al.* 2020b). The number of stretches of four consecutive proline molecules (PPPP), contained in the transactivation domain of the Meq protein, was counted. Phylogenetic analysis based on the amino acid sequences of the *meq* gene of reference strains and of selected Italian GaHV-2 strains was performed with the Maximum Likelihood method under the Jones-Taylor-Thornton model using MEGAX (Kumar *et al.* 2018). Bootstrap values, obtained with 1,000 replicates, were considered significant when equal or greater than 70.

Identification of the chicken's haplotype by PCR-SSP

The chicken's haplotype was molecularly determined, according to Zheng and colleagues (Zheng *et al.*

1999). *B-LβII* family genes were first amplified from genomic DNAs extracted from pulpy feathers, using a pair of *B-LβII* family specific degenerated primers (upstream primer: 5' CG TTC TTC TTC TRC GGT RBG AT 3' and downstream primer: 5' TA GTT GTG CCG GCA GAM CSY G 3') amplifying a 235 bp fragment conserved in all known *B-LβII* family alleles. *B-LβII* family specific PCR was carried out by adding 3 µL DNA to a 22 µL reaction mixture containing 0.125 µL GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, WI), 5 µL 5X Colorless Go-Taq Flexi Buffer, 1.75 µL MgCl2 solution, 0.5 µL dNTPs, 13 µL H_2O for molecular biology, 1 µL upstream primer and 1 µL downstream primer. Cycling conditions were as follows: 5 min of denaturation at 95°C followed by 35 cycles, each consisting of denaturation at 95 °C for 30 s, annealing at 50°C for 30 s, and extension at 72 °C for 30 s. A final elongation step at 72 °C for 5 min completed the reaction.

For the differentiation of the more commonly encountered B haplotypes, the PCR products were then subjected to a further PCR using sequence-specific primers (PCR-SSP) internal to the *B-LβII* family specific primers. Five different pairs of specific primers 4up8/1DN69, 1up32/1DN65, 3up8/2DN69, 2up8/1DN66 and 1up8/2DN66 (Zheng *et al.* 1999) designed on the basis of published sequences of standard haplotypes were used in PCR-SSP to amplify *B-LβII* family alleles associated with five of the more frequent haplotypes known B^2, B^{13}, B^{15}, B^{19} and B^{21}, respectively. The reaction mixture used was the same described above, changing the primer sets, and cycling conditions were the following: 94 °C for 5 min followed by 25 cycles each consisting of denaturation at 94 °C for 30 s, annealing at 50, 55 or 60 °C for 30 s, and extension at 72 °C for 30 s. Final elongation phase was conducted at 72 °C for 5 min. The PCR products were separated on agarose gel (2%), stained with ethidium bromide, and visualized under ultraviolet light after an electrophoretic run at 110 V and 400 mA for 35 min.

The amplicons obtained were purified, sequenced in both directions and submitted to the basic local alignment search tool (BLAST) to confirm PCR results with a similarity search.

Accession numbers

Nucleotide sequences of the *meq* gene of the detected GaHV-2 strains were submitted to the GenBank database and are available under the following accession numbers: MN518896 and MN518897. *B-LβII* family allelic nucleotide sequences were submitted to the GenBank database under accession numbers MN518898 and MN518899.

Results

Clinical signs and post- mortem lesions

Both examined chickens showed an inability to stand upright and walk, and showed spastic paralysis of legs (Figure 1). At necropsy, enlargement of the peripheral nerves and nervous plexuses was observed (Figure 2). In particular, brachial and sciatic plexuses, and brachial, sciatic, vagus and intercostal nerves were involved. The nerves had lost their typical cross-striation and showed a yellow discoloration and oedematous appearance. No lymphomas were grossly visible in the visceral organs.

At microscopic examination of the nerves, A- and B-type lesions, typical of MD according to Payne and Biggs (Payne and Biggs 1967), were detected. A-type lesions, considered of a neoplastic nature, consisted of masses of pleomorphic infiltration of small, medium and large lymphocytes (Figure 3A). B-type lesions, considered of an inflammatory nature, were characterized by interneuritic oedema and diffuse infiltration of small lymphocytes and plasma cells (Figure 3B).

Figure 1. *Clinical examination.* **A.** Bird in lateral decubitus due to spastic paralysis of the limbs. **B.** Bird showing one leg stretched forward and the other back.

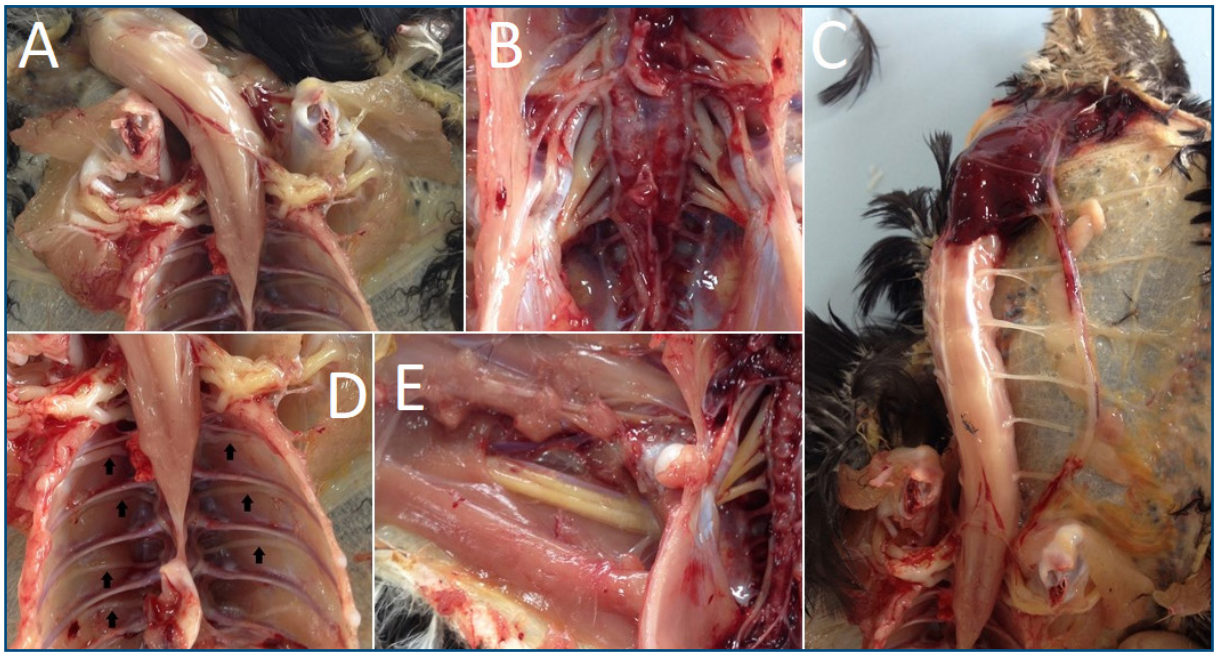

Figure 2. *Macroscopic lesions.* The following nerves and plexuses showed a yellowish discoloration, are oedematous and enlarged, and have lost their typical cross-striation: brachial plexuses and nerves (**A**); intercostal nerves (**B**); sciatic plexuses (**C**); sciatic nerve (**D**); vagus nerve (**E**).

GaHV-2 detection and molecular characterization of the *meq* gene

Feathers from both examined chickens were PCR positive for GaHV-2. The two detected viruses, named GaHV-2/Italy/Ck/419/14 and GaHV-2/Italy/Ck/420/14, showed 100% identity. The *meq* gene sequence had an overall length of 1,257 base pairs (bp), and, when compared with the *meq* sequence of the vaccine strain CVI988/Rispens (GenBank accession number DQ534538), showed an insertion of 57 bp, encoding 19 aa. Meq protein sequence had nine PPPP motifs and a proline content of 23.6%. Moreover, the detected virus shared the entire *meq* gene sequence with three strains detected in Italian backyard chickens exhibiting neurologic signs (GenBank accession numbers: MK13966, MK139664 and MK139665) (Mescolini *et al.* 2019).

The phylogenetic tree, based on *meq* amino acid sequences (Figure 4), confirmed this latter finding as the investigated viral strain formed a cluster including other strains detected in Italian backyard flocks (Mescolini *et al.* 2019).

Identification of MHC haplotypes

Genomic DNAs from the examined chickens were amplified only when the primer pair targeting B[19] haplotype was used. Amplicons of 213 bp were obtained and sequenced. Both sequences showed 100% identity with sequences of the MHC class II beta chain gene corresponding to the chicken haplotype

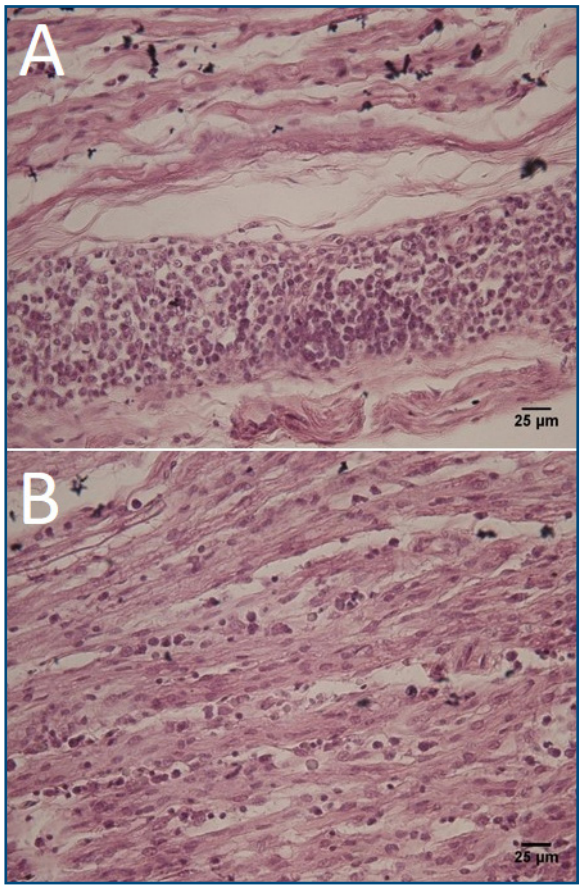

Figure 3. *Histopathological lesions.* **A.** Sciatic nerve. Pleomorphic infiltration of small, medium and large lymphocytes, compatible with A-type lesions (40×). Haematoxylin and eosin (HE). **B.** Sciatic nerve. Interneuritic oedema with diffuse infiltration of small lymphocytes and plasma cells, compatible with B-type lesions (40×). HE.

B[19] published in the GenBank database (accession numbers AB426151.1; DQ008584.2; AJ248583.1).

Discussion

The present study reports a MD outbreak in Cochin chickens following their infection with a GaHV-2 strain whose *meq* gene sequence showed typical markers of low virulence. In contrast to previous MD reports in backyard chickens, which focused on clinical and post-mortem findings (Rodriguez *et al.* 1997, Mete *et al.* 2013, Crespo and Senties-Cue 2015, Pohjola *et al.* 2015, Brochu *et al.* 2019, Cadmus *et al.* 2019, Vaught *et al.* 2019, Chacón *et al.* 2019), our study provides molecular data on the causative agent and genetic susceptibility of the affected birds.

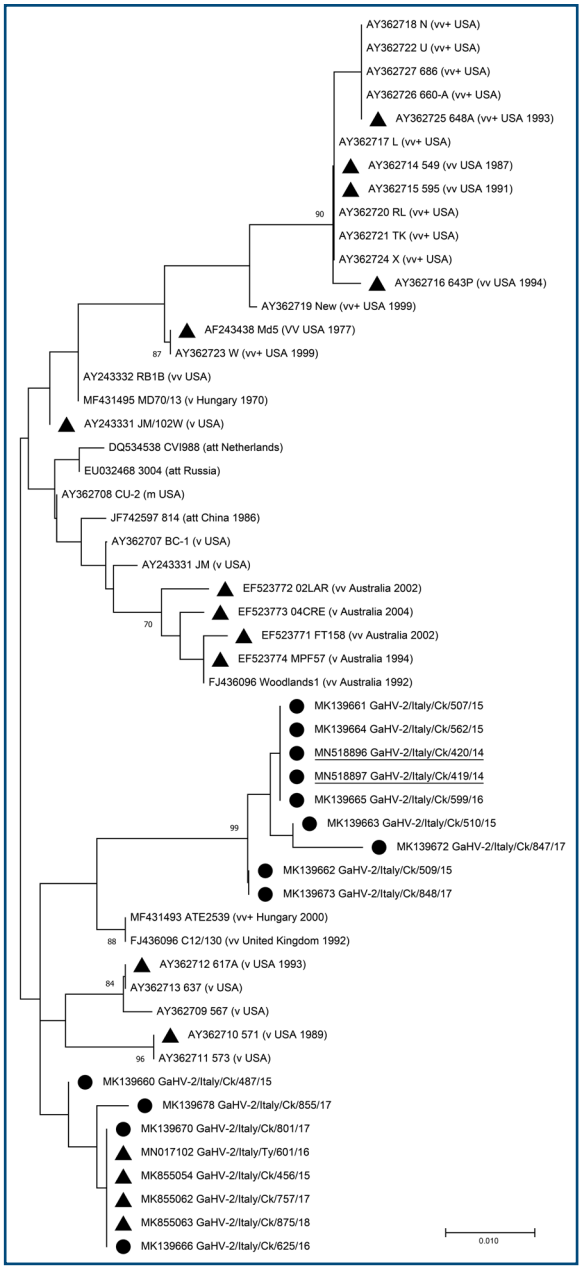

Figure 4. *Phylogenetic tree based on complete* meq *amino acid sequence of GaHV-2/Italy/Ck/419/14 and GaHV-2/Italy/Ck/420/14 (underlined in the tree) and Italian and reference strains retrieved from GenBank.* Accession numbers are located before the name of each strain along with a black dot (●), indicating strains detected in backyard flocks, or a black triangle (▲), indicating strains detected in commercial flocks (when available). Abbreviations were used for GaHV-2 pathotypes (m = mild, v = virulent, vv = very virulent, vv+ = very virulent plus) and for GaHV-2 host species (Ck = chicken, Ty = Turkey).

The low oncogenic potential of the GaHV-2 strain was confirmed by clinical, macroscopic and microscopic findings suggestive of classical nervous form of MD.

Shamblin and colleagues (Shamblin *et al.* 2004) and Renz and colleagues (Renz *et al.* 2012) demonstrated the correlation between *meq* gene molecular features and the degree of virulence of GaHV-2 strains, and proposed this as the basis to scrutinize virulence relying on *meq* gene sequencing. Viral strains with Meq containing a higher number of PPPP repeats exhibit a lower virulence, while virulent GaHV-2 strains show the lowest number of proline repeats. In this study, the GaHV-2 strain detected showed a Meq protein sequence with the highest number of PPPP repeats (n = 9) when compared with other GaHV-2 strains.

Our results, coupling sequence analysis of the *meq* gene with the observation of clinical and pathological outcomes of the infection, confirm the validity of the molecular method for GaHV-2 classification. We believe that providing data confirming the correlation between strain sequence and virulence will reinforce the notion of a pathotyping that can avoid the use of *in vivo* studies. However, at the moment, *in vivo* studies conducted as described by Witter and colleagues (Witter *et al.* 2005) remain mandatory for GaHV-2 pathotyping.

The phylogenetic analysis showed that the strain detected in the present study belong to the same cluster as other Italian low pathogenicity viruses, which are exclusively detected in backyard flocks (Mescolini *et al.* 2019) and not in the commercial sector (Mescolini *et al.* 2020a).

Trade of live ornamental purebred chickens occurs frequently between hobby farmers while prophylactic measures like biosecurity, quarantine periods or vaccination are not regularly applied.

While commercial chickens routinely receive vaccines against MD, vaccination of backyard birds is quite unusual because owners are unaware of the disease and the prophylactic measures, or because the vaccine commercial formulations are too expensive for such small flocks (Elkhoraibi *et al.* 2014). Given the circulation in the Italian rural sector of GaHV-2 strains capable of causing clinical outbreaks, veterinarians should raise awareness of this issue and promote the use of vaccines against

MD. In recent years, some Italian hobby farmers have begun to vaccinate their flocks with cell-free lyophilized turkey herpesvirus (HVT) vaccine, which is cheaper and easier to handle than cell-associated formulations, with good results on MD control in flocks where weakly virulent strains were circulating.

Results of MHC haplotype analysis showed that both affected chickens possessed the B^{19} haplotype, which is typical for susceptibility to MD, which explains the severity of the clinical and pathological effects observed in non-vaccinated young chickens infected with a low-virulence GaHV-2 strain. This finding demonstrates the importance of virus-host interaction in complicating the scenario. It has been reported that the chicken MHC haplotype B^{19} is correlated with a high susceptibility to MD, since B^{19} chickens develop more severe gross and histological lesions and show a higher level of viral replication after challenge with a very virulent GaHV-2 strain (Gao *et al.* 2015). MHC haplotype seems also to influence vaccine protective efficacy, as chickens expressing certain MHC B haplotypes seems to respond better to different vaccine strains in terms of vaccinal immunity and MD incidence after vaccination (Bacon and Witter 1992, Bacon and Witter 1993, Bacon and Witter 1994a, Bacon and Witter 1994b). More recently it has been shown that also non-MHC host genes are able to affect the vaccine-induced protection against MD (Chang *et al.* 2010, Chang *et al.* 2011, Chang *et al.* 2012, Xie *et al.* 2017). In conclusion, *ad hoc* vaccination programs could be developed when the host genetic background and the presumptive virulence of GaHV-2 circulating strains are known.

Statement of animal rights

The case report object of the present study, conducted on private pet birds with the consent of the owner, is to be considered as a veterinary clinical practice for non-experimental purposes pursuant to article 2, paragraph 1, letter b of the Italian Legislative Decree No. 26/2014 (approval issued by the Animal Welfare Committee of the University of Bologna - Protocol No. 254249).

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