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Investigation of *Salmonella*, hepatitis E virus (HEV) and viral indicators of fecal contamination in four Italian pig slaughterhouses, 2021–2022

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ABSTRACT

In the pork production chain, the control at slaughterhouse aims to ensure safe food thanks to proper hygienic conditions during all steps of the slaughtering. *Salmonella* is one of the main foodborne pathogens in the EU causing a great number of human cases, and pigs also contribute to its spreading. Pig is the main reservoir of the zoonotic hepatitis E virus (HEV) that can be present in liver, bile, feces and even rarely in blood and muscle. The aim of this study was to assess the presence of both *Salmonella* and HEV in several points of the slaughtering chain, including pig trucks. Other viruses hosted in the gut flora of pigs and shed in feces were also assayed (porcine adenovirus PAdV, rotavirus, norovirus, and mammalian orthoreovirus MRV). Torque teno sus virus (TTSuV) present in both feces, liver and blood was also considered. Four Italian pig abattoirs were sampled in 12 critical points, 5 of which were the outer surface of carcasses before processing. HEV and rotavirus (RVA) were not detected. Norovirus was detected once. *Salmonella* was detected in two of the 4 abattoirs: in the two lairage pens, in the site of evisceration and on one carcass, indicating the presence of *Salmonella* if carcass is improper handled. The sampling sites positive for *Salmonella* were also positive for PAdV. MRV was detected in 10 swabs, from only two abattoirs, mainly in outer surface of carcasses. TTSuV was also detected in all abattoirs. Our study has revealed a diverse group of viruses, each serving as indicator of either fecal (NoV, RVA, PAdV, MRV) or blood contamination (TTSuV). TTSuV could be relevant as blood contamination indicators, crucial for viruses with a viremic stage, such as HEV. The simultaneous presence of PAdV with *Salmonella* is relevant, suggesting PAdV as a promising indicator for fecal contamination for both bacterial and viruses. In conclusion, even in the absence of HEV, the widespread presence of *Salmonella* at various points in the chain, underscores the need for vigilant monitoring and mitigation strategies which could be achieved by testing not only bacteria indicators as expected by current regulation, but also some viruses (PAdV, TTSuV, MRV) which could represent other sources of fecal contamination.

1. Introduction

Salmonellosis is the second major foodborne disease in the European Union with over 87,923 confirmed cases in 2019 (notification rate: 15.7 per 100.000 population) (EFSA and ECDC, 2022). The laying hen is the most important source of human salmonellosis (EFSA and ECDC, 2022), but in some European countries, including Italy, pigs are considered the second important largest contributors to the infection (Bonardi, 2017; Graziani et al., 2013). Pigs infected by *Salmonella* can either be symptomatic or healthy carrier that, if still infected at finishing, can drive the pathogen at point of sale (Bonardi, 2017). *Salmonella* contamination can occur at any stage of pork production, in which the slaughterhouse plays an important role (Zeng et al., 2021). Before slaughtering, the presence of shedder pigs in transport trucks or the lairage environment, can increase the probability of infected animals. As observed, the time spent in holding pen for occurrence of infections in naïve pigs is as little as 2 h (Hurd et al., 2001). During the slaughtering of pigs, through the contamination of carcasses with the intestinal content

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and the feces, a risk of *Salmonella* spreading also occurs (van Hoek et al., 2012) both directly and indirectly. In fact, the presence of *Salmonella* on a carcass can originate from the same animal, for improper evisceration, and from other pigs. Cross-contamination from animals slaughtered the same day and the permanent environmental presence of *Salmonella* in the slaughterhouse as source of contamination if not properly cleaned play a major role in the spreading of this pathogen, since *Salmonella* can persist for long time in the environment (Jensen et al., 2006) becoming part of the 'house flora' in the slaughterhouse (Smid et al., 2014). Furthermore, insufficient disinfection of the equipment and utensils used can lead to cross-contamination from one carcass to another (Swart et al., 2016).

In response to these critical issues, the process hygiene criterion (European Commision, 2014) established for *Salmonella* recognized the role of carcasses contamination (EFSA Panel, 2011) as possible via of *Salmonella* spreading to the consumers and as critical point to be constantly monitored (European Commision, 2014).

The zoonotic hepatitis E virus (HEV) is another important foodborne viral pathogen transmitted to humans through the consumption of contaminated undercooked pork food products and being responsible for several sporadic cases and small outbreaks (Chelli et al., 2021). In Europe, HEV infection is caused by consumption of raw or undercooked pork products, mainly containing liver (Colson et al., 2010). HEV belongs to the *Hepeviridae* family and is classified in the Paslahepevirus genus (Purdy et al., 2022). It is a quasi-enveloped single-strand ribonucleic acid (RNA) virus (Purdy et al., 2017), highly resistant in the environment. In fact, HEV is still infectious for up to 21 days at 37 °C and for 28 days at room temperature (Johne et al., 2016). The main reservoirs of the zoonotic genotypes HEV-3 and HEV-4 are domestic pigs and wild boars (Prpic et al., 2015). Since 1997, when the first autochthonous human cases in industrialized countries were described, HEV-3 has been causing sporadic cases in human patients in Europe (Kamar et al., 2014; Sayed et al., 2015). Recently, between 2005 and 2015, an increasing number of HEV-3 cases of hepatitis E have been reported in European countries (Ricci et al., 2017). The HEV-4 genotype is also zoonotic, being considered an Asiatic genotype (Lu et al., 2006), with sporadic detections also in Europe (Colson et al., 2012). The environment of farms and abattoirs plays a crucial role in the diffusion of HEV to pigs, with the same dynamics involving the fecal contamination described above for *Salmonella*, together with the infected offal manipulation (Jensen et al., 2006; van Hoek et al., 2012; Meester et al., 2021). Infected pigs at slaughter represent then the main zoonotic transmission route of HEV to humans through the consumption of contaminated pork products (Colson et al., 2010; Colson et al., 2012).

The pig slaughter involves several stages, from farm, from transportation to unloading animals at the abattoir to obtaining the final meat product. For this reason, the occurrence of cross- and direct microbial contamination between pig carcasses in the industrial process can likely occur (Swanenburg et al., 2001). The evaluation of different types and levels of microorganisms on the carcass surfaces at the slaughtering stages represents a major point to prevent, reduce, or eliminate microbiological hazards (Bolton et al., 2013).

Similarly to *Salmonella*, the introduction of bacterial or viral pathogens into the abattoirs can lead to contamination at different processing steps over the slaughter line. The initial opening of carcasses, the removal of intestines, gallbladder, and tonsils but also improper disinfection of cutting knives or machinery for bleeding can lead to microbial spreading between carcasses. However, the role of each step in contributing to the risk is significantly variable between pathogens and particularly between viruses (HEV) and bacteria. As hygienic indicators, bacteria such as aerobic bacterial colony counts (ACCs) and *Escherichia coli* colony counts (ECCs) are commonly used but may not be adequate at predicting virus contamination. Some viruses are common in pigs, such as porcine adenovirus (PAdV) in feces and torque teno virus (TTV) in blood and could be useful as indicator of swine fecal (Dos Santos et al., 2023) and blood contamination (TTSuV) (Brassard et al., 2008). Porcine adenovirus, a double strand DNA virus, is also frequently present in asymptomatic pigs at slaughter and released in feces (Di Bartolo et al., 2012). Swine TTV, small virus with a DNA circular genome, can be detected in serum, feces, and other organs in pigs (Blois et al., 2014). Nevertheless, many other viruses with the ability to spillover to humans (rotavirus, norovirus, and mammalian orthoreovirus) could also be present in pigs and evaluating their presence in animals before entering the food chain could be useful for monitoring the introduction of viruses in the food chain.

The main aim of this study was to conduct a comprehensive investigation across four Italian slaughterhouses, focusing on the possible contamination of the abattoir environment mainly due to the difficult management of evisceration and slaughtering practices. The main focus of this study was the detection of *Salmonella* and hepatitis E virus (HEV) by swabbing key points of the abattoirs along the slaughter line, including scalding, dehairing, singeing, polishing, trimming, and washing stations, as well as carcasses. Additionally, the examination of a selected panel of viruses which may serve as indicators of fecal cross-contamination was also conducted. This panel included porcine adenovirus (PAdV) and torque teno sus virus (TTSuV). Furthermore, we probed for the presence of other viruses, such as mammalian orthoreovirus (MRV), group A rotavirus (RVA), and norovirus (NoV), considering their potential zoonotic implications and varying impacts on pig health.

2. Materials and methods

2.1. Slaughterhouses enrollment

Four slaughterhouses (named as A, B, C and D) were enrolled in this study, 2 located in Northern Italy and 2 in Central Italy, between July 2021 and October 2022. Slaughterhouses were categorized as "Large" and "Small" in terms of number of pigs slaughtered per week.

"Large" slaughterhouses (B and C; ≥20.000 pigs/week) were sampled in Northern Italy, where the age of slaughter of pig is at least 9 months and 180 kg of body live weight. "Small" slaughterhouses (A and D; ≤6.000 pigs/week) were located in Central Italy, where also younger animals (7–8 months of age) are slaughtered.

2.2. Sampling scheme

Sampling was performed during the swine slaughter chain under the supervision of the official veterinarians. In one empty holding lairage pen from each abattoir, samples were collected after cleaning. The environmental sampling was performed by using sterile foam sponges (Hygiena, CA, USA) and sampling locations were selected to be the most likely for the presence of *Salmonella* (Hill et al., 2016) and other pathogens released by pigs through feces. Each sampling site was swabbed twice, to subsequently detect viruses and bacteria.

Trucks for transport of pigs, after cleaning, were sampled onto the cargo compartment in three different points randomly selected on floor, by using the same sponge.

The same scheme was followed in each plant also including floors under evisceration site, working surfaces, knifes and swine carcasses (Table 1).

On every sampling location, approximately 100 cm^2 area was swabbed. Each carcass surface was sampled before chilling or processing, along the tummy extending to the jowl area. Both sides of the carcass were sponged (European Commission, 2005) and samples were obtained by pooling 3 tools from 3 different carcasses. This sampling scheme was used to increase the probability of detect *Salmonella* for the expected low number of positive carcasses, as previously described (Baptista et al., 2010).

For each slaughterhouse ($n = 4$), 12 samples were planned, for a total of 48 samples. Due to logistic hindrance during the slaughter chain, 3 samples were not collected, resulting in a total of 45 samples available

Table 1
Salmonella detection and viral molecular analysis results on swab samples; np = not performed (sample not available).

Slaughterhouse	Sample ID	Sampling site	*Salmonella*	HEV	TTSuV	PAdV	RVA	NoV GII	MRV	no. of microorganisms detected
A	1	Truck's floor after washing	np	np	np	np	np	np	np	
Central Italy 6000 pigs slaughtered per week	2	Lairage pens (floor) after cleaning	+	−	−	+	−	−	−	2
	3	Scalding water tank caroussel arm	−	−	+	−	−	−	−	1
	4	Upper surface at 2-knives system tank	−	−	−	+	−	−	−	1
	5	Carcass splitter saw surface	−	−	−	−	−	−	−	0
	6	Evisceration knife	−	−	+	−	−	−	+	2
	7	Evisceration step (floor)	+	−	+	+	−	−	−	3
	8	Outer surface of 3 carcasses after evisceration	−	−	−	+	−	−	+	2
	9	Outer surface of 3 carcasses after evisceration	−	−	+	−	−	−	+	2
	10	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	+	1
	11	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	+	1
	12	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	−	0
		positive/examined (%)	2/11 (18.2)	0/11	4/11 (36.4)	4/11 (36.4)	0/11	0/11	5/11 (45.5)	
B	1	Truck's floor after washing	−	−	−	+	−	−	−	1
Northern Italy 22,000 pigs slaughtered per week	2	Lairage pens (floor) after cleaning	−	−	−	−	−	−	−	0
	3	Scalding water tank caroussel arm	−	−	−	+	−	−	−	1
	4	Upper surface at 2-knives system tank	−	−	−	−	−	−	−	0
	5	Carcass splitter saw surface	−	−	−	−	−	−	−	0
	6	Evisceration knife	−	−	−	−	−	−	−	0
	7	Evisceration step (floor)	−	−	+	−	−	−	−	1
	8	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	−	0
	9	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	−	0
	10	Outer surface of 3 carcasses after evisceration	−	−	+	−	−	−	−	1
	11	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	−	0
	12	Outer surface of 3 carcasses after evisceration	−	−	−	+	−	−	−	1
		positive/examined (%)	0/12	0/12	2/12 (16.7)	3/12 (25.0)	0/12	0/12	0/12	
C	1	Truck's floor after washing	−	−	−	−	−	−	−	0
Northern Italy 20,000 pigs slaughtered per week	2	Lairage pens (floor) after cleaning	−	−	−	−	−	−	−	0
	3	Scalding water tank caroussel arm	np	np	np	np	np	np	np	
	4	Upper surface at 2-knives system tank	−	−	−	−	−	+	−	1
	5	Carcass splitter saw surface	−	−	−	−	−	−	−	0
	6	Evisceration knife	−	−	−	−	−	−	−	0
	7	Evisceration step (floor)	−	−	−	−	−	−	−	0
	8	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	−	0
	9	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	−	0
	10	Outer surface of 3 carcasses after evisceration	−	−	−	+	−	−	−	1
	11	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	−	0
	12	Outer surface of 3 carcasses after evisceration	−	−	+	−	−	−	−	1
		positive/examined (%)	0/11	0/11	1/11 (9.1)	1/11 (9.1)	0/11	1/11 (9.1)	0/11	
D	1	Truck's floor after washing	−	−	−	−	−	−	−	0
Central Italy 6000 pigs slaughtered per week	2	Lairage pens (floor) after cleaning	+	−	−	+	−	−	+	3
	3	Scalding water tank caroussel arm	−	−	+	−	−	−	−	1
	4	Upper surface at 2-knives system tank	−	−	−	−	−	−	−	0

(*continued on next page*)

Table 1 (*continued*)

	5	Carcass splitter saw surface	np	np	np	np	np	np	np	
	6	Evisceration knife	−	−	+	−	−	−	+	2
	7	Evisceration step (floor)	−	−	+	−	−	−	−	1
	8	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	+	1
	9	Outer surface of 3 carcasses after evisceration	−	−	−	+	−	−	−	1
	10	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	+	1
	11	Outer surface of 3 carcasses after evisceration	+	−	−	+	−	−	−	2
	12	Outer surface of 3 carcasses after evisceration	−	−	−	−	−	−	+	1
		positive/examined (%)	2/11 (18.2)	0/11	3/11 (27.3)	3/11 (27.3)	0/11	0/11	5/11 (45.5)	
Total		positive/examined (%; 95%CI)	4/45 (8.9; 3.5–20.7)	0/45 (0.0; 0.0–7.9)	10/45 (22.2; 12.5–36.3)	11/45 (24.4; 14.2–38.7)	0/45 (0.0; 0.0–7.9)	1/45 (2.2; 0.4–11.6)	10/45 (22.2; 12.5–36.3)	

for this study (Table 1). Sponges for *Salmonella* were transported to the laboratory at 4 °C, and processed within 24 h, and sponges for viruses were immediately stored at −20 °C until use.

2.3. Viral nucleic acid extraction from environmental swabs

Sponges were thawed and rehydrated with 5 mL of phosphate buffer saline (PBS) and incubated at room temperature for 30 min. After the incubation, swab sponges were squeezed and the resulting available suspension was collected for the nucleic acid extraction (Maunula et al., 2013). The whole volume from each swab and 150 μL fecal suspension was extracted by using the Nuclisens Magnetic Extraction System (BioMerieux, Marcy-l'Étoile, France) with a final elution in 100 μL.

2.4. Nucleic acid recovery rate calculation

Before nucleic acid extractions, suspensions were spiked with Murine Norovirus (MuNoV strain IT-1, Istituto Superiore di Sanità, Rome, Italy), as previously described (Chelli et al., 2021). The extraction recovery rate (RR) was estimated by the comparative cycle threshold (Ct) method (De Sabato et al., 2020) between MuNoV RNA detected in the spiked samples and in the controls spiked in water. Nucleic acid extractions were considered acceptable with a RR $\geq$ 1%.

2.5. Viral detection

Swab environmental samples were tested (nucleic acid volume 5 μL) for the presence of HEV, TTSuV, PAdV, RVA, NoV, and MRV, following methodologies described in previous studies. Real-time RT-PCR was used for HEV and NoV detection (Jothikumar et al., 2006; Jothikumar et al., 2005) using the RNA UltraSense™ One-Step qRT-PCR System (Thermofisher Scientific, Frederick, MD, USA), while Real-time PCR was used for TTSuV and PAdV (Brassard et al., 2010; Hundesa et al., 2009) using the Taqman™ Universal PCR Master mix (Applied Biosystems, Waltham, MA, USA). Conventional end-point RT and nested PCR were used for MRV (Leary et al., 2002) by using the Qiagen One-Step RT-PCR Kit (Qiagen, Monza, Italy) for the first round RT-PCR, and the GoTaq® G2 DNA Polymerase (Promega, Madison, WI, USA) for the second round nested PCR. For RVA, the detection was performed by both Real-time RT-PCR and end-point RT- PCR (Iturriza Gomara et al., 2002; Jothikumar et al., 2009) by using the RNA UltraSense™ One-Step qRT-PCR System (Thermofisher Scientific, Frederick, MD, USA) and the same kits used for MRV, respectively.

2.6. Isolation, identification, and serotyping of Salmonella

The detection and serotyping of *Salmonella* were performed following the indications by the ISO 6579-1:2017/AmD 1:2020 (Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella*).

Initially, each sponge was homogenized with 60 mL of buffered peptone water (BPW; Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C ± 1 °C for 18 ± 2 h. After this step, the samples were analyzed by real-time PCR as described below.

Two mL of the pre-enrichment broth (BPW) was transferred into a clean microcentrifuge tube, and centrifuged for 10 min at 10,000 ×*g* at 4 °C. The supernatant was discarded carefully, and the pellet was suspended in 200 μL of 6% Chelex 100 (Biorad, Hercules, CA, USA) and incubated for 8 min at 100 °C and. The suspension was immediately chilled on ice for 1 min, and centrifuged for 5 min at 10,000 ×*g* at 4 °C. Four μL of DNA extracted were used as template for the *Salmonella*-specific Real-Time PCR detection assay (Delibato et al., 2014).

Real-time PCR positive samples were confirmed according to ISO 6579-1. Finally, the isolated strains were serotyped according to ISO/TR 6579–3. Briefly, each *Salmonella* serotype was obtained by agglutination for the characterization of somatic, flagellar, and capsular antigens. The characterization of flagellar antigens in R-phase was not done.

2.7. Statistical analysis

Slaughterhouses were categorized as "Large" (>20.000 pigs slaughtered per week, B and C) and "Small" (A and D). The sites of swab collection were categorized in "carcass" and "other", with the latter including lairage pen, truck, floors, and utensils.

The statistical association for the same pathogen detected (*Salmonella*, NoV, TTSuV, PAdV, and MRV) across different site of samplings and different slaughterhouses that were categorized on the base of their size, was conducted by Fisher's exact test.

The correlation between the different pathogens considering all individual swab samples was calculated by Tetrachoric correlation matrix. Viruses that consistently tested negative (HEV and RVA), as well as NoV GII for which only one sample showed a positive result, were not included in this analysis. Statistical analyses were performed using the software SPSS 28.0.0 (IBM SPSS Statistics, Armonk, NY, USA). For the Tetrachoric correlation the Correlation R package version 0.8.4 was used. Statistical significance was set at $p \leq 0.05$.

3. Results

A total of 45 samples collected at 4 Italian slaughterhouses during pig slaughtering were analyzed for the detection of *Salmonella,* hepatitis E virus (HEV), and other 5 viruses (rotavirus, RVA; norovirus, NoV; torque teno sus virus, TTSuV; porcine adenovirus, PAdV, and mammalian orthoreovirus, MRV).

For the detection of viruses, the nucleic acid extraction was performed, and its recovery rate (RR) was assessed. All samples resulted in a

RR ≥ 1%, with a mean RR of 46.87% (10.66–100.00, median 43.1).

Overall, the presence of *Salmonella* was revealed in 4/45 samples (8.9%; 95%CI: 3.5–20.7), while the zoonotic foodborne HEV-3 was not detected (0/45). Negative result was obtained also for RVA (Table 1).

On the other side, the presence of PAdV, a reliable porcine fecal indicator, was detected in 11 out of 45 samples (24.4%; 95%CI: 14.2–38.7). In addition, the analysis for the detection of other viruses revealed the presence of NoV genogroup GII in 1/45 (2.2%; 95%CI: 0.4–11.6) samples, while TTSuV and MRV were detected in 10/45 and 10/45 samples, respectively (22.2% each; 95%CI: 12.5–36.3).

Overall, 19/45 samples (42.2%) resulted negative to all the tests performed (2/11 abattoir A, 7/12 B, 8/11C, and 2/11 D) (Table 1).

Considering the sampling sites of the detected pathogens, *Salmonella* was detected on samples collected on the lairage pen and on the floor at the site of evisceration in the same abattoir (A, 2/11), together with those collected in the lairage pen and on one pig carcass after the evisceration in abattoir D (2/11). None of the other samples were positive for *Salmonella*, as well as all samples from abattoirs B and C.

The molecular analysis revealed the presence of *S. Derby* in abattoir A, while *S. London* was detected in abattoir D.

PAdV was almost ubiquitous, being revealed from samples in the four abattoirs, in floors, utensils, and carcasses (4, 2 and 4 samples, respectively). Similar results were highlighted for TTSuV (3, 4, and 3) and MRV (1, 2 and 7), even if the sites distribution was slightly different for the three viruses. In particular, MRV was detected in 7 out of 20 swabbed pig carcasses (35.0%; 95%CI: 18.1–56.7), while TTSuV was detected mainly in the evisceration site (knife and floor), being present in 5 out of 8 overall samples collected in the 4 abattoirs (62.5%; 95%CI: 30.6–86.3).

The only positive NoV sample (GII) was revealed on the surface of the 2-knife system tank in slaughter C.

All four slaughterhouses showed the presence of PAdV and TTSuV, being distributed in a different way across the sampling sites. Two abattoirs (A and D) showed the presence of *Salmonella* and MRV, with the same detection rate (2 and 5 each, respectively) even if at different sampling points. Overall, the A and D abattoirs revealed a higher number of positive samples, if considering all the pathogens grouped, with respect to the lower detection rate obtained in slaughterhouses B and D (Table 1).

The prevalence of *Salmonella* was not statistically different considering the type of sample ("carcass" and "other"), however it was observed in both sample types only from "Small" slaughterhouses, and the prevalence was statistically higher in "Small" abattoir ($p = 0.049$).

The same analysis conducted on TTSuV and PAdV revealed no statistical association ($p > 0.05$) between the categories of sampling sites involved, but the positivity rate observed was higher in "Small" slaughterhouses considering both types of samples.

Similarly, for MRV the positivity was higher in "Small" slaughterhouses with statistical significance for the overall positivity ($p < 0.001$) and in association with carcasses ($p = 0.022$) and other sites ($p = 0.010$).

The statistical analysis also revealed an overall association between the positivity to at least one of the investigated pathogens and the "Small" size of the slaughterhouse, considering all samples (p = 0.02), the "carcass" samples ($p = 0.044$) and the "other" samples ($p = 0.025$).

A strong positive correlation between PAdV and *Salmonella*, detected independently from site of samplings, was observed (rho = 0.83; p < 0.001) (Fig. 1).

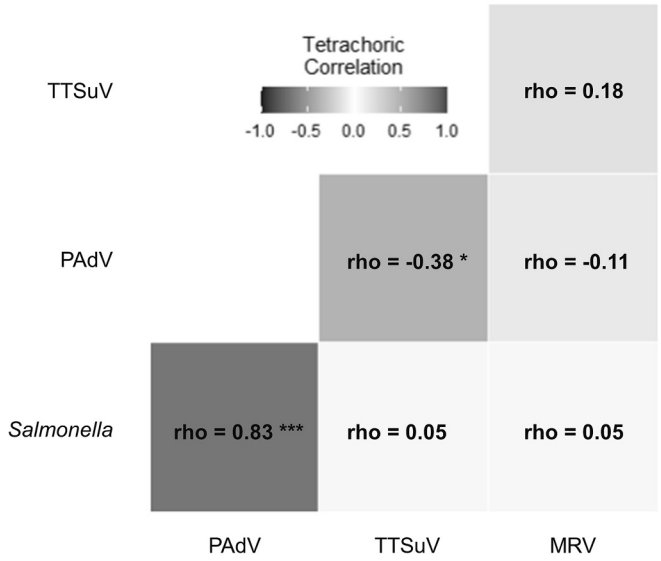

Fig. 1. Heat map of the associations between microorganisms investigated (PAdV = porcine adenovirus; TTSuV = torque teno sus virus; MRV = mammalian orthoreovirus). Black indicates positive (rho>0) or negative (rho<0) associations, with the intensity of black shading corresponding to the strength of the association. * $p < 0.05$; *** $p < 0.001$.

4. Discussion

This study was planned and performed to monitor the presence of pathogenic microorganisms, such as *Salmonella* and hepatitis E virus, additionally, we aimed to analyze various enteric viruses, such as RVA, PAdV, NoV, MRV and TTSuV as indicators of hygiene and potential zoonotic agents, at various stages of the slaughter chain.

The presence of such microorganisms may indicate possible fecal contamination from the intestinal content of pigs during the slaughtering (RVA, PAdV, TTSuV, NoV) or, in the case of TTSuV, an indication on possible contamination with blood from viremic animals, including either HEV positive or other viruses with a viremic stage.

The presence of *Salmonella* was revealed in 2 out of 4 slaughterhouses involved in this study, with a total of 4/45 positive samples, including 2 samples positive for the *S. Derby* serotype, representing one of the major serotypes responsible for human infections in Europe (EFSA and ECDC, 2019).

Salmonella was observed exclusively in "Small" slaughterhouses, with a statistically higher prevalence and with a similar presence on carcasses and other swabs from the environment of slaughter chain. Similar results were also achieved for TTSuV and PAdV whom positivity was notably higher in "Small" slaughterhouses across both sample types. This result suggests hygienic factors such as improper cleaning contributing to their higher prevalence in the smaller facilities. These facilities, likely less automated than larger sites, may have a less stringent hygiene standards. The presence of the investigated pathogens in "Small" abattoirs could be linked to the structural and technical characteristics of the abattoir. In fact, even if strictly following the GHP and GMP, small slaughterhouses have often less efficient equipment for the decontamination (i.e. singeing apparatus) and less space to avoid contact between carcasses (i.e. hooks and belts for carcasses, chilling room), even if a lower number of carcasses is processed. In contrast, larger facilities, with higher automation and a faster processing speed due to a larger number of slaughtered pigs, may benefit from more systematic management practices, ensuring a higher level of hygiene control (Primavilla et al., 2023). Nevertheless, this findings deserves further confirmation since in previous study in Italy no correlation was observed among occurrence of *Salmonella* and the size of the slaughterhouses (Primavilla et al., 2021).

Interestingly, all 4 *Salmonella* positive samples were also positive for PAdV. In particular, the tetrachoric correlation analysis revealed a robust correlation (rho = 0.83; $p < 0.001$) between the presence of PAdV and *Salmonella*, probably driven by the same matrix of contamination (feces) shared by these pathogens.

The simultaneous presence of *Salmonella* and PAdV underlines the significance of PAdV as a reliable indicator of fecal contamination for bacteria and also for the improper cleaning of environmental. As

confirmed by results, PAdV can persist not only on the floor of pen lairage after cleaning but also on the floor of the evisceration site, indicating indirectly improper cleaning and disinfection. The coexistence of these two microorganism serves as evidence, reinforcing the suitability of PAdV as an indicator for both bacteria and virus. On the other side, the ability of PAdV to persist in the environment leave open questions on the origin of the fecal contamination if either due to the co-infection with *Salmonella* or to a previous contamination still persisting in the site after the improper cleaning, possibly derived from a different source than that driving the presence of *Salmonella*.

Despite the low rate of detection, this finding represents a major concern for public health and food safety. The detection of *Salmonella* could be linked to cross-contamination with feces of pigs previously slaughtered in the abattoir, or to a possible persistence of the microorganism in certain critical points into the plant, despite the disinfection practices. The presence of *Salmonella* in feces collected on the floor of pen holdings after cleaning underlined two main critical points: firstly, infected pigs were housed together in the truck before lairage and for a few hours before slaughtering, increasing the risk of *Salmonella* transmission, which can occur within two hours (Boughton et al., 2007). Secondly, the improper cleaning and disinfection of pen floor lead to persistence of infectious *Salmonella* in the environment where new batches of animals would have been housed. One of the sponges resulted positive for MRV also, another viral pathogen mainly transmitted through feces. The presence of *Salmonella* was revealed on one sample from pig carcasses at the end of the slaughter stage. This finding is in line with the minimum accepted level of *Salmonella* presence on pig carcass at the slaughter included in the Commission Regulation (EU) 2019/229 (European Commision, 2019). These findings highlight the importance of ongoing vigilance and adherence to established regulations to ensure food safety within the slaughterhouse environment.

Conversely, the presence of HEV was not detected in any swab samples (0/45). The lack of HEV detection at environmental and carcasses level at slaughter on one hand is not surprising, in fact, even if this virus strongly persists in the environment also after disinfection, its detection can be difficult when the analysis is conducted on environmental swabs allowing to analyze only traces of pig feces. On the other hand, the contamination of carcasses with HEV was investigated sporadically. In one study, HEV resulted at high rate of detection of contaminated carcasses, and the authors also reported a significant reduction of HEV carcasses contamination after scalding, singeing, and pasteurization which can reduce the incidence of detecting the viral RNA (Jones and Johns, 2012). Conversely, a study conducted in Canada highlighted the absence of HEV on carcasses, despite the spreading of RVA, which indicated fecal cross-contamination, on the same samples (Jones and Muehlhauser, 2017).

The age of animals is another factor possibly driving the HEV negativity in our study. In fact, pigs are slaughtered at the age of 9 months in Italy, resulting less frequently in the stage of HEV shedding in feces (Monini et al., 2023). Indeed in one of the abattoir investigated, when analyzing fecal samples from 14 pig batches just prior to be slaughtered, showed consistently negative results for HEV in all batches (Monini et al., 2023).

Since HEV replication in pigs also includes a viremic phase (Chelli et al., 2021), in this study the possible blood contamination was also investigated by testing for TTSuV. Unfortunately, in absence of HEV positive samples we cannot evaluate the correlation, However, TTSuV virus was detected in 10/45 swab samples (22.2%), with only a moderate correlation with the detection of MRV (rho = 0.18; $p > 0.05$; Fig. 1) and with a negative correlation with PAdV (rho = −0.38; $p < 0.05$), confirming the different source of contamination for these viruses (blood/feces). TTSuV is commonly detected in blood and semen, but can also be detected in feces, bile, and other tissues, thus both horizontal and vertical transmission can occur (Brassard et al., 2008; Kekarainen et al., 2007; Monini et al., 2016; Sibila et al., 2009). The role of TTV in disease is unknown neither in humans nor in pigs but it was suggested that can interfere with immune response and be associated to more severe symptoms in co-infections (Ellis et al., 2008; Krakowka et al., 2008). The detection in pigs is also important since it was showed that TTSuV can be detected in humans (feces) possibly transmitted by pork food (Jimenez-Melsio et al., 2013; Monini et al., 2016).

The ubiquitous presence of TTV revealed in this study and as previously described in hospitals, water, food products suggests its high stability in the environment (Webb et al., 2020). Overall, with the above mentioned characteristics, TTSuV may serve as important indicator of feces and blood contamination for the hygienic aspects but also for monitoring both animal health and potential public health concerns associated with zoonotic transmission and food safety.

One critical step during slaughtering is represented by the evisceration. In all investigated slaughterhouses, one swab sample taken from a knife or from the floor of the evisceration workstation resulted at least positive for one of the pathogens included in our panel of fecal indicators. This result reinforces previous findings (Hdaifeh et al., 2020) about the weakness of this stage to occurrence of contamination and the need for targeted interventions to enhance hygiene protocols and minimize the risk of pathogen transmission during the step of evisceration.

Our findings highlighted the suitability of the panel of included viruses for the evaluation of the contamination on floor and utensils. In fact, the analysis showed the suitability of TTSuV and PAdV for the evaluation of possible contamination on floors, pens and utensils (knives) involved in the evisceration and split of carcasses steps. PAdV has been used as fecal indicator for pigs, being frequently present in feces of these animals (Schiff et al., 2007). Sequence analysis (data not shown) confirmed the presence of two different PAdV in abattoirs located in Northern (PAdV serotype 3) and Central Italy (PAdV serotype 5). The two serotypes are both released in feces, as expected for PadV-3 frequently associated with gastroenteritis in pigs and PAdV-5 firstly detected in nasal swabs, which has also been detected in healthy pigs and environmental samples (sewage effluent, water, and shellfish) (Gainor et al., 2022). This confirms that besides the diverse nature of serotypes of PAdV, it can be retrieved in feces of healthy pigs.

Among the viruses included in our panel, MRV represents an important zoonotic pathogen responsible for the infection of several mammal species, including humans and pigs, and causing mild gastroenteritis and respiratory disease in the infected host (Schiff, 2015). MRV has been associated with acute respiratory infections and further shown to have potential human-to-human transmission (Chua et al., 2008; Chua et al., 2011), together with cases of necrotizing encephalopathy and meningitis in infected humans reported in Europe and the United States (Ouattara et al., 2011; Tyler et al., 2004). MRV was assessed as zoonotic virus pathogen for humans and its presence confirmed the fecal contamination on carcasses at the end of the slaughtering, with the RNA detected even after the cleaning step (decontamination with hot water) of carcasses, before entering the chilling room, as also observed for *Salmonella*. This finding suggests that removal of these pathogens is challenging and confirms the need for enhanced measures and targeted interventions to mitigate the risk of *Salmonella* and other zoonotic pathogens such as MRV transmission to humans through contaminated carcasses.

Our study also investigated the presence of the enteric RVA and NoV, with the former released in feces of pigs showing a high persistence in the environment, with a zoonotic potential and being previously detected at a rate of 100% on pig carcasses at abattoir (Jones and Muehlhauser, 2017). To the other side, NoV was included in our panel of investigation since it represents a major gastroenteric virus of concern for humans. The zoonotic potential of swine NoVs is debated. Genotypes of NoVs infecting pigs and humans, even different, belong to the same genogroup GII (Cavicchio et al., 2022). Unfortunately, we could not perform the nucleotide sequence analysis due to the low RNA amount, thus far not allowing to establish the origin of the NoV GII detected. The inclusion in the panel and the negativity of the detection led us to the

hypothesis of a scarce NoV and RVA infection rate in the animals during their farming. Further studies are needed, possibly involving a higher number of samples to define better this association.

In conclusion, these findings highlighted the need to deepen investigate the presence of pathogens not only indicators of hygiene at slaughterhouse, allowing to understand better the risk factors also in pig farms that should be managed to maintain a low prevalence, since a low presence of *Salmonella* at farms will correspond to a low risk of contaminated carcasses (Pesciaroli et al., 2017). This primary prevention, together with the control measures applied at the slaughterhouse, could help to have food pork products safe for consumers.

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CRediT authorship contribution statement

Giovanni Ianiro: Investigation, Writing – original draft, Writing – review & editing. **Enrico Pavoni:** Investigation, Writing – original draft. **Luca De Sabato:** Investigation, Methodology. **Marina Monini:** Methodology, Validation. **Elisabetta Delibato:** Formal analysis, Writing – original draft. **Vitantonio Perrone:** Resources. **Fabio Ostanello:** Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. **Tarmo Niine:** Project administration, Methodology, Writing – review & editing. **Ilaria Di Bartolo:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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