**A serological survey of Brucella in two wild boar (*Sus scrofa*) populations of Lombardy (Italy)**

**Abstract max 300 words**

The aim of the present study is to measure the seroprevalence of antibodies against Brucella *abortus*, *melitensis* and *suis* in 4112 wild boars analysed in the Lombardy region, Italy, during 2017-2020. Precisely, we focused our attention on two prototypical provinces of Lombardy: Cremona and Brescia. Sex and age classes of the sampled wild boars were recorded and analysed to determine if these factors are correlated to the prevalence of antibodies against Brucella. A possibly seasonality of seroprevalence was also evaluated.

Our data showed that wild boars in the Lombardy region are exposed to brucella infection in a spatial-dependent way, and that two distinct populations are present: Cremona-one, near the Emilia Romagna Region, and Brescia-one. The seroprevalences of Brucella *abortus*, *melitensis* and *suis* in these 2 areas are different: 36.96% in Cremona and 3.13% in Brescia. According to the typing analyses performed on organs isolated from animals positive to Brucella antibodies, or animals with lesions, the totality of Brucella circulating was B. *suis*. It is likely that the Brucella *suis* circulating in Emilia Romagna moved across the Po river and arrived in Lombardy, in the Cremona area.

**Key words 4-8**

Brucella *suis*, Brucellosis, ELISA, Seroprevalence, Wild boar, wildlife

**Introduction (4000 words from intro to discussion)**

Brucellosis is a worldwide zoonosis caused by gram negative, facultative, intracellular bacteria belonging to the genus Brucella. To date, different *Brucella* species have been identified from a wide spectrum of hosts (Khurana, Sehrawat et al. 2021). Some species infect terrestrial animals: B. *abortus*, B. *melitensis*, B. *suis*, B. *ovis*, B. *canis*, B*. neotomae*, and *B. microti*, others, B. *ceti* and B. *pinnipedialis* affect marine mammals. Moreover, B. *papionis* and B. *vulpis* were respectively isolated from baboons and red foxes (Scholz, Revilla-Fernández et al. 2016). Brucella spp. can infect humans as an incidental host. The most pathogenic and invasive species for humans is B. *melitensis*, followed by B. suis, B. *abortus* and B. *canis (Khurana, Sehrawat et al. 2021)*. B. melitensis, B. *suis* and B. *abortus* are reported to be endemic in most countries (Franc, Krecek et al. 2018). The spreading of human brucellosis has radically mutated over the years, due to various sanitary, socioeconomic, and political reasons, together with the increase of international travels (Seleem, Boyle et al. 2010). Generally, Brucella is transmitted to humans through the consumption of contaminated animal products as well as unpasteurized milk and cheeses, or through direct contact with infected tissues or secretions (Moreno 2014). Brucellosis in livestock causes important economic losses in the industries, therefore, it is a notifiable disease in many countries, including Italy (Khurana, Sehrawat et al. 2021). Wild boars (*Sus scrofa*) have been identified as reservoirs of Brucella *suis* and Brucella *abortus* for both livestock and wildlife species (Godfroid 2002, Olsen, Tatum 2016) .

Wildlife disease surveillance is crucial to identify changes in wildlife disease occurrence and epidemiology and it is an essential part of the One-Health approach (Yon, Duff et al. 2019). Surveillance is required to identify new and re-emerging pathogens, to identify possible changes in host species and vectors, and to adopt appropriate measures. Many factors played a key role in the worldwide increase of emerging infectious disease (EIDs) in the last 50 years. Both anthropological and environmental factors were found to be linked to the spread of infectious diseases in Europe, like tourism, trade and climate/ environmental changes (Semenza, Lindgren et al. 2016, Semenza, Menne 2009). The Italian Lombardy region is characterized by high human population density and by an history of extensive industries and intensive agriculture, with high livestock populations. As a consequence, the wildlife and in particular wild boars, has been limited to the periphery. Due to the recent recover, growth and expansion of the wild boar population, the contacts between feral and domestic pigs were favoured (Martin, Pastoret et al. 2011). For this reason, the population of wild boars represents a risk factor for the transmission of infectious diseases, such as brucellosis, not only for other wildlife but also for livestock, human and pets (Meng, Lindsay et al. 2009). Information on the prevalence and distribution of Brucella among wild boar populations in Lombardy is currently limited. Prevalence of Brucella spp. infection in wild boars in Europe span from 0% to 60% (Cvetnić, Spicić et al. 2009, Wu, Abril et al. 2011, Grégoire, Mousset et al. 2012, Hälli, Ala-Kurikka et al. 2012, Risco, García et al. 2014).

In this context, the present study aimed to assess Brucella diffusion in wild boar in two areas of the Lombardia region (Italy) to define the risk of spreading and possible transmission to other animals and humans. For this purpose, serological, bacteriological and molecular assays were used.

**Material and methods**

Data collection

For this study blood and organs, using convenience sampling, were collected from an ongoing national wild boar monitoring program (Piano Monitoraggio Selvatici – Regione Lombardia) and were used for analyses.

This retrospective study covered a 4-year period of analysis: 2017–2020. Sex and age class of the sampled wild boars were recorded. The age of each individual was estimated using tooth eruption and tooth replacement according to Matschke and the animals were divided in 3 classes: class 0 juveniles (< 1 year old), class 1 yearlings (1-2 years old), class 2 adults (>2 years old) (Matschke).

Once established the high seroprevalence of Brucella in Cremona area, at the beginning of 2018 hunter’s season a meeting with hunters and forest rangers was reunited and it was decided to collect submandibular lymph nodes, spleen and uteri or testicles in addition to serum for pathological examinations and Brucella culture analyses for the 2018 hunter season.

Serological analyses

Serum samples were analysed by competitive ELISA to detect antibodies directed against Brucella *abortus*, *melitensis* and *suis* using the SVANOVIR Brucella-Ab C-ELISA kit (INDICAL Sweden AB) according to the manufacturer’s instructions. Briefly, 50 µl of each serum sample and controls diluted 1:10 in the buffer provided were incubated in the microtiter plate wells pre-coated with s-LPS antigen. Immediately after, were added 50 µl/well of mAb and the plate was incubated for 30 minutes at room temperature. After washing four times using the wash solution, 100 µl/well of peroxidase-conjugated antibody were added and plate was incubated for 30 minutes at room temperature. The wells were washed four times again with wash solution and 100 µl/well of chromogen (TMB Solution) were added. After 10 minutes the substrate reaction was stopped by adding 50 µl/well of Stop solution. Optical density values were measured in Tecan Sunrise Microplate Reader at 450 nm. The OD values for the negative controls had to be in the range 0.75 – 2.0. Positive controls must have a percentage of competition between 80 and 100, weak positive control must have a percentage of competition between 30 and 70, the negative controls must have a percentage of competition between -10 and 15. Samples were considered positive if the percentage of competition is equal/higher than 30, negative if not.

Detection and identification of Brucella by culture and real time-PCR

The isolation of Brucella was performed according to OIE Terrestrial Manual 2016, cap 2.1.4 par B1, B1.1, B1.2, B1.3. Briefly, animal samples were cultured directly on both Farrell medium (FM) and modified Thayer-Martin and incubated for 10 days at 37°C. Formed colonies were sub-cultured on BHI blood agar and the colonies were tested for catalase and oxidase production. Culture-positive samples were confirmed by Real Time PCR. All Brucella isolated from the samples were characterized as B. suis biovar X by MLVA??

Software and analyses

All data plotting and statistical analyses were done with GraphPad Prism 8.2 (GraphPad Software Inc., La Jolla, CA, USA). The statistically significant level was set at 0.05. Statistical analyses of seroprevalences were conducted using Fisher's Exact test.

**Results**

Between 2017 and 2020 a total of 4112 serum samples were received for testing the Brucella antibodies prevalence in two provinces of Lombardy: Brescia and Cremona. The number of sera analysed by ELISA was 3487 and 625 in Brescia and Cremona, respectively. The seroprevalences through age classes and sampling region are reported in Table 1: a global significant higher seroprevalence of Brucella *abortus*, *melitensis* and *suis* was reported in Cremona (36.96%) compared to Brescia (3.13%) (Figure 1). Of interest, as reported in Table 2 the seroprevalence of these two areas were roughly stable over the considered period (Figure 2).

Age and Sex analysis…

Seroprevalence in wild boars from Brescia (BS) and Cremona (CR) was analysed according to seasonality (Table 3).

However, positive serology for Brucella must be evaluated considering that false positives can occur due to cross reactivity to other bacteria. (Godfroid 2002). Moreover, the presence of antibodies against Brucella does not necessarily indicate an active infection at the time of analysis, but can simply reveal a previous exposition (Godfroid 2002).

A total of 92 lymph nodes was tested for the presence of Brucella spp. by bacteriological study. 18 out of these 92 samples resulted positive. Moreover, 3 out of 97 animals presented gross lesions: 2 orchitis, 1 metritis. These animals resulted negative at lymph-node culture and also at microbiological examination of the organs with lesions (uterus/testicles). On the contrast Real Time PCR from lesioned tissue (uterus/testicles) resulted positive for Brucella suis.

Interestingly, 6 animals out of 18 with lymph-nodes positive by culture were seronegative.

Relation between cultural and serological results…if possible

**Discussion**

It has been shown that wild life can be a reservoir for several zoonotic bacterial agents, including Brucella. (Yon, Duff et al. 2019). Wildlife disease surveillance is crucial to detect changes in wildlife disease occurrence and epidemiology, and it is an integral part of a One-Health approach (Yon, Duff et al. 2019). The knowledge about Brucella circulating among wildlife, such as wild boar, is important not only for the management of wild species themselves, but also for the domestic ones (Godfroid 2002, Olsen, Tatum 2016). Infected boars are able to transmit these agents to domestic pigs. Interactions between wild boars and out-door-reared pigs have shown that risk factors for transmission of Brucella were related to presence, density and spatial overlapping of wild boar and domestic pigs, piggery location and fence features (Wu, Abril et al. 2011, Risco, García et al. 2014). The transmission is favoured also by the ability of Brucella to survive in soil and water for several weeks. (Acha N ). Hunting activities represent potential sources of Brucella exposure to human (Kmetiuk, Paulin et al. 2021). For these reasons, it is crucial to be aware of the reservoir species of Brucella, in order to adopt preventive measures to limit transmission of the disease to domestic animals and humans. Therefore, only preventive actions, coordinated surveillance and research activities may help to control zoonotic foci and minimize public health risks (Martin, Pastoret et al. 2011).

The present study, focused on the seroprevalence of Brucella antibodies in two specific areas of Lombardia, Brescia and Cremona, revealed a new insight into the Brucella *spp.* distribution in wildlife.

In particular we identified two different populations of wild boars with distinct seroprevalence of Brucella. In the area of Cremona, along the Po River, the Brucella seroprevalence is similar to the seroprevalence reported for the near Emilia Romagna Region (data not published). In contrast the seroprevalence of Brucella in wild boars from Brescia area is lower than the seroprevalence of Cremona. It is likely that the wild boars from Emilia Romagna migrated across the Po river. Overall our seroprevalences were similar to those reported in other European countries: Switzerland 35.8%; (Wu, Abril et al. 2011), 25% - 46% in different Spain regions (Muñoz, Boadella et al. 2010b). Our findings are also mostly in agreement with different studies performed in Italy. Antibodies against Brucella were found in wild boars in the Piedmont Region (Bergagna, Zoppi et al. 2009), in Campania Region (Montagnaro, Sasso et al. 2010) and in Sardinia (Pilo, Addis et al. 2015). On the contrary, in the Tuscany region no antibodies against Brucella were found, likely due to the established eradication programs in domestic animals (Ebani, Cerri et al. 2003). However, the comparison of prevalence values obtained in different studies and in different areas should be interpreted with caution due to the different characteristics of the diagnostic tests used. Samples tested by PCR revealed that the Brucella present in the organ lesions was Brucella *suis*. It has been previously reported that wild boar represents a host for B. suis and plays an important role in the epidemiology of brucellosis (Cilia, Fratini et al. 2021, Cvetnić, Spicić et al. 2009, Bergagna, Zoppi et al. 2009, Godfroid 2002).

The present study shed some light on the role of wild boars in serving as a reservoir of Brucella in considered areas, but additional epidemiologic data are needed.

**Acknowledgments**

This work was supported by XXX.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Age** | **Population size** | **Positivity %** | **Lower** | **Upper** |
| Brescia | Juveniles | 440 | 3,86 | 2,06 | 5,66 |
| Yearlings | 546 | 2,56 | 1,24 | 3,89 |
| Adults | 1095 | 4,47 | 3,25 | 5,70 |
| Unknown | 1406 | 2,06 | 1,32 | 2,81 |
| Total | 3487 | 3,13 | 2,55 | 3,70 |
| Cremona | Juveniles | 116 | 28,45 | 20,24 | 36,66 |
| Yearlings | 194 | 32,99 | 26,37 | 39.61 |
| Adults | 185 | 44,86 | 37,70 | 52,03 |
| Unknown | 130 | 39,23 | 30,84 | 47,62 |
| Total | 625 | 36,96 | 33,18 | 40,74 |

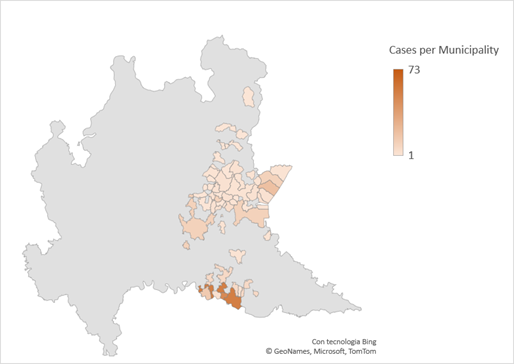
Table 1. Seroprevalence (percent) of antibodies against Brucella *abortus*, *melitensis* and *suis as* detected by ELISA in juvenile, yearling and adult wild boars from the considered areas Brescia (BS) and Cremona (CR) for the period 2017-2020.

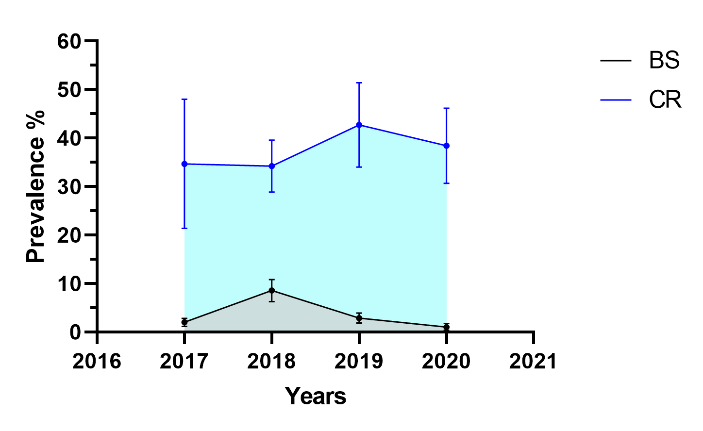
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Year** | **Area** | **Population size** | **Positivity (%)** | **Lower** | **Upper** |
| 2017 | BS | 1146 | 2,01 | 2,82 | 1,20 |
| 2018 | BS | 573 | 8,55 | 10,84 | 6,26 |
| 2019 | BS | 1000 | 2,90 | 3,94 | 1,86 |
| 2020 | BS | 768 | 1,04 | 1,76 | 0,32 |
| 2017 | CR | 49 | 34,69 | 48,02 | 21,37 |
| 2018 | CR | 301 | 34,22 | 39,58 | 28,86 |
| 2019 | CR | 124 | 42,74 | 51,45 | 34,03 |
| 2020 | CR | 151 | 38,41 | 46,17 | 30,65 |

Table 2. Seroprevalence (percent) of antibodies against Brucella *abortus*, *melitensis* and *suis as* detected by ELISA in wild boars from Brescia (BS) and Cremona (CR) per year.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | **Positivity** | **Lower** | **Upper** |
| Brescia | Jan - Apr | 3.58 | 2,20 | 4,96 |
| May - Aug | 1,40 | 2,50 | 0,29 |
| Sep - Dec | 3,31 | 4,03 | 2,58 |
| Cremona | Jan - Apr | 40,54 | 48,45 | 32,63 |
| May - Aug | 33,90 | 39,34 | 27,86 |
| Sep - Dec | 37,76 | 43,88 | 31,64 |

Table 3. Seroprevalence (percent) of antibodies against Brucella *abortus*, *melitensis* and *suis as* detected by ELISA in wild boars from Brescia (BS) and Cremona (CR) per season.

  
Figure 1. Map of the Lombardia region (Italy) showing the seroprevalence of *Brucella abortus*, *melitensis* and *suis* for the period 2017-2020.

  
Figure 2. Trend of the seroprevalence with 95% CI of antibodies against Brucella *abortus*, *melitensis* and *suis as* detected by ELISA in wild boars from Brescia (black) and Cremona (blue) over the considered years.

**Literature cited**

ACHA N, S.B., Zoonoses and communicable diseases common to man and animals.

BERGAGNA, S., ZOPPI, S., FERROGLIO, E., GOBETTO, M., DONDO, A., DI GIANNATALE, E., GENNERO, M.S. and GRATTAROLA, C., 2009. Epidemiologic survey for Brucella suis biovar 2 in a wild boar (Sus scrofa) population in northwest Italy. *Journal of wildlife diseases,* **45**(4), pp. 1178-1181.

CILIA, G., FRATINI, F., TURCHI, B., ANGELINI, M., CERRI, D. and BERTELLONI, F., 2021. Genital Brucella suis Biovar 2 Infection of Wild Boar (Sus scrofa) Hunted in Tuscany (Italy). *Microorganisms,* **9**(3), pp. 582. doi: 10.3390/microorganisms9030582.

CVETNIĆ, Z., SPICIĆ, S., TONCIĆ, J., MAJNARIĆ, D., BENIĆ, M., ALBERT, D., THIÉBAUD, M. and GARIN-BASTUJI, B., 2009. Brucella suis infection in domestic pigs and wild boar in Croatia. *Revue scientifique et technique (International Office of Epizootics),* **28**(3), pp. 1057-1067.

EBANI, V.V., CERRI, D., POLI, A. and ANDREANI, E., 2003. Prevalence of Leptospira and Brucella antibodies in wild boars (Sus scrofa) in Tuscany, Italy. *Journal of wildlife diseases,* **39**(3), pp. 718-722.

FRANC, K.A., KRECEK, R.C., HÄSLER, B.N. and ARENAS-GAMBOA, A.M., 2018. Brucellosis remains a neglected disease in the developing world: a call for interdisciplinary action. *BMC public health,* **18**(1), pp. 125-017-5016-y.

GODFROID, J., 2002. Brucellosis in wildlife. *Revue scientifique et technique (International Office of Epizootics),* **21**(2), pp. 277-286.

GRÉGOIRE, F., MOUSSET, B., HANREZ, D., MICHAUX, C., WALRAVENS, K. and LINDEN, A., 2012. A serological and bacteriological survey of brucellosis in wild boar (Sus scrofa) in Belgium. *BMC veterinary research,* **8**, pp. 80-6148-8-80.

HÄLLI, O., ALA-KURIKKA, E., NOKIREKI, T., SKRZYPCZAK, T., RAUNIO-SAARNISTO, M., PELTONIEMI, O.A. and HEINONEN, M., 2012. Prevalence of and risk factors associated with viral and bacterial pathogens in farmed European wild boar. *Veterinary journal (London, England : 1997),* **194**(1), pp. 98-101.

KHURANA, S.K., SEHRAWAT, A., TIWARI, R., PRASAD, M., GULATI, B., SHABBIR, M.Z., CHHABRA, R., KARTHIK, K., PATEL, S.K., PATHAK, M., IQBAL YATOO, M., GUPTA, V.K., DHAMA, K., SAH, R. and CHAICUMPA, W., 2021. Bovine brucellosis - a comprehensive review. *The Veterinary quarterly,* **41**(1), pp. 61-88.

KMETIUK, L.B., PAULIN, L.M.S., CASSARO VILLALOBOS, E.M., DO CARMO CUSTÓDIO DE SOUZA HUNOLD LARA,M., DE BARROS FILHO, I.R., PEREIRA, M.S., VAN WILPE BACH, R., LIPINSKI, L.C., FÁVERO, G.M., DOS SANTOS, A.P. and BIONDO, A.W., 2021. Seroprevalence of Anti-Brucella spp. Antibodies in Wild Boars (Sus scrofa), Hunting Dogs, and Hunters of Brazil. *Journal of wildlife diseases,* **57**(4), pp. 974-976.

MARTIN, C., PASTORET, P.P., BROCHIER, B., HUMBLET, M.F. and SAEGERMAN, C., 2011. A survey of the transmission of infectious diseases/infections between wild and domestic ungulates in Europe. *Veterinary research,* **42**(1), pp. 70-9716-42-70.

MATSCHKE, G.,H., Aging European Wild Hogs by Dentition.

MENG, X.J., LINDSAY, D.S. and SRIRANGANATHAN, N., 2009. Wild boars as sources for infectious diseases in livestock and humans. *Philosophical transactions of the Royal Society of London.Series B, Biological sciences,* **364**(1530), pp. 2697-2707.

MONTAGNARO, S., SASSO, S., DE MARTINO, L., LONGO, M., IOVANE, V., GHIURMINO, G., PISANELLI, G., NAVA, D., BALDI, L. and PAGNINI, U., 2010. Prevalence of antibodies to selected viral and bacterial pathogens in wild boar (Sus scrofa) in Campania Region, Italy. *Journal of wildlife diseases,* **46**(1), pp. 316-319.

MORENO, E., 2014. Retrospective and prospective perspectives on zoonotic brucellosis. *Frontiers in microbiology,* **5**, pp. 213.

MUÑOZ, P.M., BOADELLA, M., ARNAL, M., DE MIGUEL, M.J., REVILLA, M., MARTÍNEZ, D., VICENTE, J., ACEVEDO, P., OLEAGA, A., RUIZ-FONS, F., MARÍN, C.M., PRIETO, J.M., DE LA FUENTE, J., BARRAL, M., BARBERÁN, M., DE LUCO, D.F., BLASCO, J.M. and GORTÁZAR, C., 2010a. Spatial distribution and risk factors of Brucellosis in Iberian wild ungulates. *BMC infectious diseases,* **10**, pp. 46-2334-10-46.

MUÑOZ, P.M., BOADELLA, M., ARNAL, M., DE MIGUEL, M.J., REVILLA, M., MARTÍNEZ, D., VICENTE, J., ACEVEDO, P., OLEAGA, A., RUIZ-FONS, F., MARÍN, C.M., PRIETO, J.M., DE LA FUENTE, J., BARRAL, M., BARBERÁN, M., DE LUCO, D.F., BLASCO, J.M. and GORTÁZAR, C., 2010b. Spatial distribution and risk factors of Brucellosis in Iberian wild ungulates. *BMC infectious diseases,* **10**, pp. 46-2334-10-46.

OLSEN, S.C. and TATUM, F.M., 2016. Swine brucellosis: current perspectives. *Veterinary medicine (Auckland, N.Z.),* **8**, pp. 1-12.

PILO, C., ADDIS, G., DEIDDA, M., TEDDE, M.T. and LICIARDI, M., 2015. A Serosurvey for Brucellosis in Wild Boar (Sus scrofa) in Sardinia, Italy. *Journal of wildlife diseases,* **51**(4), pp. 885-888.

RISCO, D., GARCÍA, A., SERRANO, E., FERNANDEZ-LLARIO, P., BENÍTEZ, J.M., MARTÍNEZ, R., GARCÍA, W.L. and DE MENDOZA, J.H., 2014. High-density dependence but low impact on selected reproduction parameters of Brucella suis biovar 2 in wild boar hunting estates from South-Western Spain. *Transboundary and emerging diseases,* **61**(6), pp. 555-562.

SCHOLZ, H.C., REVILLA-FERNÁNDEZ, S., DAHOUK, S.A., HAMMERL, J.A., ZYGMUNT, M.S., CLOECKAERT, A., KOYLASS, M., WHATMORE, A.M., BLOM, J., VERGNAUD, G., WITTE, A., AISTLEITNER, K. and HOFER, E., 2016. Brucella vulpis sp. nov., isolated from mandibular lymph nodes of red foxes (Vulpes vulpes). *International Journal of Systematic and Evolutionary Microbiology,* **66**(5), pp. 2090-2098.

SELEEM, M.N., BOYLE, S.M. and SRIRANGANATHAN, N., 2010. Brucellosis: a re-emerging zoonosis. *Veterinary microbiology,* **140**(3-4), pp. 392-398.

SEMENZA, J.C., LINDGREN, E., BALKANYI, L., ESPINOSA, L., ALMQVIST, M.S., PENTTINEN, P. and ROCKLÖV, J., 2016. Determinants and Drivers of Infectious Disease Threat Events in Europe. *Emerging infectious diseases,* **22**(4), pp. 581-589.

SEMENZA, J.C. and MENNE, B., 2009. Climate change and infectious diseases in Europe. *The Lancet.Infectious diseases,* **9**(6), pp. 365-375.

WU, N., ABRIL, C., HINIĆ, V., BRODARD, I., THÜR, B., FATTEBERT, J., HÜSSY, D. and RYSER-DEGIORGIS, M.P., 2011. Free-ranging wild boar: a disease threat to domestic pigs in Switzerland? *Journal of wildlife diseases,* **47**(4), pp. 868-879.

YON, L., DUFF, J.P., ÅGREN, E.O., ERDÉLYI, K., FERROGLIO, E., GODFROID, J., HARS, J., HESTVIK, G., HORTON, D., KUIKEN, T., LAVAZZA, A., MARKOWSKA-DANIEL, I., MARTEL, A., NEIMANIS, A., PASMANS, F., PRICE, S.J., RUIZ-FONS, F., RYSER-DEGIORGIS, M.P., WIDÉN, F. and GAVIER-WIDÉN, D., 2019. Recent Changes in Infectious Diseases in European Wildlife. *Journal of wildlife diseases,* **55**(1), pp. 3-43.