Presence of enteric bacterial pathogens in meat samples of wild boar hunted in Campania region, southern Italy

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Abstract

Wild boars can be infected with several foodborne pathogens which may be transmitted to humans through the consumption of their meat, but currently, data of their prevalence are still limited. The present study aimed to evaluate the presence of enteric pathogens in wild boar meat samples killed in the Campania region. Twenty-eight wild boar meat samples were analyzed for the detection of *Salmonella* spp, *Y. enterocolitica*, *Campylobacter* spp., and Shiga-Toxigenic *E. coli.*

Salmonella spp. was detected and isolated in ten samples and after serotyping *S.* Veneziana, *S.* Kasenyi, *S.* Coeln, *S.* Manhattan, *S.* Thompson, and *S.* Stanleyville were identified. Twenty-one meat samples were found to be contaminated with *Y. enterocolitica*; in 6 samples the *yst*A and *yst*B genes were detected simultaneously, while in 15 only the *yst*B gene, which characterizes the bacteria belonging to the biotype 1A, was present. Shiga-Toxin producing *E. coli* was detected in 12 while *Campylobacter* spp was never detected.

In conclusion, due to the high occurrence of pathogenic bacteria detected, the present research shows that wild boars are important reservoirs for foodborne zoonoses which may be transmitted to livestock and humans. This confirms the importance of controls throughout the wild boar supply chain. In the Campania region, checks are guaranteed by the Veterinarians who work within the "management and control plan for wild boar in the Campania region" which has the twofold objective of containing the increasingly invasive presence of this animal and guaranteeing greater safety, traceability, and transparency in the consumption of meat.

Introduction

Wild boars (*Sus scrofa*) are the most widely distributed large mammals in the world (Massei *et al.*, 2015) and they are present in all continents, except for Antarctica (Sales *et al.,* 2017). Wild boars are characterized by the highest reproductive rate among the ungulates and therefore in the latest years, the population is dramatically increased (Fredriksson-Ahomaa, 2019). Moreover, in recent years, they are introducing into a wide variety of habitats including urban and peri-urban areas (Bertelloni *et al.*, 2020). Economic interest for these animals is related to the damage of crops and husbandry and the possibility of transmitting the disease to livestock and humans (Massei *et al.,* 2015). The likely exchange of disease between wild boars, domestic animals, and humans raised increased interest also among researchers (Bonardi *et al*., 2019). In contrast to domesticated animals, wild boars roam free and their diet is uncontrolled, feeding of whatever is available, including live and dead animals (Fredriksson-Ahomaa, 2019). Therefore, the natural microbial population on the skin and in the digestive tract can significantly differ between the animals. Moreover, wild boars can be infected with several foodborne pathogens which may be transmitted to humans through the consumption of their meat. In the past, the wild boar meat was eaten only by hunters and their families but nowadays it is considered a healthy and delicious food and therefore it has drawn the attention of a wider range of consumers. Unfortunately, data on the consumption of wild boar meat at a European level are still limited. The meat of wild boars is mainly consumed cooked but though some wild boar meat products are not heat-treated but only dry-cured, cold smoked, and dried (Mirceta *et al.*, 2017).

In the latest years, the four most reported zoonotic agents during gastrointestinal infection in humans were *Campylobacter* (*C.*), *Salmonella* (*S.*), Shiga toxin-producing *E. coli* (STEC), and *Yersinia* (*Y.*) (EFSA, 2021).

In 2019, 220,682 and 87,923 confirmed cases of campylobacteriosis and salmonellosis in humans were reported, respectively (EFSA, 2021). The main environmental niche for *Campylobacter* is represented by the intestinal tract of all avian species but *Campylobacter* species colonize also different apparatuses of domestic (cattle and pigs) or wild animals (birds such as ducks and gulls) and can be transmitted to humans through the consumption of this raw or undercooked meat (Facciolà *et al*., 2017). Concerning salmonellosis, in 2019, pig meat and products thereof were one of the most implicated food vehicles in strong-evidence food-borne outbreaks (EFSA, 2021).

Escherichia coli is a commensal bacterium living in the intestines of animals and humans but some pathotypes can be responsible for gastrointestinal infections: i) enteropathogenic *E. coli* (EPEC), ii) STEC, or verocytotoxin-producing *E. coli* (VTEC), iii) enterohemorrhagic *E. coli* (EHEC), iv) enteroinvasive *E. coli* (EIEC), v) enteroaggregative E. coli (EAEC or EAggEC), vi) enterotoxigenic *E. coli* (ETEC), vii) diffusely adherent *E. coli* (DAEC), and viii) adherent invasive *E. coli* (AIEC) (Bertelloni *et al.,* 2020).

Compared to other foodborne bacteria very little data on the incidence of *Y. enterocolitica* in foods and animals are available

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from EU states probably because according to the Zoonoses Directive 2003/99/EC, reports of *Yersinia* occurrence or prevalence are not mandatory and even because current methods for the pathogen's isolation and detection proved to be undermined by several limitations (Cristiano *et al,* 2021; Peruzy *et al*., 2020). Strains of *Y. enterocolitica* can be classified into six biotypes 1A, 1B, 2, 3, 4, 5 and at least 57 different serotypes. The biotype 1A has been considered as not pathogenic, though in the latest years has been associated with yersiniosis cases in humans (Strydom *et al.*, 2019).

These enteric pathogens have been detected also in wild boar meat samples, but data are still limited. Therefore, the purpose of this work was to evaluate the prevalence of the most reported zoonotic agents, i.e. *Campylobacter*, *Salmonella*, *Yersinia enterocolitica,* and STEC in wild boar populations living in the Campania region.

Materials and methods

Sampling

From October 2019 to January 2020, meat samples of 28 wild boars (W1 to W28), 14 males (average weight: 53.57 Kg), and 14 females (average weight: 47.07 Kg), aged between 4 months and 7 years, were collected in Campania region, southern Italy (Table 1). The wild boars were hunted by official hunters by the "driving" technique. The animals were immediately bled in the field and brought to hunters' private houses where the evisceration and skinning were performed. Subsequently, approx. 100 cm^2 meat was aseptically cut out the forearm area (at least 100 g each) and individually placed in sterile stomacher blender bags. All samples were transported at 4°C to the laboratory and processed within one hour after sampling.

Detection of pathogenic bacteria

For the detection of relevant foodborne pathogens, 25 grams portions of each meat sample were homogenized separately i) in 225 ml (1:10 (W/W)) buffer peptone water (BPW, CM0509, Oxoid) and incubated at 37°C for 24-h for the detection of *Salmonella* (*S.*) and STEC, ii) in 225 ml *Campylobacter* Bolton Enrichment Broth Base (Bolton Broth, 401286B2, Biolife), supplemented with Bolton Broth Selective Supplement (4240025, Biolife) and Lysed horse blood (90HLX100, Biolife) and incu-

Table 1. Gender, age (years (Y)/months (M)) and weight (kg) of the shot animals, presence of foodborne bacteria genes detected using Real-Time PCR.

Animal ID	Sex	Age	Weight (Kg)	STEC				*Y. enterocolitica*		*Salmonella* spp.	
				Genes'detection from the broth		Genes' detection from isolated colonies					
				stx$_1$/*stx*$_2$	*ea*E	*stx*$_1$/*stx*$_2$	*ea*E	*yst*A	*yst*B	iQ-Check Real-Time PCR Kits	*Salmonella* (S.) serotypes
W1	F	4Y	75		D.				D.	D.	S.Thompson
W2	F	1Y	20	D.	D.		D.			D.	S.Coeln
W3	M	1Y	25		D.				D.		
W4	F	2Y	40		D.			D.	D.		
W5	F	1Y	30		D.				D.		
W6	F	3Y	75	D.	D.	D.	D.		D.	D.	S.Manhattan
W7	F	1Y	30	D.	D.		D.	D.	D.	D.	S.Veneziana
W8	F	1Y	35		D.			D.	D.		
W9	M	1Y	40		D.						
W10	M	1Y	30	D.	D.		D.		D.	D.	S.Thompson
W11	F	1Y	30	D.	D.	D.	D.		D.	D.	S.Kasenyi
W12	F	2Y	64	D.	D.		D.		D.	D.	S.Kasenyi
W13	M	6Y	100		D.				D.		
W14	M	7Y	75	D.	D.		D.		D.		
W15	F	3Y	50		D.						
W16	F	3Y	50								
W17	M	3Y	70		D.						
W18	M	3Y	70		D.			D.	D.		
W19	F	2Y	70		D.					D.	S.Kasenyi
W20	F	4Y	70	D.	D.	D.					
W21	M	2Y	75	D.	D.			D.	D.	D.	S.Stanleyville
W22	M	3Y	80	D.	D.	D.	D.	D.	D.		
W23	M	2Y	50		D.				D.		
W24	M	1Y	30		D.				D.	D.	S.Kasenyi
W25	M	1Y	30	D.	D.				D.		
W26	M	7M	40	D.	D.		D.		D.		
W27	F	4M	20		D.				D.		
W28	M	3Y	70		D.				D.		

D: Detected.

bated at 41.5°C in the microaerophilic atmosphere for 24-h for the detection of *Campylobacter* (*C.*), and iii) in 225 ml Peptone Sorbitol Bile Broth (PSB, 17192, Sigma-Aldrich) and incubated at 25°C for 24-h for the detection of *Yersinia* (*Y.*) *enterocolitica*. From each enrichment broth, the "iQ-Check Real-Time PCR Kits" were used for the detection of *Salmonella* (BR3578123, Bio-Rad, Hercules, CA, USA), STEC (BR3578139, Bio-Rad, Hercules, CA, USA), and *Campylobacter* (BR3578135, Bio-Rad, Hercules, CA, USA), following manufacturer's recommendations. Concerning STEC, the "iQ-Check Real-Time PCR Kits", allowed the individual identification of the virulence genes stx_1 and stx_2 (Shiga toxin 1/2) and *eae* (intimin). For the detection of *Y. enterocolitica*, DNA was extracted from each enrichment broth using the Chelex-100-resin method (1422822, Bio-Rad, Hercules, CA, USA) whereby two ml of each incubated homogenate was transferred into a two ml centrifuge tube and centrifuged for 10 min at 10,000×*g* at 4°C. The supernatant was discarded, the pellet re-suspended in 300 µl of 6% Chelex 100 by vortexing, and incubated for 20 min at 56°C and again for 8 min at 100°C. The suspension was immediately chilled on ice for 1 min and centrifuged for 5 min at 10,000 ×g at 4°C. To evaluate the presence of *Y. enterocolitica* 4/O:3 and biotype 1A, a SYBR green PCR-assay was conducted, with the gene *ystA* as target for the pathogenic biotype (Peruzy *et al*., 2017). Therefore, 3 µl of DNA extract was added to 22 µl of PCR mix. The mastermix contained 12.5 µl of Qiagen QuantiTect SYBR Green PCR Kit (1x), 100 nM of both primers *yst*A-F (5′-ATCGACACCAATAACCGCTGAG-3′) and *yst*A-R (5′-CCAATCACTACT-GACTTCGGCT-3′). To evaluate the presence of the biotype 1A, the presence of the target gene *ystB* gene was examined (Peruzy et al., 2017). Three µl of DNA extract was added to 22 µl of PCR mastermix containing 12.5 µl of Qiagen QuantiTect SYBR Green PCR Kit (1x), 150 nM of both primers ystB-F (5′-GTA-CATTAGGCCAAGAGACG-3′) and ystB-R (5′-GCAACATACCTCACAACACC-3′). The fluorescence of SYBR Green and the melting curve was generated using the CFX96 system (Bio-Rad). A specific melting temperature (T_m) of 78.5±1°C indicated a positive result. While awaiting the Real-Time PCR (RT-PCR) results, the enrichment broths were stored at 4 °C. Real-Time PCR positive results for *Salmonella* spp., STEC, *Campylobacter,* or *Y. enterocolitica* were confirmed using the corresponding normalized microbiological isolation methods ISO 6579-1:2017, 13136-1:2012, 10272-1:2017 and 10273:2017. Concerning STEC, colony confirmation was performed by mean the "iQ-Check Real-Time PCR Kits".

Statistical analysis

To compare the bacterial counts, a one-way analysis of variance (ANOVA) was calculated using PAST software package (https://folk.uio.no/ohammer/past/).

Results and discussion

Wild boars are an important reservoir of zoonoses whose frequency may vary across populations living in different countries and even in different regions within the same country (Gill, 2007). Some of these can cause foodborne illness in humans, who may be infected by consuming contaminated wild boar meat. Among them, a good deal has an enteric origin, and the presence of them on the carcass surface is mainly the result of an improper evisceration (Sánchez-Rodríguez *et al*., 2018). In the present study, a total of 28 samples were analyzed for enteric pathogens' detection and except for *Campylobacter* all the other genes investigated were detected. The overall percentage of wild boars positive per at least one of the enteric pathogen genes tested was 96.43% (Table 1). No significant correlation was observed between genes detection and the gender, age, and weight of the animals.

Salmonella spp. was detected and isolated in 10 out of 28 enrichment broths analyzed (35.71%) and after serotyping six serovars were identified (*S.* Coeln, *S.* Kasenyi, *S.* Manhattan, *S.* Stanleyville, *S.* Thompson, and *S.* Veneziana). The occurrence of *Salmonella* is comparable to that we observed previously in wild boars hunted in the Campania region in 2017 (31.82%, Peruzy *et al.*, 2019) but it was higher than those observed by Atanassova *et al.,* (2008) in Germany, Peter Paulsen & Winkelmayer, (2004) in Austria, Mirceta et al., (2017) in Serbia, and Wacheck *et al*., (2010) in Switzerland. Considering the serovars, *S.* Kasenyi was the most frequently isolated (W11, W12, W19, and W24). *S.* Kanseny is frequently isolated in the wild boar population living in the Campania region (La Tela *et al*., 2021; Peruzy *et al*., 2019) but, to our knowledge, it has not been reported in any other wild boar living in other regions or other countries. Differently, *S.* Thompson detected in the present study in two animals (W1 and W10) is widespread in wild boar population and several other animal species and it has been associated with a severe human outbreak in the Netherlands in 2012 (Stella *et al.*, 2018). *S.* Veneziana, *S.* Stanleyville, *S.* Manhattan, and *S.* Coeln detected in the present work have been previously isolated in wild boars (Bonardi *et al*., 2019; Razzuoli *et al.*, 2021) and *S.* Coeln belong to the 20 most frequent serovars isolated in human cases in Europe (EFSA, 2021).

In the present study, *Y. enterocolitica* and STEC were detected in a high number of enrichment broths. Concerning *Y. enterocolitica,* six enrichment broths (21.43 %) carried both *yst*A and *yst*B genes, whilst in fifteen (53.57 %) only the gene *yst*B, used as a target for Biotype 1A (Peruzy *et al*., 2017), was detected. However, the pathogen was only isolated from one sample (W18). Pigs are considered the major reservoir where the bacterium has been commonly isolated from the tonsils and gastrointestinal tract and the contamination of the meat result from improper slaughter and evisceration techniques (Fredriksson-ahomaa *et al.,* 2006). Results of the present work confirm that wild boars could be considered an underestimated reservoir of *Y. enterocolitica,* as well (Morka *et al.*, 2018).

Concerning STEC, except for the animal W16 the gene *eae* was detected in all enrichment broths analysed. Genes stx_1 and stx_2 were always detected together. Twelve enrichment broths (42.86%) were positive for both stx_1/stx_2 and *eae* genes, whilst in fifteen enrichment broths (53.57%) only the gene *eae* was detected. Only in four and nine strains the genes stx_1/stx_2 and *eae*, respectively, were also detected from the colony's growth on the agar plates.

The gene *eae* responsible for the typical attaching and effacing lesions was used as a target for the detection of Enteropathogenic *E. coli* (EPEC), which are defined as intimin (*eae*)-containing diarrheagenic *E. coli* that do not possess the *stx* genes (Alonso *et al*., 2017). Wild boars acting as a carrier of EPEC have been previously demonstrated by Szczerba-Turek *et al.*, (2019) who detected them in 30.9% of the animals and other Spanish and Italian studies but a lower occurrence was reported (Spain = 3.3%, Alonso et al., 2017; Italy = 3.4%, Bertelloni *et al.,* 2020).

Moreover, based on results, wild boars act also as carriers of Enterohaemorrhagic *E. coli* (EHEC). In particular, the co-carriage of both *stx* and *eae* genes indicates the presence of EHEC which constitutes a subset of serotypes of STEC, (Kobayashi *et al.*, 2003). EHEC responsible for both Shigatoxins production and attaching and effacing lesions is associated with severe forms of human disease (Soare *et al.*, 2021). To our knowledge, lim-

ited data are available on the occurrence of EHEC in wild boars, however, the results of the present work contrast with those of Bertelloni *et al.* (2020) which reported a lower occurrence of EHEC (6.3%).

In the present work *Campylobacter*, the most reported gastrointestinal bacterial pathogen in humans in the European Union (EU) (EFSA, 2021), was never detected. A low occurrence was demonstrated also by Fredriksson-Ahomaa *et al*., (2020, 5%), and Atanassova *et al.*, (2008, 2.1%), whilst a higher occurrence was observed by Stella *et al.,* (2018, 16.7%).

Conclusions

In conclusion, due to the high occurrence of pathogenic bacteria detected, the present research shows that wild boars are important reservoirs for foodborne zoonoses. Therefore, based on results consumption of undercooked wild boar meat could pose a public health risk. Moreover, the spread of pathogenic bacteria in the natural environment constitutes a potential hazard also for domestic animals. In the Campania region, checks are guaranteed by the Veterinarians who work within the "management and control plan for wild boar in the Campania region" which has the twofold objective of containing the increasingly invasive presence of this animal and guaranteeing greater safety, traceability and transparency in the consumption of meat.

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