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Detection of taeniid (*Taenia* spp., *Echinococcus* spp.) eggs contaminating vegetables and fruits sold in European markets and the risk for metacestode infections in captive primates

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ABSTRACT

Due to frequent cases of alveolar echinococcosis (AE) in captive primates in Europe, 141 samples of food, which consisting of vegetables and fruits, were investigated for contamination with egg-DNA of taeniids. Each sample consisted of at least 40 heads of lettuce as well as various vegetables and fruits. The samples were purchased at different times of the year: either from September to November (autumn), originating from greenhouses or fields in the Basel region in the North of Switzerland, or in April and May (spring) when fruit and vegetables are sourced from throughout Europe from various wholesalers. Each sample was washed, and the washing water sieved through mesh apertures of 50 μm and 21 μm, respectively. The debris, including taeniid eggs, collected on the 21 μm sieve were investigated by a multiplex PCR-analysis followed by direct sequencing. In 17 (18%) of the 95 samples collected in autumn, taeniid-DNA was detected (*Taenia hydatigena* in four, *Taenia ovis* in three, *Taenia polyacantha* in two and *Hydatigera (Taenia) taeniaeformis* in five cases). Similarly, in 13 (28%) of the 46 samples collected during spring taeniid-DNA was detected (*Echinococcus granulosus* s.l. in two, *Taenia crassiceps* in one, *T. hydatigena* in two, *Taenia multiceps/Taenia serialis* in two, *Taenia saginata* in one and *H. taeniaeformis* in five cases). Although DNA of *Echinococcus multilocularis* was not found specifically in this study, the detection of other fox taeniids reveals that vegetables and fruit fed to the primates at the Zoo Basel at different times of the year and from different origin are contaminated with carnivore's faeces and therefore act as a potential source of AE infections.

1. Introduction

The larval stages (metacestodes) of several taeniids of carnivores represent aetiological agents of alveolar echinococcosis (AE), caused by *Echinococcus multilocularis*, cystic echinococcosis (CE), caused by *Echinococcus granulosus* sensu lato, and cysticercosis, caused e.g. by *Taenia crassiceps* or *Taenia martis* affecting mammals including rodents, ungulates and primates.

AE is a zoonotic disease that is widely distributed in the northern hemisphere and is emerging in large parts of Europe and Asia (Torgerson et al., 2010; Gottstein et al., 2015). The life cycle of *E. multilocularis* is maintained in Europe by rodents (*eg, Arvicola* spp., *Microtus* spp., *Myodes* spp.), which act as intermediate hosts, and red foxes (*Vulpes vulpes*), which are the most important definitive hosts. In addition, wild canids, like raccoon dogs (*Nyctereutes procyonoides*), and pet animals, such as domestic dogs, can contaminate the environment with *E. multilocularis* eggs (Eckert et al., 2011; Conraths and Deplazes, 2015).

In addition to humans, also a variety of other primates occasionally develop AE, predominantly in the liver. Several cases of fatal AE have been diagnosed worldwide in gorillas (*Gorilla gorilla*)

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(Kondo et al., 1996), an orangutan (*Pongo pygmaeus*) (Taniyama et al., 1996), *Macaca* spp. (Sato et al., 2005; Tappe et al., 2007), a Diana monkey (*Cercopithecus diana*) (Yamano et al., 2014) and lemurs (Kondo et al., 1996; Brack et al., 1997; Deplazes and Eckert, 2001; Sato et al., 2005; Umhang et al., 2013). In Switzerland, Wenker and Hoby (2011) diagnosed seven cases of AE in western lowland gorillas in Basel Zoo. Furthermore, three chimpanzees (*Pan troglodytes*), five white-handed gibbons (*Hylobates lar*) and two squirrel monkeys (*Saimiri sciureus*) located in the Walter Zoo, a private zoo in Eastern Switzerland, were recently found to be infected; all infections were confirmed at necropsy and in all animals except one chimpanzee AE was found to be the cause of primary disease.

Cystic echinococcosis (CE) is a globally distributed zoonosis with high burden of disease (Budke et al., 2006). In southern Europe, *E. granulosus* sensu stricto (genotypes 1–3), with a typically dog-sheep cycle predominates, whereas in the eastern parts of Central Europe (Baltic countries, Poland), *Echinococcus intermedius* (*Echinococcus canadensis*, pig strain, G7), maintained by a dog-pig cycle, is endemic (Bružinskaitė et al., 2009). In zoos, CE has been diagnosed and published in different primates (Boufana et al., 2012; Poglayen et al., 2015). At the Institute of Parasitology, University of Zurich there was one positive diagnosed great ape (orangutan) (Grimm F, personal communication). Bernstein (1972) described six cases of cystic echinococcosis CE in orangutans in a zoo in California. Clinical symptoms range from severe illness to asymptomatic. In the same zoo, a zoo-born chimpanzee had a perforation of the abdominal wall due to CE, and a gorilla reacted sero-positive to *Echinococcus granulosus*.

Rare cases of carnivore-transmitted cysticercosis caused by, for example, *T. crassiceps* (Heldwein et al., 2006; Flammer et al., 2014) and *T. martis* (Brunet et al., 2015; Eberwei et al., 2013), have been documented in people in Europe. Furthermore, several lethal *T. martis* (Brunet et al., 2014; De Liberato et al., 2014) and *T. crassiceps* infections were reported recently for lemurs in zoos (Luzon et al., 2010).

Captive animals can come in contact with faeces of free roaming wild or captive definitive hosts of taeniid spp. in endemic areas as fences do not prevent completely intrusion of foxes in wildlife parks (Umhang et al., 2016); in addition, food contaminated with eggs is, arguably, another probable route of infection for primates kept in zoos.

In the Zoo Basel, direct contact to the definitive hosts can be excluded and other routes of infection have to be evaluated. Faecal contamination of vegetables and fruit can occur under various circumstances during production, manufacturing, storage or transport if they are produced in conditions that allow faecal contaminated waters to be used for irrigation or washing, or if the soil is contaminated. Vegetables from such sources, if consumed raw and insufficiently washed, can be a potential source of parasitic disease (Slifko et al., 2000). Cultivation in open fields, where wild animals have access, increases the risk of contamination, especially since taeniid eggs can remain viable for several months, if conditions (humidity, temperature etc.) are suitable, as experiments showed for *E. multilocularis* eggs (Veit et al., 1995; Federer et al., 2015).

Several studies in different countries have assessed parasite contamination of vegetables and fruits and, indeed, several protozoan oocysts or cysts, and helminth eggs could be documented in Iran (Daryani et al., 2008), Libya (Abougrain et al., 2010), Egypt (Eraky et al., 2014) and Brazil (Silva et al., 2014). In India, vegetables, soil and wastewater samples were checked for helminth eggs and found in 83.3% of raw wastewater, 68.2% of treated wastewater, 68.6% of soil and 44.2% of vegetable samples (Gupta et al., 2009). In Turkey, helminth eggs, including taeniid eggs, have been found in raw vegetables collected from wholesalers (Kozan et al., 2005) and, in Norway, protozoa and helminths have been detected in fruit and vegetables (Robertson and Gjerde, 2001). Aside from direct detection of eggs by microscopy, *E. granulosus* eggs have been identified by monoclonal antibodies in environmental contamination sites in settlements in Turkana (Kenya) (Craig et al., 1988) or in soil samples in gardens of rural homesteads in southern Kazakhstan, using a modified flotation method followed by PCR identification (Shaikenov et al., 2004). Szostakowska et al. (2014) also analysed soil samples in Poland by PCR and sequencing. The same group investigated unwashed fruits and vegetables in the same endemic region of Poland and were able to amplify *E. multilocularis* DNA in 24 of 103 samples (Lass et al., 2015).

Given that, in the past, primates in zoo were fed with raw vegetables that had not been washed intensively, the probability of a potential parasite transmission with helminth eggs might be higher than it is with washed vegetables used for human consumption.

The aim of this study was to investigate cestode egg-contamination in raw vegetables and fruits fed to the gorillas at Basel Zoo, to evaluate them as a possible route of transmission of the infection with *E. multilocularis*.

2. Materials and methods

2.1. Sample collection

The total daily mass of vegetables (around 50 kg) and fruits (around 10 kg) prepared as food for the gorillas represented one sample; its quantity varied according the specific daily needs of Basel Zoo. In autumn 2013 (September to November, 95 samples), different vegetables and fruits, grown in fields and green houses in the Basel region of Switzerland, were purchased from a local farmer. The samples contained around 40 heads of lettuce (including butterhead lettuce (*Lactuca sativa* var. capitata), leaf lettuce (*Lactuca sativa* var. crispa) and batavia lettuce (*Lactuca sativa* var. longifolia); weighing approximately 14 kg and varying quantities of fennel, beetroot, allium, broccoli, red pepper, cucumber, celery, carrot, potato, onion, tomato, apple and pear. Additionally, in spring 2013 (April and May, 46 samples), vegetables and fruits of the same varieties as in autumn, originating from Europe but with unknown country/region of origin, were purchased from different markets in Basel. All vegetables and fruits were of high quality, were processed at high hygienic standards, pre-washed by the farmer (some root vegetables still contained less visible soil) and were originally prepared for human consumption. They were delivered on a daily basis to Basel Zoo as food for the gorillas.

2.2. Sample preparation

Each sample was washed in the food preparation station at Basel Zoo following the daily routine using tap water. First, all fruits and vegetables were checked, dirty spots were removed and the fruits and vegetables were sunken as a whole and washed in the sink filled with tap water for around 1 min. Thereafter, the heads of lettuce were cut in halves to facilitate subsequent rinsing of the internal leaves. The lettuce leaves were not further separated and all other fruits and vegetables were left intact, since the structure of the food was important for later feeding to the animals. The food was then placed in a large meshed plastic-container and thoroughly rinsed with a dish sprinkler. The whole washing water (~240 L collected in 60 L containers) was collected and sieved through filters with different mesh sizes. For this purpose, a tube system (diameter of 16 cm) with two filters (aperture sizes: 50 μm and 21 μm) was built. Washing debris bigger than 21 μm were

retained in the smaller filter. Subsequently, the 21 μm filter containing the debris was turned upside down and washed again with tap water and placed inside two 1.5 L bottles. Finally, at the Institute of Parasitology in Zurich, the sediment was concentrated through a series of centrifugation steps and collected in a flat tube 10 mL volume to allow examination for the presence of eggs using an inverted microscope.

Due to difficulties of reliable recognition of taeniid eggs, especially within samples collected in autumn, where a lot of pollen was present, the DNA of each sample was extracted according to Štefanić et al. (2004). A multiplex PCR for the discrimination of *E. granulosus* and *E. multilocularis* from other cestodes (e.g. *Taenia* spp., *Mesocestoides* spp.) was carried out according to Trachsel et al. (2007). The multiplex PCR primers target two mitochondrial genes; the NADH dehydrogenase subunit 1 ($Cest_1$/$Cest_2$) and the small subunit of ribosomal RNA (Cest3, Cest4 and Cest5). The pair $Cest_1$/$Cest_2$ specifically amplifies *E. multilocularis* DNA. Meanwhile, $Cest_3$/$Cest_5$ and $Cest_4$/$Cest_5$ are used to detect and discriminate between *Echinococcus* spp. causing CE and other cestodes DNA, respectively. The amplicons were directly sequenced after purification of the PCR products using the MinElute PCR purification kit (Qiagen, Hilden, Germany).

Sequencing was performed by Synergene Biotech GmbH, Biotech Center Zurich, Switzerland (http://www.synergene-biotech.com) with the primer Cest5seq for *Taenia* spp. positive samples, and with Cest4 for *E. granulosus* positive samples. Sequencing results were compared with those from GenBank nucleotide database, using BLAST tool (http://www.blast.ncbi.nlm.nih.gov).

3. Results and discussion

A total of 30 out of 141 investigated samples of vegetables and fruits purchased from local producers or markets in Basel were positive for DNA of cestode species (Table 1). From 95 samples obtained from the Basel region in autumn, 17 were taeniid DNA positive. Furthermore, 13 out of 46 samples collected in spring (European origin but from unspecified locations) were positive for taeniids including, in two cases, *E. granulosus* and, in one case, *Taenia saginata*. The *E. granulosus* sequences were short (42/57bp) with 98% and 100% identity with *E. granulosus* sequences available in GenBank (e. g. accession number: DQ822451; KJ559023). Due to the short sequences obtained with the *E. granulosus sensu lato* positive samples, further genotyping is not possible to achieve.

In total, 32 amplicons were obtained. Two samples had mixed infections (one with *H. taeniaeformis* and *E. granulosus s.l.*, and one with an unidentified cestode and *E. granulosus*).

This study, using the method described by Štefanić (2004) for the isolation of taeniid eggs combined with subsequent DNA characterisation, documents a contamination with taeniids in vegetables and fruits prepared for human consumption but used to feed zoo primates. The presence of DNA of *Taenia hydatigena*, *Taenia ovis*, the *Taenia multiceps/Taenia serialis*-complex, and *Echinococcus granulosus* suggests a contamination of the food with dog faeces. Although no DNA of *E. multilocularis* was detected in our study, the presence of typical fox species such as *Taenia polyacantha* and *T. crassiceps* indicates fox faecal contamination, which means that *E. multilocularis* eggs could be potential contaminants of the same type of vegetables. In this context, it is worth mentioning that, although the Basel region is a known *E. multilocularis* endemic area, the fox population has been reduced by fox mange in the last few years and the contamination of the environment with *E. multilocularis* eggs has been relatively low, as determined by investigating faecal samples of foxes (Frauchiger et al., 2015). So far only chewing grass and eating unwashed strawberries were considered as food-derived risk factors for alveolar echinococcosis, (Kern et al., 2004). Our results and the study in Poland (Lass et al., 2015) indicate that vegetables have to be considered as a further risk of cestode infection. Especially vegetables with rough surface and those that are difficult to wash (such as lettuce or cabbage) might be a greater potential risk.

This study was focused on finding evidence of faecal contamination with cestodes that potentially can infect zoo primates. Moreover, some of the cestodes found are also zoonotic parasites such as *E. granulosus* and *T. crassiceps*. Despite that 21 μm filter capture several kind of parasites eggs, due to the small size of the oocysts it cannot be excluded that other food-borne zoonotic agents such as *Toxoplasma gondii* and *Cryptosporidium* spp. could also be present (Dewaal et al., 2006). It is also worth to mentioned that one sample of this study was found positive for *T. saginata*, which indicates human faecal contamination. Although *T. saginata* eggs are not infective to humans, this finding suggests that the vegetables are grown or processed under suboptimal hygienic conditions and, thus, raise the alert for the transmission of geohelminths such as *Ascaris* spp. or *Trichuris* spp., protozoa such as *Giardia* spp. and other bacterial or viral pathogens (Dewaal et al., 2006).

It is worth noting that microscopic identification could not be achieved for all samples due to the large amount of dirt and pollen

Table 1
Detection of parasite DNA amplified from vegetable and fruit samples provided by local producers or by markets in month September to November (autumn) and months April and May (spring) to Basel Zoo for food for gorillas.

Region of collection/season	Taeniid species	Number of positive
Collection from Switzerland (Basel) in autumn (n = 95)	*T. hydatigena*	4
	T. polyacantha	2
	T. ovis	3
	H. taeniaeformis	5
	Cestodes not identified	3[a]
Total cestodes positive samples		**17**
Various, unspecified European countries including Switzerland/Spring (n = 46)	*T. hydatigena*	2
	T. saginata	1
	T. crassiceps	1
	H. taeniaeformis	5[b]
	T. multiceps/T. serialis	2
	E. granulosus	2[b]
	Cestodes not identified	2[a, b]
Total cestodes positive samples		**13**

[a] In these cases species identification was not possible by sequence analysis.
[b] Two species were detected in two samples (one with *E. granulosus* and *H. taeniaeformis* and one with *E. granulosus* and an unidentified cestode(s)).

particles of the same size as taeniid eggs. However, in some samples, typical taeniid eggs could be identified microscopically. Furthermore, primer specificity, especially in the case of primer Cest5, was a limitation concerning the *Taenia* species identification. According to Trachsel et al. (2007), this primer is able to amplify DNA from other cestodes such as *Mesocestoides* spp. The amplicons of multiple cestodes species present in some of the samples could hinder the species identification by direct sequencing. It also needs to be remembered that, whilst the presence of taeniid DNA is not strictly associated with the presence of viable eggs in the sample, it is an indicator of faecal contamination by taeniid's definitive hosts. Based on the sample preparation, however, it is unlikely that we isolated free DNA or worm tissue in the samples sieved for the detection of particles between around 30–40 μm in diameter. Further test development has to be initiated with the aim of detection of viable eggs. As recently shown, the most accurate and sensitive viability test for *E. multilocularis* eggs is the subcutaneous injection of free, not activated oncospheres (Federer et al., 2015). However, the potential of mRNA-detection for viability testing for metacestode infections (Kern et al., 1995) could represent an alternative approach.

The presented study confirms the risk of parasite contaminated vegetables and fruits for cestode infections in non-human primate facilities, but also documents a possible risk of infections for humans. It confirms the importance of thorough food washing not just at Basel Zoo, but also as a standard procedure in every household. Attempts to decontaminate raw vegetables by heating is problematic, as a recent study demonstrated the high heat tolerance of *E. multilocularis* eggs (survival up to 65 °C for 120 min at 70% relative humidity) (Federer et al., 2015). Moreover, such high temperatures are not suitable for raw vegetables like lettuce. An alternative for the food supply for Basel Zoo may be the use of vegetables from an area non-endemic for *Echinococcus* species, such as the Southern Ticino region of Switzerland (Hoby S. and Wenker Ch. personal communication).

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