

The presence of *Leptospira* in coypus (*Myocastor coypus*) and rats (*Rattus norvegicus*) living in a protected wetland in Tuscany (Italy)

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ABSTRACT

From September 2009 to February 2011, 122 coypus (*Myocastor coypus*) and 74 rats (*Rattus norvegicus*) were captured employing cage traps in a protected wetland in Tuscany (central Italy). Blood serum samples were collected from the animals and successively examined by the microagglutination test for several serovars of *Leptospira*: Bratislava, Ballum, Bataviae, Grippothyphosa, Icterohaemorrhagiae, Copenhageni, Mini, Pomona, Zanoni, Sejroe, Hardjo and Tarassovi. Kidney samples were collected from each animal and tested by bacteriological methods and submitted to Polymerase Chain Reaction. Thirty-four (27.87 %) coypu sera were positive to *Leptospira interrogans* serovar Bratislava, with antibody titers ranging from 1:100 to 1:400; no strain was isolated from coypu by bacteriological examination, while 12 (9.83 %) subjects were positive to PCR. All rats resulted seronegative; thirty-seven (50 %) *Leptospira* strains were isolated from rat kidneys; 30 were classified as *Leptospira interrogans* serovar Icterohaemorrhagiae and 7 as *Leptospira interrogans* serovar Ballum by the cross-agglutination test. Forty-five (60.81 %) rats resulted positive to PCR: 37 subjects positive to bacteriological examination and there were eight from which no strain were isolated from kidneys. These results would seem to suggest the minor zoonoepidemiological role of coypu in leptospirosis, which is widespread according to literature.

Key words: *Leptospira*, rat, coypu, microagglutination test, bacteriological cultures, PCR

Introduction

Leptospirosis, caused by pathogenic strains of the bacterium *Leptospira*, is maintained *in vivo* through chronic renal infection of carrier animals. Rodents and other small mammals are the most important reservoirs (LOFFLER et al., 2014), particularly

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the *Rattus* species, as the main source of *Leptospira* infection to humans and domestic animals (LEVETT et al., 2001). As described in previous Italian studies *Rattus norvegicus* is a reservoir for the serogroups Icterohaemorrhagiae and Ballum (AMADDEO et al., 1996; PEZZELLA et al., 2004).

The coypu (*Myocastor coypus*) is a large semi-aquatic rodent, and is the only member belonging to the family *Myocastoridae*. This animal is a native species of South America, particularly of subtropical areas of Brazil and Argentina. In the first half of the past century the coypu was introduced into North America, Asia, Africa and Europe (i.e. Italy) by fur ranchers. In our country, the coypu population increased significantly after 1970 and it now represents a real pest because of its negative impact on habitats and its role in the introduction and spread of several infectious agents. Moreover it is responsible for changes in the composition of indigenous communities (BARRAT et al., 2010). In the last decade, this species has spread in several countries (MICHEL et al., 2001).

Coypu live generally near rivers, streams, lakes, ponds and brackish marsh in coastal areas where they represent a potential carrier of zoonotic agents, particularly of leptospires (MONTAGNA, 2004).

Coypu often lives in the same habitats of brown rats (*Rattus norvegicus*), which is an excellent maintenance host for leptospira, particularly those belonging to the serovars icterohaemorrhagiae and Ballum, as mentioned before. In the past, only a few studies published positive data about the presence of leptospira in populations of coypus. *Leptospira*, classified as *L. interrogans* serovar Bonariensis, were first isolated from a coypu in 1949 in Argentina (ANCHEZAR et al., 1949). The serovar Wolffi was isolated from a coypu in Great Britain in 1984, and then other strains belonging to the serogroup Icterohaemorrhagiae were detected in Italy (WAITKINS et al., 1985; WANYANGU et al., 1986). In research performed in France from 1996 to 1999, three field strains were isolated from coypus, two *L. interrogans* serovar Icterohaemorrhagiae and one *L. interrogans* serovar Sejroe (MICHEL et al., 2001). In Italy strains of the Icterohaemorrhagiae serogroup have been isolated and seropositive animals detected in different regions (FARINA and ANDREANI, 1964; FARINA and ANDREANI, 1970; ARCANGELI et al., 1997; BOLLO et al., 2003).

The main aim of our research was to study the role of coypus as carrier of leptospirosis. The presence of *Leptospira* was investigated in coypus living in a natural reserve in central Italy, using serological, bacteriological and molecular methods.

Specimens of brown rats living in the same area were also analysed, because these animals are a well-known reservoir of leptospira in this habitat.

Materials and methods

Study area and sample collection. From September 2009 to February 2011, 122 coypus (*Myocastor coypus*) and 74 brown rats (*Rattus norvegicus*) were caught in a protected wetland area of central Italy, in Tuscany, located within the Fucecchio Marshes (longitude 10°48'10" east; latitude 43°44'0" north). The captures were carried out employing cage traps with corncobs and stale bread as bait.

The animals were sedated with 100 mg/mL of ketamine hydrochloride and 20 mg/mL of xylazine hydrochloride, according to the protocol described by BO et al., 1994.

Blood samples were collected by intracardiac puncture, using sterile needles and syringes; the samples were centrifuged at 1000 g for 15 min and sera were collected and stored at -20 °C. Later the animals were placed in hermetic boxes and euthanized by intracardiac puncture of Tanax® (embutramide, mebenzonium iodide, tetracayne hydrochloride solution) according to Italian legislation, and immediately submitted to necroscopic examinations. At the time of the autopsy, one kidney from each animal was removed immediately.

Bacterological analysis. Specimens from kidneys were aseptically collected and homogenised in sterile stomacher bags containing 10 mL of sterile physiological solution. Then 0.5 mL of the resultant suspension was transferred into tubes containing 6 mL of EMJH (Ellinghausen, McCulloch, Johnson and Harris) medium (Difco, Detroit, Michigan, USA) added with 5 fluorouracyl and incubated at 30 °C. The cultures were observed weekly using a dark field microscope for 4 months, before considering a specimen negative. The isolates were subcultured in three tubes containing EMJH medium to be employed in the subsequent analysis.

Molecular analyses. Genomic DNA was extracted from the kidney samples using a commercially available DNA extraction kit, DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). Then for each sample the DNA extracted was analyzed by PCR assay to amplify a 115 bp fragment of the 23S rRNA gene of *Leptospira interrogans* and a 523 bp fragment of the same gene of *Leptospira biflexa*, employing the Ri (5' CAGCGAATTAGATCTG 3'), Rb (5' TTCGCCTTCGAGATTC 3'), and F1 (5' GAACTGAAACATCTAAGTA 3') primers (WOO et al., 1997).

The PCR amplification was performed in 25 µL of reaction mixture, containing 2.5 µL of 10X Qiagen PCR buffer, 5 µL of Q Solution, 200 µM of deoxynucleoside triphosphates (dNTP), 20 pmol of Ri primer, 20 pmol of Rb primer, 40 pmol of F1 primer, 1.25 U of Taq polymerase, 2 µL of extracted DNA and 9.88 µL of sterile distilled water. The reaction was performed in an automated thermal cycler (Gene-Amp PCR System 2700, Perkin-Elmer, Norwalk, Connecticut, USA) according to the following conditions:

94 °C for 7 minutes (denaturation); 30 cycles of: 1 minute at 94 °C, 2 minutes at 44 °C and 4 minutes at 72 °C, 20 minutes at 72 °C (extension).

Amplified products were separated using 1 % agarose gel, stained with ethidium bromide and viewed using a UV light source.

Typing with antiserovar sera. The isolates belonging to the species *Leptospira* were analysed by the Microscopic Agglutination Test of Martin and Pettit (MAT) to determine their serovar. Hyperimmune reference rabbit sera against the followed serovars were employed: Icterohaemorrhagiae (Bianchi I strain), Copenhageni (Wijnberg strain), Canicola (Alarik strain) Grippotyphosa (Moskva V strain), Bratislava (Riccio 2 strain), Pomona (Mezzano strain), Hardjo (Hardjoprajitno strain), Tarassovi (Mitis Johnson strain) and Ballum (Mus 127 strain). The hyperimmune serum giving agglutination (at least 50 % of the leptospira) with the highest dilution defined the serovar of the isolate.

Serological analysis: MAT test. One hundred and ninety-six blood serum samples collected from 122 coypus and 74 rats were examined by MAT, employing the 15 antigens of *Leptospira* reported in Table 1.

Table 1. Genospecies, serovars and related strains employed in MAT test

| Genospecies | Serovar | Strain |
|----------------------------------|---------------------|----------------|
| <i>Leptospira interrogans</i> | Bratislava | Riccio 2 |
| <i>Leptospira borgpetersenii</i> | Ballum | Mus 127 |
| <i>Leptospira interrogans</i> | Bataviae | Pavia 1 |
| <i>Leptospira kirschneri</i> | Grippotyphosa | Moskva V |
| <i>Leptospira interrogans</i> | Icterohaemorrhagiae | Bianchi 1 |
| <i>Leptospira interrogans</i> | Copenhageni | Wijnberg |
| <i>Leptospira borgpetersenii</i> | Mini | Sari |
| <i>Leptospira interrogans</i> | Pomona | Mezzano |
| <i>Leptospira interrogans</i> | Zanoni | Zanoni |
| <i>Leptospira borgpetersenii</i> | Sejroe | Topo 1 |
| <i>Leptospira interrogans</i> | Hardjo | Hardjoprajitno |
| <i>Leptospira borgpetersenii</i> | Tarassovi | Mitis Johnson |

Sera with antibody titres $\geq 1:100$ were considered positive

Results

From the bacteriological cultures of the kidneys collected from the rats 37/74 (50 %) *Leptospira* strains were isolated, whereas no strain was obtained from the kidneys collected from the coypus.

PCR revealed that all the isolates belonged to *Leptospira*; and of these the serotyping revealed that 30/37 (81.08 %) strains belong to the serovar Icterohaemorrhagiae and 7/37 (18.91 %) to the serovar Ballum.

The PCR on DNA extracted from the rat kidneys detected 45/74 (60.81 %) *Leptospira* positive samples. Among these samples, 37/45 (82.22 %) specimens were also positive for bacteriological examination while 8/45 (17.77 %) were negative.

Kidney samples of 12/122 (9.83 %) coypus were *Leptospira* positive on PCR analysis. Among them, 8/12 (66.66 %) animals were seronegative and 4/12 (33.33 %) seropositive to MAT for the serovar Bratislava.

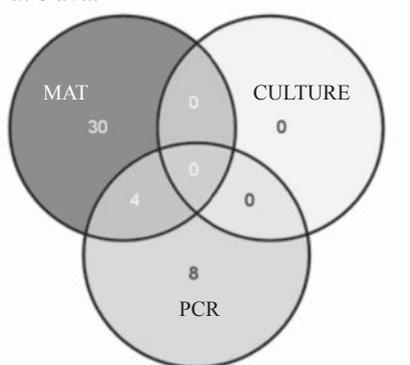


Fig. 1. Identification of positive samples by three detection methods in coypus samples (n = 122); MAT positive samples (n = 34); PCR positive samples (n = 12); Culture positive samples (n = 0)

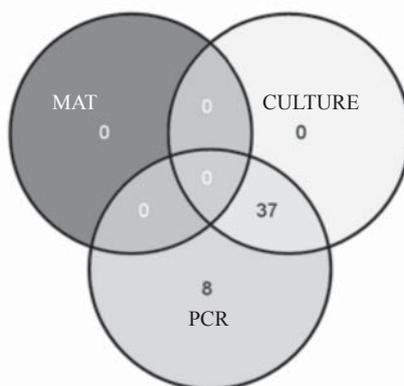


Fig. 2. Identification of positive samples by three detection methods in brown rat samples (n = 74); MAT positive samples (n = 0); PCR positive samples (n = 45); Culture positive samples (n = 37)

The serological analyses of the 122 coypus sera showed 34/122 (27.87 %) positive reactions with the same antibody titres ranging from 1:100 to 1:400 to the serovar Bratislava tested: in detail: 4/34 (11.76 %) samples were positive with the antibody titre 1:400, only 2/34 (5.88 %) sera were positive with the antibody titre 1:200; the remaining sera were positive with the antibody titre 1:100. All brown rat sera were negative (Fig. 1 and 2).

Discussion

Our research has provided information regarding the prevalence of *Leptospira* in the geographical area investigated, studying not only the *Myocastor coypus* species but also *Rattus norvegicus*, which shares the same habitat.

The high number of strains isolated from brown rats (50 %) confirmed the role of this rodent as a reservoir of *Leptospira*, particularly of the serovars Icterohaemorrhagiae and Ballum, as previously described in literature (AMADDEO et al., 1996; PEZZELLA et al., 2004).

PCR assays, carried out on DNA samples extracted from kidney tissues of brown rats positive to bacteriological analysis, were all positive. Moreover, 8 rat kidney samples, negative to bacteriological tests, were instead positive to PCR. This could be due to the fact that PCR is more sensitive than culture examination.

On the other hand the negative serological response observed in all rats analysed is not completely clear; a likely hypothesis could be that chronically infected animals, such as maintenance hosts, often do not have specific circulating antibodies or have titres too low to be detected.

Regarding the coypu samples, all the kidney samples were negative to bacteriological examination. Twelve of them were positive to PCR, which, as previously mentioned, could be due to the higher sensitivity of PCR compared to culture.

The high percentage of coypu positive serological reactions to the serovar Bratislava (27.86 %) could be caused by the increasing diffusion of leptospira belonging to the Australis serogroup among several domestic and wild animal species (CERRI et al., 2003) However Bratislava strains were not isolated either from the coypu kidneys or from brown rat kidneys, from the same area. Another hypothesis could be that the serological positive results to this serovar are due to non-specific reactions or cross-reactions with the serovar Icterohaemorrhagiae, considering the low antibody titres observed and the fact that no leptospira were detected in the coypus kidneys. PCR performed on kidney tissue samples collected from seropositive and seronegative coypus showed 4 and 8 positive results, respectively. These data confirmed the presence of *Leptospira* in the kidney tissues of coypus where the concentration of leptospira was probably too low to be detected through cultural analysis.

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The present research seems to confirm the minor zoonoepidemiological role of coypus in the study area; these data are in agreement with two previous studies carried out in Italy (FARINA and ANDREANI, 1964; FARINA and ANDREANI, 1970).

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SAŽETAK

Od rujna 2009. do veljače 2011. u zamke su bile uhvaćene 122 nutrije (*Myocastor coypus*) i 74 štakora selca (*Rattus norvegicus*) u zaštićenoj močvari u Toskani u središnjoj Italiji. Prikupljeni uzorci krvnog seruma bili su pretraženi mikroaglutinacijskim testom na nekoliko serovarova leptospira: Bratislava, Ballum, Bataviae, Grippothyphosa, Icterohaemorrhagiae, Copenhageni, Mini, Pomona, Zanoni, Sejroe, Hardjo i Tarassovi. Uzorci tkiva bubrega, uzeti od svake životinje, bili su pretraženi bakteriološki i lančanom reakcijom polimerazom. Tridesetčetiri (27,87 %) uzorka seruma nutrije bila su pozitivna na vrstu *Leptospira interrogans* serovar Bratislava s titrom protutijela od 1:100 do 1:400. Bakteriološkom pretragom iz nutrija nije bio izdvojen nijedan izolat, dok je 12 uzoraka (9,83 %) bilo pozitivno PCR-om. Svi štakori bili su serološki negativni, ali je 37 (50 %) izolata leptospira bilo izdvojeno iz tkiva njihovih bubrega. Od toga je 30 izolata pripadalo vrsti *Leptospira interrogans* serovaru Icterohaemorrhagiae, a 7 serovaru Ballum pretragom križnim aglutinacijskim testom. Ukupno su 45 štakora (60,81 %) bila pozitivna pretragom PCR-om, a 37 bakteriološkom pretragom. Od osam štakora nije bio izdvojen nijedan izolat leptospira iz bubrega. Ti rezultati upućuju na neznatnu zoepidemiološku ulogu nutrija u širenju leptospiroze.

Ključne riječi: *Leptospira*, štakor, nutrija, mikroaglutinacijski test, bakteriološka pretraga, lančana reakcija polimerazom
