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# Characterisation of *Yersinia enterocolitica* strains isolated from wildlife in the northwestern Italian Alps

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## Abstract

**Introduction:** Yersiniosis is a zoonosis causing gastroenteritis, diarrhoea, and occasionally reactive arthritis and septicaemia. Cases are often linked to meat consumption and the most common aetiological agent is the Gram-negative bacilliform *Yersinia enterocolitica* bacterium. The occurrence of *Yersinia* spp. among wild animals has mostly been studied in wild boar, but it has seldom been in other species. **Material and Methods:** A total of 1,868 faecal samples from animals found dead or hunted were collected between 2015 and 2018 in the Valle d'Aosta region of the northwestern Italian Alps. Alpine ibex faecal samples were collected during a health monitoring program in 2018. Bacteria were isolated *via* PCR and confirmed as *Y. enterocolitica* biochemically. Strain antimicrobial susceptibility was tested by Kirby–Bauer disc diffusion, and the presence of virulence factors and antimicrobial resistance genes was investigated using whole-genome sequencing. **Results:** *Yersinia enterocolitica* strains of biotype 1A were detected in six faecal samples from red deer (0.93%), roe deer (0.49%) and red foxes (0.7%). Strains found in beech martens (3.57%) and Alpine ibex (2.77%) belonged to biotypes 1B and 5, respectively and harboured the pYPTS01 plasmid that had only been detected in *Y. pseudotuberculosis* PB1/+. All the isolates were resistant to ampicillin and erythromycin. **Conclusion:** The biovar 1A strains exhibited different virulence factors and behaved like non-pathogenic commensals. The strain from an Alpine ibex also harboured the self-transmissible pYE854 plasmid that can mobilise itself and the pYPTS01 plasmid to other strains. The beech marten could be considered a sentinel animal for *Y. enterocolitica*. Phenotypic resistance may account for the ability of all the strains to resist β-lactams.

**Keywords:** *Yersinia enterocolitica*, wildlife, virulence factors, antibiotic resistance.

## Introduction

*Yersinia enterocolitica* belongs to the *Yersinia* genus of the Enterobacteriaceae family and is the causative agent of an important zoonotic disease called yersiniosis. Human infection occurs in all age groups, but clinical illness is more frequently reported in children and young adults, with asymptomatic infection being common in adults. This bacterium is a gastrointestinal pathogen that usually causes fever, enteritis, enterocolitis, and diarrhoea which can be bloody, while reactive arthritis and septicaemia are relatively rare. Extraintestinal spread can occur under certain conditions, such as immunosuppression and iron overload (8).

The trend of human yersiniosis cases was stable in 2015–2019, and at the end of this period it was the fourth most commonly reported zoonosis in humans in the European Union, with 6,961 confirmed cases reported. The most common bioserotype of *Y. enterocolitica* was 4/O:3, and the second most common was 2/O:9 (12). Despite the relative frequency of cases, a common surveillance programme for *Y. enterocolitica* has not yet been introduced in the European Union and animal monitoring data submitted to the European Food Safety Authority (EFSA) are collected without any harmonised scheme (12).

The classification of *Y. enterocolitica* strains has traditionally been based on a combination of biochemical and serological tests. Six biotypes (1A, 1B,

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2, 3, 4 and 5) and more than 70 serotypes have been identified so far (8). This bacterium is ubiquitous, frequently being isolated from animals, soil, various sources of water and food products, including vegetables, animal-derived foodstuffs, seafood, raw milk and pasteurised dairy products (12). Most of the isolates recovered from animals, untreated water and environmental samples belong to biotype 1A and are non-pathogenic with no clinical importance (20, 30). Infections normally occur through the ingestion of contaminated food. *Suidae* are considered the principal reservoir of *Y. enterocolitica*, and pork meat and processed meat products are the main source of human infection (12). *Yersinia enterocolitica* is present in several animals, including birds, fish, insects, frogs and mammals (30). The wild mammals which carry *Y. enterocolitica* are wild boar (4, 15, 22, 29), red deer (20, 25, 29), roe deer (5, 29), fallow deer (29), Alpine ibex (20), canids (23, 24, 25) and mustelids (23, 24).

The course of a *Y. enterocolitica* infection is influenced by virulence determinants located on the chromosome and the pYV plasmid. The genes located within the plasmid that are directly responsible for the pathogenicity are *yadA*, encoding the *Yersinia* adhesin (YadA), and the Yop virulon, encoding *Yersinia* outer membrane proteins (Yops). The adhesin mediates binding to human epithelial cells (HEp-2) and microvilli (3, 8), triggers an inflammatory response in epithelial cells by inducing the production of interleukin-8 (IL-8) and plays a central role in promoting serum resistance, acting as a C4-binding protein (C4bp) receptor. The Yop virulon has four components: a type III secretion system, translocator Yops, a control element, and effector Yops (3, 8). The secretion system forms pores in the membrane of eukaryotic target cells, then effector proteins are transported through the pores to the cytosol of target cells (3, 8). Effector Yops cause the activation of proinflammatory cytokines and the inhibition of the signalling pathways of target eukaryotic cells that can result in the repression of the host immune system.

The main chromosomal virulence genes of *Y. enterocolitica* are attachment and invasion locus (*ail*), invasin (*inv*), mucoid *Yersinia* factor A (*myfA*), high pathogenicity island (*HPI*) and *Yersinia* stable toxins (*yst*). With a mediating function, *ail* is involved in the attachment and invasion of host cells and is also fundamental to serum resistance during exponential growth because it binds to the complement component C4bp, and thereby facilitates bacterial survival (3, 8). For the bacteria to translocate into M cells, *inv* is required, as it also is for the colonisation of Peyer's patches (3). Invasin binds to integrins, creating an integrin cluster, and triggers the remodelling of the actin cytoskeleton leading to the internalisation of *Y. enterocolitica* in epithelial cells. The toxin gene *yst* has been classified into *ystA*, y*stB* and y*stC*, which are similar to the heat-stable toxin type I of *Escherichia coli*. The *Yersinia* stable toxins trigger the activation of guanylate cyclase, the increase in cyclic guanosine monophosphate levels in epithelial cells, and the accumulation of fluids in the intestinal lumen, resulting in diarrhoea. Pathogenic strains harbour the *ystA* gene, whereas biotype 1A strains usually encode *ystB* (3, 8). The *myfA* factor is an adhesin maximally expressed at 25°C that acts during the first phase of infection and is involved in the initial colonisation and internalisation of host cells. It is responsible for the formation of a layer of extracellular material surrounding the bacterium surface (3, 8).

The iron restriction present in the body fluid of mammals is a host defence mechanism against potential pathogens and the presence of an iron acquisition system is therefore fundamental for the survival of the pathogen in its host (28). Therefore, most bacteria produce and secrete siderophores under conditions of low iron availability, which are iron chelators capable of binding exogenous iron and transporting it into the bacterial cells (3). The highly pathogenic *Y. enterocolitica* biotype 1B usually harbours the *HPI*, which encodes proteins involved in the biosynthesis, regulation, and transport of the yersiniabactin siderophore (25). This compound affects the uptake and utilisation of iron by bacterial cells and may promote their growth under iron-limiting conditions in host tissues (3). An alternative source of iron for different microorganisms are haeme-containing compounds (28). Some *Y. enterocolitica* strains possess the haemin uptake operon which encodes four proteins including *hemR*, an outer membrane protein receptor that removes iron bound to haeme proteins, allowing its uptake from the extracellular environment (25, 28).

The aim of our study was to investigate the presence of *Y. enterocolitica* in wild animals through analysis of faecal samples collected in the Valle d'Aosta region between 2015 and 2018 and to evaluate both the degree of pathogenicity and the antibiotic resistance of the isolated strains by means of whole-genome sequencing (WGS).

## Material and Methods

**Sample collection.** A total of 1,868 faecal samples of different wild animals were collected between 2015 and 2018 in the Valle d'Aosta, an Italian region located in the northwestern Alps between Switzerland and France (Fig. 1). The specimens were obtained from different wild species found dead or shot during the hunting seasons. The faecal samples were placed in sterile tubes and stored at −80°C before being tested. The wild animals investigated for the presence of *Yersinia enterocolitica* by means of real-time PCR were hooded crows (*Corvus cornix*, n = 79), rock partridges (*Alectoris graeca*, n = 3), rooks (*Corvus frugilegus*, n = 6), a heron (*Ardea alba*, n = 1), tawny owls (*Strix aluco*, n = 2), a golden eagle (*Aquila chrysaetos*, n = 1), European starlings (*Sturnus vulgaris*, n = 2), a barn owl (*Tyto alba*, n = 1), little owls (*Athene noctua*, n = 2), buzzards (*Buteo buteo*, n = 3), cormorants (*Phalacrocorax*

*carbo*, n = 3), jays (*Garrulus glandarius*, n = 185), goshawks (*Accipiter gentilis*, n = 3), a sparrow (*Passer domesticus*, n = 1), common kestrels (*Falco tinnunculus*, n = 4), mallards (*Anas platyrhynchos*, n = 4), sparrow hawks (*Accipiter nisus*, n = 5), black grouse (*Lyrurus tetrix*, n = 18), red foxes (*Vulpes vulpes*, n = 285), wolves (*Canis lupus*, n = 8), wild boar (*Sus scrofa*, n = 107), roe deer (*Capreolus capreolus*, n = 406), red deer (*Cervus elaphus*, n = 213), chamois (*Rupicapra rupicapra*, n = 312), beech martens (*Martes foina*, n = 28), a weasel (*Mustela nivalis*, n = 1), badgers (*Meles meles*, n = 71), stoats (*Mustela erminea*, n = 4), a pine marten (*Martes martes*, n = 1), marmots (*Marmota marmota*, n = 10), a red squirrel (*Sciurus vulgaris*, n = 1), hares (*Lepus europaeus*, n = 58) and hedgehogs (*Erinaceus europaeus*, n = 4). In addition, Alpine ibex (*Capra ibex*) faecal samples (n = 36) obtained during a wildlife health monitoring program in 2018 were included in the molecular screening. Sex, age, and sampling locations were recorded for every animal that tested positive for *Y. enterocolitica* (Table 1).

**Deoxyribonucleic acid (DNA) extraction and real-time PCR conditions.** An initial enrichment step was performed to increase the PCR sensitivity. The faecal samples were mixed with a specific volume of *Yersinia* PSB broth (Microbiol, Cagliari, Italy), in such a way as to achieve a weight/volume percentage of 5, and then incubated at 5°C for a week. After incubation, 1 mL of the faecal suspensions was withdrawn and transferred to a tube.

The samples were then centrifuged for 5 min at 12,000 × *g* and after removing the supernatant, the pellet was mixed with 100 µL of PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the samples were incubated for 10 min at 95°C and centrifuged for 5 min at 12,000 × *g*. Finally, the supernatant containing the DNA was withdrawn.

The detection of *Y. enterocolitica* strains was performed by real-time PCR. All the analyses were carried out with the StepOnePlus Real-Time PCR System (Thermo Fisher). Detection employed the TaqMan approach, targeting the *ail* gene (9, 31). A real-time PCR protocol was applied which was adopted from Lambertz *et al*. (31), with primers and TaqMan probe manufactured by Thermo Fisher Scientific. The PCR mixture consisted of 12.5 µL of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 500 nM of each primer, 200 nM of the probe and 5 µL of template, in a total volume of 25 µL. The PCR cycling conditions were an initial denaturation step at temperature of 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 60 s. An internal positive control (IPC) was added to every PCR run using a commercially available TaqMan exogenous IPC kit (Eurogentec, Seraing, Belgium). If the IPC cycle threshold value was higher than 38, the template was then diluted 1:10 with $ddH_2O$ and subjected to a second amplification, using the real-time PCR.

**Isolation and identification of *Y. enterocolitica*.** The faecal suspensions which tested positive for *Y. enterocolitica* by real-time PCR, were inoculated on cefsulodin-irgasan-novobiocin agar (Microbiol) and then incubated at 30°C for 24 h. The colonies morphologically compatible with *Y. enterocolitica* were subcultured on Columbia blood agar (Becton Dickinson, Franklin Lakes, NJ, USA) and MacConkey agar (Microbiol) and then incubated at 30°C for 24 h. Afterwards, the colonies from each plate were subjected to biochemical characterisation using API 20E and API 50CH strips (BioMérieux, Marcy l'Étoile, France) according to the manufacturer's protocol.

**Antimicrobial susceptibility.** The antimicrobial resistance of 8 *ail*-positive *Y. enterocolitica* strains recovered from different wild animal species was tested. The analysis was performed with the Kirby–Bauer disc diffusion technique using Mueller–Hinton agar plates (Microbiol). Both the test protocol and the analysis of the results were carried out according to the Clinical and Laboratory Standard Institute guidelines (M02-A12, 2019).

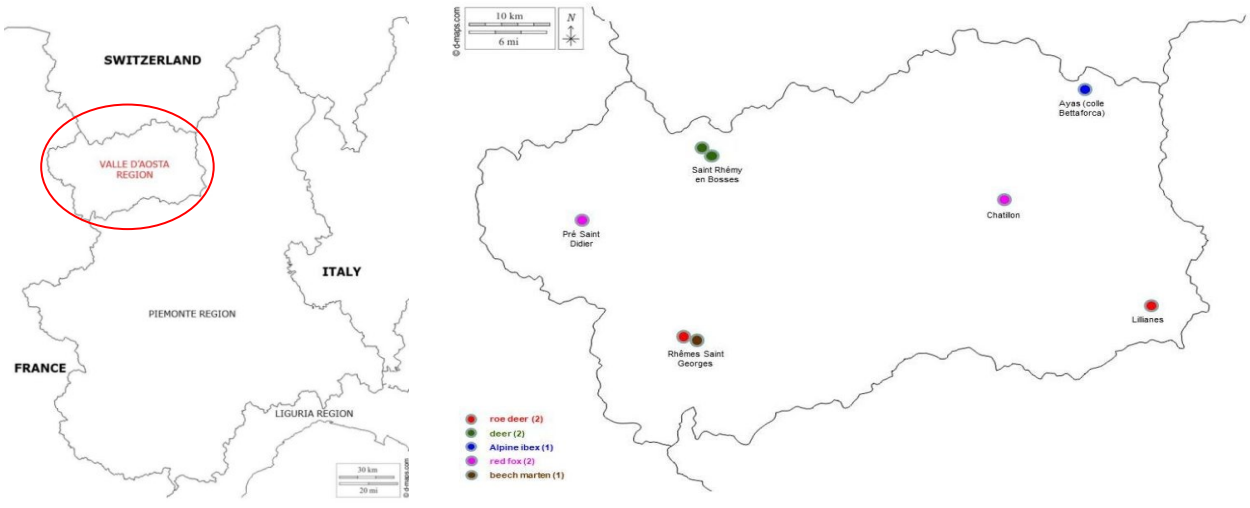


**Fig. 1.** Geographical distribution of *Y. enterocolitica*-positive samples in the Valle d'Aosta region of the northwestern Italian Alps

**Table 1.** Identification and characterisation of wild animals which tested positive for *Y. enterocolitica* and the strains isolated from their faeces in the Valle d'Aosta region

| Source | Sex | Age | Sampling locations | Subspecies | Bio/serotype | *ail* | *inv* | *myfA* | *ystA* | *ystB* | *yadA* | *Yops* | *hemR* | *HPI* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Roe deer | female | A | Lillianes | *enterocolitica* 8081 | 1A/O:5 | + | | | | + | | | | |
| Roe deer | female | A | Rhêmes Saint Georges | *enterocolitica* 8081 | 1A/O:8 | + | | | | + | | | | |
| Red deer | female | A | Saint Rhémy en Bosses | *enterocolitica* 8081 | 1A/O:5 | + | | | | + | | | | |
| Red deer | female | S | Saint Rhémy en Bosses | *enterocolitica* 8081 | 1A/O:1.2 | + | | | | + | | | | |
| Alpine ibex | female | A | Ayas | *palearctica* 105.5R(r) | 5/O:3 | + | + | + | + | | + | + | + | |
| Red fox | male | A | Châtillon | *palearctica* 105.5R(r) | 1A/NT | + | + | + | + | | | | + | |
| Red fox | female | A | Pré Saint Didier | *palearctica* 105.5R(r) | 1A/O:9 | + | + | + | + | | | | + | |
| Beech marten | male | Y | Rhêmes Saint Georges | *palearctica* 105.5R(r) | 1B/O:5 | + | + | + | + | | + | + | + | |

A – adult; S – subadult; Y – young; + – present

The following antimicrobials were tested: ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), erythromycin (15 µg), gentamicin (10 µg), streptomycin (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), enrofloxacin (5 µg), cephalothin (30 µg), tetracycline (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), and chloramphenicol (30 µg).

**Genome sequence analysis.** The selected isolates were subjected to DNA extraction with the EXTRACTME Genomic DNA isolation kit (Blirt, Gdańsk, Poland) following the manufacturer's protocol. DNA concentrations were determined with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Whole genome sequencing runs were performed on a MiSeq System (Illumina, San Diego, CA, USA) using paired-end libraries prepared employing the Illumina DNA Prep Kit with 150 bp read length. The reads were first made the subjects of Galaxy tool FastQC Read Quality Reports, accessed *via* the Galaxy public server (1), to provide quality control checks on raw sequence data. The reads were then trimmed using the Galaxy Trimmomatic tool (6). Unicycler v. 0.4.1.1 was used *via* Galaxy to assemble the genomes. The contigs were then analysed by means of KmerFinder 3.2 to perform genomic taxonomy (11), ResFinder 4.1 to identify antimicrobial resistance genes (7), PlasmidFinder 2.0 to detect and analyse the plasmids (10) and VirulenceFinder 2.0 to identify the virulence factor (17). Finally, a phylogenic comparison was performed using EnteroBase tools (33) to assign a whole genome multi locus sequence type (wgMLST) starting from the WGS data and the derived tree was visualised with GrapeTree (34).

## Results

The pathogen was detected in eight wild animals with an overall prevalence of 0.43% (8/1,868). These strains were isolated from the faeces of two red deer, two roe deer, two red foxes, a beech marten and an Alpine ibex collected in the Valle d'Aosta region.

The prevalence of *Y. enterocolitica* in red deer was determined at 0.93% (2/213). Two strains were isolated from two red deer hunted near Saint Rhémy en Bosses (Fig. 1) in 2015. These strains both belonged to biotype 1A, but their serotypes were different, being O:5 and O:1.2 (Table 1). They belonged to *Y. enterocolitica* subspecies *enterocolitica* 8081 and harboured the *ail* (GenBank accession no. JX972143.1) and *ystB* (GenBank accession no. MK734422.1) genes (Table 1). These *Y. enterocolitica* strains were resistant to ampicillin, erythromycin and cephalothin and susceptible to streptomycin, sulfamethoxazole with trimethoprim, cefotaxime, enrofloxacin, ciprofloxacin, nalidixic acid, chloramphenicol and tetracycline (Table 2). Concerning cefuroxime, the strain belonging to serotype O:5 was resistant, whereas the strain belonging to serotype O:1.2 was susceptible.

Two strains of *Y. enterocolitica* were found in two roe deer in 2015 and the prevalence in this species was assessed at 0.49% (2/406). One roe deer was hunted near Lillianes (Fig. 1), while the other was shot near Rhêmes Saint Georges. As was also the case with the isolates from red deer, the isolated strains both belonged to biotype 1A, but their serotypes were different, being O:5 and O:8 (Table 1). Sequencing of their genomes highlighted that the strains both belonged to the *Y. enterocolitica* subspecies *enterocolitica* 8081 and possessed the *ail* and *ystB* genes (Table 1). These *Y. enterocolitica* strains were both resistant to ampicillin, erythromycin and cephalothin and susceptible to streptomycin, sulfamethoxazole with trimethoprim, cefotaxime, enrofloxacin, ciprofloxacin, nalidixic acid, chloramphenicol, tetracycline and cefuroxime (Table 2).

Regarding canids, two strains were detected in two red foxes and the prevalence in this species was 0.7% (2/285).

**Table 2.** Antimicrobial resistance patterns in *Y. enterocolitica* strains isolated from wildlife

| Antibiotic | 1A/O:5 (roe deer) | 1A/O:8 (roe deer) | 1A/O:5 (red deer) | 1A/O:1.2 (red deer) | 5/O:3 (Alpine ibex) | 1A/N.T. (red fox) | 1A/O:9 (red fox) | 1B/O:5 (beech marten) |
|---|---|---|---|---|---|---|---|---|
| Ampicillin | R | R | R | R | R | R | R | R |
| Amoxicillin/ Clavulanic acid | S | S | S | S | R | S | S | S |
| Erythromycin | R | R | R | R | R | R | R | R |
| Gentamicin | S | R | S | S | S | S | S | S |
| Streptomycin | S | S | S | S | S | S | S | S |
| Trimethoprim/ Sulfamethoxazole | S | S | S | S | S | S | S | S |
| Enrofloxacin | S | S | S | S | S | S | S | S |
| Ciprofloxacin | S | S | S | S | S | S | S | S |
| Nalidixic acid | S | S | S | S | S | S | S | S |
| Cephalothin | R | R | R | R | R | R | R | S |
| Tetracycline | S | S | S | S | S | S | S | S |
| Cefotaxime | S | S | S | S | S | R | S | S |
| Chloramphenicol | S | S | S | S | S | S | S | S |
| Cefuroxime | R | R | R | S | S | R | S | R |

R – resistant; S – susceptible

These foxes were found dead near Pré Saint Didier and Châtillon (Fig. 1) in 2015. In their necropsies, thoracic trauma, cachexia, generalised lymphadenopathy and severe sarcoptic mange were observed. The isolated strains were biotyped as 1A, but only one was serotyped, and the other was not typable (NT) (Table 1). Genome sequencing revealed that both strains belonged to the *palearctica* 105.5R(r) subspecies and harboured the chromosomal *ail*, *inv* (GenBank accession no. Z48161.19), *myfA* (GenBank accession no. Z21953.1), *hemR* (GenBank accession no. AM286415.1) and *ystA* (GenBank accession no. U09235.1) virulence genes (Table 1). These *Y. enterocolitica* strains were shown to be resistant to ampicillin, cephalothin and erythromycin and susceptible to streptomycin, sulfamethoxazole with trimethoprim, enrofloxacin, ciprofloxacin, nalidixic acid, chloramphenicol and tetracycline (Table 2). Regarding cefotaxime and cefuroxime, the strain belonging to the serotype not typable was able to resist them, whereas the other was not.

*Yersinia enterocolitica* was detected in a beech marten found dead close to Rhêmes Saint Georges (Fig. 1) in 2015, and the bacteria had prevalence of 3.57% in this species (1/28). The necropsy of this mustelid revealed the gastritis, haemorrhagic enterocolitis and pulmonary haemorrhages associated with liver and lung congestion. The isolated strain belonged to the bioserotype 1B/O:5 (Table 1) and the *palearctica* 105.5R(r) subspecies. The analysis of the virulence factors revealed the presence of both chromosomal and plasmid-encoded virulence genes. This strain harboured the virulence plasmid, pYPTS01 (GenBank accession no. CP001049), containing the *yadA* (GenBank accession no. X13881) and *Yops* (GenBank accession no. AF102990) genes, as well as the chromosomal *ail*, *inv*, *myfA*, *hemR* and *ystA* virulence factors. This strain exhibited resistance against ampicillin, erythromycin and cefuroxime, and susceptibility to the other antibiotics (Table 2).

The isolation of *Y. enterocolitica* in a clinically healthy Alpine ibex located near Ayas (Fig. 1) was achieved in 2018 and this signified a prevalence of 2.77% in this species (1/36). The strain was identified as bioserotype 5/O:3 (Table 1) and the genome sequence analysis revealed that it belonged to the *palearctica* 105.5R(r) subspecies and possessed both chromosomal and plasmid virulence factors. The chromosomal virulence markers were *ail*, *inv*, *myfA*, *hemR* and *ystA*, while the pYPTS01 virulence plasmid harboured the *yadA* and *Yops* genes. In addition, the self-transmissible pYE854 plasmid (GenBank accession no. AM905950) was observed. This strain showed antibiotic resistance to ampicillin, cephalothin, erythromycin and amoxicillin with clavulanic acid, and susceptibility to other antibiotics (Table 2).

**Table 3.** Whole-genome comparison of the bioserotyped *Y. enterocolitica* strains isolated from wild animals with the multi locus sequence type (MLST), core genome (cg) MLST and whole genome (wg) MLST approaches

| Source | Bio/serotype | MLST | cgMLST | wgMLST |
|---|---|---|---|---|
| Roe deer | 1A/O:5 | 649 | 4721 | 5608 |
| Roe deer | 1A/O:8 | 650 | 4724 | 5610 |
| Red deer | 1A/O:5 | 19 | 4994 | 6124 |
| Red deer | 1A/O:1.2 | 362 | 4722 | 5609 |
| Alpine ibex | 5/O:3 | 13 | 4727 | 5614 |
| Red fox | 1A/NT | 12 | 4995 | 6125 |
| Red fox | 1A/O:9 | 12 | 4723 | 5606 |
| Beech marter | 1B/O:5 | 12 | 4725 | 5611 |

The whole genome core sequences of the eight isolated *Y. enterocolitica* strains were compared to each other and to the genomes of *Y. enterocolitica* available from EnteroBase. None of them formed a distinct cluster (Table 3) and showed similarity to other *Y. enterocolitica* strains (data not shown). Finally, the presence of antibiotic resistance genes was investigated using WGS, which revealed the presence of the streptogramin A acetyltransferase gene (GenBank accession no. AF170730) in all the strains.

## Discussion

The main reservoir of *Y. enterocolitica* is swine, and the strains associated with human disease have

frequently been isolated from the tonsils and faecal samples of slaughtered pigs (12, 14, 25). Wild boar act as a potential reservoir of *Y. enterocolitica*, although there is no epidemiological link between this wild species and domestic pigs. The presence of *Y. enterocolitica* isolated from wild boar hunted in Italy has been reported. The wild boar population increase in the last decades having brought these animals into greater proximity to human populations, thereby intensifying human–wildlife interactions (such as hunting), suggests that the main source of infection is anthropogenic (22). In our study, *Y. enterocolitica* was detected in roe deer, red deer, Alpine ibex, red fox and beech marten, but not in wild boar. The presence of this wild suid only in a small area of the Valle d'Aosta region and the low number of wild boar faeces samples collected compared to samples from other wild ungulate species could explain the absence of *Y. enterocolitica* in this species.

The large number of faecal samples in our study suggested that the detection of *Y. enterocolitica* strains be by means of a real-time PCR targeting the *ail* gene, preceded by an initial enrichment step to increase the PCR sensitivity. The *ail* gene is an ideal target as one of the chromosomal encoded genes required for the survival of *Y. enterocolitica* in the host (8) and is usually present in strains associated with pathogenicity in humans (9, 14, 31). However, the *ail* gene has also been detected in some biotype 1A strains considered non-pathogenic (15, 24, 32) and other genes have frequently been used as a target to identify pathogenic *Y. enterocolitica*, such as *inv*, *ystA*, *ystB* and *myfA* (3, 15, 22, 25). Thus, in our study, the usage of the *ail* gene alone as a virulence marker was considered rather as a first screening test to detect potentially pathogenic strains of *Y. enterocolitica*, which in turn should be further investigated to determine pathogenicity.

In our study, six out of eight strains belonged to biotype 1A. This biotype is usually highly heterogeneous and includes many serogroups, and its strains are normally considered to be non-pathogenic commensals as they do not harbour the pYV plasmid or the main chromosomal virulence factors (3, 8, 30). The presence of *Y. enterocolitica* in red deer and roe deer has already been studied by Joutsen *et al*. (20), who isolated several strains identified as the 1A/O:8 and 1A/O:5 bioserotypes in Switzerland. Aschfalk *et al.* (2) detected the 1A/O:8, 1A/O:6 and 1A/O:5 bioserotypes in Norway and Bancerz-Kisiel *et al.* (5) found four strains biotyped as 1A/NT in Poland. The wild ungulates reported in these studies were all in good health before being hunted; commensurately, the strains isolated in Switzerland and Poland harboured the *ystB* gene. In our study, the presence of *Y. enterocolitica* strains identified as bioserotypes 1A/O:5 and 1A/O:1.2 was observed in two clinically healthy red deer. In addition, two strains isolated from roe deer belonging to the 1A biotype and O:5 and O:8 serotypes were detected. These strains harboured both the *ail* and the *ystB* genes. Based on our results and those previously reported, biotype 1A strains belonging to the O:5 and O:8 serotypes may circulate among red deer and roe deer in Europe. Diarrhoea in the rectal intestines of wild ungulates was not observed, suggesting that the gene encoding the thermostable enterotoxin was not expressed. This is consistent with another report in which several biotype 1A strains were isolated from diarrhoeic stools of paediatric patients, swine throat swabs and various aquatic sources, some of which that hybridised with the probe for the thermostable enterotoxin failed to produce enterotoxin, suggesting the presence of a silent *ystB* gene (27).

The presence of *Y. enterocolitica* belonging to biotype 1A has already been reported in red foxes. Nowakiewicz *et al*. (24) detected six strains in Poland in this wild species and in beech martens and raccoons. Most of these strains harboured the *ail* gene, but unfortunately the bioserotype was not identified. In addition, Nikolova *et al.* (23) found different strains of *Y. enterocolitica* in foxes that were serotyped as O:3. In our study, both strains isolated from red foxes harboured the same virulence factors: *inv*, *myfA*, *ail*, *hemR* and *ystA*. The last gene is present in pathogenic *Y. enterocolitica* (3, 4, 5, 25), while it is seldom detected in biotype 1A strains, which usually encode the *ystB* gene (3, 22, 13). The presence of the *inv*, *myfA* and *ail* virulence genes is supposed to make these strains potentially pathogenic and the presence of *ystA* might trigger diarrhoea in the host. No signs of gastroenteritis or diarrhoea were observed during the necropsy of the red foxes, suggesting that these *Y. enterocolitica* strains had behaved as non-pathogenic commensals even when the host's immune defences were weakened. Therefore, their ability to trigger an infection did not appear to be related to the host's immune system. Our results seem to be consistent with those of other studies, in which pathogenic biotype 1A strains differed considerably from non-pathogenic ones because they had a greater ability to penetrate cultured epithelial cells, survive within macrophages, and escape from phagocytes and epithelial cells by a mechanism that did not appear to kill the host cell or compromise membrane integrity (16, 30).

The presence of *Y. enterocolitica* in a beech marten has already been reported, as two strains that harboured the *ail* gene were detected in Poland (24), while a strain serotyped as O.3 was found in Bulgaria (23). In our study, a pathogenic strain was isolated from the faeces of a beech marten and identified as the 1B/O:5 bioserotype. The necropsy of the animal revealed gastritis and haemorrhagic enterocolitis, which were most likely caused by the pathogenic *Y. enterocolitica* isolated from the gastrointestinal tract. The beech marten may have died due to the bioserotype 1B/O:5 strain. The species could be considered a sentinel animal for *Y. enterocolitica*, as they are used to living near inhabited centres and coming into contact with domestic animals which are well-known hosts of this bacterium. The genome of the strain was sequenced and the presence of both chromosomal virulence genes (*inv*, *myfA*, *hemR* and *ystA*) and the pYPTS01 plasmid containing the *Yops* and *yadA* genes was observed. This is the first report in which the pYPTS01 plasmid has

been observed in *Y. enterocolitica*, because it had only been detected in *Y. pseudotuberculosis* PB1/+ prior to this research (18).

The presence of *Y. enterocolitica* in Alpine ibex has already been studied. Joutsen *et al.* (20) isolated a strain of *Y. enterocolitica* from the faeces of an Alpine ibex hunted in Switzerland. The strain was identified as bioserotype 3 or 5/O:3 and harboured both the *ail*, *ystA* and *myfA* virulence factors and the pYV plasmid containing the *yadA* gene. In our study, a *Y. enterocolitica* strain was isolated from the faeces of a clinically healthy Alpine ibex which belonged to the 5/O:3 bioserotype. The *ail*, *inv*, *myfA*, *hemR* and *ystA* virulence factors and the pYPTS01 plasmid containing the *yadA* and *Yops* genes were detected, suggesting that a *Y. enterocolitica* strain of the 5/O:3 bioserotype may circulate among the Alpine ibex located in the northwestern Alps. This strain possessed the same plasmid detected in the strain isolated from the beech marten previously described, further confirming that *Y. enterocolitica* harboured the pYPTS01 plasmid. In addition, the self-transmissible pYE854 plasmid, which appears to have the same functional characteristics as the fertility factor of *E. coli*, was detected (19). This is the first report in which a *Y. enterocolitica* strain was observed to harbour both the pYE854 and pYPTS01 plasmids. The former plasmid can mobilise others if they contain a specific region called p1340, one such with this content being the latter plasmid (18). Hammerl *et al.* (19) mobilised *Yersinia* pYV virulence plasmids containing the p1340 region *in vitro* by the action of the pYE854 conjugative plasmid, and they hypothesised that this process was likely to occur in nature from a pathogenic strain of *Yersinia* to another non-pathogenic strain (18). Our results may confirm this hypothesis since the co-presence of these elements in a wild-type strain of *Y. enterocolitica* was observed.

The development of antibiotic resistance is usually associated with genetic changes, either in the acquisition of resistance genes or in mutations in elements relevant to the activity of the antibiotic. However, resistance called phenotypic resistance can also be achieved without any genetic alteration. This capacity of *Y. enterocolitica* has already been studied, revealing its natural resistance to certain types of antibiotics such as ampicillin, amoxicillin and the first generation of cephalosporins (21). Sacchini *et al.* (25) conducted a study in central Italy on the antibiotic resistance of *Y. enterocolitica* strains found in intensive pig farming. The results revealed them to have the greatest resistance to ampicillin, followed by resistance to streptomycin. Modesto *et al*. (22) analysed different strains of *Y. enterocolitica* isolated from the livers of several wild boar hunted in northwestern Italy, reporting that all the *Y. enterocolitica* isolated exhibited resistance against ampicillin, streptomycin and tetracycline, whereas they were susceptible to chloramphenicol and enrofloxacin. Bucher *et al.* (9) analysed the antibiotic resistance of *Y. enterocolitica* strains isolated from human and porcine samples in southern Germany. A high percentage of resistance to ampicillin and erythromycin was shown and no difference was detected between the resistance patterns of human and porcine strains. Similar results were also reported among strains isolated from pigs slaughtered at a Swiss abattoir (14). In another report, 14 strains isolated from wild boar and 78 from fattening pigs were characterised. They were resistant to ampicillin, erythromycin and amoxicillin with clavulanic acid and susceptible to cefotaxime, ciprofloxacin, chloramphenicol, gentamicin, and nalidixic acid (15). Our results are consistent with those previously reported, because all the isolated strains were susceptible to tetracycline, chloramphenicol, nalidixic acid, ciprofloxacin, enrofloxacin, trimethoprim/sulfamethoxazole and streptomycin and resistant to ampicillin and erythromycin. Investigation of the presence of antibiotic resistance genes confirmed all isolated strains to possess the streptogramin A acetyltransferase gene, which confers resistance against erythromycin (26). Contrastingly, none of the strains harboured β-lactamase genes which confer resistance against penicillin (ampicillin) and cephalosporins (cephalothin, cefotaxime and cefuroxime). Therefore, the ability of all strains to resist β-lactam antibiotics could be linked to their phenotypic resistance.

In conclusion, the biotype 1A strains of *Y. enterocolitica* isolated in this study exhibited different virulence factors and behaved like non-pathogenic commensals even when the host's immune system was weakened. Therefore, further investigations of the gene expression of their virulence markers should be performed. The beech marten could be considered a sentinel animal for *Y. enterocolitica*, as a research subject may have died because of the pathogenic strain containing the pYPTS01 plasmid, which had only been observed in *Y. pseudotuberculosis* PB1/+ strains in earlier research. The final noteworthy implication follows from the establishment that a strain of *Y. enterocolitica* isolated from a clinically healthy Alpine ibex harboured the pYPTS01 and pYE854 plasmids. The second of these, the self-transmissible plasmid, can mobilise itself and other plasmids to other strains, which in turn could acquire new pathogenicity mechanisms. Further investigation of the horizontal gene transfer among different *Yersinia* species in wildlife should be carried out.

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