Molecular survey on the occurrence of avian haemosporidia, *Coxiella burnetii* and *Francisella tularensis* in waterfowl from central Italy

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**ABSTRACT**

The aim of the present study was to evaluate the occurrence of some avian Haemosporidia, *Coxiella burnetii* and *Francisella tularensis* in waterfowl from Tuscany wetlands. One-hundred and thirty-three samples of spleen were collected from regularly hunted wild birds belonging to 13 different waterfowl species. DNA extracted from each sample was submitted to PCR assays and sequencing to detect the pathogens. Thirty-three samples (24.81%) were positive with PCR for at least one pathogen: 23 (17.29%) for *Leucocytozoon* spp., 6 (4.51%) for *Plasmodium* spp., 4 (3%) for *C. burnetii*, 2 (1.5%) for *Haemoproteus* spp. No specific *F. tularensis* amplifications (0%) were detected. To the best of our knowledge, this study firstly reports data about haemosporidian and *C. burnetii* infections in waterfowl from Italy.

1. Introduction

Avian haemosporidia are a group of protozoan parasites, among which *Plasmodium*, *Haemoproteus* and *Leucocytozoon* genera are encountered. These parasites are transmitted by blood sucking dipteran vectors, with birds acting as intermediate hosts (Valkiunas, 2005). In detail, after the inoculation of sporozoite stages by the vectors during the bloodmeal, exoerythrocytic schizonts develop in different tissues (i.e. spleen, liver, lungs, heart, brain …) giving gametocytes into the blood cells. These latter stages are infective for the vectors.

Haemosporidians occur worldwide except in Antarctica (Beadell et al., 2009) and most infections are relatively benign, probably due to long-term host parasites evolutionary associations (Bennet et al., 1993). The occurrence of these protzoa has been reported from several birds’ species (Schmid et al., 2017; Dimitrov et al., 2018; Ferreira-Junior et al., 2018; Valkiunas et al., 2019; Schumm et al., 2019) and from dipteran species (Ionică et al., 2017; Žižký et al., 2017; Martin et al., 2019; Schoener et al., 2019).

Domestic Anatidae are highly susceptible to haemosporidia (Valkiunas, 2005) and wild waterfowl have been reported as infected in several countries (Lovén et al., 1980; Reeves et al., 2015; Ramey et al., 2015; Meixell et al., 2016).

To the best of our knowledge, a unique survey by Sacchi and Prigioni (1986) evaluating occurrence of haemosporidia in wild waterfowl by microscopy in Italy yielded no positive results.

*Coxiella burnetii* is the etiologic agent of Q fever, a worldwide zoonotic bacterial disease. Even though domestic ruminants are considered as the main reservoirs for this pathogen, several wild mammals have been found to be hosts of this microorganism. *C. burnetii* has been found in birds (Babudieri and Moscovici, 1952; Stein and Raoult, 1999; Ebani et al., 2016), too, but little is known about the transmission patterns among avian populations. Animals as well as humans usually become infected through oral and inhalation routes, but the transmission of *C. burnetii* is also possible via tick bites (Porter et al., 2011).

*Francisella tularensis* is the causative agent of the severe zoonotic disease tularemia. It is a Gram negative bacterium, with a wide range of hosts including invertebrates and mammals. Birds have been experimentally infected with *F. tularensis* (Mörner and Mattsson, 1988) and naturally acquired infections have been reported in different avian species (Green and Wade, 1929; Green and Shillinger, 1932; Nakamura, 1950; Stahl et al., 1969; Mörner and Mattsson, 1983). However, studies about tularemia in avian populations were conducted many years ago and updated information is not available. Moreover, symptoms and lesions in birds are not known and some authors suggested that these animals may acquire the infection without developing disease (Mörner and Mattsson, 1988).

The transmission of *F. tularensis* may occur through haematophagous arthropods, as well as through direct or indirect contact with...
Table 1
PCR primers and conditions employed in the assays for the detection of each pathogen.

| Pathogens                      | Amplicons (target gene) | Primers sequence (5’ – 3’) | PCR conditions | References                        |
|-------------------------------|-------------------------|----------------------------|----------------|-----------------------------------|
| * Haemoproteus spp. Plasmodium spp. | 617 bp (cytochrome b) | HAIM1F (CATATATTAAGAATTATAGGAG) | 94 °C - 30 s | [Hellgren et al., 2004]           |
| Leucocytozoon spp.           |                         | HAIM2R (ATGACAGAAGAAATACCATTC) | 50 °C-30 s    |                                   |
| ** Haemoproteus spp. Plasmodium spp. | 480 bp (cytochrome b) | HAIM1F (ATGTTGCTTTGATATAATGCATG) | 94 °C - 30 s | [Bensch et al., 2000]             |
| Leucocytozoon spp.           |                         | HAIM2R (GCTATTTTGTAGTATATTG) | 50 °C-30 s    |                                   |
| Coxiella burnetii            | 478 bp (cytochrome b) | HAIM1L (ATGTTTGTATAGTATACATTACATG) | 72 °C-45 s (25 cycles) | [Hellgren et al., 2004] |
| Francisella tularensis       | 687 bp (IS1111a)       | Trans-1 (TATGTATCCACTCCAGGAT) | 94 °C-30 s | [Berri et al., 2009]             |
|                             |                         | Trans-2 (CCCCCAACACCTCCCTTATTC) | 64 °C-1 min |                                   |
|                             |                         |                             | 72 °C-1 min |                                   |

*Primary amplification; ** Secondary amplification.

Table 2
Number of specimens and detail of positive reactions for Coxiella burnetii, Haemoproteus spp., Leucocytozoon spp. and Plasmodium spp. in relation to the tested avian species.

| Animal species | No. tested specimen | Positive reactions |
|----------------|---------------------|--------------------|
| Aueriformes    |                     |                    |
| Anas crecca    | 63                  | 15 Leucocytozoon spp. |
| Anas platyrhynchos | 21            | 3 C. burnetii |
| Anas penelope  | 19                  | 2 Plasmodium spp.  |
| Anas clypeata  | 10                  | 1 Haemoproteus spp.|
| Anas acuta     | 3                   | 3 Leucocytozoon spp.|
| Tadorna tadorna | 2                 | 4 Plasmodium spp.  |
| Anas strepera  | 2                   | 1 C. burnetii |
| Anas querquedula | 1                | 2 Leucocytozoon spp.|
| Aythya fuligula | 1                  | Negative |
| Aythya fuligula | 1                  | Negative |
| Anser anser    | 1                   | Negative |
| Chrodriforines |                     |                    |
| Gallinago gallinago | 6           | 1 Haemoproteus spp.|
| Grauforines    |                     |                    |
| Fulica atra    | 1                   | Negative |
| Total          | 133                 | 35                 |

hunting seasons (September–January) in the Province of Pisa, in the wetland “Padule di Fucecchio” (43°48’N 10°48’E/43.8’N 10.8’E/43.8), located on the migration route of waterfowls. All the animals were intended for human consumption, so the carcasses were immediately refrigerate until processing (about within 24 h). Bird’s carcasses were submitted to assessment of body condition and examined for ectoparasites. Viscera were removed from each bird, maintained at 4 °C and sent to the laboratories. Impression smears were prepared from spleen of each animal for microscopical examination. The remaining portions were stored at −20 °C for molecular analysis. It was not possible to obtain blood specimens to investigate blood stage parasites and sellers allowed us to collect spleens, only. However this tissue was reported as sensitive in detecting haemosporidian parasites (Scaglione et al., 2016; Valkiunas and Iezhova, 2017). Spleen allows the detection of C. burnetii and F. tularensis, too, because hematogenous spread of these bacteria lead to involvement of several organs including this one (Bell, 1980; Maurin and Raoult, 1999).

2.2. Microscopical examination

The spleen impression smears were prepared on glass slide, fixed in methanol and stained with modified Wright’s solution. Each smear was examined through 100 fields at low magnification (400X), then at least 100 fields were observed at high magnification (1000X).

2.3. Molecular analysis

Extraction of total DNA was carried out from up to 10 mg of each spleen sample using the DNeasy® Blood & Tissue kit (Qiagen, Milano, Italy) and following the manufacturer’s instructions. DNA specimens were stored at 4 °C until used as template for the PCR assays.

Different PCR protocols were carried out to detect DNA of pathogens. Haemoproteus spp., Plasmodium spp., Leucocytozoon spp. DNA were detected, with a nested PCR protocol, according to Hellgren et al. (2004) and Bensch et al. (2000). The extracted DNA was first subjected to an amplification common to Leucocytozoon, Haemoproteus and Plasmodium, and then, its product was submitted to two parallel PCRs, one amplifying both Haemoproteus and Plasmodium while the another one amplifying Leucocytozoon only.). C. burnetii and F. tularensis DNA were investigated, using primers and procedures previously described, respectively (Milutinović et al., 2008; Berri et al., 2009). Target genes, primers sequences and PCR conditions are summarized in Table 1.

PCR amplifications were performed using the EkoTagq PLUS 2x Master Mix (Lucigen Corporation, Middleton, Wisconsin, USA) and an automated thermal cycler (Gene-Amp PCR System 2700, Perkin Elmer, Norwalk, Connecticut, USA).
PCR products were analysed by electrophoresis on 1.5% agarose gel at 100 V for 45 min; gel was stained with ethidium bromide and observed. SharpMass™ 100 Plus Ladder (Euroclone, Milano, Italy) was used as DNA marker.

Samples resulted positive for *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. were sequenced on both strands by the commercial laboratory BMR-Genomics (Padua, Italy), using the inner primers. The sequence obtained was assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.233 and compared with those available in GenBank® using the BLASTn® program (http://www.ncbi.nlm.nih.gov/BLAST). Sequences obtained by overlapping both sense and antisense strands were exclusively used in the article.

3. Results

All animals of the study were in good nutrition conditions and no lesions and ectoparasites were observed during the carcasses’ manipulation. No microorganisms or parasite stages were observed in smears.

Among the 133 tested samples, 33 (24.81%) were PCR positive for at least one pathogen. More in details, 23 (17.29%) birds were positive for *Leucocytozoon* spp., 6 (4.51%) for *Plasmodium* spp., 4 (3%) for *C. burnetii*, 2 (1.5%) for *Haemoproteus* spp. No specific *F. tularensis* amplifications (0%) were detected (Table 2).

Sequence analysis for *Leucocytozoon* spp. revealed that the majority of the isolates fell in two broad groups.

One group (here called Leucocytozoon “lineage duck 1”) was composed of 9 isolates, showing 100% homology with parasites sequenced from previous studies (L. Kvleu MG593842; L. TS-2014a voucher MN-08-A-0235, KJ577823; L. MMSL02, KU295418; L. TUSW04 JQ314223).

The other group (Leucocytozoon “lineage duck 2”) was composed of 6 isolates, exhibiting 100% homology with different parasites sequenced (DUCK 18, KM386326; L. BWTE20, KU295418; L. TUSW04 JQ314223).

One isolate exhibited 100% identity with L. DUCK40 KM386348.

One isolate exhibited 98% homology with L.duck. D Duck32 KM386340 e Duck34 KM386342. The phylogenetic tree in Fig. 1 illustrates the leucocytozoon sequencing results.

![Fig. 1. Phylogenetic tree showing the Leucocytozoon sequencing results.](image-url)
Sequences analysis for *Plasmodium* found 100% homology with *Plasmodium circumflexum* for 3 samples and *Plasmodium polare* for one sample; one specimen scored 100% similar to *P. Sybor2* isolate. One sample showed 99% homology with the sequence P. Sybor2. Two amplicons were identified as *Haemoproteus* spp.

A mixed infection by *Leucocytozoon* and *P. circumflexum* was observed in one *A. penelope* whereas a mixed infection by *Leucocytozoon* and *C. burneti* was detected in one *A. crecca*.

More detailed data about hosts and agents are reported in Tables 3 and 4.

### 4. Discussion

The investigated subjects showed a 23.3% prevalence of infection by haemosporidia, with *Leucocytozoon* being the most abundant parasite detected via PCR (17.29%). In similar investigations this genus was the more frequently encountered in *Anseriformes*, both domestic (Ramey et al., 2012) and wild (Ramey et al., 2015; Reeves et al., 2015; Smith et al., 2016; Seimon et al., 2016). The L. “lineage duck 1” showed 100% identity with parasites detected from *Anser domesticus* (L. Kvlue MG593842, Turkey), *Anser indicus* (L. TS-2014a voucher MN-08-A-0235, KJ577823, Mongolia) A. platyrhynchos (L. MMSL02, KU295418, Alaska) and *Cypsiurus colombianus* (L. TUSW04 JQ314223, Alaska). The “lineage duck 2” exhibited 100% identity with isolates coming from *Anas* sp. (L. DUCK 18, KM386326, Pacific America) and *Anas discors* (L. BWT20, KU363710, North America). These findings suggest that the lineages observed in the present study are well adapted to Anatidae birds. Additionally, since the same lineages were observed also in other continents, the isolates described in Italy don’t seem to be confined only in Europe. Such findings needs further studies to strengthen this hypothesis.

*Plasmodium* was identified in 6 animals (4.51%), 2 *A. crecca* and 4 *A. penelope*. In these animals 2 species and different sequences were recognized. This haemosporidian genus, in contrast with *Leucocytozoon* and *Haemoproteus*, is not considered as a host specific parasite (Reeves et al., 2015; Smith et al., 2016). *P. circumflexum* has been recorded to have a low host specificity (Dimitrov et al., 2015), and in the present study was reported in a mixed infection with *Leucocytozoon*. *P. polare*...
was identified in one specimen from A. crecca. This haemosporidian species has been previously isolated from great tits (Parus major), from Switzerland (Roeyen et al., 2013). Haemosporinae was identified only in 2 samples (1.5%), from a G. gallinago and a A. plathyrrhynchos; however, isolated sequences did not allow us to recognize the species.

Data from Italy are scanty and consist of a study, carried out by microscopy, in which Leucocytozoon and Haemoproteus were not detected in Anatidae and Charadriidae from North Italy (Sacchi and Prigioni, 1986). At the best of our knowledge, this is the first report of haemosporidian infection in A. penelope, A. strepera, A. querquendula, T. tadorna and G. gallinago.

Similar studies have been conducted on waterfowl in the USA (Ramey et al., 2015; Reeves et al., 2015; Meixell et al., 2016) in a lower number of bird species and in injured birds from Japan (Inumaru et al., 2010), who reported a case of tularemia with typical Pasteurella tularensis infection. Data on haemosporidian infections from Italy are very limited. A recent investigation detected four animals (3 A. crecca and 1 A. penelope) positive (3%) for C. burnetii, whereas no F. tularensis-positive reactions were found. C. burnetii and F. tularensis may be transmitted by haematopagous arthropods, mainly ticks, but other transmission routes are possible. C. burnetii-positive birds could have contracted the pathogen by ticks’ bite, but it is easier to consider the oral route. In fact, waterfowl may travel large distances per day and reach areas where infected animals, wild and domestic, are present. Birds infected by C. burnetii play a relevant epidemiological role, because they can disperse the bacteria in the environment through their feces and they may be source of coxiellae for ticks during the blood meal.

On the basis of previous epidemiological studies in mammals and data relative to human infections, F. tularensis seems to not be largely prevalent in Italy (Pascucci et al., 2015; Ebani et al., 2016, 2017; Graziani et al., 2016; Rocchigiani et al., 2018), thus the negative results of the tested waterfowl could reflect the true epidemiological status. F. tularensis has been proven to be able to infect different avian species, thus birds could be cause of infection for humans. Hunters are more at risk of infection having direct or indirect contact with wild birds, as suggested by Padeskhi et al. (2010), who reported a case of tularemia with typical ulceroglandular form in a hunter who acquired the infection through a nail scratch from a buzzard (Buteo buteo).

Data about the transmission of C. burnetii and F. tularensis among avifauna are scant in scientific literature. Two studies have been carried out on ticks collected from birds in Italy: Toma et al. (2014) detected C. burnetii DNA in 42/127 analysed ticks collected from migratory birds of different species, but not waterfowl; Pajoro et al. (2018) did not detect C. burnetii and F. tularensis in any ticks collected from 124 wild birds. A recent investigation has been carried out on ticks collected from migratory birds of other species collected from 673 migratory birds along the Mediterranean birds. A recent investigation has been carried out on fecal samples – collected from 124 wild birds. A recent investigation has been carried out on ticks collected from migratory birds of different species, but not waterfowl; Pajoro et al. (2018) did not detect C. burnetii and F. tularensis in any ticks collected from 124 wild birds. A recent investigation has been carried out on ticks collected from migratory birds of other species collected from 673 migratory birds along the Mediterranean

5. Conclusions

The present study firstly reports data about haemosporidia and C. burnetii infections in waterfowl from Italy. In particular, these findings would add more data about haemosporidia species and strains circulating in avian population. Moreover, it has been confirmed the involvement of wild birds in the epidemiology of C. burnetii, suggesting that these animals may be direct and indirect source of infection for people. In fact, humans could be at risk when manipulating infected live birds or carcasses, as well as they could contract the infection because of the environment contamination with coxiellae excreted through birds’ droppings.

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Conflicts of interest

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work.

References

Babuideri, B., Moscosi, L., 1952. Experimental and natural infection of birds by Coxiella burnetii. Lancet 169, 195–196.

Beadell, J.S., Goyan, R., Gebhard, C., Hoitling, F., Melo, M., Schmidt, B.K., Perkins, S.L., Graves, G.R., Fleischer, R.C., 2009. Host associations and evolutionary relationships of avian blood parasites from West Africa. Int. J. Parasitol. 39, 257-266.

Bell, F., 1980. Tularemia. In: Steele, J.H. (Ed.), CRC Handbook Series in Zoonoses. Section A: Bacterial, Rickettsial, and Mycotic Diseases. CRC Press, Boca Raton, Florida, pp. 161–163.

Bennett, G.F., Peirce, M.A., Ashford, R.W., 1993. Avian hematozoa—mortality and pathogenicity. J. Nat. Hist. 27, 993–1001.

Bennett, V.Y., Sjürgiman, M., Hasselquist, D., Östman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. Proc. R. Soc. Lond. B 267, 1583–1589.

Berri, M., Rekiki, A., Boumedine, A., Rodolakis, A., 2009. Simultaneous differential detection of Chlamydia phila abortus, Chlamydia phila pecorum, and Coxiella burnetii from aborted ruminant’s clinical samples using multiplex PCR. BMC Microbiol. 9, 130. https://doi.org/10.1186/1471-2180-9-130.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. Proc. R. Soc. Lond. B 267, 1583–1589.

Berti, V.V., Rocchigiani, G., Nardoni, S., Bertelloni, F., Vasta, V., Papini, R.A., Verin, R., Poli, A., Mancianti, F., 2017. Molecular detection of tick-borne pathogens in wild red foxes (Vulpes vulpes) from Central Italy. Acta Trop. 172, 197–200.

Birkenheuer, F., de Souza Ramos, D.G., Pacheco, M.A., Ritter, A., de Souza Ribeiro, F., Escalante, A.A., Braga, E.M., 2018. A new pathogen spillover from domestic to wild animals: Plasmodium juxtanuculare infects free-living passerines in Brazil. Parasitology 145, 1949–1958.

Bonacci, R., Ferrari, F., et al., 2016. Zoonoses in Italy in the M. S., and coworkers (2016) detected local transmission of avian malaria and related haemosporidian parasites (Apicomplexa, Haemosporida) at a Special Protection Area of Natura 2000 network. Parasitol. Res. 117, 2187–2199.

Bennett, V.Y., Sjürgiman, M., Hasselquist, D., Östman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. Proc. R. Soc. Lond. B 267, 1583–1589.

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