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SHORT COMMUNICATION

Isolation and characterization of pseudorabies virus from a wolf (*Canis lupus*) from Belgium

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Abstract Aujeszky's disease is an economically important disease in domestic pigs caused by the alphaherpesvirus pseudorabies virus (PRV). However, also wild boars are a natural reservoir for the virus, and this can lead to infection of wildlife carnivore species. Three wolves held in the wildlife park of Han-sur-Lesse in the province of Namur in Belgium were suspected to be infected with PRV based on the nervous symptoms they showed after being fed with wild boar offal. The diagnosis was confirmed for a female wolf by a positive real-time PCR detecting PRV. The virus was isolated from the brain tissue of the wolf and characterized by restriction fragment length polymorphism analysis and phylogenetic analysis. The obtained *Bam*HI restriction fragment pattern of the wolf isolate was similar to that of the reference strain Kaplan, thereby characterizing it as a type Ip isolate. Type I PRV strains, and particularly subtype Ip, are predominant in European wild boar. Phylogenetic analysis based on the sequence of a fragment of glycoprotein C showed that the Belgian isolate belonged to cluster B and that the sequence was identical to that of wild boar isolates from southwestern Germany, eastern France, and Spain. This study is the first report of Aujeszky's disease in wolves and shows that they are susceptible to PRV by eating infected wild boar offal leading to fatal neurological disease. This illustrates the possible implications of PRV-infected wild boar for the conservation of wolves and other carnivore species.

Keywords *Canis lupus* · Wolf · Wild boar · Aujeszky's disease · Pseudorabies virus · Genetic characterization

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Introduction

Aujeszky's disease is an economically important disease caused by the porcine alphaherpesvirus pseudorabies virus (PRV) or suid herpesvirus 1. Members of the family *Suidae* are the only natural hosts of the virus and infection can cause respiratory, reproductive, and neurological symptoms in an age-dependent way (Pomeranz et al. 2005). In several European countries, the virus is successfully eradicated in domestic pigs due to large scale vaccination programs. However, surveillance studies have shown that the virus is still circulating in the European wild boar population (Müller et al. 2011). For Belgium, the latest published results report a seroprevalence between 15 and 22 % in the south of the country (Czaplicki et al. 2006). The presence of PRV in wild boars poses a risk for reintroduction of the virus into the domestic pig population and can also lead to infection of wildlife species. Infection of nonnative hosts with a wild-type PRV strain is uniformly lethal (Pomeranz et al. 2005), and fatal PRV infection has already been observed in several

carnivore species such as bears, coyotes, foxes, African wild dogs, and a panther after feeding on infected offal from domestic pigs or wild boars (Bitsch et al. 1969; Schultze et al. 1986; Glass et al. 1994; Raymond et al. 1997; Zanin et al. 1997; Haddane and Essalhi 1998). Although it is known that wolves (*Canis lupus*) use wild boar as a part of their natural diet (Nores et al. 2008) and can consequently be exposed to the virus, this is the first report of a case of Aujeszky's disease in this species.

Materials and methods

Virus identification and virus isolation

Brain tissue samples were collected from the wolf suspected to be infected with PRV. A real-time PCR targeting a fragment of the gB and gE gene was done for diagnosis of the disease. The brain material was used for virus isolation on PK15 cells and immunofluorescence staining using a PRV-specific fluorescein-conjugated antiserum was performed to confirm the presence of PRV (for details, see Online Resource 1).

Restriction fragment length polymorphism analysis

The restriction fragment pattern of the PRV isolate was determined after it was passaged once on ST cells. The supernatant was ultra-centrifuged at 100,000*g* and 4 °C for 90 min. DNA was extracted from the virus pellet and 1 μg of DNA was digested with 15 U *Bam*HI (Roche). The same procedure was applied to PRV reference strains Bartha and Kaplan. The digests were loaded on a 0.7 % agarose gel and run overnight at 50 V (for details, see Online Resource 1).

Sequencing and phylogenetic analysis

A ±800 bp fragment of the gC gene was amplified with the DyNazyme Ext DNA polymerase (Thermo Fisher Scientific, USA) using previous published primers (Hahn et al. 2010; Müller et al. 2010) and genomic DNA from the first passage of the wolf isolate. The PCR product was used as a template for sequencing using the PCR primers with the BigDye Terminator Sequencing Kit (Applied Biosystems, USA), and the reaction products were analyzed on a 3130 Genetic Analyzer (Applied Biosystems). The obtained sequence (Genbank accession number: KF415193) and previous described sequences from European wild boar (Müller et al. 2010; Steinrigl et al. 2012) were used for phylogenetic analysis (for details, see Online Resource 1).

Results and discussion

The wolves concerned were housed in the wildlife park of Han-sur-Lesse (50° 7′ N, 5° 11′ W) in the province of Namur in Belgium. After being fed with offal from wild boar hunted in the reserve, three wolves showed typical nervous symptoms, including incoordination, abnormal positioning of the head, and circling. They were euthanized after the symptoms appeared and necropsied at the Regional Association of Animal Health and Identification. Apart from unilateral pulmonary congestion, no macroscopic abnormalities were found. The brain of a female wolf was sent to CODA-CERVA for further diagnosis.

Diagnosis of Aujeszky's disease was confirmed by real-time PCR since the brain tissue of the wolf tested positive for the gB and gE gene of the virus (data not shown). Because vaccine strains can be discriminated from wild type strains based on the absence of gE, these results indicate that the wolf was infected with a wild-type PRV strain.

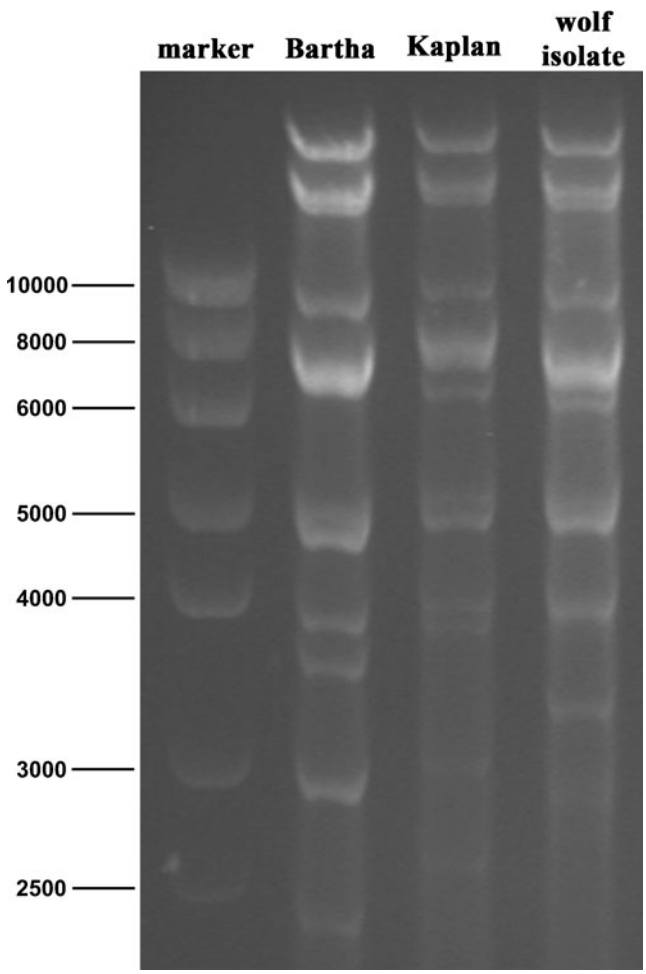

Fig. 1 *Bam*HI restriction fragment length polymorphism pattern of PRV reference strains Kaplan and Bartha and the Belgian PRV strain isolated from a Belgian wolf. Smartladder (Eurogentec, Belgium) was used as a molecular marker

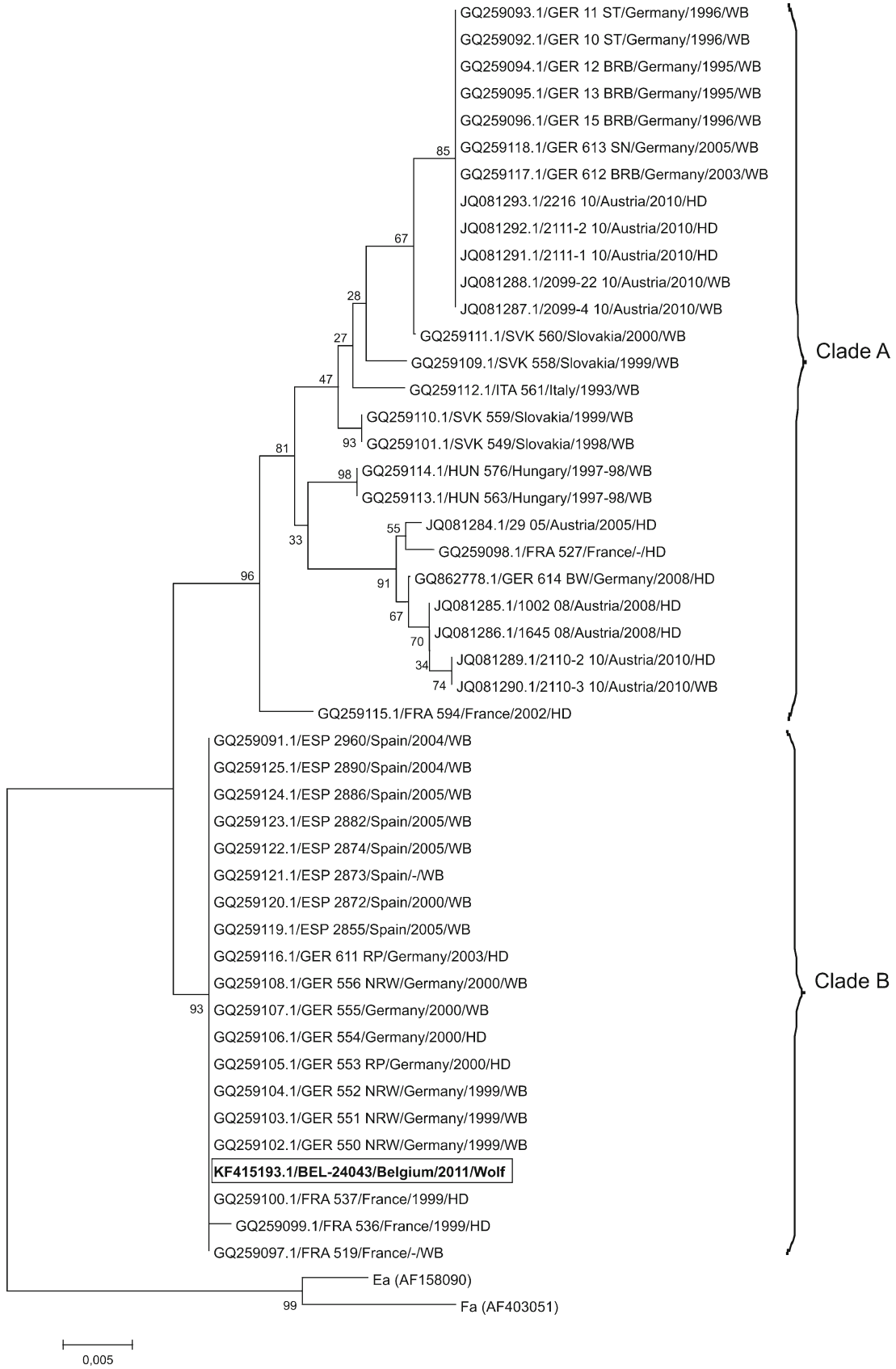

Fig. 2 Phylogenetic tree of the Belgian wild boar isolate and European wild boar isolates constructed using the neighbour joining method. The bootstrap test (500 replicates) and the composite maximum likelihood substitution model were used. The Chinese strain Ea and Fa were used as outgroup

Genetic characterization of the PRV isolate was done using two routinely used molecular techniques to study genetic relationships among PRV isolates: restriction fragment length polymorphism (RFLP) analysis and sequencing followed by phylogenetic analysis. Conventional RFLP analysis of genomic DNA using *Bam*HI was initially the standard method for characterization of PRV isolates and allows to discriminate four major genotypes and several subtypes (Herrmann et al. 1984). In Central Europe, mainly type I and type II genotypes are present. Type I strains are typically found among older domestic isolates and wild boar strains, while type II strains are mainly seen in domestic pigs (Capua et al. 1997; Müller et al. 2010). In Northern Europe, type III strains are predominant (Herrmann et al. 1984). The PRV isolate from the Belgian wolf showed a similar pattern as the Kaplan reference strain, thereby identifying the isolate as a type Ip strain (Fig. 1), the most common type present in European wild boar (Müller et al. 2010).

More recently, also sequencing and phylogenetic analysis has been used for molecular typing of PRV strains based on the analysis of a ±800 bp fragment of glycoprotein gC, a nonessential component of the virion envelope (Goldberg et al. 2001). Sequencing of this fragment has allowed differentiation of wild boar isolates into two clades, A and B (Müller et al. 2010). The sequence of the gC fragment of the Belgian PRV isolate from the wolf belonged to clade B (Fig. 2). Clade B compromises isolates from southwestern Germany, eastern France, and Spain, indicating that the Belgian isolate is closely related to wild boar strains circulating in neighboring countries. In contrast, clade A is more diverse and comprises isolates from Slovakia, Hungary, eastern Germany, Italy, and France.

This is the first report of Aujeszky's disease in wolves and indicates that they are susceptible to PRV infection. As in other canids, PRV infection of wolves leads to a fatal neurological disease. Since wild boar composes one of the main food sources of European wolves (Nores et al. 2008) and PRV infection seems to be more widespread among them than originally thought (Müller et al. 2011), the presence of wild boar carrying PRV could have severe implications for the conservation of wolves and other carnivore species like bears, foxes, coyotes, and wild felices that use wild boar as part of their diet.

Management of PRV in wild boars, that occasionally show severe symptoms upon PRV infection (Gortázar et al. 2002; Schulze et al. 2010), could improve their overall health status but could also limit the risk of transmission of the virus to other susceptible wildlife and into the domestic swine population. This is however challenging since vaccination of wildlife is difficult. A possible solution could be found in oral immunization of wild boar what has successfully been practiced against classical swine fever virus (Kaden et al. 2000). As seen in the lethal outcome of a PRV infection in nonhost species, this would however require the development of an effective and safe oral vaccine. Recently, it was shown that oral immunization of wild boar with an attenuated live vaccine efficiently protected against PRV infection (Maresch et al. 2012), but the safety aspect has to be further evaluated. Other alternatives could be found in the use of inactivated or subunit vaccines.

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