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Virus distribution and detection in corn snakes (*Pantherophis guttatus*) after experimental infection with three different ferlavirus strains

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ABSTRACT

Ferlaviruses are important pathogens of snakes. However, factors influencing the pathogenicity of individual isolates as well as optimal protocols for virus detection in tissues of infected snakes have been insufficiently studied. The objectives of this study were to compare virus detection using previously described PCR and cell culture protocols following infection with three genetically distinct ferlaviruses in corn snakes (*Pantherophis guttatus*) as a model species. Groups of 12 corn snakes were each inoculated intratracheally with a genogroup A, B, or C ferlavirus. Tracheal washes and cloacal swabs were tested for virus shedding on days 16 and 28. Three animals were each euthanized on days 4, 16, 28, and 49. Beside immunohistochemistry of lung tissue, several organs (lung, intestine, pancreas, kidney, brain) were tested for the presence of ferlavirus. Distinct differences were noted in the pathogenicity of the three viruses, with a genotype B isolate causing the greatest pathology. PCR was more sensitive in comparison to cell culture, but results varied depending on the tissues. Ferlaviruses spread rapidly into the tissues, including the brain. Overall average detection rate was 72%, and was highest on day 16. There were differences between the groups, with the most virulent strain causing 100% positive samples at the end of the study. Some snakes were able to clear the infection. Shedding via cloaca was seen only on day 28. For ante-mortem sampling, a tracheal wash sample is recommended, for post mortem diagnosis, a pooled organ sample should be tested.

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1. Introduction

Ferlavirus infections (formerly described as ophidian paramyxovirus infections) in snakes were first documented in 1976 following a 1972 outbreak in Switzerland (Fölsch and Leloup, 1976). This outbreak was connected to respiratory disease and deaths in some of the snakes. The virus associated with this outbreak was isolated in cell culture (Clark et al., 1979) and named Fer-de-Lance virus (FDLV). FDLV has since been completely sequenced (Kurath et al., 2004) and is the type species for the genus *Ferlavirus* in the family *Paramyxoviridae* (ICTV, 2014, http://www.ictvonline.org/virusTaxonomy.asp). Since that outbreak, ferlaviruses have been detected in a variety of snake species including elapids and colubrids as well as boids and pythonids, and both private collections and zoological collections can be affected (Jacobson, 2007). The disease can be devastating in reptile collections, and the most evident clinical signs are generally associated with respiratory disease. Neurological signs, emaciation, and anorexia are also described frequently (Fölsch and Leloup, 1976; Jacobson et al., 1981; Jacobson, 2007). Pulmonary lesions indicating severe pneumonia can be confirmed histologically, with hyperplasia of the respiratory epithelium, interstitial infiltration, secondary bacterial infection and severe inflammatory reactions (Jacobson et al., 1992, 1997; Stacy and Pessier, 2007). Beside snakes, ferlavirus infections have also been detected in lizards (Ahne and Neubert, 1991; Essbauer and Ahne, 2001; Jacobson et al., 2001; Marschang et al., 2002, 2009) and tortoises (Marschang et al., 2009; Papp et al., 2010b). Although in some cases the consequences of an infection seem to be less dramatic in comparison to snakes, pneumonia has also been described.

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Work on the comparison of ferlaviruses has shown both serological and genomic differences between strains (Blahak, 1994; Ahne et al., 1999; Francke et al., 2001). Recent publications have divided the ferlaviruses into four different genogroups based on partial genome sequences of viruses from snakes, lizards, and chelonians (Marschang et al., 2009; Papp et al., 2010a,b, 2013; Abbas et al., 2011). The genogroups have been called A, B, C, and “tortoise”. Viruses belonging to genogroups A, B, and C have all been found in a variety of squamate hosts, and viruses belonging to A and B have also been found in a chelonian host (Marschang et al., 2009; Papp et al., 2010a,b, 2013; Abbas et al., 2011), while the “tortoise” genotype is represented by a single virus isolate from a Hermann’s tortoise (*Testudo hermanni*) with pneumonia (Marschang et al., 2009). Based on the information available, there is no species specificity of the ferlaviruses, although snakes in general appear to be most susceptible. Little is known on possible differences in the virulence between different strains.

Ferlaviruses have been detected in different organs including the kidney, small intestine, lung, trachea, liver, and the heart, as well as from choanal and cloacal swabs and tracheal wash samples (Blahak, 1994; Papp et al., 2010a; Pees et al., 2010; Abbas et al., 2011). Brain tissue has been examined a few times and detection of ferlaviral RNA by in situ hybridization has been reported (Sand et al., 2004; West et al., 2001). However, studies using virus isolation or PCR have reported no success in detecting virus in this organ (Blahak, 1994; Papp et al., 2010; Woo et al., 2014). Virus isolation in cell culture is well established for ferlaviruses, and was also used for the first isolation in 1979 (Clark et al., 1979). In 1999, Ahne et al. described the use of reverse-transcription PCR protocols targeting portions of the L (large polymerase protein) and HN (hemagglutinin neuraminidase) genes for the detection of ferlaviruses (Ahne et al., 1999). Additional primers and protocols have also been described (Franke et al., 2001; Marschang et al., 2009). Today, PCR protocols for the detection of ferlaviruses in reptiles are established in most commercial laboratories dealing with reptile samples.

Only one study so far has examined the clinical impact of an experimental infection with a ferlavirus in squamate reptiles. In that study, six Aruba island rattlesnakes (*Crotalus unicolor*) were inoculated with a virus isolate via the trachea (Jacobson et al., 1997). Several animals were subsequently sacrificed and the virus was isolated from the lung tissue. All animals demonstrated pneumonia and the last snake died spontaneously on day 22 after infection. No tissues other than the lung were examined for the presence of ferlaviruses in that study and the virus used was not further characterized.

Even though ferlavirus infections are important causes of respiratory disease in snakes, and reptiles are regularly checked for the presence of this virus, little is known about the pathogenesis, the incubation period of the virus after infection, the organ distribution and the shedding via the body orifices. Additionally, with reference to these points, it is still unknown if there are differences between different virus strains, e.g. between the genogroups that have been described so far. It would therefore be of great importance for the understanding of the infection as well as the optimization of diagnostic procedures to increase the knowledge on the pathogenesis and organ distribution of ferlavirus infections.

The aim of this study therefore was to compare the detection of different ferlaviruses after experimental infection using cell culture as well as PCR using corn snakes (*Pantherophis guttatus*) as a model species.

This study met the local as well as international guiding principles for biomedical research, and was approved by the local authority (animal trial no. TVV 61/13).

2. Material and methods

A total of 42 adult corn snakes (*P. guttatus*) were included in the study (Table 1). All animals were acquired from a commercial company and originated from unremarkable collections. Further details on the age and origin of the animals were not known. Prior to inclusion in the study they underwent a thorough health check including assessment of the clinical status (body condition, overall condition, clinical examination following established standards). Further, the snakes were examined for ecto- and endoparasites (visual inspection, fecal sample as well as cloacal wash sample for native assessment as well as flotation for parasite eggs). Swabs from choana and cloaca as well as tracheal wash samples were checked for bacterial and fungal pathogens, using cytology and aerobic culture media. In addition, a combined cloacal swab and fluid from a tracheal wash sample was checked for the presence of ferlaviruses following the established protocol for L gene detection (Ahne et al., 1999). No screening for other possible pathogens (e.g. reptarenaviruses, nidoviruses, adenoviruses) was done.

Only snakes without clinical signs and negative for ferlavirus as well as other pathogens were used for the study. All snakes were housed for at least two months before the beginning of the study, and examinations were repeated before the start of the acclimatization period (day 6).

The snakes were housed in terraria with a size of approx. 140 × 78 × 65 cm, in groups of six animals per terrarium. Terraria were supplied with a suitable ground material (turf), hiding places and a water basin. Temperature and humidity were recorded using commercial data loggers (microlite II, imec, Heilbronn, Germany) and kept within a range suitable for the species (20–28 °C, hot spots to 35 °C, 40–70% rel. humidity). Depending on the sampling day concept, snakes were fed every 7–14 days with one mouse each. Within the sham-infected group, food was accepted continously by most snakes (83–100%). In infection group 1 (genogroup A), food intake was also consistent at 90–100%. In contrast, in group 2 (genogroup B) the food intake decreased from 100% rsp. 83% before infection beginning on day 11 after infection (44%). In group 3 (genogroup C), food intake decreased to 50% from day 20. The terraria for the infection group were placed in separate rooms with anterooms used as hygiene lock (separate rooms providing appropriate hygienic facilities through which access to the infection groups was possible). To prevent cross infections between the groups, only one transmission study with one virus was carried out at a time, and the control group study took place in a different room at a different time.

Snakes were divided into three infection groups with 12 animals each, and one sham-infected control group with six animals. Details of size, body masses and sex are listed in Table 1.

The experimental setup consisted of an acclimatization period of six days, followed by the day of virus inoculation, and a 49 day post infection (p.i.) period.

Three virus strains were used in separate groups for the inoculations, details and references are provided in Table 2. All virus strains were cultured on VH2-cells according to an established protocol (Abbas et al., 2011). The total number of passages for each virus prior to inoculation in the corn snakes was

Table 1
Animals used in the study: size, body mass, sex, for each group as an average and max/min.

Study	No. animals	Length (cm)	Body mass (g)	Male/female
Trial 1	12	126 (119–141)	358 (219–519)	7/5
Trial 2	12	121 (104–140)	350 (232–498)	6/6
Trial 3	12	123 (106–150)	451 (288–667)	4/8
Control group	6	133 (126–140)	524 (410–761)	4/2

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