Analysis of seroprevalence data on Hepatitis E virus and *Toxoplasma gondii* in wild ungulates for the assessment of human exposure to zoonotic meat-borne pathogens

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ABSTRACT

Seroprevalence data for *Toxoplasma gondii* and Hepatitis E virus (HEV) in wild boar (*Sus scrofa)*, roe deer (*Capreolus capreolus),* red deer *(Cervus elaphus),* mouflon (*Ovis aries/musimon*) and chamois *(Rupicapra rupicapra)* hunted/culled in northern Italy were used to fit seroprevalence distributions describing the exposure and co-exposure of the species to the two pathogens. The higher proportion of *T. gondii* and HEV seropositive animals was observed in wild boars with point estimate seroprevalence of 49% (N=331) and 15% (N=326) respectively. Data allowed comparisons by area (pre-Alpine Vs Alpine environment) for roe deer, red deer and mouflons. Contrasts between the distributions describing the uncertainty in seroprevalence suggest roe deer, red deer and mouflons have higher probability of being seropositive to *T.gondii* in pre-Alps. When considering HEV, few seropositive animals were detected and contrasts were symmetrically centred to zero for roe deer and red deer; mouflons shown higher probability of being seropositive in Alpine environment. HEV seropositive animals also included chamois (P=5.1%, N=97) in the Alpine districts, confirming circulation of HEV in remote areas. Evidence of HEV and *T. gondii* co-exposure was limited except for wild boars where it was observed in 30 samples representing 60% of the overall HEV-positive samples.

Seroprevalence data of single infection and co-infection are extremely useful to investigate circulation of zoonotic pathogens in wild animals and estimate the foodborne risk of human exposure, however, these type of data do not directly translate into the presence/absence of the pathogen in seropositive and seronegative animals. At benefit of future development of quantitative risk assessments aiming at estimating the risk of human infection/co-infection via consumption of game meat, we developed and made available an online application that allows estimating the probability of the pathogen(s) being present as a function of seroprevalence data.

Keywords: quantitative risk assessment, zoonosis, game meat, food safety, risk analysis

1. INTRODUCTION

Consumption and demand of game products in Europe has increased in last decades (Navarro-Gonzalez et al., 2016). While representing a small proportion of the overall meat market (Vikas et al., 2019), game meat is sometimes considered as an alternative to the meat sourced from farm animals for reasons ranging from sustainability (Holechek et al., 2020) to good biochemical composition (Paulsen et al., 2011; Tomasevic et al., 2018). Distinctive taste and aroma clearly represent an additional key driver for consumers preference and a recent study also identified an important niche market for raw game meat (Demartini et al., 2018).

Growing interest in game meat amongst consumers raises the challenge of estimating the public health risks posed by the zoonotic pathogens of which wild animals are reservoirs (EFSA, 2013). In the context of food safety, use of risk assessment frameworks, and Quantitative Microbiological Risk Assessment (QMRA) models in particular, is the standard practice to support scientific-based risk management strategies (FAO/WHO, 2010).

When considering the biological risks posed by consumption of meat of domestic animals, availability of prevalence data, together with experimental studies investigating the changes in microbial load along the food chain, have informed “farm-to-fork” QMRAs for different animal species/foodborne pathogens (EFSA, 2010, 2020; Smith et al., 2013; Van Damme et al., 2017). However, when considering wild game meat, characterisation of the foodborne risks posed by microbiological hazards is limited to few studies (Coburn et al., 2005; Franssen et al., 2017). One of the key parameters needed to estimate the risk of human exposure to meat-borne pathogens is the prevalence of infection but particularly in wild animal populations, accurate prevalence data are difficult to obtain. Moreover, when presence of pathogens are investigated, data are often presented as serological surveys, in which case, the evidence is useful for confirming circulation of the pathogens in the animal population(s), but cannot be directly used to estimate the prevalence of infection.

Toxoplasma gondii and Hepatitis E Virus (HEV) are amongst the key zoonotic pathogens that can be transmitted to human through consumption of raw or undercooked meat products of both wild animals and livestock (EFSA, 2017, 2018) but for which systematic monitoring of wildlife (and livestock) is not uniformly regulated across European countries. Several surveys have confirmed circulation of these pathogens in different wild animal populations across Europe (De Sabato et al., 2020; Fanelli et al., 2020; Sgroi et al., 2020; Trogu et al., 2020). The development of "Forest-to-fork" (Paulsen et al., 2014) QMRAs should be encouraged to understand the actual risk of human exposure to HEV and *T. gondii* from consumption of wild game meat and other possible exposure pathways such as handling and processing of carcasses (Dubey et al., 2021; Schielke et al., 2015; Yugo & Meng, 2013).

At this respect, it should be noted that while the scientific evidence suggests many popular wild animal species such as wild boars (*Sus scrofa*) or red deer *(Cervus elaphus)* as susceptible to infection to both these zoonotic pathogens, to our knowledge, there are not published studies reporting paired data for both the pathogens within the same animals. This type of evidence would be extremely useful for identification of the animal species with an increased risk of exposure to both the pathogens. In fact, common to *T. gondii* and HEV is the foodborne oral (ingestion) route of human infection through consumption of raw or undercooked meat products of infected animals. Indeed, *T. gondii* and HEV are not heat resistant and cooking temperatures >70°C can effectively inactivate the pathogens (Imagawa et al., 2018; Mirza Alizadeh et al., 2018). As the evidence suggests that both the pathogens are circulating in wild animals populations, availability of paired data could therefore also serve to inform quantitative risk assessments aimed at evaluating not only the probability of foodborne exposure to *T. gondii* and HEV in isolation but also the probability of co-exposure to both the pathogens.

With the intention of providing new evidence for qualitative or quantitative risk assessments models to estimate the risk of human exposure or co-exposure to *T. gondii* and/or HEV form consumption of wild game meat, the main objective of this study was to describe/compare *T. gondii* and HEV exposure and co-exposure seroprevalence data of 5 ungulate species. Namely, wild boar (*Sus scrofa)*, roe deer (*Capreolus capreolus),* red deer *(Cervus elaphus),* mouflon (*Ovis aries/musimon*) and chamois *(Rupicapra rupicapra)* hunted in North Italy. In addition, considering that *T. gondii* and HEV studies on wild animal populations are often designed as serological survey (Almería et al., 2021; Bier et al., 2020; Burri et al., 2014; Kobayashi et al., 2021; Roqueplo et al., 2017; Tsachev et al., 2021; Zeng et al., 2020), a general method based on conditional probabilities is proposed to maximise the uptake of seroprevalence data to inform QMRA models aimed at estimating the risk of human exposure and co-exposure to meat-borne pathogens.

2. MATERIAL AND METHODS

2.1. Study area. Samples were collected in the province of Bergamo (North of Italy) over an area spanning on over 148.000 ha from the pre-Alpine districts at 300 to over 3000m asl in the Orobie Alps (45°40'- 46°10' N, 9°25'- 10°20' E). Considering the differences in terms of anthropization between the two environments, the study area has been divided into the macro-areas: "pre-Alpine" and "Alpine" district respectively (Figure 1).

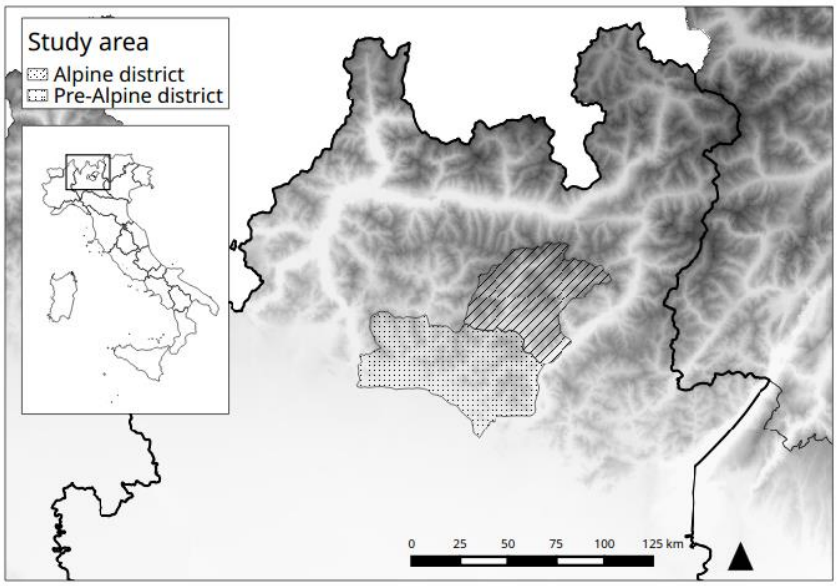

Figure 1. Map of the study area in Northern Italy showing the Alpine and pre-Alpine districts where samples were collected.

2.2. Sampling. Fresh blood samples were collected in field by hunters or gamekeepers from major blood vessels during animal bleeding or from the cardiac clot at the hunting control centers. As part of this study, 331 serum samples (pre-Alps=331, Alps=0) of wild boars, 323 (pre-Alps=258, Alps=65) roe deer, 96 (pre-Alps=37, Alps=59) red deer, 50 (pre-Alps=32, Alps=18) mouflons and 104 (pre-Alps=0, Alps=104) chamois were analysed for detection of antibodies against both *T. gondii* and HEV. However, for some samples (i.e. 95 roe deer, 7 chamois and 5 wild boars) detection of antibodies against HEV was not possible due to the limited amount of blood collected in field sampling. These samples were excluded from the co-exposure analysis. All animals were hunted/culled between 2017 and 2018 during autumnal hunting seasons and regional depopulation plans.

2.3. Serological analysis. After centrifugation at 1200 rpm for 15 minutes, sera were stored at -20°C until the analysis. Highly hemolyzed sera were excluded from the investigation. Samples were firstly tested for the presence of anti-*T. gondii* immunoglobulins G using a commercial ELISA kit (ID Screen Toxoplasmosis Indirect ELISA, IDVET, Montpellier, France). Presence of specific antibodies allows the binding of the P30 antigen coated to the microwells. Subsequently sera were tested by a species-independent enzyme-linked immunosorbent assay (HEV ELISA 4.0v, MP Diagnostics-Biomedicals, Singapore), able to simultaneously detect immunoglobulins M, G, and A formed against HEV recombinant protein ET2.1. This commercial kit was previously used in wild boar and cervids

(Rutjes et al., 2010; Thiry et al., 2017). Both the ELISA tests were performed according to manufacturer recommendations using recommended cut-off values and with the presence of antibodies evaluated by measuring the optic density of the colorimetric reaction (spectrophotometer—450 nm).

2.4. **Pathogen-specific seroprevalence by animal species.** The non-probabilistic nature of sampling for data generation prevented use of inferential analysis based on standard errors such as p-values and confidence intervals. For this reason, data were analysed and compared by using an approach that allows explicit modelling of the uncertainty in the seroprevalence estimates as a function of the observed data. Assuming the seroprevalence of HEV and *T. gondii* in a given animal population is P, the variability in the number of positive samples s in n animals can be estimated from the binomial process:

$$s \sim Binomial(n, P) \quad \text{Eq.1}$$

However, when the unknown parameter is P, the Beta distribution:

$$P = Beta(\alpha; \beta) \quad \text{Eq.2}$$

can be used to describe the uncertainty in P as a function of the observed number of positive samples s out of n. Indeed, α and β in equation 2 are parameterised as: $\alpha = s + 1$ and $\beta = n - s + 1$. Therefore, the uncertainty in the seroprevalence estimates for HEV and *T. gondii* were described by simulating 100,000 values of a Beta distribution parameterised according to equation 2 for each species. In order to compare the seroprevalence of the two sampling areas, the same distribution was parameterised considering the samples for each animal species desegregated by area. Contrasts calculated as the difference between the simulated values of the pathogen-specific seroprevalence uncertainty distributions in the two study areas were computed and results of different species compared. With the contrasts calculated as the difference between seroprevalence values in pre-Alpine and Alpine districts, a difference greater than zero indicates the seroprevalence is higher in pre-Alps. Considering the origin of the samples (culling of wild boars and hunting of chamois took place in pre-Alpine and Alpine districts only respectively), contrasts could be computed only for mouflons, roe deer and red deer.

2.5. Co-exposure seroprevalence. In addition to the seroprevalence of *T. gondii* and HEV in the different animal species, another key outcome from a food safety perspective is the occurrence of animals that are seropositive to both the pathogens. Being capable of modelling the probability of an animal being: (i) negative to both *T. gondii* and HEV (PNEG), (i) seropositive to HEV (PHEV), (ii) seropositive to *T. gondii* (PTG) and finally (iii) seropositive to *HEV* and *T. gondii (PTGHEV)* could be extremely useful in the context of quantitative probabilistic modelling of exposure to meat borne

pathogens. Hence, seroprevalence data were used to fit a Dirichlet distribution. The Dirichlet distribution of joint density function:

$$f(P_1, \dots, P_k) = \frac{\Gamma(\sum_{i=1}^{k} a_i)}{\prod_{i=1}^{k} \Gamma(a_i)} \prod_{i=1}^{k} P_i^{a_i - 1} \quad \text{Eq.3}$$

can be considered as the multivariate equivalent of the Beta (Vose, 2008); hence, it can be used to model the uncertainty in a set of probabilities $\{P_1 \dots P_2\}$ of a multinomial process. Again, simulated seroprevalence data (100,000 iterations) were presented.

Modelling of co-exposure could be done for the samples were detection of antibodies against both *T. gondii* and HEV was possible, hence, the dataset included 326 serum samples of wild boars, 97 chamois, 96 red deer, 50 mouflons and 228 roe deer. Again, to evaluate the differences in the seroprevalence estimates by area, contrasts were calculated as the difference between the simulated multinomial values of PNEG, PTG, PTHEV and PTGHEV in pre-Alpine and Alpine districts for animal species of which samples were available for the two areas (i.e. mouflons, roe deer and red deer).

2.6. General framework for computing the prevalence of infection given seroprevalence data.

Detection of antibodies does not necessarily correlate with the ongoing *T. gondii* and HEV infection and therefore, the actual presence of viable virus or parasite in animals. In fact, several experimental or epidemiological studies investigating the presence of the parasite and the virus seropositive livestock species shown how this relationship is likely dependent upon the anatomical part that is analysed for *T. gondii* (Opsteegh et al., 2016) and the course of infection for HEV (Pavio et al., 2010). In particular for *T. gondii*, important differences in detection of viable cysts in seropositive animals are also observed when considering different animal species (Opsteegh et al., 2016). Considering the strength of this association has mainly been explored in livestock and high differences can be expected between species, it would be speculative to attempt extrapolations of correlation results observed in livestock to wild animals. Nonetheless, a general framework for modelling the prevalence of infection in the animal population given results of seroprevalence data is proposed and made available, as part of the present study, through an online application developed using the shiny package in R and made available here: https://mcrvc.shinyapps.io/SeroApp/.

The app consists in two sections, section 1, “Seroprevalence (1 pathogen)” can be used to compute the uncertainty distribution for the seroprevalence of one pathogen according to equation 1. In this case, the only parameters that are required are the total number of samples tested (*n*) and the number of positive samples observed (*s*).

The second section, “Seroprevalence (2 pathogens)” can be used to model the Dirichlet distributions describing the uncertainty in a set probabilities within a multinomial process (Equation 2). In this case,

the inputs that are required are the number of animals observed for each possible outcome (i.e. Negative to X & Y, Positive to X, Positive to Y and Positive to X & Y).

In both the sections, the uncertainty in the prevalence of infected animals (P_{INF}) given seroprevalence (P_{SERA^+}) is computed as:

$$P_{INF(X)} = \left[P_{SERA_X^+} * P(INF_x|SERA_X^+)\right] + [(1 - P_{SERA_X^+}) * P(INF_x|SERA_X^-)] \quad \text{Eq.4}$$

when considering 1 single pathogen *X* and

$$P_{INF(X)} = \left[P_{SERA_X^+} * P(INF_x|SERA_X^+)\right] + [(1 - P_{SERA_X^+}) * P(INF_x|SERA_X^-)] + [(P_{SERA_{XY}^+} * P(INF_x|SERA_X^+)] \quad \text{Eq.5}$$

$$P_{INF(Y)} = \left[P_{SERA_Y^+} * P(INF_Y|SERA_Y^+)\right] + [(1 - P_{SERA_Y^+}) * P(INF_Y|SERA_Y^-)] + [(P_{SERA_{XY}^+} * P(INF_Y|SERA_Y^+)] \quad \text{Eq.6}$$

$$P_{INF(XY)} = P_{INF(X)} * P_{INF(Y)} \quad \text{Eq.7}$$

When considering two pathogens *X* and *Y*.

In all the equations, $P\left(INF_{X;Y}\middle|SERA_{X;Y}^+\right)$ and $P\left(INF_{X;Y}\middle|SERA_{X;Y}^-\right)$ are the conditional probabilities of seropositive and seronegative animals to pathogen X or Y being infected.

For purpose of illustration, presence of HEV-RNA in sera of seropositive and seronegative wild boars was inferred from data presented in a Spanish study (de Deus et al., 2008) where out of the 27 HEV-positive samples, 21 were from seropositive animals (N=64) to at least one immunoglobulin while 6 were from seronegative; hence, $P(INF|SERA^+) = 0.33$ and $P(INF|SERA^-) = 0.08$. Evidence of *T. gondii* in muscular tissues of wild boar given seroprevalence data was estimated considering the concordance data in pigs between detection of antibodies by modified agglutination test (MAT) on cardiac fluid and demonstration of viable *T. gondii* in heart by mouse bioassay; specifically: $P(INF|SERA^+) = 0.69$ and $P(INF|SERA^-) = 0.06$ (Opsteegh et al., 2016). With no wild boar-specific data available, this worked example assumes data on pigs can be used for wild boars. Unavailability of correlation data for the other animal species considered in this study is at present preventing performing the same estimations. The app however allows quantification of these probabilities once specie-specific correlation data will be available.

RESULTS

The number of seropositive and seronegative animals for *T. gondii* and *HEV* disaggregated by area and species are summarised in Table 1.

	Wild boar		Roe deer		Red deer		Chamois		Mouflon	
	T. gondii (+/-)	*HEV* (+/-)	*T. gondii* (+/-)	*HEV* (+/-)	*T. gondii* (+/-)	*HEV* (+/-)	*T. gondii* (+/-)	*HEV* (+/-)	*T. gondii* (+/-)	*HEV* (+/-)
Pre-Alps	164/167	50/276	120/138	1/162	10/27	0/37	--/--	--/--	9/23	0/32
Alps	--/--	--/--	10/55	0/65	9/50	0/59	4/100	5/92	3/15	1/17
Total (%)	164/167 (49.5%)	50/276 (15.3%)	130/193 (40.2%)	1/227 (0.4%)	19/77 (19.8%)	0/96 (0.0%)	4/100 (3.8%)	5/92 (5.1%)	12/38 (24.0%)	1/49 (2.0%)

Table 1. Number of seropositive and seronegative animals to *T. gondii* and HEV by species and area.

The number of animals seronegative to *T. gondii* and *HEV* (NEG), seropositive to *T. gondii* (PTG), seropositive to *HEV* (PHEV) and seropositive to both *T. gondii* and *HEV* (PTGHEV) are presented as Cleveland plot in Figure 2.

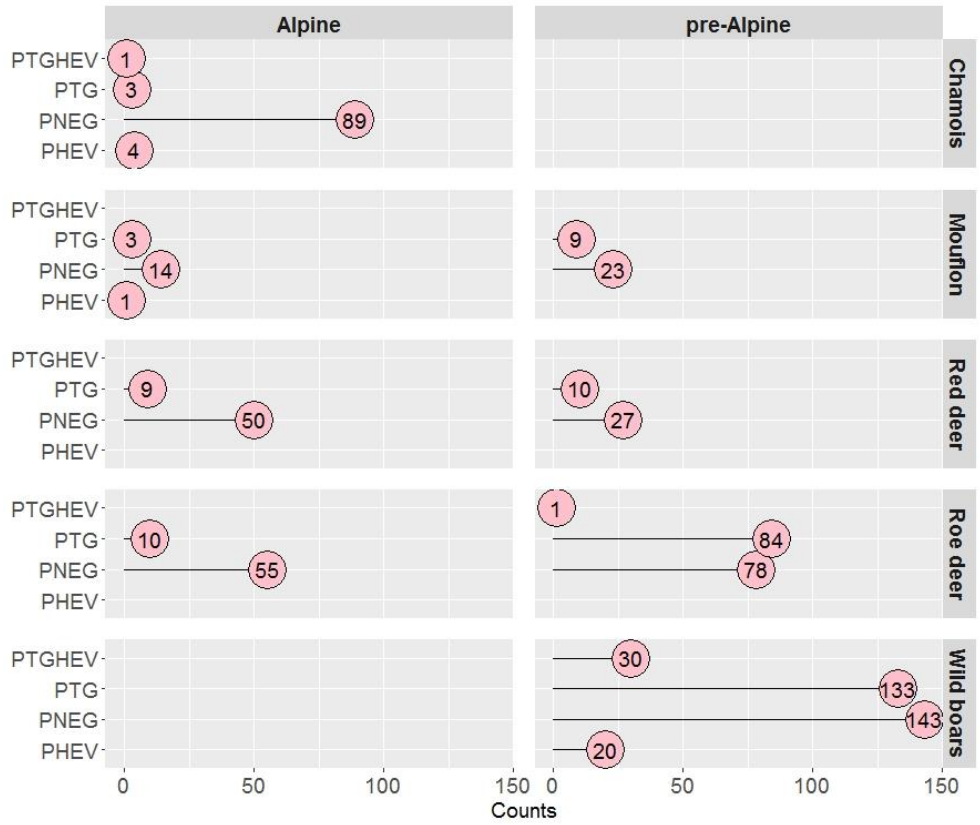

Figure 2. Cleveland plot outlining the number of animals negative to T. gondii and HEV (PNEG), positive to T. gondii only (PTG), positive to HEV only (PHEV) and positive to either T. gondii and HEV (PTGHEV). Counts are presented by area (Pre-Alps and Alps)

The seroprevalence estimates for the individual combinations species-pathogen are reported in table 2 (column pre-Alpine + Alpine) with the seroprevalence estimates for mouflons, red deer and roe deer also reported as disaggregated by area.

		pre-Alpine		Alpine		pre-Alpine + Alpine	
Species	**Outcome**	**Median**	**(5^{th}-95^{th} perc.)**	**Median**	**(5^{th}-95^{th} perc.)**	**Median**	**(5^{th}-95^{th} perc.)**
Wild boars	*T. gondii*	0.49	0.45-0.53	n.a.	n.a.		
	HEV	0.15	0.12-0.19	n.a.	n.a.		
Roe deer	*T. gondii*	0.46	0.41-0.52	0.16	0.1-0.24	0.4	0.36-0.45
	HEV	0.01	0-0.03	0.01	0-0.04	0.01	0-0.02
Red deer	*T. gondii*	0.28	0.17-0.4	0.09	0.16-0.25	0.2	0.14-0.27
	HEV	0.02	0-0.08	0.01	0-0.05	0.01	0-0.03
Mouflon	*T. gondii*	0.29	0.17-0.43	0.19	0.08-0.36	0.25	0.16-0.35
	HEV	0.02	0-0.09	0.09	0.02-0.23	0.03	0.01-0.09
Chamois	*T. gondii*	n.a.	n.a.	0.04	0.02-0.09		
	HEV	n.a.	n.a.	0.06	0.03-0.10		

Table 2. Location parameters (Median, 5^{th} and 95^{th} percentiles) of the distributions describing the uncertainty in the seroprevalence estimates obtained by parameterising the Beta distributions with s (number of positive samples) and n (total number of samples analysed). Results for the different animals in relation to T. gondii and HEV are presented by area.

Contrasts calculated as the difference between the simulated seroprevalence uncertainty distribution of animal species in pre-Alpine on Alpine districts are outlined as density plots in figure 3.

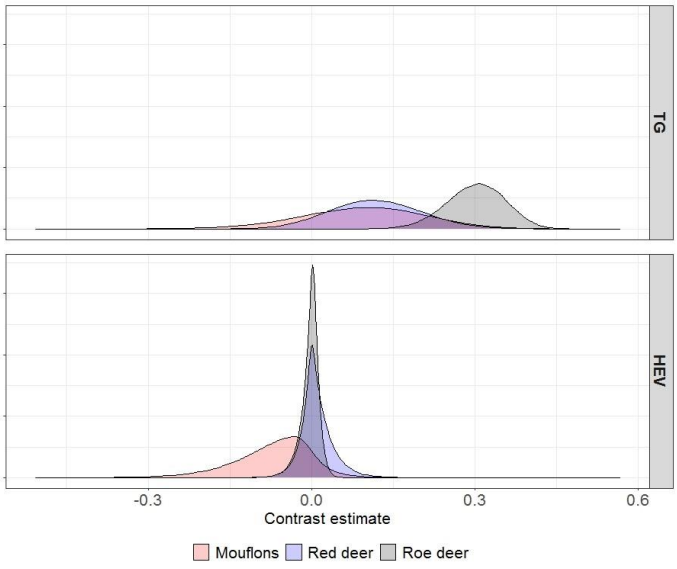

Figure 3. Density plots representing the comparison of the distribution of the contrast estimates for T. gondii and HEV seroprevalence in Pre-Alps on Alps by animal species. Contrasts represent the distributions of the differences between the seroprevalence distributions for Pre-alps on that of Alps, hence, differences greater than zero indicate higher seroprevalence values on Pre-alps.

Contrasts represent the distributions of the differences between the seroprevalence distributions for pre-Alps on that of Alps. The shape of contrasts distributions for all animal species for which contrasts

could be computed (i.e. mouflons, red deer and roe deer) indicate the seroprevalence of *T. gondii* is higher in samples from the pre-Alpine districts as compared to Alpine. When considering the seroprevalence in relation to HEV, the contrasts distributions for roe deer is about centred to zero indicating the difference between the uncertainty distributions describing the seroprevalence in the two areas is not substantial. On the other hand, mouflons in pre-Alps seems to be less likely to be seropositive as compared to those in the alpine environment while red deer seems to be slightly more likely to be seropositive in pre-Alpine districts.

The Dirichlet distributions describing the probability of an animal being negative (PNEG), seropositive to *T. gondii* only (PTG), *HEV* only (PHEV) and both *T. gondii* and *HEV* (PTGHEV) are reported by animal species and area (exception made for wild boars and roe deer as samples were from one area only) in Table 3.

		pre-Alpine		**Alpine**		**pre-Alpine + Alpine**	
Species	**Outcome**	**Median**	**(5th-95th perc.)**	**Median**	**(5th-95th perc.)**	**Median**	**(5th-95th perc.)**
Wild boar	PNEG	0.44	0.39-0.48				
Wild boar	PTG	0.41	0.36-0.45				
Wild boar	PHEV	0.06	0.04-0.09				
Wild boar	PTGHEV	0.09	0.07-0.12				
Roe deer	PNEG	0.47	0.41-0.54	0.81	0.73-0.88	0.58	0.52-0.63
Roe deer	PTG	0.51	0.44-0.57	0.16	0.09-0.24	0.41	0.36-0.46
Roe deer	PHEV	0	0-0.02	0.01	0-0.04	0	0-0.01
Roe deer	PTGHEV	0.01	0-0.03	0.01	0-0.04	0.01	0-0.02
Red deer	PNEG	0.69	0.56-0.79	0.81	0.72-0.88	0.78	0.71-0.84
Red deer	PTG	0.26	0.16-0.39	0.16	0.09-0.24	0.2	0.14-0.27
Red deer	PHEV	0.02	0-0.07	0.01	0-0.05	0.01	0-0.03
Red deer	PTGHEV	0.02	0-0.07	0.01	0-0.05	0.01	0-0.03
Mouflons	PNEG	0.67	0.53-0.79	0.69	0.51-0.83	0.71	0.60-0.80
Mouflons	PTG	0.27	0.16-0.41	0.17	0.07-0.33	0.24	0.15-0.34
Mouflons	PHEV	0.02	0-0.08	0.08	0.02-0.21	0.03	0.01-0.09
Mouflons	PTGHEV	0.02	0-0.08	0.03	0-0.13	0.01	0-0.05
Chamois	PNEG			0.89	0.84-0.94		
Chamois	PTG			0.04	0.01-0.08		
Chamois	PHEV			0.05	0.02-0.09		
Chamois	PTGHEV			0.02	0-0.05		

Table 3. Location parameters (Median, 5th and 95th percentiles) of the distributions describing the uncertainty in the seroprevalence estimates obtained by parameterising the Dirichlet-multinomial distributions with the number of negative to both T. gondii and HEV, positive to t. gondii only, positive to HEV only and positive to T. gondii and HEV. Results for the different animals in relation to T. gondii and HEV are presented by area. PNEG=Seronegative; PTG= seropositive to T. gondii; PHEV= seropositive to HEV and PTGHEV= seropositive to both T. gondii and HEV.

Contrast between simulated values for seroprevalence estimates in Pre-Alpine and Alpine districts are outlined in Figure 4.

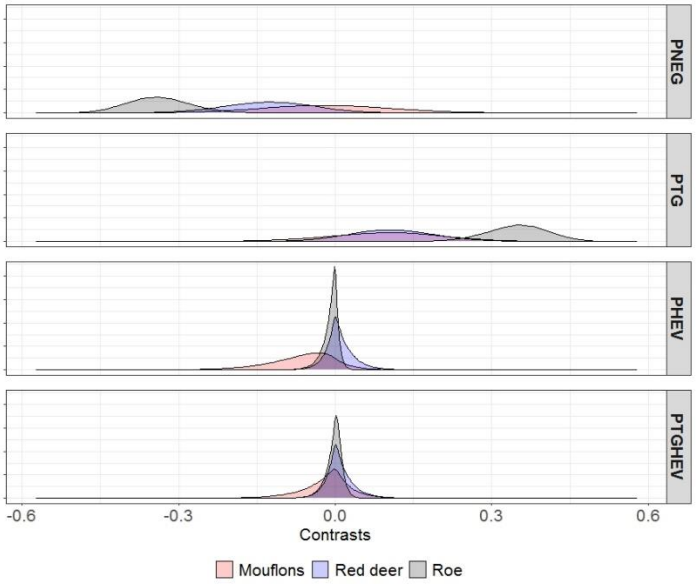

Figure 4. Density plots representing the comparison of the distribution of the contrast estimates for all the simulated serostatus included as possible outcome in the Dirichlet-Multinomial distribution: PNEG=Seronegative; PTG= seropositive to T. gondii; PHEV= seropositive to HEV and PTGHEV= seropositive to both T. gondii and HEV. Contrasts represent the distributions of the differences between the seroprevalence distributions for Pre-alps on that of Alps, hence, differences greater than zero indicate higher seroprevalence values on Pre-alps.

Although based on slightly less animals due to unavailability of paired *T. gondii* and HEV data, contrasts between the simulated seroprevalence status in Pre-Alps and Alps when considering the outcomes PTG and PHEV in the Dirichlet-Multinomial process shown the same trends described for the single-pathogen seroprevalence estimates (Figure 4). Contrasts in relation to the probability of co-exposure (PTGHEV) are all centred and symmetrical to zero indicating the distributions describing the uncertainty in the probability of being seropositive to both the pathogens do not differ by area. The location parameters (Median, 5th and 95th percentiles) of the uncertainty distributions describing the actual presence of *T. gondii* and HEV in wild boars given seroprevalence data (Equation 5-7) are presented in Table 4.

	Prevalence Median (5th-95th perc)	**Seroprevalence Median (5th-95th perc)**
P(TG)	0.37 (0.34-0.40)	0.41 (0.36-0.45)
P(HEV)	0.09 (0.08-0.1)	0.6 (0.04-0.09)
P(TGHEV)	0.03 (0.028-0.04)	0.09 (0.07-0.12)

Table 4. Estimated probabilities for the presence of HEV and T. gondii in wild boars given seroprevalence data. P(TG)=probability of T. gondii being present, P(HEV)= Probability of HEV being present, P(TGHEV) probability of both T. gondii and HEV being present. For easier comparison, seroprevalence data are also reported in last column.

Results shown how the probability of the pathogen being actually present in wild boars is lower than the seroprevalence when considering *T. gondii* but slightly higher when considering HEV. This can be explained by the combined effect of a high proportion of HEV seronegative animals in the wild boars

279 population and $P(INF|SERA^{-})$, the expected probability of HEV being present in seronegative
280 animals.

281 **3. DISCUSSION**

282 Serological results of this study suggest circulation of HEV and *T. gondii* in both the study area with
283 substantial differences being observed between animal species and between the Alpine and pre-
284 Alpine environments for the species where results could be compared by area (i.e. roe deer, red deer
285 and mouflons).

286 Considering *T. gondii*, wild boars and roe deer shown the highest (and very similar) seroprevalence
287 estimates followed by red deer and mouflons. All the seroprevalence estimates for the different
288 animal species were higher in pre-Alpine districts when compared to the Alpine ones, here, the
289 observed low seroprevalence estimates are consistent with previous evidence within the same
290 mountain range (Gaffuri et al., 2006) and other Alpine districts (Formenti et al., 2016). Although not
291 explored as part of this study, it can be hypothesized that higher seroprevalence estimates in the pre-
292 Alpine environment could be explained by vicinity of human activities and livestock and hence, greater
293 opportunities for exposure to oocyst-contaminated feed, water, or direct/indirect contact with
294 domestic animals species (i.e. cats). Of particular interest when considering seroprevalence data of *T.*
295 *gondii* is the seroprevalence in roe deer (0.46) and wild boars (0.49) sharing the same pre-Alpine
296 environment. Although the prevalence of *T. gondii* infection is often lower in herbivores than in
297 omnivores and carnivores (Ferroglio et al., 2014), the seroprevalence estimates described in this study
298 suggest a widespread exposure to the parasite across all animal species in pre-Alps. The
299 seroprevalence point-estimate values for mouflons and red deer are lower but also very similar, 0.29
300 and 0.28 respectively. Altogether, these results suggest that proper epidemiological studies would
301 help identify common sources of *T. gondii* exposure across animal species.

302 Higher detection rate of anti-HEV immunoglobulins in wild boar when compared to other animal
303 species in particular is not surprising considering this species is a well-known animal reservoir
304 (Fredriksson-Ahomaa, 2019). Although the uncertainty around the seroprevalence estimates is high,
305 some seropositive animals (chamois and mouflons) were found in the Alpine environment, confirming
306 circulation of HEV in remote areas. This is consistent with recent data on chamois, red deer and ibex
307 reported from other Alpine districts (Palombieri et al., 2020; Trogu et al., 2020). However, in spite of
308 red deer being considered as a true reservoir for HEV (Kukielka et al., 2016; Van der Poel, 2014), none
309 of the red deer analysed as part of this study shown evidence of exposure to HEV supporting in
310 principle the hypothesis of red deer being accidental hosts (Anheyer-Behmenburg et al., 2017).
311 Contrasts between uncertainty distributions shown higher probability for mouflons in Alps to be

seropositive as compared to those in pre-Alpine environment, however, this result should be considered cautiously considering the limited sample size and therefore, the influence that the only positive found in the alpine environment had on the shape of the uncertainty distribution.

Few animals shown evidence of co-exposure to *T. gondii* and HEV and although the simulated results of the multinomial process led to wide uncertainty distributions due to the low sample sizes, (especially when results are disaggregated by area), results of the contrasts seems to suggest the probability of co-exposure is not different by area for the considered animal species. However, in wild boars, evidence of co-exposure was observed for 30 (9%) samples representing 60% of the overall HEV-positive samples (N=50). Wild boars samples were only from the pre-Alpine environment and therefore comparisons by area could not be made; however, these results suggests a dedicated epidemiological study would again be useful to unveil possible common environmental risk factors explaining exposure to both HEV and *T. gondii* amongst wild boars.

While seroprevalence data are useful to understand circulation and exposure to pathogens in different areas (EFSA, 2017; Rostami et al., 2017), as mentioned in the introduction, these type of data do not directly translate into the presence/absence of the pathogen in seropositive and seronegative animals. Indeed, molecular identification of both *T. gondii* and HEV have shown contrasting results with respect to serological status (Anheyer-Behmenburg et al., 2017; Bachand et al., 2019; Dubey et al., 2020a, 2020b; Kozyra et al., 2020; Opsteegh et al., 2016). Considering it is precisely the presence/absence of the pathogens in meat that matters from a food safety perspective, we have made available as part of this study, a probabilistic approach that allows estimating the probability of the pathogen(s) being actually present based on seroprevalence data.

The method, based on a set of conditional probabilities, requires prior knowledge of the key parameters: $P(INF|SERA^{+})$, and $P(INF|SERA^{-})$, the conditional probabilities of seropositive and seronegative animals being infected. This relationship, particularly if considering HEV where the risk for the meat to be contaminated (and thus posing an actual risk to consumers and handlers) is mainly posed by animals at viraemic phase (Crotta et al., 2021), is likely to be species-specific and yet to be elucidated for the animal species considered in this study. Hence, for using of seroprevalence data to inform risk assessment models, further studies investigating the occurrence of HEV and *T. gondii* in seropositive and seronegative animals are strongly encouraged to provide the evidence needed.

From the worked example on wild boars presented in this study, it is clear how the availability of this evidence is important, particularly for the pathogens where the seroprevalence in the population is low, the probability of the pathogen being present in seronegative animals has a large effect. The method proposed here can therefore be considered as the first step towards practical use of

seroprevalence data for quantitative risk assessment model aimed at estimating the risk of human exposure and co-exposure to meat borne pathogens from consumption and handling of game meat.

It should be noted that the methods presented in the online application as part of this study are extremely flexible and can be used for any pathogen/animal or even pathogen/food product combination. Indeed, the first section of the app simulates values of a Beta distribution describing the uncertainty in the probability of a success in a Binomial process, as such, it can be used to model the uncertainty around P for any given *n* (the number of samples) and *s* (the number of positive samples). The second, simulates values of a Dirichlet distribution, the Beta-equivalent for a Multinomial process, as such, it can be used to model the uncertainty in the occurrence of negative, positive to X, positive to Y and positive to XY pathogen, where X and Y can be any pathogen.

CONCLUSIONS

Our analyses shown evidence of exposure of *T. gondii* and HEV amongst animals within both the Alpine and pre-Alpine environments. Although the analyses rely on the serological results that cannot directly demonstrate an ongoing infection, the observed seroprevalence values suggest circulation of the pathogens also in the animal species typically living in remote areas. The proposed approach to estimate the prevalence of infection/co-infection from seroprevalence data maximises the use of the serological data that are often collected as part of wildlife health surveillance plans to inform risk assessment models.

Ethics. This research did not involve any purposeful killing of animals nor were animals shoot specifically for providing samples for this study. All biological samples analysed were from animals legally hunted during hunting seasons in accordance with the Italian Law (N. 157 of 11/02/1992) and Habitat Directive 92/43/EEC of 21 May 1992 as part of the Regional depopulation plans 2017 and 2018. Therefore, the analysis of biological samples did not required an additional approval of the ethics committee.

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