Illegal Wildlife Imports More than Just Animals—Baylisascaris procyonis in Raccoons (Procyon lotor) in Norway

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ABSTRACT: In autumn 2011, 11 illegally imported animals were seized from a farm in southern Norway. These included four raccoon dogs (Nyctereutes procyonoides), four raccoons (Procyon lotor), and three South American coatis (Nasua nasua), all considered alien species in Norway. An additional two raccoons had escaped from the farm prior to seizure. The seized animals were euthanized and postmortem examination revealed that the four raccoons had moderate to high numbers of the zoonotic nematode Baylisascaris procyonis in their intestines, ranging from 11 to 115 nematodes per small intestine, with a mean of 53. The identity of the nematodes was confirmed using molecular analysis of ITS-1, ITS-2, cytochrome C oxidase 1, and 18S. Echinococcus multilocularis was not detected in any of the 11 animals. Toxocara and Toxascaris sp. eggs were detected in the feces of two raccoons, and two coatis had coccidia oocysts (80 and 360 oocysts per gram). Domestic dogs and other wildlife on the farm had potential access to the animal pens. Given that the eggs can remain infective for years in the environment, local veterinary and health authorities will need to remain vigilant for symptoms relating to infection with B. procyonis.

Key words: Baylisascaris, emerging diseases, illegal trade, Norway, PCR, raccoon, zoonosis.

In late autumn 2011, 11 illegally imported animals were found on a farm in southern Norway. Four raccoon dogs (Nyctereutes procyonoides), four raccoons (Procyon lotor), and three South American coatis (Nasua nasua) were seized and physically euthanized by local environmental management authorities. The animals were sent to the Norwegian Veterinary Institute in Trondheim for postmortem examination. Investigation by local authorities revealed that two other raccoons had escaped from the farm. One of them had been captured and killed in October 2011 and was not sent for postmortem examination. The second raccoon is presumed to still be at large. The farmer refused to provide information regarding the origin of the animals.

Because the main priority of environmental management authorities was to investigate if these animals had been bred, two of the raccoon dogs were examined upon arrival. The remaining two raccoon dogs, four raccoons, and three coatis were stored at −20°C until postmortem examination. Eleven animals were necropsied following standard veterinary pathologic procedures. Fecal samples were taken from the colons (Table 1). The small intestines were divided into five equal-length segments, and segments two and four were sent for additional parasitologic examination. All animals were heavily overweight to grossly obese (Singh et al., 2004). The parasite burden in the small intestine of the raccoons was moderate to high, with nematodes almost completely filling the intestinal lumen. Small intestinal nematode counts were 11, 25, 61, and 115. The mean nematode count for the four raccoons was 53. Two of the raccoons had milk spots on the hepatic surface that proved to be granulomas suggestive of larval migration on histologic examination of samples taken from one of the animals. The results from the other two raccoon livers were inconclusive because of advanced autolysis. There were no other significant findings in the other animals examined.

We used a modified McMaster’s method for fecal egg counts (Taylor et al., 2007).
Segments two and four of the small intestine were examined using a sedimentation counting technique (SSCT, Umhang et al., 2011), examining the sieves as well as the sediment for parasites. Parasites found were stored in 70% ethanol at 4°C until further examination. A 2-mm broad transverse slice from the midsection of four nematodes was placed in Eppendorf tubes at 4°C for later molecular analysis. The morphology of 16 nematodes was examined.

The general morphology of the nematodes, measuring 8–16 cm long, identified them as Ascarididae (Fig. 1a). Three types of eggs were identified during McMasters examination (Table 1). The predominant egg type was thick walled with fine granular surface, was slightly elliptical, and contained a single large, round-celled embryo, consistent with Toxocara/Baylisascaris eggs (Fig. 1b). In total 192 eggs were measured to verify size. On the basis of morphology and size of the predominant type of eggs, Baylisascaris procyonis was suspected (Averbeck et al., 1995), but Toxocara and Toxascaris eggs were also seen (Fig. 1b). Parasite eggs were not found in any of the raccoon dogs or in one coati. The remaining two coatis had a low number of coccidian oocysts (80 and 360 oocysts per gram) but no further attempt was made to identify these oocysts to genus.

The nematodes were fixed in 70% ethanol and examined after clearing overnight in polyvinyl lactophenol (Waldeck GmbH & Co. KG, Münster, Germany). The nematodes were identified as B. procyonis based on lip morphology (Fig. 1c, d; Kazacos and Turek 1982; Averbeck et al., 1995).

DNA extraction was performed using a Genemole automated DNA extractor (Mole Genetics, Oslo, Norway) with an overnight lysis step. DNA extracts were stored at −20°C. Molecular identifications were performed using three sets of primers producing sequences from the following regions: ITS-1, 5.8 S rRNA, and ITS-2 (amplified using universal primers ITS-1 and ITS-2); 18S (amplification using Nem18SF [Hasegawa et al., 2009] and BspREW [Ø. Øines, unpubl.]; 5′-ACCA-GACAAATCACTCCACCA-3′); and mitochondrial cytochrome C oxidase 1 (CO1) amplification using JB3 and JB4.5 primers; Bowles et al., 1992). All PCR reactions were set up in 40-μL reactions containing 40 pmol/μL of each primer, 1× Taq buffer containing 1.5 mM MgCl2 and 200 μM of each dNTP, and 2U of Taq polymerase (GE Healthcare, Buckinghamshire, UK). A Dyad thermocycler (MJ Research/Biorad, Hercules, California, USA) was used for the amplification, with initial denaturing at 95°C for 2 min, 35 cycles with denaturing at 95°C for 30 sec, annealing at 46°C for 30 sec, and an elongation step at 72°C for 1 min. The final elongation step was 72°C for 10 min. DNA negative controls were included in all PCR setups. There were no positive controls for the ITS and 18S PCR.

### Table 1. Fecal examination results (using a modified McMasters method) for four raccoons (Procyon lotor) illegally imported into Norway. The animals had been frozen prior to postmortem examination and sampling. Raccoons, in their natural habitat, are reported to shed on average between 20,000 and 26,000 Baylisascaris procyonis eggs per gram (EPG) of feces (Kazacos 2001). Individual counts of Toxocara and Baylisascaris eggs were not made as it was not possible to accurately measure the eggs to differentiate between the two parasites.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Volume feces examined (g)</th>
<th>Results (EPG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1.5</td>
<td>31,681 Toxocara/Baylisascaris</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800 Toxascaris</td>
</tr>
<tr>
<td>Male</td>
<td>1.0</td>
<td>25,800 Toxocara/Baylisascaris</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 Toxascaris</td>
</tr>
<tr>
<td>Female</td>
<td>3.0</td>
<td>83,600 Toxocara/Baylisascaris</td>
</tr>
<tr>
<td>Female</td>
<td>3.0</td>
<td>100 Toxocara/Baylisascaris</td>
</tr>
</tbody>
</table>
multilocularis was used as positive control in the CO1-PCR. The PCR products were visualized using 1% agarose gel (Affymetrix/USB, Cleveland, Ohio, USA) stained with GelRed™ (Biotium, Hayward, California, USA), run at 95 volts in tris/borate/EDTA (TBE) 1× buffer for approximately 90 min, then visualized using a ChemiDoc Xrs+ imaging system (Bio-Rad).

The PCR reactions produced a single band for each reaction, and the products were cleaned using Nucleospin (Macherey-Nagel, Düren, Germany) using the protocol for PCR-mix cleanup. Sequencing was outsourced to Macrogen Europe (Amsterdam, Netherlands) using the PCR primers. Chromatograms were edited manually in Contig Express (Vector Nti® Advance 11.5, Invitrogen, Carlsbad, California, USA). Four or more chromatograms, from forward and reverse primers and duplicate DNA samples, were assembled in Contig Express sequences and then analyzed. Flanking areas, containing primer hybridization sites at the beginning and end, were omitted in order to avoid the introduction of new bases from the primers. The sequencing of ITS PCR product yielded an 878-base pair (bp) sequence that was deposited in GenBank (accession JQ403615). The 18S PCR produced a 934-bp sequence (KC172105) and the CO1 PCR produced a 393-bp long sequence (KC172104). BlastN searches were performed on each sequence generated. The top matches from each BlastN search were imported and aligned in AlignX (vector Nti Advance 11.5, Invitrogen, Carlsbad, California, USA) with the fragments. The alignments were exported into MEGA (Tamura et al., 2011) and clustering was performed using NJ (neighbor-joining; Saitou and Nei, 1987) searches. The 934 bp 18S fragment (KC172105) was 100% identical to the GenBank entry B. procyonis U94368. The smaller mtCO1 PCR (393 bp) fragment we obtained from the sample (KC172104) was identical to the same region of CO1 of GenBank entry JF951366 (B. procyonis). The longer 878-bp fragment (JQ403615) covered the region that flanked the ribosomal 5.8S,
including partial ITS-1 and ITS-2 loci. There were no available sequences from *B. procyonis* in the databases that covered this entire fragment. Identification was based on the 3’ end of the molecule (ITS-2), which revealed homology with two ITS-2 sequences, both from *B. procyonis*. The 344-bp ITS-2 sequence of GenBank entry AB051231 was 100% identical to the 3’ region of JQ403615, which covered the ITS-2 (from bp 566). The molecular data supported the diagnosis of *B. procyonis*.

This is the first identification of *B. procyonis* in Norway. The parasite is found in raccoons in North America and Germany, with reported prevalence varying between regions (Kazacos 2001; Beltrán-Beck et al., 2011). Raccoon dogs and raccoons are found in the wild in Germany and *E. multilocularis* (the causative agent of alveolar hydatidosis in humans) is endemic in wild canids in Germany. Given the unknown origin of the animals, our first priority was to rule out infection with *E. multilocularis*, because this parasite has not been identified in mainland Norway (Wahlström et al., 2011). We did not find *E. multilocularis* in any of the 11 animals investigated.

Both *Toxocara canis* and *B. procyonis* can cause larva migrans in humans, although concern is greater for *B. procyonis* because of the more aggressive nature of the immune response (Sorvillo et al., 2002). The larger size of *Baylisascaris* larvae combined with more aggressive migration, including to neurologic and ocular predilection sites, seems to cause more tissue destruction than *Toxocara* (Kazacos, 2001). All four raccoons had *B. procyonis* in their small intestine, and it would seem reasonable to assume that the escapees also had this infection, given that the vast majority of wild raccoons harbor this parasite. Prevalences of 68–82% have been reported in raccoons in North America (Kazacos, 2001). Environmental contamination with *B. procyonis* eggs remains the greatest concern. These eggs are extremely environmentally resistant and have been known to remain infective for more than a decade when stored at 4 C as well as after storage in formalin (Sorvillo et al., 2002; Shafir et al., 2011). Domestic dogs can also act as the definitive host (Lee et al., 2010), and the farmer also bred a variety of dogs that had access to the animal pens. As a security measure we recommended that anthelmintic treatment be given to all the dogs on the farm as well as those that had been to the farm previously.

The Governmental Agency for Nature Management and the Public Health Institute were informed of the findings after the molecular diagnosis was made. The local Food Safety Authority was contacted and given advice with regard to decontamination of the pen. They were advised to remove the topsoil (top 10 cm) and send it for incineration to limit further environmental contamination. Unfortunately, because of the lack of available molecular phylogeographic data on *B. procyonis*, we were not able to trace the origin of the *B. procyonis*, which might have helped in identifying the geographic origin of the animals. The investigation into tracing the origin of the animals is ongoing and molecular methods were being considered by the Governmental Agency for Nature Management.

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**LITERATURE CITED**


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