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High incidence of *Yersinia enterocolitica* (Enterobacteriaceae) in Alpine Accentors *Prunella collaris* of the Tatra Mountains

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Abstract. Cloacal and pharyngeal swabs were sampled from 33 Alpine Accentors. A total of 32 specimens were *Yersinia* positive, with 73% of birds being positive for *Y. enterocolitica* and 51% for *Yersinia* spp. A comparison of host characters and environmental conditions showed these to be consistent with the different life strategies of *Y. enterocolitica* and other *Yersinia* species. *Y. enterocolitica* is more successful at colonizing the birds' digestive tracts — the occurrence of *Y. enterocolitica* was significantly higher in the cloacal than the pharyngeal swabs. The occurrence of *Y. enterocolitica* was high in summer, especially in the nesting period (July). In juveniles (including nestlings), there was a 100% prevalence of *Y. enterocolitica*, whereas only two out of nine juveniles were *Yersinia* spp. positive. There was no significant difference between the occurrence of *Y. enterocolitica* in anthropogenic and natural habitats, but the occurrence of *Yersinia* spp. was much greater in the former than in the latter habitats. The presence of the *ail* gene associated with pathogenic *Y. enterocolitica* strains was not confirmed in any of the samples examined.

Key words: Alpine Accentor, *Prunella collaris*, yersiniosis, *Yersinia enterocolitica*, PCR, *ail* gene

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INTRODUCTION

Yersinia enterocolitica is a gram-negative coccobacillus of the family Enterobacteriaceae, facultative anaerobes with both types of chemoorganotrophic metabolism — respiratory and fermentative. Yersiniosis is a zoonotic disease found worldwide. Humans may also be vulnerable to this disease; however, infections are rare. Clinical signs include dehydration, inactivity, listlessness, diarrhea, difficulty breathing, anorexia, and weight loss. There are two typical syndromes that may characterize infected birds: i. infected birds may experience a very rapid and acute onset of disease where sudden death may occur ii. birds may experience a slower onset of disease where the clinical signs may take weeks to manifest. In this case, the disease may or may not resolve for weeks, months, or years (Hewett et al. 1998). Infected animals transmit this bacterial organism through fecal and urine contamination of food and water. Rodents are often considered a natural reservoir of *Yersinia* spp. (Kapperud 1975, Bercovier et al. 1978, Kaneko & Hashimoto 1981, Fukushima et al. 1990) along with birds (Kato et al. 1985, Shayegani et al. 1986, Fukushima & Gomyoda 1991, Niskanen et al. 2003). Wild-living birds have been thought to play a significant role in the maintenance and dissemination of *Y. enterocolitica* in the environment because of their great mobility.

The Alpine Accentor is a bird species exclusively restricted to high mountain areas (Davies et al. 1996, Herr 1996). It is a ground-feeding omnivore with a complex and variable mating system (Nakamura 1995, Davies et al. 1995). The Alpine Accentor is a typical of high altitude vegetation in the Palearctic region (Cramp 1988) and thus could be considered as a bird model species for the monitoring of the high altitude environment.

The occurence of *Yersinia enterocolitica* in the Alpine Accentor was detected during previous studies of the composition of the gut microflora

and the relations between different bacterial species (Sedlárová 2004, Janiga et al. 2007). The main objective of this study is to describe the prevalence of yersiniosis in the Alpine Accentor, the host and environmental conditions influencing the occurrence of these bacteria in high mountain environments.

MATERIAL AND METHODS

The samples were collected from the end of March to the end of October 2006 in high mountain localities of two Slovak national parks: the High Tatra NP and the NP Low Tatras. Localities are situated at an altitude of 1750–2634 m a.s.l. The study sites were classified into two different categories according to the level of anthropogenic impact: i. anthropogenic habitats (close to tourist facilities, mountain refuges or peaks with high visitor traffic) or ii. natural habitats (not influenced by tourist visitation). Prevalence of yersiniosis in both types of habitats was compared. Samples were divided into two groups according to the weather conditions during sampling: i. dry-warm period — sunny weather without any precipitation; wet-cold period — cloudy weather with precipitations. Prevalence of yersiniosis in both types of weather conditions was also compared. Free-living Alpine Accentors were captured using food traps and mist-nets. They were weighed with 0.2 g accuracy using a Pesola spring scale and standard morphometric measurements taken. Birds were sexed by the presence of a cloacal protuberance in males (Nakamura 1990). Two types of samples were obtained — pharyngeal swabs and cloacal swabs. Samples of bacteria were taken using sterile transport swabs suitable for both aerobes and anaerobes (DispoLab, Copan Italia, Brescia, Italy). After measuring and sampling, the birds were released.

Isolated bacterial cultures from cloacal and pharyngeal swabs were enriched in Trypton-soya broth at 24°C for two days (Nikolova et al. 2001). Enriched cultures were plated on selective medium, e.g. Cefsulodin-Irgasan-Novobiocin agar and cultivated at 24°C (Devenish & Schieman 1981, Head et al. 1982, Hussein et al. 2001).

For the identification of *Y. enterocolitica*, a reference strain (CCM 5671) *Y. enterocolitica* subsp. *enterocolitica*, serovar 0:3, biovar 4 was obtained from the Czech collection of microorganisms, Masaryk University, Brno.

One inoculation loop of bacterial culture was suspended in 200 μl of lysis buffer (TE buffer + 1% Triton X-100, pH = 8). The suspension was incubated at 95°C for 10 minutes and then centrifuged at 12 000 x g for 5 minutes. Isolated DNA was diluted to a concentration of 20 ng/μl in TE buffer (10mM TRIS-Cl, 1 mM EDTA, pH = 8) and stored at -20°C.

Y. enterocolitica was identified among isolated bacterial cultures using the PCR method. Other bacterial cultures were not determined to the species level and will be referred to as *Yersinia* spp. PCR for detection of *Y. enterocolitica* DNA was performed using primers Y1 and Y2 for amplification of a 330 bp fragment of the 16S rRNA gene of *Y. enterocolitica* (Neubauer et al. 2000). A1 and A2 primers were used to amplify a 430 bp fragment of the *ail* gene, found exclusively in pathogenic *Y. enterocolitica* strains (Wannet et al. 2001). DNA samples were amplified in a total volume of 25 μl which included 40–50 ng of total genomic DNA, 200 μM each dATP, dCTP, dGTP and dTTP, 3mM $MgCl_2$, Bio Therm Star DNA Polymerase. Amplification was performed in a Techne thermal cycler. PCRs were conducted using the following conditions: denaturation at 95°C for 10 min, 36 cycles: 94°C for 45 s, 58°C for 45 s, 72°C for 1 min, and the final extension at 72°C for 7 min. The PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

A χ^2 statistics was used to test the hypothesis of independence of frequencies of selected factors (contingency, Cramer´s V, Pearson's r and Kendall´s Tau B coefficients were calculated and used to check the validity and orientation of χ^2 data). Morphometric data of different groups of birds were compared by one-way ANOVA (Statgraphics 5.0).

RESULTS

Cloacal and pharyngeal swabs were sampled from 33 Alpine Accentors (15 adult males, 9 adult females and 9 juveniles) in 15 localities of High and Low Tatras. A total of 32 specimens were *Yersinia* positive either in the pharynx or in the cloaca. *Yersinia* strains were not recorded, neither in the pharynx nor in the cloaca, in only one individual, which was captured on the Rysy peak.

The comparison of distribution, host parameters and environmental conditions showed different life strategies of *Y. enterocolitica* versus other non-identified *Yersinia* species. There was a highly

Table 1. The prevalence of yersiniosis in the Alpine Accentors according to examined bacteria, season, sex and age of hosts (number of examined birds with relative values in the brackets). PH — pharynx, CL — cloaka, + — positive, - — negative.

Yersinia species	No birds sampled (N)	PH & CL - (%)	PH + (%)	CL + (%)	PH & CL + (%)	χ^2	p
Y. enterocolitica	(33)	9 (27)	9 (27)	7 (21)	8 (24)	33.7	0.0001
Yersinia spp.	(33)	16 (49)	8 (24)	6 (18)	3 (9)		
Y. enterocolitica	July (13)	0 (0)	7 (54)	1 (8)	5 (38)	12.8	0.046
	August (9)	3 (33)	1 (11)	4 (44)	1 (11)		
	October (6)	2 (33)	1 (17)	1 (17)	2 (33)		
Yersiniosis Total	Females (9)	0 (0)	2 (22)	0 (0)	7 (78)	2.9	0.4
	Males (15)	1 (7)	3 (20)	3 (20)	8 (54)		
Y. enterocolitica	Juveniles (9)	0 (0)	5 (56)	1 (11)	3 (33)	8.0	0.04
	Adults (24)	9 (37)	4 (17)	6 (25)	5 (21)		
Yersinia spp.	Juveniles (9)	7 (78)	0 (0)	2 (22)	0 (0)	6.4	0.9
	Adults (24)	9 (37)	8 (33)	4 (17)	3 (13)		

significant difference between the occurrence of *Y. enterocolitica* and *Yersinia* spp. in pharyngeal and cloacal samples (Table 1). Whilst *Y. enterocolitica* occured in 73% of all swabs (pharyngeal and cloacal), *Yersinia* spp. was positive in 51% of all cases.

There was a significant difference of *Y. enterocolitica* occurrence in different time periods as well as pharyngeal versus cloacal swabs (Table 1). High occurrence of *Y. enterocolitica* was especially recorded in summer, which is the nesting period for accentors (July). No such relationship was established for *Yersinia* spp. ($\chi^2 = 11.3$, df = 6, p = 0.08).

No significant difference in the occurrence of *Y. enterocolitica* ($\chi^2 = 0.5$, df = 3, p = 0.91) as well as of total yersiniosis (Table 1) was found between adult male and female accentors; but a significant difference in the occurrence of *Y. enterocolitica* was found between adult and juvenile birds. In juveniles (including nestlings) there was a 100% prevalence of *Y. enterocolitica*, in adults the prevalence was 63% (Table 1). *Yersinia* spp. show a different pattern of occurrence in juvenile and adult birds (Table 1) than *Y. enterocolitica*. *Yersinia* spp. only occurred in two out of nine juveniles. The mother of those two positive nestlings was also positive for *Yersinia* spp.

Table 2 shows the occurrence of yersiniosis in adults in relation to their morphological characters. Although female accentors tend to be smaller than males, no statistically significant difference in body mass was established when prevalence (*Y.e.*) and sex were considered as two independent variables (two way ANOVA — F(*Y.e.*) = 0.02, p = 0.9, F(sex) = 2.5, p = 0.1, df = 19), the same results was found for *Yersinia* spp. (two way ANOVA — F(*Y.sp.*) = 0.01, p = 0.9, F(sex) = 2.7, p = 0.1, df = 19). Consequently both sexes were pooled together and tested against *Yersinia* prevalence as a single group. Those birds positive for *Y. enterocolitica* tended to be morphologically larger (but not heavier). This relationship was significantly indicated by tarsus length.

Table 2. Morphological characters of adult Alpine Accentors examined for yersiniosis. N — number of birds. F-test of one-way ANOVA.

	Positive + Negative -	Morphological variables	Mean ± SE	N	*F*	p
Yersinia spp.	+	Body mass (g)	40.7 ± 1.3	14	0.06	0.81
	-		41.2 ± 1.6	9		
	+	Tarsus length (mm)	30.6 ± 0.3	15	1.5	0.23
	-		31.2 ± 0.4	9		
Y. enterocolitica	+	Body mass (g)	41.2 ± 1.3	15	0.11	0.74
	-		40.4 ± 1.7	8		
	+	Tarsus length (mm)	31.2 ± 0.3	15	4.0	0.05
	-		30.2 ± 0.4	9		

Table 3. The prevalence of yersiniosis in the Alpine Accentors according to potential effects of anthropogenic influence (number of examined accentors with relative values in brackets).

	Positive + Negative -	N	Antropogenic habitats (%)	Natural habitats (%)	χ^2	p
Yersinia spp.	-	16	5 (31)	11 (69)	3.7	0.05
	+	17	11 (64)	6 (35)		
Y. enterocolitica	-	9	6 (67)	3 (33)	1.6	0.2
	+	24	10 (42)	14 (58)		

The relationship was not found in *Yersinia* spp. (Table 2).

The influence of weather on the frequency of occurrence of *Yersinia* was also tested. The occurrence of yersiniosis (both *Y. enterocolitica* and *Yersinia* spp.) was not significantly influenced by type of weather (dry-warm vs. wet-cold periods: $\chi^2 = 1.0$, df = 1, p = 0.8 for *Y. enterocolitica*; $\chi^2 = 1.8$, df = 1, p = 0.6 for *Yersinia* spp.). Occurrence of *Yersinia* spp. was much higher in anthropogenic (65%) than in natural habitats (35%). There is no significant difference in occurrence of *Y. enterocolitica* between anthropogenic and natural habitats (Table 3).

Besides using the PCR method for determination of *Y. enterocolitica*, the presence of the *ail* gene associated with the pathogenic strains of *Y. enterocolitica* was also examined. The presence of the *ail* gene was not confirmed in any of the examined samples.

DISCUSSION

Alpine Accentors show unusually high prevalence of yersiniosis (*Y. enterocolitica* — 73%, *Yersinia* spp. — 51%) in comparison with bird species tested by other authors (see Table 4). These results are not in coincidence with preliminary studies concerning the composition of Alpine Accentors gut microflora (Sedlárová 2004, Janiga et al. 2007), because of the different cultivation method. In this study we used selective cultivation medium for *Yersinia* recovery.

Y. enterocolitica is a psychrophilic bacterium, capable of persisting in cold environments. Gill & Reichel (1989) referred about the ability of this organism to grow at -2°C. This might be the reason for its successful expansion in high mountain environments and for its high prevalence in Alpine Accentors. The occurrence of *Y. enterocolitica* in cold environments has been confirmed in many studies. In the cool and cold months (November to June), organisms were isolated from 847 of 1314 (64.5%) mice and 137 (82%) of moles, but in the warm months (July to September), organisms were isolated from 75 of 216 (34.4%) mice and from 2 of 7 (28.6%) moles (Fukushima et al. 1990). Chernyanskii (1981), in a study carried out in arctic regions of Russia, recovered four isolates of *Y. enterocolitica* from Siberian Ruddy Vole *Clethrionomys rutilus*. Human yersiniosis has generally occurred more frequently during the cold seasons (Arvastoson et al. 1971, Winblad 1973, Fukushima et al. 1987). Isolation of these organisms in pigs is also more frequent during cold seasons (Zen-Yoji et al. 1974, Tsubokura et al. 1976, Weber & Knapp 1981). Fukushima et al. (1984) reported that the seasonal incidence of isolation of *Yersinia* spp. in dogs was remarkable and that isolations were frequent in months with average temperatures of under ca. 10°C.

We found an interesting relationship between the occurrence of pharyngeal and cloacal yersiniosis. The occurence of *Y. enterocolitica* in cloacal samples increased throughout the summer season, whereas the occurrence of *Yersinia* spp. decreased (Table 1). The number of positive pharyngeal samples was approximately equal in both examined groups, whereas the number of positive cloacal samples was different with high level of significance. *Y. enterocolitica* is apparently more successful at colonizing the gastrointestinal tract of the examined birds (Table 1). Fukushima et al. (1990) tested 911 mice and 136 were found positive for *Yersinia* spp. in the oral cavity and 622 in the rectum; among 135 moles 30 were *Yersinia* spp. positive in oral cavity and 92 in rectum).

The highest prevalence of *Y. enterocolitica* was found during the nesting period (July) with 100% occurrence of *Y. enterocolitica* in adult birds, juveniles and nestlings. Because the total prevalence of *Y. enterocolitica* in young birds was 100%, and in adult birds 63%, the nesting period is the time of the highest manifestation of yersiniosis. This could be expected, since the immune system of juveniles is not mature yet. The transmission of yersiniosis in Alpine Accentors might be associated

Table 4. The prevalence of *Y. enterocolitica* in different bird species according to previous studies. N — total number of tested birds.

Bird species	N	% *Y. enterocolitica* positive animals	Source
Anas platyrhynchos	19	84.2	Boer & Stigter (1984)
Anas platyrhynchos	24	4.2	Shayegani et al. (1986)
Branta leucopsis	105	18.0	Niskanen et al. (2003)
Branta canadensis	42	2.4	Shayegani et al. (1986)
Meleagris gallopavo	39	5.1	Shayegani et al. (1986)
Phasianus colchicus	32	65.6	Boer & Stigter (1984)
Phasianus colchicus tohkaidi	33	15.2	Kato et al. (1985)
Bambusicola thoracica thoracica	36	2.8	Kato et al. (1985)
Calidris alpina	25	4.0	Niskanen et al. (2003)
Tringa totanus	30	3.3	Niskanen et al. (2003)
Larus argentatus	16	12.5	Kapperud & Olsvik (1982)
Columba palumbus	24	20.8	Boer & Stigter (1984)
Columba livia domestica	47	2.1	Kato et al. (1985)
Streptopelia orientalis	118	3.4	Kato et al. (1985)
Bubo virginianus	79	2.5	Shayegani et al. (1986)
Buteo jamaicensis	21	4.8	Shayegani et al. (1986)
Hypsipetes amaurotis	57	7.0	Kato et al. (1985)
Phoenicurus phoenicurus	22	4.5	Niskanen et al. (2003)
Turdus merula	43	4.7	Niskanen et al. (2003)
Molothrus ater	20	5.0	Shayegani et al. (1986)
Corvus kvailantii and *Corvus corone*	117	6.0	Kato et al. (1985)
Corvus corone	4	50.0	Kapperud & Olsvik (1982)
Cyanopica cyana	21	23.8	Kato et al. (1985)
Passer montanus	14	7.1	Kato et al. (1985)
Sturnus cineraceus	57	10.5	Kato et al. (1985)

with the feeding ecology of the host, which feeds mainly on insects during this period, in contrast to the granivorous diet adopted later in the year. The nestlings are mainly fed with insects (larval and adult craneflies, harvestmen and spiders), which might be the cause of the 100% prevalence of *Y. enterocolitica*. Hamasaki et al. (1989) collected cloacal swab from 528 free-living adult birds representing 15 species. *Yersinia* spp. was isolated from five of 15 species of birds examined. Positive birds were insectivorous. *Yersinia* sp. was not isolated from 10 species of other birds. This trend could not be observed in *Yersinia* spp., which supports the idea of a competitive relationship between *Yersinia* spp. and *Y. enterocolitica*.

We have confirmed the natural occurrence of *Y. enterocolitica* in the high mountain environment of the Tatras throughout the year. *Y. enterocolitica* was found equally in high altitude localities with and without anthropogenic influence, in contrast to other *Yersinia* spp., which were found at the localities influenced by human activity with significantly higher occurrence. *Y. enterocolitica* is able to persist in the high mountain environment and enter the host's body with ingested food. Sporadically captured Dunnocks *Prunella modularis* have also been examined for yersiniosis with positive tests for *Y. enterocolitica*. High prevalence of yersiniosis found in *P. collaris* indicates that this species might be an important reservoir of *Yersinia* spp. in mountain habitats. The importance of wild animals living in mountaineous areas for the transmission of yersiniosis has been recognized by Fukushima et al. (1990) and Fukushima & Gomyoda (1991). Keet (1974) isolated *Y. enterocolitica* serovar 0:8 from a mountain stream in the United States and linked this finding to a case of human infection. Similarly in Japan many of the human patients lived in mountainous areas and used untreated or imperfectly treated stream water as their drinking water (Saito et al. 1994).

Antagonistic host-parasite interactions lead to coevolution of host defenses and parasite virulence. We investigated the relationship between the size of hosts and prevalence of yersiniosis. The prevalence of yersiniosis was not (in general)

positively related to the host body size (indicated mainly by body mass). The frequency of occurrence of *Y. enterocolitica* was positively related with host body size (indicated by tarsus). Larger host individuals tend to have more variable spectrum of bacterial microflora (Janiga et al. 2007) but they need not to be more infected by specific strain or species of parasite than smaller individuals.

All captured birds showed good physical condition and none of them displayed symptoms of yersiniosis inspite of the fact that these birds must survive in harsh climate conditions. For this reason, yersiniae must be apparently a common element of Alpine Accentor's microflora in high mountain biotopes. This suggestion is confirmed by the fact that none of our samples was positive for the *ail* gene. Our results are in accordance with the work of Niskanen et al. (2003), concerning the occurrence of yersiniosis.

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STRESZCZENIE

[Występowanie bakterii *Yersinia enterocolitica* u płochacza halnego w słowackich Tatrach]

Yersinia enterocolitica jest chorobotwórczą bakterią gram ujemną należącą do rodziny jelitowców (Enterobacteriaceae). Przenosi się przez pokarm i wodę zanieczyszczone kałem lub moczem zarażonych osobników. Uważa się, że gryzonie i ptaki mogą być naturalnym rezerwuarem tych bakterii.

Podczas badań pobrano wymazy z kloaki i gardła 33 płochaczy halnych złapanych w 15 miejscach słowackich Tatr. Zebrany materiał posiewano na podłoże selektywne dla bakterii *Yersinia*. Następnie przy pomocy analiz genetycznych wyróżniano *Yersinia enterocolitica* od innych bakterii *Yersinia* (sp.). Próbki zbadano także na obecność genu *ail*, który występuje w patogennych szczepach *Y. enterocolitica*. W analizach osobno potraktowano *Y. enterocolitica* oraz inne, nieoznaczone bakterie *Yersinia*.

Stwierdzono bardzo wysokie zarażenie bakteriami *Yersinia* badanych płochaczy. Tylko u jednego osobnika nie stwierdzono tych bakterii ani w wymazie z kloaki, ani z gardła. Dla pozostałych ptaków przynajmniej jedna z tych próbek była pozytywna. Częstość występowania *Y. enterocolitica* i *Yersinia* sp. w kloace i gardle różniła się istotnie (Tab. 1). *Y. enterocolitica* znacznie częściej stwierdzana była w kloace (Tab. 1). Częstość występowania bakterii nie różniła się pomiędzy płciami, choć takie różnice zanotowano między ptakami dorosłymi i młodymi (Tab. 1). Porównano także, czy zarażenie jest związane z parametrami kondycji (Tab. 2). Ciężar ciała nie był związany z obecnością bakterii, zaś osobniki zarażone *Y. enterocolitica* charakteryzowały się dłuższym skokiem. Nieoznaczone gatunki *Yersinia* były stwierdzane częściej w środowiskach antropogenicznych, zaś dla *Y. enterocolitica* takich zależności nie zaobserwowano (Tab. 3). W żadnej z badanych prób nie wykryto genu ail.

Autorzy pracy przedstawili także występowanie tej bakterii u innych ptaków (Tab. 4) i stwierdzili, że obserwowane przez nich zarażenie płochaczy jest jednym z najwyższych zanotowanych do tej pory u ptaków. *Yersinia* jest bakterią mogącą przetrwać w zimnym klimacie, może być powszechna w górach i to może tłumaczyć tak wysoki poziom zarażenia badanych ptaków.