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Short communication:
Prevalence of IgG against hepatitis E virus, *Salmonella* spp., and *Toxoplasma gondii* in meat juice samples from wild boars hunted in Southern Italy

Kurzmitteilung:
Prävalenz von IgG gegen den Hepatitis E-Virus, Salmonella spp. und Toxoplasma gondii in Fleischsaftproben von in Süditalien gejagten Wildschweinen

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Summary

Free-ranging wild boars can represent a reservoir for zoonotic foodborne pathogens. They pose a threat to public health not only under food safety aspects through consumption of undercooked game meat, but also on an epidemiological level through transmission of diseases from wildlife to livestock. The aim of this study was to investigate the prevalence of immunoglobulin G (IgG) against hepatitis E virus (HEV), *Salmonella* spp., and *Toxoplasma (T.) gondii* in meat juices samples of wild boars in Southern Italy using commercial ELISA assays. We detected IgG prevalence rates of 58 % for HEV (tested with Priocheck® HEV Ab porcine), 65 % (tested with Salmotype® pig screen) and 7 % (tested with Priocheck® Salmonella Ab porcine) for *Salmonella* spp., and 14 % for *T. gondii* (tested with Priocheck® Toxoplasma Ab porcine). The high prevalence data in muscle juice samples underline the impact of wild boars as an important reservoir for zoonotic agents also in Southern Italy.

Keywords: wild boars, foodborne pathogens, ELISA, meat juice

Zusammenfassung

Wildschweine können ein Reservoir für Zoonoseerreger darstellen. Dies ist einerseits lebensmittelhygienisch beim Verzehr von nicht vollständig durchgegartem Wildbret, wie aber auch epidemiologisch, vor dem Hintergrund einer möglichen Übertragung von Erregern aus der Wildschweinpopulation in die Hausschweinepopulation bedeutend. Das Ziel dieser Studie war es, die Prävalenz von Immunglobulin G (IgG) gegen Hepatitis E-Virus (HEV), *Salmonella* spp. und *Toxoplasma (T.) gondii* im Fleischsaft von Wildschweinen aus Süditalien mittles kommerzieller ELISA-Tests zu untersuchen. Dabei wurden Seroprävalenzen von 58 % für HEV (getestet mit HEV PrioCHECK® Ab porcine), 65 % bzw. 7 % für Salmonellen (getestet mit Salmotype® pig screen; Priocheck® Salmonella Ab porcine) und 14 % für *T. gondii* (getestet mit PrioCHECK® Toxoplasma Ab porcine) gefunden. Die hohen Seroprävalenzdaten unterstreichen die Bedeutung der Wildschweine auch in Süditalien als wichtiges Reservoir für Zoonoseerreger.

Schlüsselwörter: Wildschwein, Lebensmittel assoziierte Pathogene, ELISA, Fleischsaft

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Introduction

Wild boars can act as important reservoirs for foodborne pathogens (Wacheck et al., 2010) including hepatitis E virus (HEV), *Salmonella* spp., and *Toxoplasma gondii (T. gondii)*. Zoonotic HEV has become a great concern in industrialized countries, where non-travel related cases of food-borne transmission due to undercooked pork and wild boar meat have been documented (Matsuda et al., 2003; Banks et al., 2004; Masuda et al., 2005; de Deus et al., 2008; Garbuglia et al., 2013). These sporadic cases are mainly caused by HEV genotype 3 and 4 (Emerson and Purcell, 2003). Hepatitis remains a common waterborne disease in developing countries where genotype 1 and 2 are often spread via fecal-oral transmission among humans.

Salmonella are worldwide among the leading causes of foodborne outbreaks, being responsible in 2012 for 91,034 confirmed cases in Europe (notification rate 22.2 cases per 100,000 population) (Anonymous, 2014). Wild pigs represent a common reservoir for *Salmonellae* as shown by Wacheck et al. (2010), who reported a cultural detection rate of 12 % among Swiss hunted boars. Moreover, according to data reported by EU member states in the context of the Zoonoses Directive (2003/99/EC) 2004–2011, 18 % of wild boar fecal samples tested positive for this zoonotic pathogen (Anonymous, 2013).

Toxoplasmosis is a parasitic infection caused by the protozoan *T. gondii.* While the parasite can infect warm-blooded animals that carry tissue cysts, domestic and wild cats are the only host fecally spreading an environmentally resistant form (oocysts). Humans can become infected through consumption of undercooked meat containing intermediate tissue cysts or water/food contaminated with oocysts from *Felidae* feces or through congenital infection in pregnant women. While toxoplasmosis presents with mild symptoms and is generally self-limiting in immune-competent people, complications in immune-compromised individuals are possible (Tenter et al., 2000; Dubey et al., 2010). *T. gondii* is common in wild boar in Europe, with seroprevalences ranging between 8 % and 38 %, and its importance as a risk for human health has been highlighted by EFSA's opinions on modernization of meat inspection in farmed wild boars (Anonymous, 2013).

Zoonotic foodborne pathogens are an important matter of concern in view of public health and therefore, the aim of this study was to investigate the prevalence of antibodies against HEV, *Salmonella* spp., and *T. gondii* in hunted boars by means of commercial ELISA assays intended for meat juice.

Material and Methods

Sampling. During two subsequent hunting seasons (September to December 2012 and September to November 2013), state gamekeepers and hunters collected a total of 200 diaphragm muscle samples from wild boars *(Sus scrofa)*. Most hunted animals were aged over 20 months and male. For each animal, location of hunting was recorded. Samples originated from three neighbouring regions in Southern Italy: Calabria (no. 137), Campania (no. 13), and Basilicata (no. 50). After the chest cavity had been opened, diaphragm tissue (approximately 10 g per sample) was collected under sterile conditions, placed into sterile bags, and sent to the laboratory where samples were stored at –20 °C until processing. To obtain meat juice, muscles were thawed and squeezed manually. Depending on sample size, up to 2,000 µl were obtained and kept at –20 °C until testing.

Detection of antibodies against HEV. Meat juices were tested for anti-HEV immunoglobulin G (IgG) using Priocheck® HEV Ab porcine test (Prionics, Schlieren, CH), a commercial ELISA kit (sensitivity 91 %; specificity 94 %). Briefly, 10 µl of samples were diluted in 90 µl of dilution buffer and brought onto a test plate coated with recombinant HEV antigen of ORF2 and ORF3 of the genotypes 1 and 3. Positive, negative, and cutoff controls were included in each run. After incubation for 60 min at 37 °C, microwell plates were washed four times with 300 µl of washing fluid and 100 µl of conjugate were added. Conjugate incubation (30 min at 37 °C) was followed by washing and addition of 100 µl of chromogenic (TMB) substrate. Reaction was stopped after 30 min at room temperature by adding 100 µl of stop solution. Color development was measured at 450 nm (reference filter at 620 nm) (Tecan Group Ltd., Männedorf, CH). Interpretation of results followed manufacturer's instructions. Thus, samples with an optical density (OD_{450}) above or equal to the cutoff value were considered positive. Samples with ambiguous results were retested.

Detection of antibodies against Salmonella. Anti-*Salmonella* IgG were detected using SALMOTYPE® Pig Screen ELISA test (sensitivity 78 %; specificity 99 %) (Labor Diagnostik Leipzig, DE). Following manufacturer's instruction, 10 µl of meat juice were diluted in 90 µl of dilution buffer, brought onto a coated microwell plate (LPS O-antigens 1, 4, 5, 6, 7, and 12) and incubated for 60 min at room temperature. A positive and a negative control were included in each run. After washing three times with 300 µl of washing solution, we added 100 µl of anti-IgG HRP conjugate to each well. After an incubation of 30 minutes at room temperature, the plate was washed and 100 µl of TMB substrate were added. The reaction was halted with 100 µl of stop solution after 10 minutes at room temperature. The OD_{450} was measured immediately (Tecan Group Ltd). Interpretation of results followed the manufacturer's instruction: samples with an OD_{450} percentage value above or equal to 20 were considered positive, values between 10 and 20 were considered ambiguous and the respective samples were retested.

All samples were also tested using a second ELISA kit (Priocheck® Salmonella Ab porcine, no data about sensitivity and specificity available (Prionics AG) according to manufacturer's instructions. Briefly, 50 µl of juice were diluted in 100 µl of dilution buffer. Following 1 minute-incubation, 10 µl were transferred to the coated test plate (LPS O-antigens 1, 4, 5, 6, 7, 12) containing 90 µl of dilution buffer. A positive, a negative, and a validation control were added in each run. Test plate was incubated for 90 minutes at room temperature. Conjugate addition (100 µl of conjugate solution) followed 300 µl-washing for 6 times. The plate was then incubated for 60 minutes at room temperature and washed 6 times. Subsequently, 100 µl of chromogenic substrate (TMB) were dispensed to the microwell plate and the reaction was stopped after 15 min incubation at room temperature. OD_{450} was measured (Tecan Group Ltd) and results were expressed as per-

cent positivity (PP). PP values equal or above 40 % were considered positive.

Detection of antibodies against T. gondii. Antibodies against *T. gondii* were determined using Priocheck® Toxoplasma Ab porcine ELISA kit (sensitivity 98.0 %; specificity 99.6 %) (Prionics AG) following the manufacturer's instruction. Briefly, 20 µl of meat juices were pre-diluted in 180 µl of diluent and then 100 µl were transferred to the coated test plate (cell culture derived tachyzoite antigen). A positive, a weak positive, and a negative control were used in each run. After an incubation time of 60 minutes at room temperature, the plate was washed four times with 300 µl of washing fluid and the conjugate (100 µl) was added. Conjugate incubation (60 min at room temperature) was followed by washing and addition of the chromogenic substrate (TMB). The reaction was stopped after 15 min at room temperature by adding 100 µl of stop solution. The OD_{450} was measured (Tecan Group Ltd) with a 620 nm reference filter. Results were expressed as percent positivity (PP). Values obtained above or equal to the cutoff of 15 PP were considered positive.

Statistical analysis. Hypothesized risk factor (hunting regions) association with seropositivity was computed: chi-squared analyses were used for this purpose (significance level for p <0.05) (GraphPad Software, Inc., La Jolla, USA).

TABLE 1: *Detection rates of anti-hepatitis E virus, anti-Salmonella and anti-Toxoplasma gondii antibodies (IgG) in individual meat juice samples from wild boars hunted in three regions of southern Italy using different ELISA assays.*

	Priocheck® HEV Ab porcine		Salmotype® pig screen		Priocheck® Salmonella Ab porcine	Priocheck® Toxoplasma Ab porcine
	Positive	Ambiguous[1])	Positive	Ambiguous[1])	Positive	Positive
Calabria (n = 137)	82 (60 %)	0 (0)	93 (68 %)	10 (7 %)	4 (3 %)	17 (12 %)
Campania (n = 13)	8 (62 %)	0 (0)	8 (62 %)	1 (8 %)	0 (0)	3 (23 %)
Basilicata (n = 50)	25 (50 %)	0 (0)	29 (58 %)	10 (20 %)	10 (20 %)	8 (16 %)
Total (n = 200)	115 (58 %)	0 (0)	130 (65 %)	21 (11 %)	14 (7 %)	28 (14 %)

[1]): Only meat juice samples are included that remained ambiguous after retesting.

Results and Discussion

Using Priocheck® HEV Ab porcine, 115 out of 200 individual meat juice samples (58 %) collected from hunted wild boars were tested positive for the presence of anti-HEV IgG (Table 1). The overall high prevalence of anti-HEV IgG in the animal population tested suggests that the virus is endemic in Southern Italy and highlights the role that these animals may play in transmission of HEV to susceptible animals such as domestic pigs. However, the occurrence of anti-HEV antibodies seems to be not equally distributed over the Italian territory. Martinelli et al., (2013) reported a detection rate of 10% for Central/Northern Italy.

In Europe, for wild boars different anti-HEV IgG detection rates have been documented. Seroprevalence of 44 % were shown for Poland (Larska et al., 2014), 30 % of seropositive boars were found in Germany (Adlhoch et al., 2009), 26 % in Spain (Boadella et al., 2012), 14 % in France (Carpentier et al., 2012), and 12 % in the Netherlands (Rutjies et al., 2010). We did not find statistically significant associations with hunting regions.

By screening for anti-*Salmonella* antibodies using SALMOTYPE® pig screen 130 out of 200 samples (65 %) tested positive (Table 1). By screening all meat juice samples with Priocheck® Salmonella Ab porcine a much lower prevalence, with only 14 out of 200 samples (7 %) testing positive, was found (Table 1). Discrepancies between these tests have already been demonstrated elsewhere (Farzan et al., 2007; Vico et al., 2010). In Italy, the Salmonella status of wildlife has been determined using cultural and serological approaches at different stages of infection. Zottola et al. (2013) reported a high seroprevalence of 67 % in wild boars hunted in the nearby Lazium region (Central Italy), although only 11 % of them were fecal shedders. Chiari et al. (2013) investigated the prevalence in northern Italy and detected a prevalence rate of 25 % as consequence using bacterial culture. Prevalence rates of 11 % and 12 % were found in Spanish boar sera (Closa-Sebastià et al., 2011) and cultured wild boar tonsils in Switzerland (Wacheck et al., 2010), respectively.

Screening for antibodies against *T. gondii* gave 28 positive samples (14 %) using Priocheck® *Toxoplasma* Ab porcine with no statistically significant associations with hunting regions. The prevalence rate measured in our study seems to be consistent with results from other Italian regions. For instance, antibodies against *Toxoplasma* have been detected in central Italy (Lazium region) with a rate of 14 %; sera were screened by Immunofluorescent Antibody Test (Ranucci et al. 2013). In Northwestern Italy (Piemonte region), a prevalence rate of 16 % was reported in skeletal muscle using molecular methods (Ferroglio et al., 2014).

Spain reported a high seroprevalence of 44 % using a modified agglutination test (Closa-Sebastià et al., 2011), compared to the lower seroprevalence of 23 % in France (Beral et al., 2012) and with the 7 % positive meat juice sampled in Switzerland (P-30-ELISA) (Berger-Schoch et al., 2011).

Conclusion

The seroprevalence data from muscle juice samples of 200 hunted animals in three different regions presented in this study underline the impact of wild boars as an important reservoir for zoonotic agents (HEV, *Salmonella* spp. and *T. gondii*) also in Southern Italy. A total of 5 % of the tested animals demonstrated multi-hazards exposition. Therefore, wild boars pose a threat to public health not only under food safety aspects through consumption of undercooked game meat, but also on an epidemiological level through transmission of pathogens from wildlife to livestock, especially when new interfaces are generated, for example through non-confinement breeding systems.

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