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ORIGINAL PAPER

Raccoons (*Procyon lotor*) in Germany as potential reservoir species for Lyssaviruses

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Abstract Raccoons can be found almost everywhere in Germany since their first successful introduction in 1934. Although the animal is a well-known reservoir species for rabies in the USA, during the last European fox rabies epizootic, only a few rabid raccoons were reported from Germany. In recent years, the raccoon population density has increased tremendously, especially in (semi) urban settings. Presently, Germany is free of terrestrial wildlife rabies. To assess the potential risk that the raccoon population in Germany could act as a reservoir species upon reemergence of rabies, the susceptibility of the local raccoon population was investigated. Wild-caught animals were inoculated with the most likely lyssavirus variants to infect the local population. It was shown that the raccoons were fully susceptible for a dog and raccoon rabies virus isolate. Also, five of six raccoons inoculated with a fox rabies virus isolate showed clinical signs. However, none of the raccoons infected with European Bat Lyssavirus type 1 succumbed to rabies; meanwhile, all these raccoons seroconverted. It is concluded that the highest risk for the raccoon population in Germany to become infected with lyssaviruses is through the importation of rabies infected dogs.

Keywords Raccoon · Rabies · Susceptibility · Germany · RABV · EBLV-1

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Introduction

In Europe, the red fox (*Vulpes vulpes*) is considered the only reservoir species for terrestrial wildlife rabies; a viral disease caused by lyssaviruses. However, since the late 1980s, another host species has emerged in northeastern Europe, the raccoon dog (*Nyctereutes procyonoides*). Yet, it is unclear if this species is capable of facilitating disease persistence independently from the fox population in this area (Cliquet et al. 2011). Interestingly, there are also other potential rabies reservoir species living in Europe without evidence that these animals play any role in the spread of the disease. For example, golden jackals (*Canis aureus*) are known to have been a reservoir species in Israel (Yakobson et al. 2006). Jackals are relatively widespread in southeast Europe living predominantly in small and scattered populations (Arnold et al. 2012). Rabies cases in jackals have been reported incidentally from this area (Johnson et al. 2007; Hikmet Ün, pers. comm.), but so far they cannot be considered a reservoir species. The small Indian mongoose (*Herpestes auropunctatus*) has been introduced on some islands off the Croatian coast and has invaded also parts of the mainland (Cirovic et al. 2011). This invasive animal species is considered the principal wildlife reservoir for rabies on several Caribbean islands (Slate 2011), but up till now no rabies cases in mongoose have been reported from Croatia, Bosnia–Herzegovina, and Montenegro. Interestingly, spill over rabies cases in Egyptian mongoose (*Herpestes ichneumon*) have been reported from Israel (Yakobson et al. 2004), a country with fox-mediated rabies just like Croatia. Finally, the most frequently reported rabid wildlife species in the USA, the raccoon (*Procyon lotor*) (Blanton et al. 2011), can be found almost everywhere in central Europe since the initial successful introduction through the release of four animals in Hesse—Germany, 1934 (Beltran-Beck et

al. 2012). In Germany, extremely high raccoon population densities have been observed, especially in (semi) urban areas, as high as 100 animals/km^2 (Hohmann et al. 2001; Michler 2004; 2007). During the last fox rabies epizootic, only few rabies cases in raccoons were identified; the virus has never been able to establish itself within the German raccoon population (Vos et al. 2012). The reasons for this have never been studied in detail. It seems that the raccoon density started to increase exponentially only after the disappearance of fox rabies due to the distribution of oral rabies vaccine baits. So during the last rabies outbreak among foxes, the density of the German raccoon population was apparently still too low to sustain an independent infection cycle. Another explanation is a possible reduced susceptibility of raccoons to the fox rabies virus variants circulating in Europe, as suggested by Artois et al. (1989). Interspecific variability in susceptibility for different rabies virus variants is well known and has also been described for raccoons infected with fox isolates (Winkler and Jenkins 1991). This effect could potentially be enhanced by the relatively low genetic diversity of the German raccoon population due the small number of "founder events" (Gramlich et al. 2011).

In an effort to assess the risk of the raccoon population becoming a potential rabies reservoir species in Germany, we investigated the susceptibility of raccoons to different lyssaviruses, including different rabies virus (RABV) isolates, most likely to infect the raccoon population. Although no indigenous terrestrial rabies case has been reported in Germany since 2006 (Müller et al. 2012), fox rabies could reemerge in Germany from neighboring countries still infected. Another potential mode of transmission could be a sustained spillover infection from illegally imported rabies-incubating dogs (*Canis lupus familiaris*). Several cases of imported dog rabies in Europe including Germany have been reported in recent years (Johnson et al. 2011). Furthermore, raccoons are also kept as pets in Europe and imported animals from North America incubating rabies form another, albeit small, risk. Finally, especially in (semi) urban settings, raccoons often use attics of buildings as resting sites. These are also sometimes used by bats during different periods of the year. Hence, it is possible that raccoons can come into contact with a bat infected with a bat lyssavirus. The most likely scenario would be a spillover with European Bat Lyssavirus Type 1 (EBLV-1) from its major host, the serotine bat (*Eptesicus serotinus*). This bat species is known for using buildings as roosting sites, and EBLV-1 spillover infections from bats to terrestrial mammals have been documented in the past (Müller et al. 2004; Tjørnehøj et al. 2006; Dacheux et al. 2009). Although the number of animals investigated in this study was low, the German raccoons were susceptible for all investigated virus isolates except EBLV-1. In contrast to Artois et al. (1989), it is concluded that the raccoon population could become a reservoir species for terrestrial rabies in case of RABV reemergence in Germany.

Material and methods

Animals

Raccoons

To rule out any interference of genetic variation in susceptibility, it was necessary to use free-living animals from the German raccoon population instead of imported animals from commercial sources. The study protocol was evaluated and approved by the responsible committees of the appropriate authorities in the federal state of Saxony-Anhalt (nr 42502-2-1034IDT) and the veterinary authorities in Hesse. The animals were caught using wooden box traps during two periods (August and October 2011) in a suburb of the city of Kassel, Hesse. Animals were transported to the experimental animal facility at IDT and kept in individual cages within isolation units during the entire experiment. Animals were fed daily with 150 g dried fodder for dogs (Good Deal Hundevollnahrung; Voror Dog Vertrieb, Enger—Germany) and 100 g fruits and/or vegetables. Water was offered ad libitum.

Mice

Three week old BALB/c-mice (FLI-stock) were used for the determination of the mouse intracerebral lethal dose 50 ($MICLD_{50}$) of the virus isolates used for inoculation of raccoons.

The experiments were evaluated by the responsible animal care, use, and ethics committee of the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) and gained governmental approval (registration no. LALLF M-V/TSD/7221.3-2.1-002/11). General care was provided as required.

Viruses

In order to test the susceptibility of German raccoons based on the most likely risk scenarios explained above one EBLV-1 isolate of German origin and three virulent primary host adapted RABV-isolates from a European red fox, Eurasian dog and North American raccoon were selected. Details of the isolates used for this study are shown in Table 1. To reach sufficient and comparable high titres for inoculation, all isolates were cell adapted by four serial passages in murine neuroblastoma cells (Na42/13) and diluted with minimum essential medium. The titre was determined and expressed in tissue culture infective dose per milliliter ($TCID_{50}$)/ml) (King 1996).

Table 1 The virus isolates used to experimentally infect raccoons

Virus	Species	Origin	Year	$TCID_{50}$/ml	$MICLD_{50}$/ml
RABV	Dog	Azerbaijan	2002	$10^{5.7}$	$10^{5.9}$
RABV	Red fox	North Rhine Westphalia, Germany	1998	$10^{6.0}$	$10^{4.2}$
RABV	Raccoon	Alabama, USA	1981	$10^{5.7}$	$10^{6.0}$
EBLV-1	Serotine bat	Schleswig Holstein, Germany	2001	$10^{6.1}$	$10^{6.0}$

Determination of $MICLD_{50}$

For $MICLD_{50}$ determination, tenfold dilution series (10^{-1} to 10^{-6}) of the virus isolate stocks used for raccoon infections were prepared and 30 µl of each dilution was intracranially injected in three week old BALB/c-mice (six mice per group). The animals were checked daily for clinical signs. After development of clinical signs of rabies, the mice were euthanized, and the brains were prepared for immunohistological confirmation of rabies infections using a slightly modified method described previously (Brookes et al. 2007). $MICLD_{50}$ per ml virus stock were calculated according to Spearman and Kärber (Aubert 1996).

Inoculation

All 22 raccoons divided over four groups received 0.8 ml of one of the isolates in the *M. masseter* by the intramuscular route (2×0.4 ml). During virus administration and blood sampling, the animals were immobilized with 2.5 mL ketamine (Ketamine 100 mg/mL, WDT; Garbsen—Germany) and 2.5 mL xylazine (Sedaxylan 20 mg/mL, WDT; Garbsen—Germany). The raccoons were observed daily during the first week postinfection; afterwards, the animals were observed more frequently. Upon detection of the first clinical signs of a neurological disorder, the raccoons were euthanized administering 5 ml pentobarbital (Release® 300 mg/mL, WDT, Garbsen—Germany) intracardially after immobilization as described previously.

Assays

Different regions of the raccoons′ brain, Ammon′s horn, medulla oblongata, cerebellum, were tested for the presence of the rabies virus antigen using the direct fluorescence antibody test (Dean et al. 1996). Blood samples were taken prior to infection by claw clipping and, if possible, on the day of death. From the EBLV-1 infected raccoons, an additional blood sample was taken 49 days post infection, halfway through the observation period. Blood samples were examined for the presence of rabies virus neutralizing antibodies (VNA) using the rapid fluorescence focus inhibition test (RFFIT) (Smith et al. 1973) with adaptations as described by Cox and Schneider (1976), either using CVS-11 or EBLV-1 as test virus depending on the virus inoculum. The salivary glands (*G. submandibularis*) were investigated using the rabies tissue culture infection test for viable virus particles (Webster and Casey 1996)

Results

$MICLD_{50}$ determined in three week old BALB/c mice showed that the virus isolates from dog, raccoon, and serotine bat exhibited comparable virulence in the mouse model which also correlated with similar tissue culture infectious titres (Table 1). Also, the infectious virus titre of the red fox isolate was comparable. However, the $MICLD_{50}$ of this isolate was more than 50-fold decreased compared with the others.

The results of the susceptibility experiments in raccoons are summarized in Table 2. All raccoons inoculated with the dog (n=6) or raccoon isolate (n=5) succumbed to rabies. One of the six raccoons infected with the fox isolate survived until it was euthanized 45 days postinfection. Subsequent testing revealed no indication of rabies. All EBLV-1 infected raccoons survived and did not show any signs of neurological disorders. The incubation periods of all animals that succumbed to infection ranged between 8 and 20 days. The shortest incubation periods were observed for the dog isolate, followed by the raccoon isolate, and finally the fox isolate. While viral antigen was evenly distributed in the brain sections of raccoon RABV infected animals, the intensity of fluorescence in the Ammon′s horn was lower in fox RABV-infected raccoons, and viral antigen was even absent in this part of the brain in some dog RABV-infected raccoons. Only the medulla tested positive for viral antigen in all rabies positive animals (Table 2). Viable virus was not detected in any of the salivary glands of animals that succumbed to infection. All animals tested seronegative prior to infection, but all samples taken on the day of death or euthanasia had detectable levels of rabies VNA, including the raccoons infected with EBLV-1. In this group, the level of antibodies generally increased from day 0 postinfection to day 49 postinfection; while at euthanasia (day 98 postinfection), titres had decreased again (Fig. 1). RFFIT titres of the EBLV-1 infected animals could not be expressed in International Units, therefore a comparison with the other serological results is not possible. Only four animals were found dead without showing any clinical signs (Table 2).

Table 2 Results of experimental study, virus detection in brain (FAT), serum virus neutralizing antibodies (VNA), (RFFIT), and day of death post infection

Animal	Virus	Sex	VNA (IU/ml)	FAT			Day of death
				A	C	M	
1	RABV dog-isolate	Male	nd	–	+++	+++	11*
2		Male	nd	++++	++++	++++	11*
3		Male	6.6	–	–	++	11
4		Female	0.9	++++	+++	++++	8
5		Male	1.0	–	++	+++	11
6		Male	nd	+++	+++	++++	11*
7	RABV fox-isolate	Male	0.8	–	–	–	45
8		Male	0.4	+	+++	++	20
9		Female	1.9	+	+++	++	15
10		Male	5.7	+	++	+++	13
11		Male	1.3	+	++	+++	14
12		Female	18.4	+	++++	+++	12
13	RABV raccoon-isolate	Female	nd	+++	++++	++++	12*
14		Male	2.1	++++	+++	++++	13
15		Male	2.7	+++	+++	+++	13
16		Male	1.0	++	++	++++	13
17		Female	1.5	+	+++	++	14
18	EBLV-1 serotine-isolate	Male	pos.	–	–	–	survived
19		Female	pos.	–	–	–	survived
20		Male	pos.	–	–	–	survived
21		Female	pos.	–	–	–	survived
22		Female	pos.	–	–	–	survived

A Ammon′s horn, *C* cerebellum, *M* Medulla oblongata, − negative, + very weak positive, ++ weak positive, +++ moderate positive, ++++ strong positive

*Animals were found dead, otherwise animals were euthanized *nd* not determined, *pos*. positive (details provided in Fig. 1)

The other animals that were euthanized all developed a wide range of different clinical signs; licking and biting of legs, vocalization, spasms, salivation, jaw rigor, paralysis, apathy, uncoordinated movements, and no food uptake.

Discussion

In this study, we assessed whether the German raccoon population is susceptible to different RABV variants and EBLV-1 in order to evaluate the possibility of lyssavirus establishment in this native population. Based on experimental data, Artois et al. (1989) concluded that although wild-caught raccoons in France were susceptible to a European fox rabies variant, it would be very unlikely that the European raccoon population would become infected under natural conditions. This conclusion was based on the extremely high doses needed to infect the animals. Sikes and Tierkel (1961) already showed that raccoons were highly resistant to a lyssavirus infection with a fox salivary gland

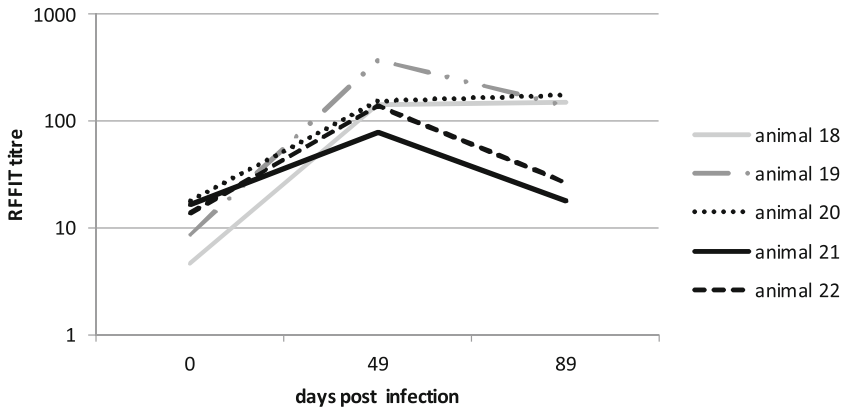

Fig. 1 RFFIT titres of individual raccoons infected with EBLV-1 at three different time points, i.e., 0, 49, and 98 days postinfection

isolate. However, the outcome of infection is not only determined by viral dose. Other factors like amount of virus-containing saliva transmitted, site of infection, and severity of the wound can also influence the outcome. During the last fox epizootic, rabies cases in raccoons were reported from Germany, indicating that raccoons were to some extent susceptible under natural conditions for the circulating fox rabies virus variant (Vos et al. 2012). The fact that these cases did not cause a sustained spillover infection in the raccoon populations may have been due to the lower population densities at that time or indicate other unknown host barriers. For instance, the susceptibility of the German raccoons to rabies infection may be influenced by the genetic features of this founder population as shown for North American haplotypes (Srithayakumar et al. 2011).

Given the fact that at the moment only certain bat lyssaviruses are indigenous in Germany, the most likely event that a German raccoon will come in contact with a lyssavirus is through contact with an infected bat, especially with EBLV-1. It can therefore be a reassuring thought that raccoons are highly refractory to EBLV-1 infection; none of the animals succumbed to infection. Contrary to some experimentally EBLV-1 infected foxes and ferrets, none of the raccoons showed any signs of neurological disorder associated with rabies (Vos et al. 2004a, b; Cliquet et al. 2009). However, the results of this study show that the German raccoons are susceptible to RABV infection, including a European fox variant. In contrast to the other two RABV isolates, one raccoon infected with the fox isolate survived infection. Also, Artois et al. (1989) did not obtain a 100 % mortality rate using a similar dose of a fox isolate (Table 3). In the current epidemiological situation, Germany and all neighboring countries, except for Poland, are considered free of fox-mediated rabies. Also, the remaining rabies foci in Poland are located in the eastern part of the country. Hence, the risk of reemergence of fox rabies and subsequently possible incursion into the raccoon population is considered low. Unfortunately, the occurrence of rabies cases in Europe through importation of pets, especially dogs, is regularly observed (Johnson et al. 2011). In many of these cases, these RABV-infected animals have had multiple contacts with humans, pets, and other animals prior to detection. Hence, there is potential risk that such an infected animal could also encounter a raccoon and transmit the disease, especially in urban settings. Furthermore, in contrast to the previous epidemiological situation in Germany, the raccoon population density has increased considerably in the last decade and has reached levels were it could sustain an independent transmission cycle.

The inoculation dose that was used in this study and similar experimental studies is much higher than the natural infectious dose, as indicated by the very short incubation periods observed (Table 3). In captive studies, the incubation period in raccoons infected with a raccoon variant ($10^{4.2}$ $MICLD_{50}$) ranged from 10 to 107 days (Winkler and Jenkins 1991). McLean (1975) mentioned an average incubation period of 39–79 days for raccoons infected with the raccoon rabies variant ($10^{3.5–4.0}$ $MICLD_{50}$) after natural exposure. Tinline et al. (2002) estimated the modal incubation period for raccoons to be 5 to 6 weeks with a maximum of 19 weeks. The incubation period for the RABV isolates in this study was very short and ranged from 8 to 20 days. The same dog isolate was also used as a control virus in young foxes, and also here the animals succumbed within 2 weeks (Müller et al. 2009).

The fact that no virus was detected in the salivary glands leaves room to speculate that raccoons are susceptible but are not able to transmit the virus to other animals. However, the absence of virus in the salivary glands indicating no virus shedding in saliva must also be seen as a result of the high inoculation dose used. The raccoons died before the virus could reach the salivary glands. Artois et al. (1989) observed slightly longer incubation periods and were able to isolate virus from the salivary glands from the infected raccoons albeit at titres much lower than the infectious dose.

Hence, it can be concluded that in contrast to EBLV-1, the German raccoon population is susceptible to RABV-infection. The fact that during the last fox epizootic only

Table 3 Experimental studies in raccoons with different rabies variants

Variant	Number of animals	MICLD50	Mortality (%)	Incubation period (days)	Reference
Raccoon	5	$10^{5.3}$	100	<25	Hamir et al. 1996
Dog	5	$10^{6.4}$	100	<17	Hamir et al. 1996
Dog	5	$10^{5.2}$	100	10–26	Rupprecht et al. 1986
Dog	7	$10^{5.0}$	100	19–25	Rupprecht et al. 1992
Striped skunk	2	$10^{5.9}$	0	–	Hill et al. 1993
Striped skunk	3	$10^{4.8}$	0	–	Hill and Beran 1992
Fox	3	$10^{3.2}$	100	14–15	Hill and Beran 1992
Fox	2	$10^{5.4}$	100	18–25	Artois et al. 1989
Fox	6	$10^{4.4}$	67	19–22	Artois et al. 1989

few rabies cases in raccoons were reported from Germany cannot be explained exclusively by reduced susceptibility of raccoons for the fox rabies virus variant. However, when comparing the outcome of this study with other experimental studies (Table 3), no indication for a genetic-based reduced susceptibility of the German raccoon population for rabies can be found. It can be hypothesized that during this last epizootic, the contact rates of foxes and raccoons were very low. Based on the outcome of this study and the present rabies epidemiological situation in Germany, the highest risk that the raccoon population will become infected with rabies is by importation of dog rabies. However, the actual risk of establishment of an exogenous rabies virus variant (i.e., originally from another species) in the raccoon population will depend upon many factors including frequency of exposure of the naive population and virus and host characteristics. Although the immediate risk may not seem worrisome, it must be stressed that no experience and no tools are readily available to control a rabies outbreak among raccoons in Germany, especially in urban settings (Vos et al. 2012).

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