Tenacity of mammalian viruses in the gut of leeches fed with porcine blood

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Leech therapy is currently considered to be of high therapeutic value in medicine. However, feeding leeches with fresh animal blood during the maintenance and reproduction phase bears the risk of transmission of zoonotic viruses to the patient. We hypothesize that this would be abolished by subjecting leeches to quarantine measures prior to use. The required duration of quarantine would depend on the maximum survival time of pathogens in contaminated leeches. In order to be able to estimate this survival time reliably, experiments were conducted with enveloped and non-enveloped mammalian viruses possessing either RNA or DNA. Leeches were fed porcine blood contaminated with bovine parvovirus (BPV), feline calicivirus (FCV), equine arteritis virus (EAV) and equine herpesvirus type 1 (EHV-1) and kept in aquaria at 10 °C. From week 6 after feeding onwards, some leeches were held at 30 °C. Before feeding and at different time points thereafter, blood samples were taken from the leeches to determine residual virus infectivity. Prototype mammalian viruses were able to survive in inoculated leeches for considerable periods of time. When leeches were kept at 10 °C throughout, reisolation of infectious virus from the leeches' abdominal cavity blood was no longer possible at 23 (FCV), 23 (EAV), 27 (EHV-1) and 29 (BPV) weeks after inoculation. Shifting the temperature to 30 °C in week 6 slightly reduced the duration of detection of infectious viruses to 15 (EAV and EHV-1), 21 (FCV) and 27 (BPV) weeks. These data indicate that the ability of mammalian viruses to survive in leeches theoretically poses a possible risk for patients unless adequate precautionary measures are adopted. Application of a quarantine period, e.g. 31 weeks (i.e. including an additional safety period) at 10 °C, may be a suitable measure to significantly decrease this risk.

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INTRODUCTION

Hirudo medicinalis, medicinal leeches, belong to the phylum Annelida. Leeches are temporary blood-sucking parasites and, during the time of sucking, secrete a wide variety of substances, some of which are of benefit for the host. An example is hirudin, which is best known for its anticoagulative effect. Hirudin is used in the treatment of inflammation of the middle ear (Seleznev *et al.*, 1992), is used as a systemic anticoagulant and may prove useful for *in vitro* blood collecting systems. Medical use of leeches also includes treatment of black eyes (Seleznev *et al.*, 1992).

Due to these beneficial secretions, leeches have been successfully applied to many ailments in humans for over 2000 years. The first documented attempts to use *H. medicinalis* for medicinal purposes date back to the time of

Hippocrates. Leech therapy is also used in traditional Chinese medicine. Paintings of medicinal leeches have been found in pharaohs' tombs. The Solomon Parables also describe leech treatment in ancient medicine. The Roman physician Galen classified leech therapy as a method for achieving healthy balance. The use of leeches peaked between 1830 and 1850 in Europe, but subsequent shortages in delivery led to a decline in use. Currently, 100 000 patients in Germany per year (about 0.1 % of the total population) regularly use leeches for therapy, in particular to treat vascular diseases. Leech therapy has also become popular in veterinary medicine in recent years.

Most leeches feed as blood-sucking parasites on their preferred host (Mann, 1962). If the preferred host is not available, most leeches will feed on other classes of host. Some feed on the blood of humans and other mammals, whilst others parasitize fish, frogs, turtles or birds.

Abbreviation: TCID₅₀, 50 % tissue culture infective dose.

Sanguivorous leeches can ingest an amount of blood several times their own weight at one meal. After feeding, the leech retires to a dark spot to digest its meal. Digestion is slow, which enables the leech to survive very long fasting periods of up to several months (Sawyer, 1981).

Although leeches may harbour zoonotic bacteria and/or viruses obtained by sucking from previous hosts, only a very limited number of cases of diseases transmitted by leeches have been reported. Examples of pathogens involved include *Streptococcus* sp., *Clostridium tetani*, classical swine fever virus and *Aeromonas hydrophila* (Adams, 1988; Dickinson & Lent, 1984; Kaestner, 1982; Shope, 1957). However, a high incidence of infections, with *Aeromonas* spp. being one of the major pathogens, was reported after the application of medicinal leeches (Bauters *et al.*, 2007). Leeches used for medicinal purposes in Germany are from natural habitats mainly in the Near East or are bred in German aquaculture plants. Before use, leeches from both sources are kept isolated for a distinct time period beginning from import or last blood meal in order to enable self-inactivation of possibly incorporated pathogens. An appropriate length of quarantine will depend on the maximum duration that pathogens remain viable inside the leeches, but such experiments have rarely been reported. Bacteriophages and bacteria, for example, persisted in large numbers for at least 6 months in the gut of experimentally infected leeches (Nehili *et al.*, 1994). Hence, this study aimed to determine the survival time of prototypic mammalian viruses in leeches.

Groups of leeches were inoculated with two non-enveloped viruses, bovine parvovirus (BPV) and feline calicivirus (FCV), and two enveloped viruses, equine arteritis virus (EAV) and equine herpesvirus type 1 (EHV-1), by feeding them virus-contaminated porcine blood and they were maintained in aquaria. At the beginning of the experiment and at distinct times after inoculation, some leeches were euthanized and analysed for residual viral infectivity. We also tested whether different maintenance temperatures had an influence on virus survival time in leeches.

METHODS

Leeches. *H. medicinalis* leeches were obtained from Biebertaler Blutegelzucht GmbH and kept in the laboratory in aquaria at 10 °C until further use.

Viruses and cell cultures. The viruses used were two non-enveloped viruses (BPV, strain Haden; and FCV, strain CAL) and two enveloped viruses (EHV-1, strain RACH; and EAV, strain Bucyrus). Virus propagation was carried out in primary fetal calf lung cell cultures (BPV), feline embryo cells (FCV) or Vero cells (EHV-1 and EAV). Cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5 % fetal calf serum (FCS) and antibiotics. Logarithmic virus dilution series were prepared in MEM with 5 % FCS, and 100 µl of each dilution was inoculated in quadruplicate onto cell monolayers grown in 96-well microplates to determine virus titres. After incubation at 37 °C and 5 % CO₂, 50 % tissue culture infective doses (TCID₅₀) were estimated according to the methods of Spearman (1908) and Kärber (1931).

Determination of virus survival in leeches. Leeches were inoculated by being fed virus-contaminated blood. Test virus suspensions were mixed 1:10 with warm porcine blood (obtained at a local abattoir; Schlachthof) and vortexed for 3 min to obtain a homogeneous virus suspension at a dose of at least 10⁵ TCID₅₀ ml⁻¹. In a preceding experiment, blood from the same source was tested by tissue culture inoculation and found to be free of cytopathic viruses. Contaminated blood was then fed to the leeches by filling condoms with ~200 ml contaminated blood and allowing the leeches to suck through the skin of the condoms. Leeches were placed in water-filled aquaria at a constant temperature of 10 °C. After 6 weeks, the water temperature was increased to 30 °C in some aquaria.

Immediately after the leeches were fed and at 1–2-week intervals up to the end of the experiment, three leeches each were euthanized by freezing at –70 °C for 1 h. After thawing at 37 °C, the abdominal cavity was opened with scissors and the blood was collected in a syringe. To recover the viruses, the blood was frozen at –20 °C for 20 min, thawed at room temperature and then centrifuged at 2500 g for 5 min. The supernatant was used to determine residual virus titres. Titres were expressed as mean titres from three leeches per time point. Additionally, at late time points, 1 ml blood from each of the leeches was inoculated onto a cell monolayer grown in tissue culture flasks, with a resulting detection limit of 0.5 log TCID₅₀ ml⁻¹.

Cryosafe tubes were filled with 1 ml contaminated blood or virus suspension in PBS as controls. Control tubes were placed in the respective aquaria and tested for residual virus infectivity at the same time intervals as the leeches.

RESULTS

In the experiments at 10 °C, survival times of EHV-1 and EAV in leeches were greater than in PBS. However, in each case, the maintenance of virus infectivity was lower in contaminated blood than in leeches (Fig. 1). Viruses were inactivated in the leeches to levels below the detection limit of the cell culture assay as late as 23 weeks (FCV), 23 weeks (EAV), 27 weeks (EHV-1) and 29 weeks (BPV) when the leeches were kept at 10 °C throughout. Increasing the maintenance temperature to 30 °C in week 6 resulted in only a slightly shorter survival time of the viruses: titres then dropped below the detection limit within 15 weeks (EAV, EHV-1), 21 weeks (FCV) and 27 weeks (BPV) (Fig. 2).

As shown in Fig. 1, infectious BPV could be detected in at least one of three leeches until week 27 after feeding. Virus suspended in PBS (control) was detected until week 37 (end of experiment) and remained at a titre as high as 5.0 log₁₀ TCID₅₀ ml⁻¹. Blood controls contained infective virus until week 17. FCV survived in leeches until week 21, whilst virus was detected until week 23 in the PBS controls and until week 13 in the blood controls. Infectivity of EAV was observed in leeches until week 21. In blood and PBS controls, viable virus was present until weeks 11 and 15, respectively. EHV-1 virus titre persisted in leeches until week 25, but was only detected until weeks 19 and 21 in PBS and blood controls, respectively.

Leeches maintained at 30 °C from week 6 onwards after feeding harboured infectious BPV in at least one of three leeches up to 25 weeks after inoculation (Fig. 2). Virus

Fig. 1. Virus titres in the abdominal cavity of leeches fed with virus-contaminated porcine blood and maintained in aquaria at a temperature of 10 °C throughout. ▲, Virus control in PBS; ■, virus control in blood; —, blood samples from leeches (arithmetic means of three calculations). a, Contaminated blood before feeding; b, blood directly after feeding; dl, detection limit (0.5 log TCID₅₀ ml⁻¹).

controls with porcine blood remained infective until week 13. However, BPV in the PBS control remained infectious to the end of the experiment (week 31). FCV was isolated from leeches until week 19. FCV virus suspensions in PBS and porcine blood remained infectious until weeks 15 and 11, respectively. EAV was shown to remain infectious in leeches until week 13 after feeding, with the blood and PBS controls both remaining positive until week 8. EHV-1 infectivity was detected until week 13 in the blood recovered from the leeches, as well as in both controls.

DISCUSSION

The application of leeches in human medicine is currently considered to be of high value, in particular for the treatment of vascular diseases. However, medical application may pose a risk of patients becoming infected with different zoonotic agents. The reason for this hypothesis is that leeches maintained in leech plants have to have blood meals before being distributed to medical practitioners. Both imported leeches from the Near East, which have

Fig. 2. Virus titres in the abdominal cavity of leeches fed with virus-contaminated porcine blood and maintained in aquaria at temperatures of 10 °C for the first 6 weeks after feeding and at 30 °C thereafter. ▲, Virus control in PBS; ■, virus control in blood; ×, blood samples from leeches (arithmetic means of three calculations). dl, Detection limit (0.5 log TCID₅₀ ml⁻¹).

sucked blood from wild animals, as well as domestic-bred leeches, which are mostly fed with porcine blood, have an inherent risk of carrying pathogens, such as hepatitis E virus, from swine blood. This risk may be decreased by isolation of the leeches for a distinct quarantine time. The required duration of quarantine is presently unknown but depends strongly on the potential survival time of mammalian viruses in the gut of leeches. Therefore, a study was undertaken to determine how long mammalian viruses can remain infectious after inoculation of leeches.

The survival of viruses depends mainly on the tenacity of the viruses used and the ambient temperature but also on

different unknown environmental conditions. Accordingly, test viruses were chosen for this study based primarily on their tenacity but also for their ability to replicate in permanent cell lines, to propagate to high titres and to induce pronounced cytopathic effects. BPV, FCV, EAV and EHV-1 were selected. EAV and EHV-1 are known to be of low tenacity (Liess & Kaaden, 2003; Mahnel, 1983; Rolle & Mayer, 2002; Schliesser & Strauch, 1981), but their stability towards harmful environmental factors is higher than that of, for example, human immunodeficiency virus. FCV was included in this study because of its high tenacity, which is comparable to that of adenoviruses and reoviruses, which are widespread in animals. BPV is one of the most stable

mammalian viruses. It withstands treatment with a variety of chemical substances and has a thermal stability comparable to that of papillomaviruses (Brown, 1981). BPV remains infective for more than a year at 4 °C, for 13 months at room temperature, for 5 months at 37 °C, for 1–2 days at 56 °C and for 1–3 h at 80 °C (Siegel, 1976; Wigand *et al.*, 1981). In fact, BPV could be reisolated from the leeches for the longest time when compared with the other test viruses. Few studies on the tenacity of viruses in leeches have been published previously. Reported survival times of 12–20 weeks for classical swine fever and myxoma viruses (Shope, 1957) are in good agreement with the results of this study.

Surprisingly, the enveloped viruses EHV-1 and EAV, known to be very sensitive to environmental conditions, both showed a rather long survival time. In the case of EHV-1, this time was shorter (25 weeks) than that for BPV (27 weeks) but longer than that of FCV and EAV (21 weeks). A human virus that has similar environmental resistance to EHV-1 and FCV is hepatitis B virus (HBV). From the data of this study, it could be concluded that HBV might also maintain infectivity for some 21–25 weeks. The results from assessment of the virus control in porcine blood indicated that, in almost all experiments, survival times were longer in leeches than in the blood samples. This suggests that substances exist in the leeches that might conserve virus infectivity. These substances may relate to or even be identical to those that are secreted into the intestines of the leeches after a blood meal in order to keep the blood fresh for a long time. Of note, the tenacity of viruses in leeches may thus differ from their tenacity towards disinfectants. Concentrations used to disinfect non-enveloped viruses usually exceed the concentrations needed to inactivate enveloped viruses. The results of this study showed that this general assumption cannot be extended to the conditions present in leeches. It is essential for further studies to include not only stable non-enveloped viruses but also enveloped viruses that are otherwise considered to be less stable. In contrast, the genome of the viruses (DNA or RNA) appeared not to influence the maintenance of virus infectivity in leeches.

Consequently, it is also essential to differentiate between enveloped and non-enveloped rather than between RNA and DNA viruses if rules for the quarantine times of leeches after feeding in advance of medical application are to be put in place. If this is not applicable, recommendations for quarantine times should be based on studies with extremely stable viruses. BPV belongs to this group of viruses. In fact, this study revealed the longest survival time for this agent in leeches. FCV, which represents a stable virus when considering its tenacity towards disinfectants (Al-Khleif *et al.*, 2009), turned out to be less suitable as a test virus given that EHV-1 survived in leeches for longer. We suggest that studies with BPV are the most meaningful to determine the appropriate time that leeches need to be kept in quarantine to prevent possible transmission of viruses. In our studies, the survival time of BPV in leeches

kept at 10 °C was 27 weeks and slightly less if the leeches were kept at 30 °C starting from week 6. Time savings by application of higher temperatures will therefore be unexpectedly low. It is probable that the extraordinarily high heat stability of BPV, a characteristic of this virus (Herbst *et al.*, 1990), is responsible for this phenomenon. In conclusion, our studies indicated that, if another 4 weeks are added, a quarantine period of a total of 31 weeks at 10 °C between the last blood feed and the distribution of the leeches to medical practitioners should sufficiently diminish the risk of transmitting viral infections by leech therapy.

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