



Epidemiology of leptospirosis in North-Central Italy: Fifteen years of serological data (2002–2016)

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ABSTRACT

Leptospirosis is a re-emerging bacterial zoonosis. North-Central Italy is characterized by a geographic area that promote *Leptospira* circulation. Data on sero-epidemiological survey carried out from 2002 to 2016 in North-Central Italy were reported and discussed. Overall, 709 out of the 8488 (8.35%) tested sera were positive for *Leptospira* at the cut-off titer (1:100) and 218 (2.57%) at higher titer ($\geq 1:400$). The highest percentages of positivity was recorded for coypus (22.86%), swine (19.74%) and bovine (13.03%). Pomona and Australis resulted the serogroup more often detected, followed by Sejroe and Icterohaemorrhagiae; while, a low number of positive sera was detected for serogroups Ballum, Canicola and Tarassovi. Percentage of positive sera for each year slightly decreased from 2002 to 2008 and rose from 2009. High percentages of positive reactions were recorded in 2014 (17.23%), 2015 (19.61%) and 2016 (38.05%). In conclusion, the results of this investigation reported an increase of leptospirosis in North-Central Italy. Furthermore, several animals resulted infected as accidental hosts by unusual *Leptospira* serovars. These data could suggest a change in host range for some serovars, that may promote the adaptation to new hosts.

1. Introduction

Leptospirosis represents one of the most important widespread re-emerging bacterial zoonosis [1–3]. Several outbreaks accounting for thousands of deaths worldwide highlight the importance of leptospirosis as a severely neglected infectious disease [4,5]. Leptospirosis has a wide distribution and occurs overall in tropical, subtropical and temperate zones, favoured by a large variety of both wild and domestic mammals which can play the role of natural reservoirs of pathogenic *Leptospira* spp. [1,6]. Some animals are asymptomatic renal carriers of this bacterium and they contribute to maintain the infection in a particular environment by constantly shedding *Leptospira* with urine [7,8]. Accidental contact with urine of colonized or infected animals by *Leptospira* serovars causes the incidental infection and produces clinical diseases. While, specific *Leptospira* serovars which show close relationship with particular animal species develop host-maintained infection. The maintenance host generally does not develop symptoms, except after long time, but it acts as a natural source of a specific serovar [6,8]. In fact, *Leptospira* epidemiology is strictly related to the presence and widespread of the maintenance hosts species [9]. In recent years, some serovars seem to be prevalent and emerging, especially

among wild animals, but also in domestic species. This occurrence suggests that the epidemiology of leptospirosis may change over time in animals as well as in humans [10].

Central Italy, and in particular Tuscany, is a geographic area characterized by some peculiarities which promote the presence and persistence of *Leptospira* in hosts and in environment: a) presence of wild animals which could represent potential reservoirs; b) presence of domestic animals raised in semi-extensive or extensive farms, which promotes contact with wild species; c) a significant presence of hunting activity; d) abundance of wetlands such as marshes, ponds and irrigation canals.

The main purpose of the present work was to refer data on a sero-epidemiological survey carried out in Central Italy, particularly in Tuscany, on serum samples collected from 2002 to 2016 in order to assess the prevalence of leptospirosis in domestic and wild animals and to compare it with the data from a previous epidemiological surveillance investigation carried out in the same area between 1995 and 2001 [9].

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2. Material and methods

From January 2002 to December 2016, 8488 serum samples were analysed for anti-*Leptospira* antibodies. Sera were collected from healthy and ill animals belonging to 23 different species: sheep (*Ovis aries*, 2682 sera), swine (*Sus scrofa*, 1332 sera), bovine (*Bos Taurus*, 1328 sera), dog (*Canis lupus familiaris*, 1144 sera), wild boar (*Sus scrofa*, 479 sera), goat (*Capra hircus*, 327 sera), european brown hare (*Lepus europaeus*, 162 sera), red fox (*Vulpes vulpes*, 94 sera), horse (*Equus caballus*, 74 sera), roe deer (*Capreolus capreolus*, 74 sera), coypus (*Myocastor coypus*, 70 sera), fallow deer (*Dama dama*, 65 sera), donkey (*Equus asinus*, 2 sera), ferret (*Mustela putorius furo*, 1 serum), cat (*Felis catus*, 4 sera), red deer (*Cervus elaphus*, 56 sera), wolf (*Canis lupus*, 43 sera), rats (*Rattus norvegicus*, 34 sera), mouflon (*Ovis musimon*, 8 sera), mouse (*Mus musculus*, 8 sera), Guinea pig (*Cavia porcellus*, 2 sera), alpaca (*Vicugna pacos*, 1 serum) and bear (*Ursus arctos*, 1 serum). Furthermore, 329 sera were of human origin and collected from subjects with clinical symptoms referable to leptospirosis; sera samples were sent from hospitals and only one sample from each acute patient was analysed. All sera were collected in North-Central Italy. Samples from domestic animal (dog, cat, donkey, ferret, horse, Guinea pig, alpaca) were collected by different practitioner veterinarians during routinely clinical practice and sent to the Laboratory of Infectious Disease of Department of Veterinary Medicine of Pisa University. Samples from breeding animals (sheep, swine, goat, bovine) were collected at farm from live animals or at slaughterhouse during jugulation. Samples from wild animals were collected from thoracic cavity of hunted animals (wild boar, European brown hare, roe deer, fallow deer, red deer) or hatted in the roadside and found dead animals (red fox, wolf, mouflon, bear). Rodents (coypus, rats, mouse) were caught with Tomahawk traps, sedated and blood samples collected as previously described [11].

Serological investigation was carried out with Microscopic Agglutination Test (MAT) [12]. The following serovars were used as live antigens: Icterohaemorrhagiae (strain Bianchi, serogroup Icterohaemorrhagiae), Canicola (strain Alarik, serogroup Canicola), Pomona (strain Mezzano, serogroup Pomona), Tarassovi (strain Mitis Johnson, serogroup Tarassovi), Grippotyphosa (strain Moscow V, serogroup Grippotyphosa), Bratislava (strain Riccio 2, serogroup Australis), Castellonis (strain Castellon 3, serogroup Ballum) and Hardjo (strain Hardjoprajitno, serogroup Sejroe). The employed antigens were references strains obtained by the National Centre for Leptospirosis, Istituto Superiore di Sanità, Rome, Italy. *Leptospira* culture were maintained in *Leptospira* Medium Base Ellinghausen-MacCullough-Johnson-Harris (EMJH – Difco, Becton, Dickinson and Company, Sparks, MD, USA), sub-cultured every 7–10 day and checked for purity, mobility and agglutination power until the use. Titers of 1:100 were considered positive; 2-fold serial dilutions were tested to determine the endpoint titer.

3. Results

Overall, 709 out of the 8488 (8.35%) sera resulted positive for *Leptospira* at the breakpoint titer (1:100). All samples collected from the following species resulted negative: alpaca, donkey, guinea pig, mouse, rat, ferret, cat, fallow deer, red deer, roe deer, mouflon, bear and wolf. Two hundred and eighteen sera (2.57%) resulted positive at high titer ($\geq 1:400$). Table 1 reports the number of positive sera grouped by animal species. The highest percentages of positive sera were recorded for coypus, swine and bovine at low (22.86%, 19.74% and 13.03%, respectively) and high titer (5.61%, 6.31% and 3.16%, respectively). None horse sera showed positivity to titer of 1:400 or higher. Coypus, wild boar, fox and hare resulted the unique wild species that showed positive reactions. In particular, 22.86%, 8.56%, 3.04% and 1.85% of sera resulted positive at titer $\geq 1:100$ for coypus, wild boar, fox and hare, respectively. Among the 329 human sera examined, 10 (4.26%) resulted positive and 3 (1.06%) showed a titer of 1:400 or higher.

Table 1

Number of positive sera to *Leptospira* at low ($\geq 1:100$) and high titers ($\geq 1:400$).

Species	Examined	Titer			
		$\geq 1:100$	%	$\geq 1:400$	%
Bovine	1328	173	13.03	42	3.16
Dog	1144	99	8.65	27	2.36
Goat	327	9	2.75	6	1.83
Horse	242	7	2.89	0	0.00
Wild boar	479	41	8.56	13	2.71
Hare	162	3	1.85	0	0.00
Coypus	70	16	22.86	4	5.71
Sheep	2682	84	3.13	38	1.42
Swine	1332	263	19.74	84	6.31
Fox	94	4	3.04	1	0.91
Human	329	10	4.26	3	1.06
Total	8488	709	8.35	218	2.57

Table 2

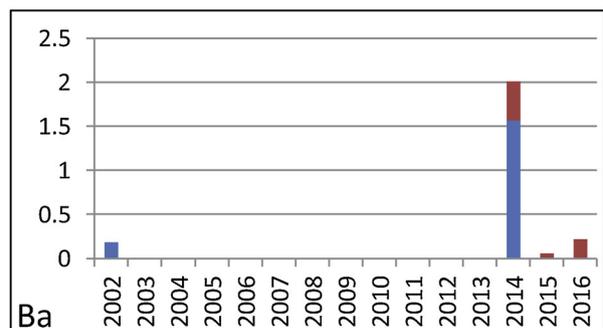
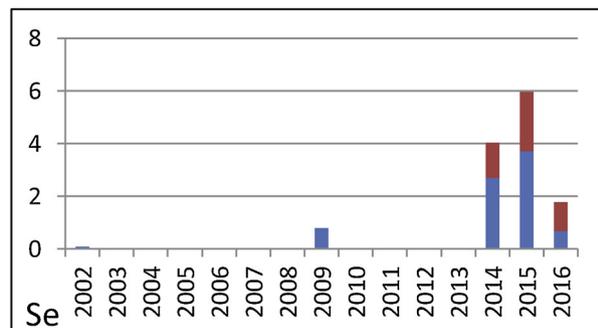
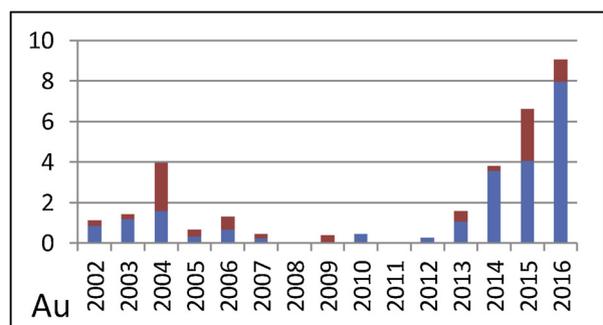
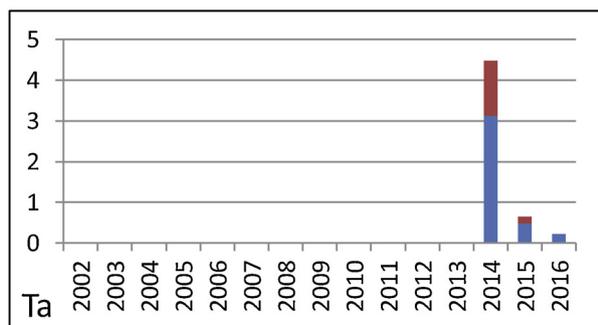
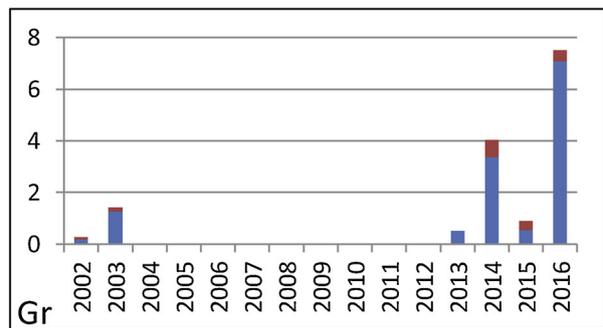
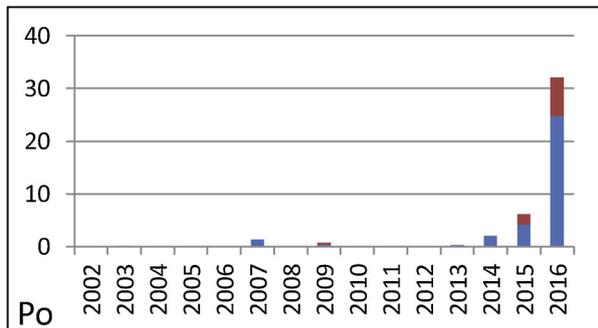
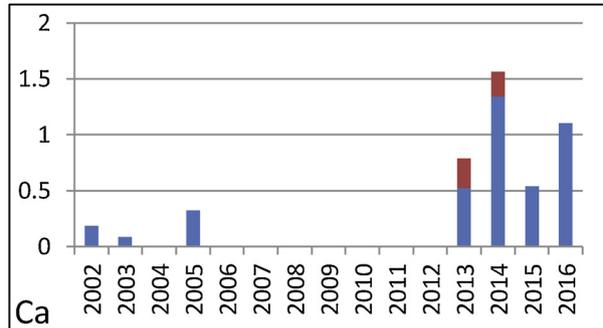
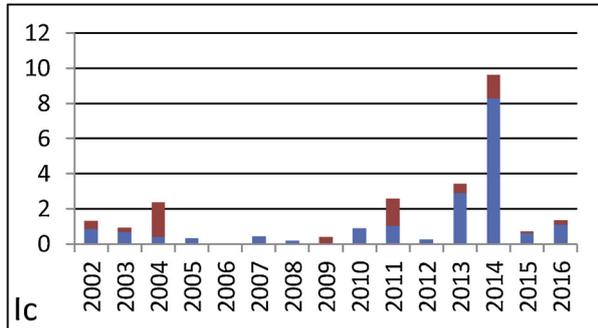
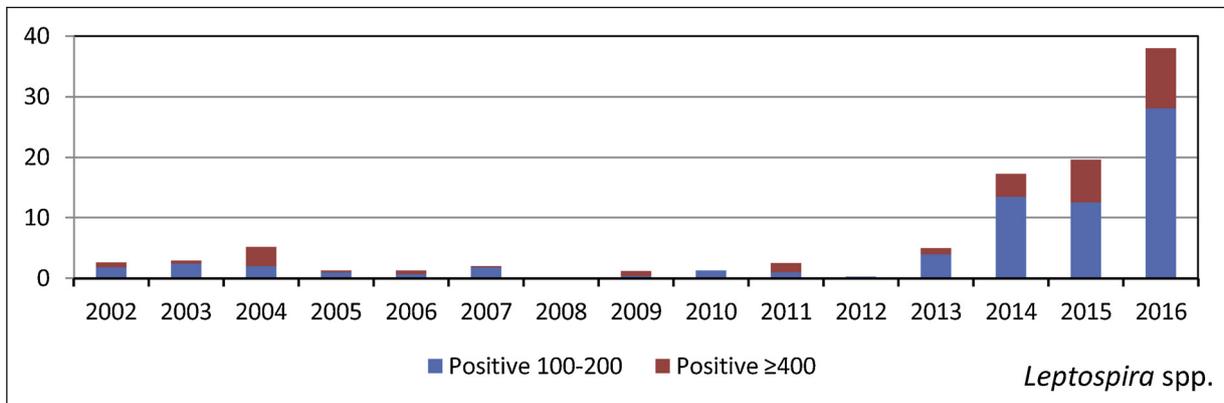
Numbers of positive serological reactions detected for anti-*Leptospira* antibodies at low ($\geq 1:100$) and high titers ($\geq 1:400$) in relation to animal species and serogroup.

Animal species	Titer	<i>Leptospira</i> spp.								Total	Positive to more serovars
		Ic	Ca	Po	Gri	Tar	Au	Se	Ba		
Bovine	≥ 100	21	4	55	48	3	3	94	4	232	44*
	≥ 400	4	1	3	5	1	1	30	2	47	3*
Dog	≥ 100	58	14	16	4	1	48	0	0	141	33*
	≥ 400	16	1	2	0	1	19	0	0	39	11*
Goat	≥ 100	9	0	0	0	0	0	0	0	9	0
	≥ 400	6	0	0	0	0	0	0	0	6	0
Horse	≥ 100	3	0	1	0	0	2	1	0	7	0
	≥ 400	0	0	0	0	0	0	0	0	0	0
Wild boar	≥ 100	3	1	4	1	18	12	14	1	54	11*
	≥ 400	1	0	1	0	3	1	7	1	14	1
Hare	≥ 100	0	0	0	0	0	1	0	2	3	0
	≥ 400	0	0	0	0	0	0	0	0	0	0
Coypus	≥ 100	1	0	0	14	0	8	0	0	23	7*
	≥ 400	0	0	0	2	0	3	0	0	5	3
Sheep	≥ 100	20	1	30	20	8	14	19	5	117	21*
	≥ 400	2	0	17	6	3	1	12	1	42	3*
Swine	≥ 100	2	6	160	0	2	131	1	0	302	40*
	≥ 400	0	0	45	0	1	41	0	0	87	3
Fox	≥ 100	1	0	0	0	0	3	0	0	4	0
	≥ 400	0	0	0	0	0	1	0	0	1	0
Human	≥ 100	7	2	1	2	0	2	0	1	15	2*
	≥ 400	2	0	0	1	0	0	0	0	3	0
Total	≥ 100	125	28	267	89	32	224	129	13	907	158
	≥ 400	31	2	68	14	9	67	49	4	244	24*

Note: Ic: serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae; Ca: serogroup Canicola, serovar Canicola; Po: serogroup Pomona, serovar Pomona; Tar: serogroup Tarassovi, serovar Tarassovi; Gri: serogroup Grippotyphosa, serovar Grippotyphosa; Au: serogroup Australis, serovar Bratislava; Ba: serogroup Ballum, serovar Castellonis; Se: serogroup Sejroe, serovar Hardjo.

* Some sera resulted positive to more than 2 different serovars.

Table 2 shows the distribution of positive sera grouped by serogroups and animal species. Pomona and Australis resulted the serogroups more often detected, with 267 and 224 positive reactions (titer 1:100), respectively. Moreover, for the same serogroups, 68 and 67 sera, respectively showed positivity to high titer ($\geq 1:400$). A low number of positive sera was detected for serogroups Ballum, Canicola and Tarassovi (13, 28 and 32 sera, respectively). Hare was the only species in which positivity to serogroups Icterohaemorrhagiae was never found. Similarly, goat resulted the only species in which serogroup Australis was never detected. Positivity to serogroups Icterohaemorrhagiae and Canicola was found mainly in dog sera.



■ Positive 100-200 ■ Positive ≥400

(caption on next page)

Fig. 1. Seroprevalence of *Leptospira* spp. in North and Central Italy from 2002 to 2016: Percentage of positive sera (y-axis) detected for each *Leptospira* serogroups tested for each year of investigation (x-axis).

Note: Ic: serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae; Ca: serogroup Canicola, serovar Canicola; Po: serogroup Pomona, serovar Pomona; Tar: serogroup Tarassovi, serovar Tarassovi; Gri: serogroup Grippityphosa, serovar Grippityphosa; Au: serogroup Australis, serovar Bratislava; Ba: serogroup Ballum, serovar Castellonis; Se: serogroup Sejroe, serovar Hardjo.

Serogroups Pomona and Australis resulted more often associated with swine. Most of the sera positive to Tarassovi were from wild boar. Positivity to serogroups Grippityphosa and Sejroe was detected especially in bovine sera.

Among human sera, positivity to six serogroups was recorded: Icterohaemorrhagiae, Canicola, Pomona, Grippityphosa, Australis and Ballum. Icterohaemorrhagiae was the most detected serogroup. Positive samples were never found among human sera from 2004 to 2012. In 2013, 2014 and 2016 positive sera were 1/20, 4/9 and 1/11, respectively; all these sera showed an antibody titer < 1:400.

Percentage of positive sera for each year slightly decreased from 2002 to 2008; in particular in 2008 only 1 out of 597 (0.17%) sera resulted positive at titer of 1:100. From 2009, the percentage of positive sera increased; particularly, in 2014, 2015 and 2016 a high percentage of positive sera was recorded: 17.23%, 19.61% and 38.05%, respectively. Fig. 1 reports the percentage of positive sera to *Leptospira interrogans* and to each serogroup detected in each year of investigation.

Table S1 (supplementary material) reports the distribution of positive sera at titer \geq 1:100 and \geq 1:400 in relation to animal species, *Leptospira* serogroups and year of detection.

As regard the 244 sera that exhibited a titer \geq 1:400, 145/244 (59.43%) had a titer of 1:400, 57/244 (23.36%) had a titer of 1:800, 28/244 (11.47%) had a titer of 1:1600, 9/244 (3.69%) had a titer of 1:3200, 2/244 (0.82%) had a titer of 1:6400, 1/244 (0.41%) had a titer of 1:25600 and 2/244 (0.82%) had a titer of 1:102400.

3.1. Bovine

The total apparent prevalence detected in bovine sera was 13.03%. In cattle 7.08% (94/132) and 2.26% (30/1328) sera scored positive for serogroup Sejroe at titer \geq 1:100 and \geq 1:400, respectively. A percentage of 4.15% (55/1328) and 0.23% (3/1328) were positive for Pomona at titer \geq 1:100 and \geq 1:400, respectively. As concern serogroup Grippityphosa, 3.61% (48/1328) and 0.38% (5/1328) sera scored positive at titer \geq 1:100 and \geq 1:400, respectively. Low level of positivity was scored for serogroup Icterohaemorrhagiae, Canicola, Tarassovi, Australis and Ballum.

3.2. Dog

The total apparent prevalence in dog was 8.65% (2002–2016). Percentage of 5.07% (58/1144) and 4.19% (48/1144) of sera resulted positive to serogroup Icterohaemorrhagiae and Australis at titer \geq 1:100, respectively. Percentages of 1.40% (16/1144) and (0.17% 2/1144) of sera scored positive for serogroup Pomona at titer \geq 1:100 and \geq 1:400, respectively. As regards Canicola, 1.22% (14/1144) and 0.09% (1/1144) sera resulted positive at titer \geq 1:100 and \geq 1:400, respectively. Low number of positive sera was observed for Grippityphosa and Tarassovi with 0.35% (4/1144) and 0.09% (1/1144) of positive sera at titer \geq 1:100, respectively. No positivity was encountered for serogroup Sejroe and Ballum.

3.3. Swine

The total apparent prevalence detected in swine was 19.74% (2002–2016). For Pomona, 12.01% (160/1332) and 3.38% (45/1332) of swine sera have been detected positive at titer \geq 1:100 and \geq 1:400, respectively. While for Australis, 9.83% (131/1332) and 2.33% (311/1332) of sera scored positive at titer \geq 1:100 and \geq 1:400,

respectively. On the other hand, only 0.15% (2/1332) and 0.08% (1/1332) of sera were detected positive for Tarassovi at titer \geq 1:100 and \geq 1:400, respectively. As regard serogroup Canicola, 0.45% (6/1332) of sera scored positive at titer 1:100/1:200 (3 at titer of 1:100 and 3 at titer of 1:200). No positive sera were detected for serogroups Grippityphosa and Ballum, and only 0.15% (2/1332) and 0.08% (1/1332) of sera resulted positive for Icterohaemorrhagiae and Sejroe, respectively.

3.4. Horse

The total apparent prevalence obtained in horse was 2.89%. Positive serological reactions were detected only for Icterohaemorrhagiae (1.24%–3/242 sera), Australis (0.83%–2/242 sera), Pomona (0.41%–1/242 sera) and Sejroe (0.41%–1/242 sera).

3.5. Sheep and goat

The total apparent prevalence observed in sheep was 3.13%. A percentage of 0.71% (19/2682) of sera scored positive to serogroup Sejroe at titer \geq 1:100 and 0.44% (12/2682) were positive at titer \geq 1:400. Relative high number of positivity was reported for serogroups Pomona (1.12% 30/2682), Icterohaemorrhagiae (0.75%–20/2682) and Grippityphosa (0.75%–20/2682) at titer \geq 1:100. Furthermore, positive reactions were found in sheep sera for Australis (0.52%), Tarassovi (0.30%), Ballum (0.19%) and Canicola (0.04%) at titer \geq 1:100.

For goat sera, only 2.75% (9/327) of samples resulted positive for Icterohaemorrhagiae at titer \geq 1:100, among them 6 were positives at titer \geq 1:400.

3.6. Wild boar

The total apparent prevalence detected in wild boar was 8.56%. For Tarassovi, 3.76% (18/479) and 0.63% (3/479) of sera scored positive at titer \geq 1:100 and titer \geq 1:400, respectively. Percentages of 2.51% (12/479) and 0.21% (1/479) of sera resulted positive to serogroup Australis at titer \geq 1:100 and titer \geq 1:400, respectively. Only 0.84% (4/479) and 0.21% (1/479) of sera resulted positive for serogroup Pomona at titer \geq 1:100 and titer \geq 1:400, respectively. As concerns serogroup Sejroe, 2.92% (14/479) and 1.46% (7/479) of sera scored positive at titer \geq 1:100 and titer \geq 1:400, respectively. Furthermore, positivity to other serogroups was detected at titer \geq 1:100: 0.62% (3/479) of sera for serogroups Icterohaemorrhagiae, 0.21% (1/479) for Canicola, 0.21% (1/479) for Grippityphosa and 0.21% (1/479) for serogroup Ballum.

3.7. Other wild animals

Excluding wild boar, seropositivity in wild animals was recorded only for hares, foxes and coypus. Only 1.85% (3/162) of hare's sera scored positive in this survey, 0.62% (1/162) for Australis (titer 1:200) and 1.23% (2/162) for Ballum (titer 1:100 and 1:200, respectively). Low number of fox sera examined in this investigation resulted positive for *Leptospira*, in particular for serogroups Icterohaemorrhagiae (1.06% - 1/94) and Australis (3.19% - 3/94). Only 1.06% (1/94) of sera resulted positive at titer \geq 1:400, for Australis. As regard coypus, a high percentage of sera, 32.86% (23/70), analyzed in this investigation scored positive to *Leptospira*. Positivity was recorded for Grippityphosa (20.00% - 14/70), Australis (11.43% - 8/70) and Icterohaemorrhagiae

(1.43% - 1/70). Furthermore, 4.29% (3/70) and 2.86% (2/70) of sera exhibited a titer \geq 1:400 for serogroup Australis and Grippotyphosa, respectively.

3.8. Human

The total apparent prevalence observed in man was 4.26%. Icterohaemorrhagiae was the most detected serogroup from human sera (2.12%–7/329). However, positivity to all tested serogroups, with exception of Tarassovi and Sejroe, was recorded.

4. Discussion

Leptospirosis is a worldwide public health and veterinary problem, frequently underestimated, characterized by a downward trend [13]. Climatic changes, modifications of ecological niches, emergence of new potential maintenance-hosts could represent the most important factors involved in *Leptospira* epidemiology. The environmental and geographic features of North-Central Italy area can be considered as the optimal conditions for *Leptospira* spreading [14–22]. Tuscany, the main region involved in this serological survey, is characterized by some peculiarities which promote the presence and persistence of *Leptospira* in hosts and in the environment. Increasing presence of wild animals, potential reservoirs, presence of animals raised in semi-extensive or extensive farms, hunting activity and presence of wetlands such as marshes, ponds, lakes and irrigation canals are the main factors involved in enzootic trend of leptospirosis in Italy.

In this study, serological results obtained whit a panel of eight *Leptospira* serovars were reported. Strains employed as live-antigens was chosen considering the serovars more often detected, by isolation or serology, in Italy [10,14,18,19,23–25]; indeed, positivity to other serovars were rarely reported [26]. Moreover, these strains were employed routinely in the Laboratory of Infectious Disease of Department of Veterinary Medicine of Pisa University for research and diagnosis, and, for this reason, all serum samples considered were tested with same MAT antigens.

In this survey, the total apparent prevalence of antibodies against *Leptospira* registered during 2002–2016 was 8.65%. In a previous investigation, carried out in the same area during 1995–2001, the total apparent prevalence was 6.81% [9]. It is not possible to exclude that this increase could be due to the different number of available samples and the proportion and representation of the different animal species included in the study. However, the increase could be also related to a changing of some environmental conditions (rainfall, temperature, etc.) which promoted the rise and spreading of new serovar/strains or the re-emerging of endemic strains. The observed increase of positive sera was mainly related to the last years of this investigation (2013–2016) and could be due to an increase of rainfall in the investigated area, especially in wetlands.

Serological investigations highlighted different apparent prevalence trends for each of the eight *Leptospira* serovars tested. Apparent prevalence of positive sera decreased from 1995 to 2001 to 2002–2016 for serogroups Icterohaemorrhagiae (from 22.24% to 13.78%), Australis (from 55.04%–24.69%) and Sejroe (from 22.08%–14.22%). Furthermore, apparent prevalence increases for Canicola (from 0% to 3.08%), Pomona (from 0.64% to 29.43%), Grippotyphosa (from 0% to 9.81%), Tarassovi (from 0% to 3.52%) and Ballum (from 0% to 1.43%) [9]. The results obtained from previous and from this investigation could be different for some reasons. In the previous work [9], carried out in the same investigated area, employing the same *Leptospira* serovars and strains as antigens, the threshold titer was 1:400, conversely in this investigation the threshold titer used was 1:100. Furthermore, the number of tested sera for each animal species could have influenced the detected total apparent prevalence.

Considering the distribution of positivity detected year by year, it is possible to observe a decrease in the percentage of positive sera

detected from 2002 to 2008 and an increase starting from 2009. In particular, from 2014 it was registered an impressive increase of *Leptospira* positive samples (Fig. 1a).

Annual fluctuations in *Leptospira* spreading and seropositivity is well documented [27] and could be related to many factors as climate changing, rains and drought springs. Fluctuations in observed data could be related not only to environmental modifications, but also to hosts changing: variations in exposure to *Leptospira* by animal, introduction of new wild animal species, changing in herds management. As concerns this last point, in last years breeding management changed from indoor intensive to extensive or semi-extensive with outdoor access in order to improve animal welfare. Furthermore, it could be related to modifications of *Leptospira* epidemiology: introduction of new serovars/strains or change in host specificity range by classical serovars/strains.

As for serogroup Icterohaemorrhagiae, seropositivity reflected more or less the observed global trend. However, positivity to this serogroup was constantly detected, even if during some years with only few positive samples (Fig. 1b). This is an expected result, indeed, Icterohaemorrhagiae is maintained by rats and it is the serogroup most often involved in animal and human infection in many parts of the world [28].

Only few samples scored positive to serogroup Canicola during the first years of investigation and no positive sera were detected between 2006 and 2012 (Fig. 1c). It seems that this serovars re-emerged from 2013. In nature, Canicola is maintained by dog and vaccination programs carried out for about 50 years in this species led to its disappearance [29]. Data obtained in this investigation showed in recent years an increase of positivity to Canicola in animals different to dogs. This finding could suggest a possible change in host range of this serogroup.

serogroup Pomona seemed to be almost disappeared from the investigated area for more than fifteen years (Fig. 1d). Ten positive reactions were recorded from 2002 to 2013, furthermore, a previous investigation conducted in the same geographical area [9], reported an apparent prevalence of 0.64% (4 out of 9885 sera) between 1995 and 2001. From 2014, the number of positive sera increased, and Pomona was the most detected serogroup in 2016. In the last years of the investigation, a large number of swine sera were analysed, and this could have influenced the improved detection of positivity to Pomona. However, a considerable number of positive reactions was found also in sera of species other than pig, supporting the hypothesis of the rise of this serogroup in investigated area.

Circulation of serogroup Grippotyphosa in the investigated area was slightly documented during this investigation. This is in accordance with previous reports conducted in the same region and in Italy [9,10]. From 2013, a slight increase in the detected number of positive reactions was registered (Fig. 1e). This trend is in accordance with many studies conducted in Europe, where Grippotyphosa is considered an emerging serovar [30–33]. Considering the studied area, it is not possible to exclude that this occurrence could be related to the import from East Europe of wild animals for hunting purposes, in particular hares [34,35].

Positivity to serogroup Tarassovi was never detected from 2002 to 2013 (Fig. 1f). This trend is in line with other National surveys [9,10], indeed, in past years, Tarassovi showed a very limited diffusion in Italy. In 2014, a peak of positivity was registered which could resemble an epidemic event. Positive reactions were found mainly in wild boar, but also in domestic animals. In 2015 and 2016, the number of positive sera decreased and probably it will return to zero. These results suggest that Tarassovi is not disappeared from North and Central Italy territory, and infections could occur, since Tarassovi is probably maintained in the environment by *reservoirs* other than swine, as suggested by previously reported data in Italy [10].

As regard serogroup Australis, its trend reflects the global trend observed. Every year, positivity to this serogroup was detected, with

exception of 2008 and 2011 (Fig. 1g). This finding could probably be expected considering the high number of positive *reservoir* hosts present in investigated area, as hedgehog and wild boar [36,37]. Generally, Australis is reported as an emerging serogroup, but considering the obtained data it could be considered as endemic in investigated area.

Considering the abundance of cattle and, especially, sheep herds in the studied area, the low number of positivity for serogroup Sejroe for many years was unexpected. Indeed, this serogroup were frequently detected in Italy and sometimes it was involved in clinical outbreaks [9,10,26]. As for the other serogroup, after a silent period of about 12 years, from 2014, an increase of positive sera for Sejroe was registered, suggesting a restart of the circulation of this serogroup in investigated area. More focused investigations should be probably required to better understand this finding. It may be supposed it could be related to the increase of outdoor herds, especially for dairy cows, related to the increased attention for animals' welfare. Furthermore, animal species different to domestic ruminants could be infected and this could contribute to the rise of serogroup Sejroe.

During the investigated period, serogroup Ballum showed a very limited diffusion. In 2014, a slight increase of seropositivity was registered, but it was a limited event. In Italy, this serovar was rarely detected in serological investigations [9,10,14,19,23], even if it was recently isolated from small mammals in Tuscany [25]. These observations could suggest a low virulence for animals and man of Ballum strains circulating in North and Central Italy territory.

4.1. Bovine

The total apparent prevalence in bovine increase from 0.005% (1995–2001) [9] to 13.03% (2002–2016). In cattle, higher prevalence of serogroup Sejroe has been detected. These data confirm that bovine represents the main maintenance-host for *Leptospira* belonged to this serogroup () [7]. Relative high number of positive reactions was also detected for serogroup Pomona and Grippotyphosa in cattle sera. Severe infections in cattle due to serogroup Pomona is uncommon and usually occur in young animals [7]. Nevertheless, based on a recent National survey, Pomona resulted the second representative serovar in cattle in Italy [10]. The relative high number of positivity could be related to the semi-extensive or extensive farms. These types of breeding promote the contact with wild animal, in particular wild boars. Positivity to serogroup Grippotyphosa resulted higher than that observed in previous investigations conducted in Italy [9,10]. However, in recent years, in other European countries, serovar Grippotyphosa was often detected in cattle and occasionally involved in clinical leptospirosis outbreaks [27,30,31]. In accordance with these Authors, the obtained data seem to identify Grippotyphosa as an emerging serovars/serogroup in cow. The low level of positivity detected for the other serogroup was in accordance with other studies [9,10].

4.2. Dog

The total apparent prevalence in dog increased from 5.42% (1995–2001) to 8.65% (2002–2016) [9]. Icterohaemorrhagiae and Australis resulted the most represented serogroup. These results are in accordance with data available in literature [7,9,10]. As regard Pomona, infection by *Leptospira* belonged to this serogroup in dog produce a severe disease characterized by lethargy, fever, inappetence, diffuse haemorrhage, renal and liver failure [38,39]. In Europe, infections in dogs caused by this serogroup are rare and reported only in few countries of East-Europe, such as Romania [7]. For this reason serovar Pomona was not included in dog vaccines [40]. The obtained data seem to suggest an increasing incidence of this serogroup in dog during last years (Table S1) [9,10,14]. Taking into account the increase of Pomona positivity in dog and the severe symptoms, the research on this serovar could be intensify. Low level of positivity was detected for serogroup Canicola. This result confirms the decreasing of this serovar in many

European countries during the last years due to the use of vaccines [7,41]. Low number of positive sera was observed for Grippotyphosa and Tarassovi. In Europe, Grippotyphosa is considered an emerging serovar in dogs [14,30,32,33] and was included in leptospirosis dog vaccine. Contrary to what is generally observed in Europe, data obtained by this investigation suggest a limited spreading of Grippotyphosa in dogs among investigated area. In Europe, Tarassovi is rarely reported in dog [10,18]. This is in accordance with the obtained data, since only one serum was found positive to this serogroup, even if at high titer (1:800) suggesting a recent and probably acute infection. Serovars Hardjo and Ballum are rarely detected in dog as suggested by several investigations [7,10,41].

4.3. Swine

The total apparent prevalence in swine increased considerably from 9.16% (1995–2001) [9] to 19.74% (2002–2016). It is known that swine is the maintenance host for serovar Pomona (serogroup Pomona), serovar Tarassovi (serogroup Tarassovi) and serovar Bratislava (serogroup Australis) [7]. As expected, Pomona and Australis were the serogroup more often detected in this species. Bratislava is considered an emerging serovar and it could be the cause of abortion and other reproductive disorder in swine. However, some strains become “pig-adapted”, causing subclinical infections [42]. In different European countries, during last years, Bratislava was the more detected serovar in pig sera samples [10,43]. Despite the fact that swine represents the *reservoir* host for Pomona, in last years, the seroprevalence of this serovar in pig was low [9,10,43]. It is noteworthy that, in this investigation, the seroprevalence of Pomona in swine was increasing, in contrast with other surveys. The low percentage of positivity detected for Tarassovi confirm that this serogroup seems to disappear, as suggested by other investigations [9,10,43]. The cause could be the wide use of vaccination program in swine farm [44,45]. As regard Canicola, some studies demonstrated that swine could be infected by *Leptospira* belonged to this serogroup and that intraspecies transmission is possible [43,46]. For this reason, pigs are considered a new possible potential maintenance host for serogroup Canicola, although its real epidemiological role is not still clarified [47,48]. The results of this investigation confirm the circulation of this serovars among swine in Italy too. About the prevalence detected for serogroups Grippotyphosa, Ballum, Icterohaemorrhagiae and Sejroe, obtained data are in accordance with other studies [9,10,43].

4.4. Horse

The total apparent prevalence obtained in horse was characterized by a remarkable decrease from 11.08% (1995–2001) [9] to 2.89% (2002–2016). This species is susceptible to a wide range of incidental infections, that are often characterized by absence of clinical symptoms. Bratislava is the most common serovar detected in horses, but also Grippotyphosa, Pomona, Icterohaemorrhagiae, Autumnalis, Sejroe, Canicola, and Ballum serogroups are occasionally reported [7,49]. The results of this investigation confirm this evidence. The low number of positive sera detected in horse could be related to few clinical samples or few specimens with clinical manifestations. Also, the sera could have been collected after equine uveitis manifestation. Equine uveitis, also known as “moon blindness”, is an ocular disease consequent to *Leptospira* infection in horse, that occurs after the acute phase of leptospirosis when the antibody titer decrease [49].

4.5. Sheep and goat

The total apparent prevalence observed in sheep decreased from 12.13% (1995–2001) [9] to 3.13% (2002–2016). These results could seem unexpected. Indeed, in investigated area sheep flocks are breed extensively or semi-extensively and this could promote contact with

wild animals. However, the obtained data are in line with a recent survey conducted by the Italian reference center for leptospirosis [10]. Despite sheep represent the second maintenance host for serovar Hardjo [50], infection by this serovar could induce subclinical or clinical disease [7], with abortion, stillbirth, birth of weak lambs, agalactia and infertility [51–53]. Although the results of this investigation do not show a high number of positive samples for serogroup Sejroe, almost all have a titer $\geq 1:400$. These data could suggest the occurrence of clinical infection in sheep confirming the high virulence of serovar Hardjo for this animal species. Sheep could be infected also by other serovars [7]. In this investigation, positivity was also reported for serogroups Pomona, Icterohaemorrhagiae and Grippotyphosa. These results are in disagreement with previous investigations carried out in the same area [9] and in Italy [10,26]. Pomona and Icterohaemorrhagiae could cause disease in sheep, in particular they have been associated with reproductive disorders [54,55]. Furthermore, other positive reactions were found in sheep sera for Australis (0.52%), Tarassovi (0.30%), Ballum (0.19%) and Canicola (0.04%) at titer $\geq 1:100$. Seropositivity for these *Leptospira* serogroups was previously reported in sheep where they could represent the cause of accidental infections [56]. Even if the presence of these serovar was reported in other studies, the reactivity to all tested serovars detected in sheep during this investigation seems unusual. It could be probably explained by the presence of wild boars, hares, hedgehogs, rodents (as mice and rats) and other wild animals, which represent maintenance hosts for different serovars, in the areas where the sampling was conducted [24,30,34,41,57–59].

As regard goat sera, data obtained in this investigation confirm that goats are not very susceptible to *Leptospira* infection, as reported in literature by other study, where seropositivity was described for serovar Hardjo [52,60,61], Icterohaemorrhagiae [61,62] and Poi [26].

4.6. Wild boar

The total apparent prevalence in wild board increased from 2.39% (1995–2001) [9] to 8.56% (2002–2016). The highest prevalence was detected for serogroups Tarassovi and Australis. Similarly to swine, wild boar is a maintenance host for serovars Pomona, Tarassovi and Bratislava [7]. These results disagree with other studies previously conducted in the same area of investigation and in Italy. In Tuscany, from 1995 to 2001 no positive sera for Tarassovi has been detected in wild board [9,15]. In Italy, recent surveys indicate the same trend: no Tarassovi seropositivity or few positive samples (2 out of 1987 sera) were found in wild boar [10,23,63]. However, high seroprevalence for this serovar has been reported in different European Countries [64–66]. Wild boar could represent the *reservoir* host of this serovar, disappeared in other domestic animal, and it could contribute to maintain Tarassovi strains in environment. Furthermore, the Tarassovi high prevalence in investigated area could be connected to the import in Tuscany of wild boar from East Europe Countries for hunting [35,64,65]. The second most representative serogroup in wild boar was Australis. that is one of the most worldwide spread *Leptospira* [27], consequently, this result was expected. Indeed, this serovar is frequently detected in wild boar in Italy [9,10,15,23,63] and in Europe [64–68]. Unexpected data has been recorded concerning positivity to Pomona, considering that wild boar is a potential *reservoir*, as reported by other studies carried out in Europe [67]. These data were unexpected, but similar to those reported by other Authors in Italy [10,23,63]. As concerns serogroup Sejroe, that is generally associated to cattle and sheep [7], its detection in wild boar seems singular. In Italy, no Sejroe seropositivity has been founded previously in wild boar [10,23,63]. However, Hardjo seems to be the most prevalent serovar in East Europe in wild boar [67]. For this reason, the results of this investigation finding could be related to the import of animals from East Europe for hunting purposes. Nevertheless, in investigated area there are a lot of free-range farms of cattle and sheep and it is plausible to assume that wild boar became infected after

direct or indirect contact with these animals. Moreover, half of positive sera showed an antibody titer higher than 1:400, suggestive of recent and possible acute infection. For all these reasons it could not be excluded a possible involvement of wild boar in epidemiology of *Leptospira* belonging to serogroup Sejroe, as maintenance or incidental host. Low positivity was detected to the other serogroups: Icterohaemorrhagiae, Canicola, Grippotyphosa and Ballum. These animals could easily come in contact with *Leptospira* due to their lifestyle and positivity to many different serovars is sporadically, but constantly reported [10,23,64–68].

4.7. Other wild animals

As regard the other wild species investigated, positivity has been detected only in samples of hares, foxes and coypus. It is well documented that hare could be infected by different *Leptospira* serovars, especially Grippotyphosa [23,34,57,69]. Fox was reported as incidental host for different serovars, such as Icterohaemorrhagiae, Ballum and Bratislava [10,59,70].

For both these species, a low percentage of positive sera was detected in this survey, compared to the other reported studies. This could suggest a weak involvement of these two animal species in *Leptospira* epidemiology in investigated area.

As regard coypus, some studies highlighted the circulation of the same serovars among these animals and its possible role as reservoir host [19,71–73]. In accordance with these studies, a high percentage of sera, 32.86% (23/70), analyzed in this investigation scored positive to *Leptospira*. The high titers recorded in some sera for serogroup Australis and Grippotyphosa, could suggest a recent infection, confirming the circulation of these serogroup in coypus. Positivity to Australis and Icterohaemorrhagiae was frequently reported, while positivity to Grippotyphosa, which is considered an emerging serovar in Europe, could open new interesting epidemiological scenarios.

No positive reactions were detected in sera from wild ruminants: roe deer, red deer, fallow deer and mouflon. The data of this investigation confirm the marginal role that these animals have in the epidemiology of *Leptospira* according to the low number of positivity reported in literature [9,10,18,74,75].

No positivity was detected in sera collected from other wild animals (wolves, rats, bear). However, small number of samples were analyzed for these species and it is not possible to advance robust epidemiological hypothesis.

4.8. Human

The total apparent prevalence observed in man slightly decreased from 5.60% (1995–2001) [9] to 4.26% (2002–2016). Humans are incidental hosts for *Leptospira* and they could be infected by serovars maintained by animals in a particular geographical region. In past years in Europe, human leptospirosis had a fluctuating trend, but different confirmed cases were always registered. In particular in Italy about 40 human cases were annually recorded from 2008 to 2015 [76,77]. According to these studies, Icterohaemorrhagiae remained the most detected serovar from human sera, but positivity to almost tested serovars, was recorded. This finding could suggest a change also in human leptospirosis related to a modification of *Leptospira* epidemiology in investigated area.

5. Conclusion

Leptospirosis is probably the most widespread, (re-)emerging and prevalent zoonotic disease in the world. However, due to the difficult to exactly diagnose the disease clinically and by laboratory test, sometime, it could be not recognized and consequently severely neglected. For this reason, the true spread and increase of leptospirosis remains probably unknown [13]. Considering that many domestic and wild mammals

represent natural carriers of pathogenic leptospires, acquire epidemiological information on animal leptospirosis could be helpful for both human and breeding animal health. Despite that isolation and strains characterization should have a highest diagnostic value, serology represents for some disease, such as leptospirosis, the best instrument for epidemiological purpose. The results of this investigation provide information on *Leptospira* epidemiology in a defined geographical area involving many different animal species, maintenance end accidental hosts, and humans. Moreover, a long period of time was considered and this allowed to put in evidence a fluctuation in *Leptospira* positivity recovery, as also suggested by other Authors; this trend involved both the total percentage of positive animals registered year by year and the serogroup encountered. The obtained results seem to highlight an increase of *Leptospira* in North-Central Italy and a change in serogroup potentially involved in animal and human infection.

Several animals resulted infected by unusual *Leptospira* serovars and this finding could suggest a change in host range for some serovars, that may promote the adaptation to new hosts. Constant serological monitoring results essential to control the evolution of the dynamics of *Leptospira* epidemiology and it could represent the basis to lead future investigations focused on specific animals and that must include both serological that isolation or molecular techniques.

Conflict of interest statement

All authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.cimid.2019.04.001>.

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