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Presence and survival of African swine fever virus in leeches

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ABSTRACT

This research is focused on the study of African swine fever virus (ASFV) survival in the leech *Hirudo medicinalis*.

To detect the infection route of the virus in leeches, two introduction methods were used: blood-feeding from infected swine hosts and exposure to ASFV-contaminated water (leeches cultivated with ASFV).

This study found that the survival of ASFV in leeches was much longer than that in leech-free medium.

The persistent presence of the virus in leeches and leech excretion was detected in both groups. The virus excreted from both groups of leeches in the terminal stages of the experiment was able to induce productive infection in porcine alveolar macrophages.

In an attempt to understand ASFV, transmission to pigs was conducted through the ingestion of leech-cultivated water and leeches that had fed on ASFV-infected animals or that were cultivated environmentally with the virus.

The ingestion of some samples at 60–80 days after cultivation demonstrated successful ASFV transmission via per os infection.

In conclusion, leeches can serve as a possible reservoirs for ASFV in the absence of its main hosts – pigs and some ticks of the genus *Ornithodoros*.

1. Introduction

African swine fever virus (ASFV) can be transmitted by contaminated water (Boklund et al., 2018). Moreover, water sources may be a crucial source for the transmission of ASF virus (Kukielka et al., 2016). However, it was shown that ASFV was rapidly inactivated (usually within several days) in the water environment (Turner, Williams, 1999). Thus, searching for aquatic ASFV biological reservoirs that are able to preserve and transmit the virus in the absence of viremic hosts remains a very important focus. Until now, the source of ASF persistence in water has never been shown. One possible source could be the urine or feces of infected animals (Chenais et al., 2017). However, the virus cannot survive for a long period in the environment without a natural reservoir that can facilitate ASFV transmission and maintenance. Transmission, of course, is more considerable in wild boar populations, as domestic pigs can support several transmission cycles (Alkhamis et al., 2018). Despite this, the role of water sources in the involvement in the virus transmission cycle should not be excluded. It is also possible that the virus survives for a long time in unknown water reservoirs. In natural conditions, leeches can be an important biological host for environmental bacteria, protozoa and viruses, which colonize their digestive tract (Boughalmi et al., 2013). The leech was first investigated as a potential reservoir for viruses by Boynton (1913). He found that rinderpest virus could be transmitted to cattle by the water leech *Hirudinaria manillensis*. One of the principal articles revealing the role of leeches as potential reservoirs for viruses was published in 1957 (Shope, 1957); it suggested that classical swine fever and *Myxoma* viruses could be harbored by leeches. Leeches as mechanical vectors for fish viruses were described in a review by Ahne and co-authors (2002). It has also been reported that *Hirudo medicinalis* can harbor a fish virus causing infectious pancreatic necrosis (Salimi, Abdi, 2016). Marine leeches can serve as mechanical vectors for the fibropapilloma-associated turtle herpesvirus (Greenblatt et al., 2004). The presence and survival of some bacteriophages inside the guts of leeches were studied by Nehili et al (1994). The ability of some mammalian viruses to survive in the *Hirudo medicinalis* gut was described by Al-Khleif et al (2011) and Wilken, Appleton (1993). Unfortunately (for scientific investigations), although leeches may harbor mammalian

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viruses obtained by a blood meal from the aforementioned hosts, only a few cases of viruses transmitted by leeches have been reported (Shope, 1957; Al-Khleif et al., 2011).

This research is focused on African swine fever (ASF) virus survival within the leech (*Hirudo medicinalis*) and the possible existence of an ASFV reservoir among prevalent blood sucking freshwater inhabitants – the leeches. This is supported by the fact that the leeches are blood sucking animals with the ability to consume blood from a wide spectrum of vertebrate hosts, with pigs being among the most frequent (Schnell et al., 2012); pigs are omnivorous and usually eat anything, from grain to carrion. Pigs consume an extensive amount of invertebrates, including leeches (Schley, Roper, 2003).

The aim of our research was to study the ability of leeches to persistently harbor and excrete ASFV into the environment and the features of this excretion, as well as the persistence of infectability of the excreted viruses.

2. Methods

2.1. Animals

Adult *Hirudo medicinalis* leeches were obtained from the "Hirudo Med" Medical Center, Yerevan, Armenia. For this experiment, seven pigs were used as virus donors, and all successfully introduced ASFV to all the leeches. The leeches were maintained in a tightly closed glass aquaria filled with stale tap water (Nehili et al., 1994). All aquaria were covered to protect the leeches from direct sunlight.

Data on the leeches involved in the experiment are presented in Table 1.

The leeches used for hemadsorption (HAD) detection were also used to investigate the possibility of virus isolation for the infection of swine AMs. The leeches (group 1, n = 96; group 2, n = 96; control group 1, n = 10; control group 2, n = 10) were used for HADU detection and virus isolation to analyze their ability to cause infection in porcine alveolar macrophages (AMs). Group 1 included leeches fed directly on ASFV-infected pigs (i.e. only feeds once). The leeches were permitted to feed for 10–30 minutes. Group 2 included hungry leeches cocultivated with virus isolated from spleen extracts of ASFV-infected swine (dose of ASFV 4.0 lg $HADU_{50}$/ml). Ten leeches fed on human blood (i.e. only feeds once) and 10 hungry leeches were used as control groups 1 and 2, respectively, and were kept in the laboratory under the same conditions as the experimental leeches.

Thermal conditions for the leeches were established according to McCreesh et al (2014), excluding the most tepid conditions.

Leeches were cultivated along with ASF virus for 24 h, then isolated from virus-contaminated water, washed in tap water and cultivated in virus-free water. Leeches from groups 1 and 2 were used for HADU detection as well as isolation of the virus for swine AM infection. They were sacrificed freezing in a Petri dish until they were frozen solid (Al-Khleif et al., 2011). They were then cleaved into anterior and posterior halves, paraffinized and sectioned with a microtome (5 μm).

Leech mortality in all groups (both control and experimental) was compared at the end of the experiments (160 days). Leeches excretion and molted skin were separated as sediments by centrifugation at 1000 rpm for 10 minutes.

ASF virus presence was tested in all leeches and all samples of water throughout all stages of the experiment.

2.2. Transmission of African swine fever virus in the process of natural feeding

Six pigs were fed a mixed diet containing bodies of frozen leeches and leech-cultivation water samples. Two groups of 3 animals were infected by oral administration with samples that had been cultivated for 60–80 and 140–160 days. Each pig received at least 30 ml of leech-cultivation water and 1 frozen leech from each group.

2.3. Virus

Virus titration was performed as described previously and expressed as log 10 HAD_{50}/ml for non-adapted cells (Enjuanes et al., 1976). ASFV was obtained from spleens originating from infected pigs. The virus (Arm07) genotype II (Arm07) was first isolated in 2007 from the spleen of a swine infected with ASFV (Gallardo et al., 2018). The virus belonged to genotype II, which is distributed in Trans-Caucasian countries. Pigs (7 animals) were housed in separate stables, where they had access to a commercial feed twice per dayand to clear water at all times. Pigs were infected by intramuscular injection with 10^4 hemadsorbing doses (HAD_{50})/ml. The dose of the virus in the organ extracts was 5.5–6.0 l g $HADU_{50}$/ml (at 6th day post infection). The level of ASFV in the blood of infected pigs before leech (group 1) feeding on the skin was 5.5 ± 0.5 lg $HADU_{50}$/ml. The level of ASFV in the leech-cultivation water (further tested at ten-day intervals throughout the experiment) (group 2) was 4.0 ± 0.1 lg $HADU_{50}$/ml. The animal experiments were permitted by the Institutional Review Board/Independent Ethics Committee of the Institute of Molecular Biology of NAS RA (reference number IRB00004079).

Table 1
Groups of the leeches involved in experiment.

leech that sucked the blood of an infected pig (group 1)						leech (hungry) that cultivated in environment with ASF(group 2)					
days	rtPCR	HADU	Histology	Freeze	Control (human blood)	days	rtPCR	HADU	Histology	Freeze	Control (hungry)
10	2	2	1	1	–	10	2	2	1	1	–
20	2	2	1	1	–	20	2	2	1	1	–
30	2	2	1	1	–	30	2	2	1	1	–
40	2	2	1	1	–	40	2	2	1	1	–
50	2	2	1	1	–	50	2	2	1	1	–
60	2	2	1	1	–	60	2	2	1	1	–
70	2	2	1	1	–	70	2	2	1	1	–
80	2	2	1	1	–	80	2	2	1	1	–
90	2	2	1	1	–	90	2	2	1	1	–
100	2	2	1	1	–	100	2	2	1	1	–
110	2	2	1	1	–	110	2	2	1	1	–
120	2	2	1	1	–	120	2	2	1	1	–
130	2	2	1	1	–	130	2	2	1	1	–
140	2	2	1	1	–	140	2	2	1	1	–
150	2	2	1	1	–	150	2	2	1	1	–
160	2	2	1	1	10	160	2	2	1	1	10
Sum	32	32	16	16	10	Sum	32	32	16	16	10

2.4. Isolation of DNA and implementation of qPCR

A volume of 0.2 ml of all investigated samples was submitted to nucleic acid extraction using a Viral Genomic DNA/RNA Extraction Kit (MyBioSource, San Diego, California, USA; catalogue number MBS846571), following the manufacturer's instructions.

The presence of ASFV in our samples was measured by quantitative real-time polymerase chain reaction with a Rotor Gene Q instrument (Qiagen, USA). DNA (2 µl, corresponding to 40 ng) was added to 18 µl PCR mix. The final reaction mix contained 500 nM primers and 250 nM probe (PrimeTime qPCR Assay, IDT, USA), 10 mM $MgCl_2$, 200 µM each dNTPs, 1 U Hot-start Taq polymerase and 1x PCR Buffer (Dia-M, Moscow, Russia). The primers and fluorescent-labeled probe used were as follows:

ASFV gene:

Fluorescent probe - 6-FAM/TAMRA

Sequence 1 – TGC TCA TGG TAT CAA TCT TAT CG

Sequence 2 – CCA CTG GGT TGG TAT TCC TC

Sequence 3 - /56-FAM/ TTC CAT CAA AGT TCT GCA GCT CTT /36-TAMSp/

β-actin gene:

Fluorescent probe - TET/ZEN/IBFQ

Sequence 1 - CTC GAT CAT GAA GTG CGA CGT

Sequence 2 -GTG ATC TCC TTC TGC ATC CTG TC

Sequence 3 -/5TET/AT CAG GAA G/Zen/G ACC TCT ACG CCA ACA CGG /3IABkFQ/

The β-actin gene was used as a housekeeping gene.

2.5. Alveolar macrophage culture

Three-month-old pigs were euthanized, and the lungs were removed. Cells obtained during bronchoalveolar lavage were resuspended in sterile Hank's balanced salt solution. They were centrifuged at 600 *g* for 10 min and resuspended in RPMI 1640 with 5% fetal bovine serum at a cell concentration of 0.5–1 × 10^6 ml. After 3 h at 37 °C in a humidified, CO_2 incubator, the adhered cells were washed three times with RPMI to remove contaminating non-adherent cells and then re-incubated in RPMI 1640 with 10% FBS (Forman et al., 1983).

All cells and samples were stained withFeulgen-Naphthol Yellow staining procedure (Gaub et al., 1975). The infection of swine AM cells was performed by maximal dilutions of all cultured media and leech bodies (1 × 10^{-1} $HADU_{50}$/ml logarithmic dilutions). Before infection, the experimental samples were tested by HAD and rtPCR methods for the detection of ASF virus.

2.6. Statistical analysis

All in vitro experiments were conducted in triplicate. The significance of virus-induced changes was evaluated by a two-tailed Student's t-test; p values < 0.05 were considered significant. SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

3. Results

3.1. Experimental design and virus measurement

All leeches were divided into two groups. The leeches in the first group that were to be infected with ASFV (transmission trough blood meal) were applied to the skin of the lower abdomen of pigs infected with ASFV. In this experiment, the pigs were used 6 days postinfection (Fig. 1A). This stage was chosen to ensure the highest levels of viremia (Fig. 1B). Leeches were fed for approximately 10 min, and then the feeding was interrupted by forcible manual removal. Several hours later, the feeding area developed multiple petechiae, and the pigs were euthanized.

The leeches in the second group were intended for cocultivation with ASFV. Leeches in this group were cocultivated with a 10-fold dilution of infected pig spleen extract (dose of ASFV 4.0 lg $HADU_{50}$/ml).

ASFV in both groups was successfully transmitted to the leeches (confirmed by HADU from body extracts, Fig. 1C).

The leech cultivation temperature range was set between 23 °C and 32 °C (modeling of seasonal changes of summertime conditions for virus survival in the wild), with 30 days at 22 °C–25 °C, 80 days at 26 °C–32 °C and 60 days at 24 °C–26 °C (Fig. 1D).

Our study did not document any evidence of effects of ASFV on the leech average life expectancy since the leech mortality in the control groups (1 leech - 10% from the 1 st control group; 1 leech - 10% from the 2nd control group) did not differ from that of the experimental groups (7 leeches (7.3%) from the 1 st group; 8 leeches (8.3%) from the 2nd group).

3.2. Presence of African swine fever virus in the leeches following feeding and cocultivation

Data on virus detection by the HADU method in all samples, both from the leech bodies and the culture media, are shown in Table 2. As seen in the table, the virus was detectable in all samples up to 150 days after the experiment. On day 160, the virus was detected in 50% of leeches, whereas the culture media did not show any evidence of virus.

In the control groups (without leeches), inactivation of ASFV in similar (to experiment) conditions occurred at approximately 10–35 days. The baseline ASF virus titer in the culture medium was registered as 4.0 lg HADU/ml. Our data showed that virus inactivation was directly dependent on medium temperature, and ASFV could remain infective for at least 12–14 days when kept in the dark at 30 °C (Fig. 2A). The longest period in which the virus remained detectable by HADU was 30–35 days (Fig. 2A).

Viral DNA measured by rtPCR was detectable until 3–5 weeks of incubation (depending on medium temperature).

As shown in Fig. 2B, ASFV was detected in all leeches for up to 150–160 days after feeding. Following cocultivation, ASFV was detected for up to 140–150 days.

Fig. 2B shows the rtPCR detection data of ASFV DNA in the leeches fed on infected pig blood at the 150th day of follow-up. As a comparison, logarithmic dilutions of the ASF virus isolated from infected pig spleen are shown. The lowest spleen ASFV dilution corresponded to 3 lg $HADU_{50}$/ml, and the level of viral DNA (evaluated by rtPCR) in a leech was approximately 10,000 times less.

Using the HADU method, it was shown that during the initial stage of the experiment, the level of virus in the blood-fed leeches was expectedly higher than that in the virus-cocultivated leeches (Fig. 2C). Then, 60–80 days after the initiation of the experiment (Fig. 2C), the virus content decreased in the body of leeches that fed on infected pig blood. Nonetheless, virus in the blood-fed leech was present until the end of the experiment (on day 160) (Table 2). On day 150, the virus was detected in the majority of the probes studied. The rtPCR data were also concordant with the data obtained by the HADU method (Fig. 2D, Table 2).

As in the control group, the baseline ASF virus titer in the culture medium (with cocultivated leeches) was also 4.0 lg $HADU_{50}$/ml. The virus titer in the culture media decreased during the process of cocultivation with leeches; nonetheless, the virus was detectable by both the HADU (Fig. 2E) and rtPCR methods (Fig. 2F) in both of the leech groups until the end of the experiment. These results showed that when ASFV was cultivated with leeches, the survival rate of the virus was significantly increased. African swine fever viral genome copies in culture medium and sediments in both groups were determined using rtPCR. Fig 2G represents a change that occurs in levels of viral genome copies both in water and in sediment in group I during cultivation. The data indicated that the sediment (in group I) generally have achieved higher ASFV DNA copy numbers than the cultivation medium.

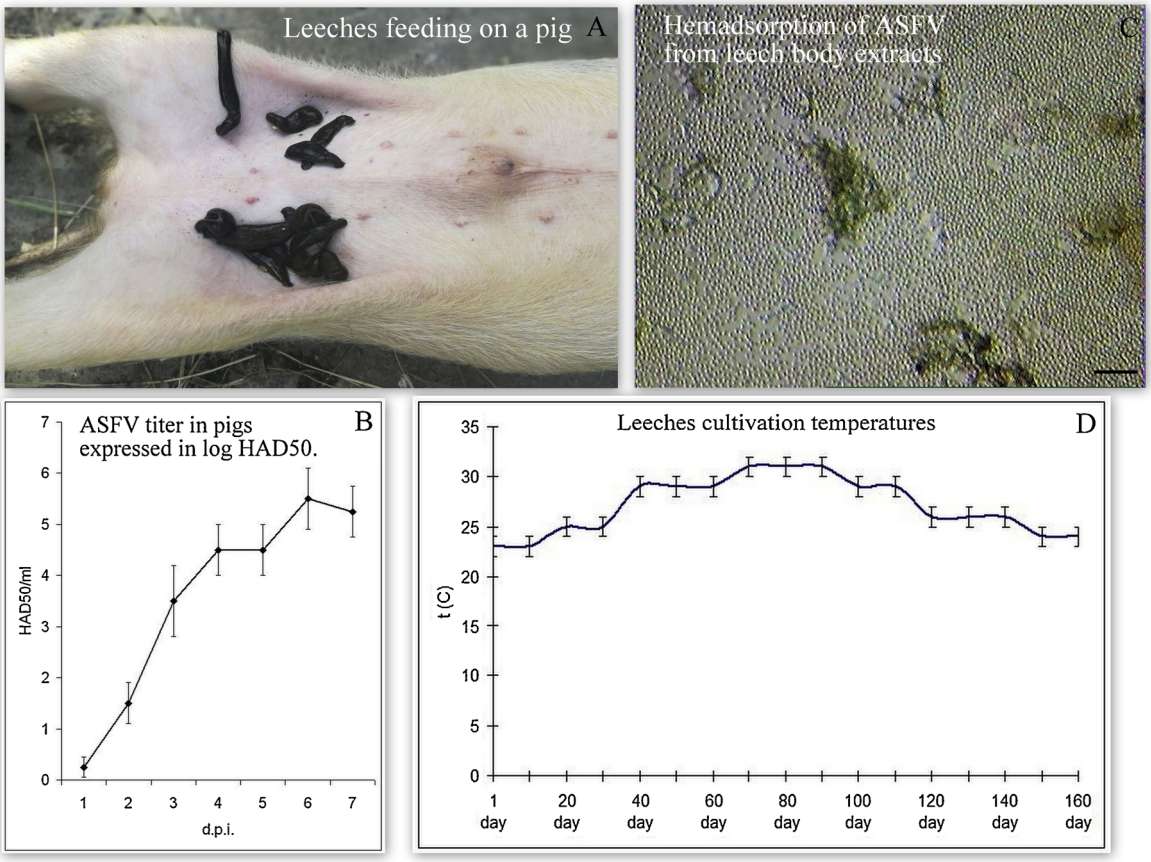

Fig. 1. Experimental design.
A. *Hirudo medicinalis* (medicinal leech) feeding on a pig; B. African swine fever virus titers in porcine blood (HADU) representing disease dynamics. C. Hemadsorption of African swine fever virus from leech body extracts (group 2 at 60 days after cultivation; 1×10^{-1} $HADU_{50}$/ml logarithmic dilution). Scale bar = 100 μm. D. Cultivation temperatures of the environment (water) during the experiment.

Table 2
Presence of african swine fever virus in leeches.

leech that sucked the blood of an infected pig (group 1)			leech that cocultivated in environment with ASFV (group 2)		
days	Detection virus by rtPCR	Detection virus by HADU	days	Detection virus by rtPCR	Detection virus by HADU
10	+ +	+ +	10	+ +	+ +
20	+ +	+ +	20	+ +	+ +
30	+ +	+ +	30	+ +	+ +
40	+ +	+ +	40	+ +	+ +
50	+ +	+ +	50	+ +	+ +
60	+ +	+ +	60	+ +	+ +
70	+ +	+ +	70	+ +	+ +
80	+ +	+ +	80	+ +	+ +
90	+ +	+ +	90	+ +	+ +
100	+ +	+ +	100	+ +	+ +
110	+ +	+ +	110	+ +	+ +
120	+ +	+ +	120	+	+ +
130	+ +	+ +	130	+	+
140	+	+ +	140	+	+
150	+ +	+ +	150	+	–
160	–	+	160	–	–

+ + presence of the ASFV in both leeches.
+ ASFV present in one leech.
- ASFV absent.

3.3. Ability of virus to infect cells

These experiments were designed to determine whether ASFV (virus obtained from cultivation water and body extracts from both investigated groups) is capable of subsequent virus transmission (productive) to normal cells. Primary cultures of porcine AMs were infected with the highest dilutions, 1×10^{-1}, of the cultured media. These experiments were performed after incubation for 130–160 days.

Feulgen-positive cytoplasmic DNA assemblies of ASFV were detected in AMs infected by virus obtained from the leech bodies and leech-cultivation water samples in both groups (leeches that fed on the viremic blood – Fig. 3A, and leeches cultivated with ASFV – Fig. 3B). ASFV DNA assemblies were developed 24–48 hours postinfection.

An increase in the ASFV titer was observed at 24–48 hours following inoculation with water from cultivated leeches as well as extracts of the bodies. After 48 h of passage on the AMs, the productivity of infectious ASFV titers increased approximately 1.5–2.8-fold (data from rtPCR analysis; Fig. 3C, D). Next, HADU measurement in porcine AMs infected by virus obtained from the leech bodies and leech-cultivation water samples in both groups was performed. The data indicated a 2- to 10-fold increase in the HADU number after 48 h of experimental infections in both groups (Fig. 3E).

3.4. Ability of virus to infect pigs

The experimental data regarding whether leeches that fed on ASFV-

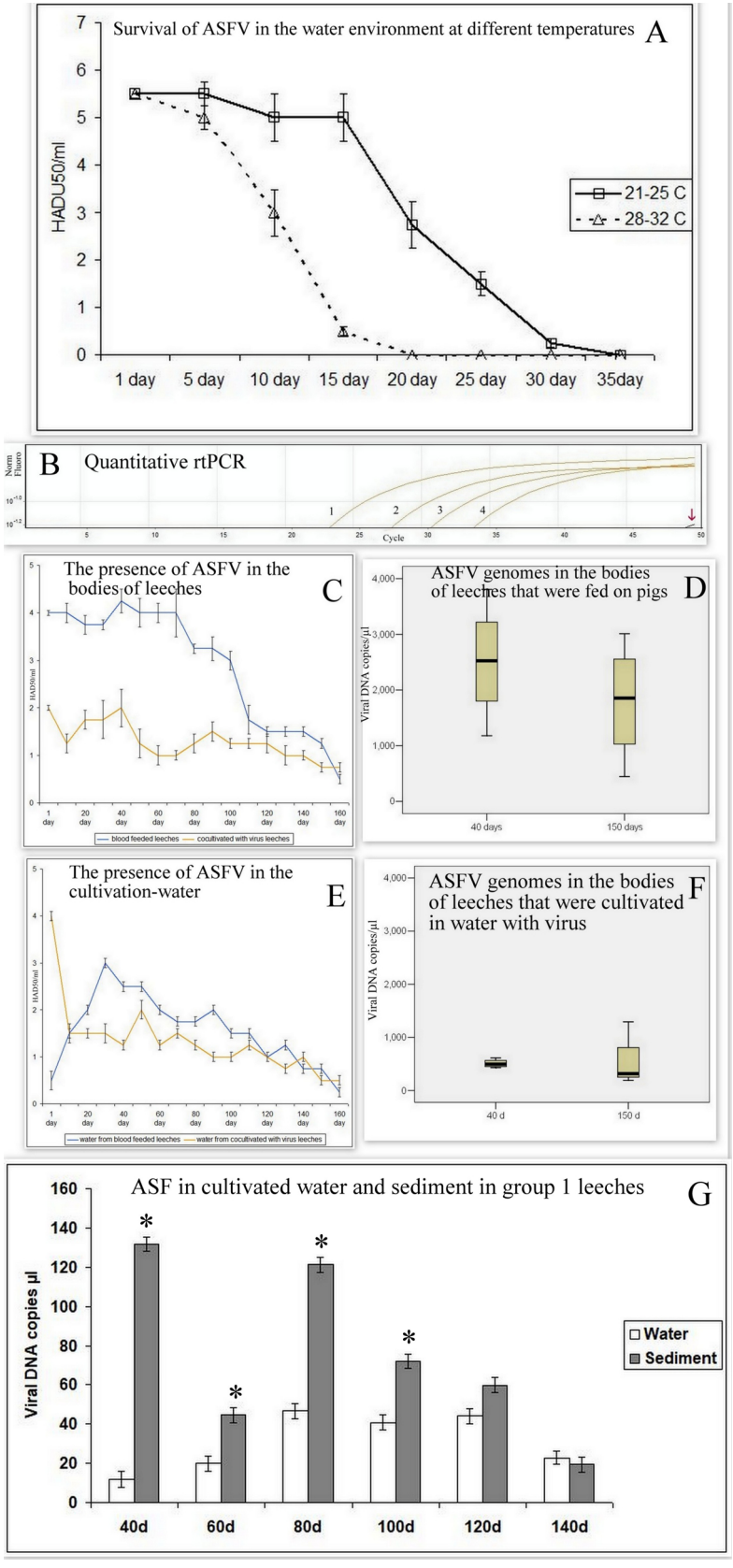

Fig. 2. Changes in African swine fever virus load during cultivation with leeches.

A. Survival of African swine fever virus in the water environment at different temperatures.

B. Quantitative rtPCR. ASFV genome copy synthesis is performed with 10-fold serial dilutions: 1 – 1×10^6 $HADU_{50}$/ml; 2 – 1×10^5 $HADU_{50}$/ml; 3 – 1×10^4 $HADU_{50}$/ml; 4 – 1×10^3 $HADU_{50}$/ml. Arrow - ASFV genome copies obtained from a leech at day 150; the leech was cultivated with ASFV in a water environment. C. The presence of ASFV in the bodies of leeches that were fed on pigs and cultivated in water with ASFV (measured by the HADU method). D. The presence of ASFV in the bodies of leeches that were fed on pigs, as measured by rtPCR. E. The presence of ASFV in the cultivation-water of leeches that were fed on pigs and cultivated in water with ASFV (measured by the HADU method). F. The presence of ASFV in the bodies of leeches that were cultivated in water with ASFV, as measured by rtPCR. G. The presence of ASFV in the cultivation-water and sediment of leeches that were fed on pigs; *significant compared with water samples ($p < 0.05$-$p < 0.01$).

infected swine or cultivated in the environment with ASFV could subsequently transmit virus to pigs by natural feeding are presented in Table 3.

Our data indicated that the natural transmission of ASF virus by infected leeches through the oral administration of leeches to pigs was possible, but it was not a regularly reproducible phenomenon, especially in the late stages of the experiment.

There was a long latency period (12–15 days) between oral

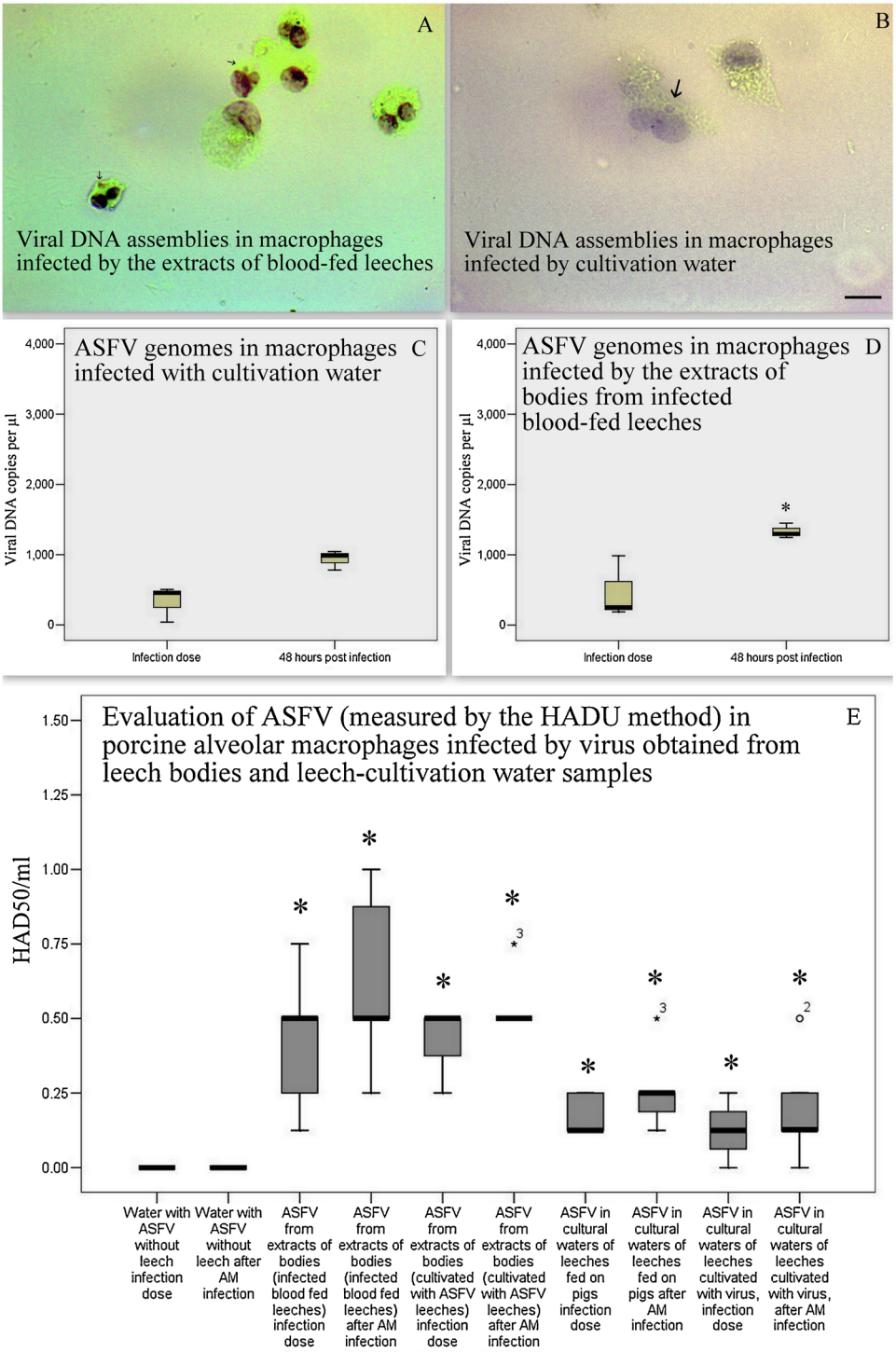

Fig. 3. Transmission of ASFV by Infected Leeches (after natural feeding) and Leeches cultivated with infected blood to porcine alveolar macrophages.

A. Feulgen-positive viral DNA assemblies replication in porcine alveolar macrophages infected by the extracts of bodies from infected blood-fed leeches (150 days). B. Feulgen-positive DNA viral replication in porcine alveolar macrophages infected with water that was cultivated with ASFV (150 days). Scale bar = 10 µm.[1]

C. D. ASFV genomes (measured by rtPCR) in porcine alveolar macrophage cultures. C. Alveolar macrophages were infected with cultivation water (10-fold dilution, 150 days). D. Alveolar macrophages were infected by the extracts of bodies from infected blood-fed leeches (150 days) (*$p < 0.05$).

E. Evaluation of ASFV (measured by the HADU method) in porcine alveolar macrophages infected by virus obtained from leech bodies and leech-cultivation water samples in both groups. Measurements were performed at 48 h postinfection; *significant compared with water samples ($p < 0.05$-$p < 0.01$).

Table 3
Transmission of ASF virus by infected leeches and leeches-cultivated water to pigs by oral administration.

Time since virus introduction (days)	Pig №	ASF transmission
60-80	1	Negative
	2	Successful
	3	Successful
140-160	4	Negative
	5	Negative
	6	Negative

administration and the appearance of the first clinical symptoms (loss of appetite and high temperature). An experimental study showed that pigs developed symptoms that were strikingly similar to classical ASF (except an abnormally long latent stage).

Attempts to transmit ASFV in the process of pig feeding were ineffective; all three pigs were ASFV-free.

[1] There is absence of asterisks on Fig. 2G. I tried to replace the figure by edited one but I couldn't do it.

4. Discussion

The data obtained from the results showed that ASF virus remains active up to 140 days after cocultivation with leeches under relatively high temperatures. It can be expected that cocultivation at decreased temperatures may provide increased survival of the virus (data consistent with Al-Khleif et al., 2011).

This result allows us to consider that leeches are a suitable reservoir for AF virus between epidemic outbreaks. Our study results explain seasonal changes in the prevalence of ASFV infection in outdoor pigs and wild boar populations (increased ASFV cases in spring-autumn) (Pautienius et al., 2018).

Our results also indicate that ASFV survives significantly longer in leeches than in any other environmental sample (except biological *ornithodorid* ticks vectors and *suid* hosts). ASF virus survival occurs in high-temperature medium (compared to data of Al-Khleif et al., 2011). It is still unknown how leeches maintain viral infectivity; Al-Khleif et al. (2011) suggested that some substances exist in the leech body that might conserve virus infectivity.

Persistent virus survival in leeches and its excretion into the surrounding environment can be supported by virus replication in the leech tissues or the ingested blood or by long-term preservation of the virus in tissues, with its release in low doses. Blood fed to the leeches can be an optimal medium for the replication of various intracellular parasites, particularly *Plasmodium* parasites (Nehili et al., 1994). However, no analogous data regarding virus replication exist. Effective (high output of new virus particles) reproduction of ASF virus within the blood obtained from pigs is less probable since the titers of excreted virus in the two groups were very similar (except during the initial stages of experiment).

Detection of ASF virus in the culture water can be mediated either by leech molting or/and excretion. Molting in leeches occurs every 2–3 days (Paxton, 2005), and sediment with molted skin was ASFV-positive by rtPCR analysis (data not shown).

We conclude that leeches may play an important role in the maintenance of African swine fever virus. Additionally, it is possible that viruses can enter the environment as a result of excretion processes (Mann, 1962). The leeches *Hirudo medicinalis* and its sister taxon *H. verbana* are widely distributed in Europe and western Asia. Despite the fact that the species are now separated based on their genome and morphology, they share similar behavioral patterns (Kutschera, Elliott, 2014). Thus, leeches can be an important source for virus persistence in the vast territories of Eurasia.

The persistence of ASFV in leeches for at least five months suggests that the virus is well protected from environmental factors. Such long-term survival could be of high importance as a mechanism for the preservation of ASFV in nature (Shope, 1957). Our data suggest that the diversity of aquatic ecological niches for ASFV is not yet fully understood. Studies by Schley, Roper (2003) showed that wild boars feed on a wide range of animal species, including *Hirudinea*. Some regions, such as Armenia, are characterized by outdoor-bred pigs (Beltrán-Alcrudo et al., 2018), and persistent survival of the virus within leeches, presumably, can be a cause of additional seasonal outbreaks (as leeches often appear in the diet of wild boar as well as outdoor-bred pigs). In the wild in Eurasian regions, boars are the main reservoir of ASF virus. To date, it is considered that boars are often infected through contact with boar cadavers that died from African swine fever (Probst et al., 2017). The data obtained in our study indicated that leeches are also a crucial source of ASF virus, and this may significantly affect the course of an ASF epidemic in Eurasian regions.

Our data indicate that the ingestion of some samples 60–80 days after cultivation can result in successful ASFV transmission via per os infection. In the water without leeches, infective virus particles and detectible amounts of viral genomes were absent.

The failure to infect pigs by samples obtained from late (140–160 days) cultivations is probably associated with a critically low infective dose for oral administration (Howey et al., 2013; Maurer and Griesemer, 1958). Low doses of ASFV in cultivated water also decreased the possibility for transmission (in the late stages of the experiment). Contaminated feed is a low-probability event compared with contaminated liquid ingestion (Niederwerder et al., 2019)

5. Conclusion

1 The presence of ASF virus in leeches prevailed for a period much longer than the time of virus survival in leech-free medium. Interestingly, the virus survived in both the leeches fed on infected porcine blood and the leeches that were obtained from the cocultivation environment.
2 ASF virus was detected in cultivated waters from blood-fed as well as virus-cultivated leeched for a long period of time.

In summary, leeches serve as potential reservoirs for the survival of the African swine fever virus in the absence of other hosts, such as pigs (the main hosts), as well as ticks of the genus *Ornithodoros*, which are biological vectors of the virus.

Declaration of Competing Interest

There are no conflicts of interest in the present study.

Acknowledgments

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