VIRAEMIC PIGS ENTERING THE FOOD CHAIN ARE THE MOST LIKELY SOURCE OF HEPATITIS E VIRUS (HEV) IN PORK MEAT: MODELLING THE FATE OF HEV DURING SLAUGHTERING OF PIGS.

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Viraemic pigs entering the food chain are the most likely source of hepatitis E virus (HEV) in pork meat: modelling the fate of HEV during slaughtering of pigs.

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ABSTRACT

Hepatitis E Virus (HEV) is an emerging foodborne pathogen and consumption of raw or undercooked pork products has been associated with increased risk of human infection. This work represents the first attempt to evaluate the risk of HEV being present on pig carcasses and in meat at the end of the slaughtering process considering the steps of bleeding, scalding, dehairing, singeing, polishing, evisceration and trimming. Based on available knowledge on the epidemiology and biology of HEV, the risk pathways leading to the presence of HEV on carcasses as a consequence of (i) faecal contamination of the skin from environment and contacts with contaminated faeces during transport and lairage, (ii) contact with viraemic blood at bleeding and (iii) faecal/bile cross-contamination during evisceration were assessed qualitatively. The pathway through which HEV could be present in meat of viraemic pigs, as conveyed by residual blood in muscular tissue after bleeding was instead modelled quantitatively. Of the three risk pathways evaluated qualitatively, only the occurrence of HEV on carcasses as a consequence of accidental rupture of the gut or gallbladder at evisceration was found to be non-negligible, but with a very low likelihood of occurrence. The quantitative output for the expected amount of HEV in meat of viraemic pigs shows minimum and maximum values of 0.10 and $1.1x10^4$ genome copies (gc)/g respectively with $4.8x10^2$ and $5.3x10^3$ gc/g at 95^{th} and 99^{th} percentile of the cumulative distribution. These results are consistent with the existing evidence that levels of HEV RNA in meat samples are usually low even in the presence of high viral loads in livers of the same animals. Results of the sensitivity analysis confirm highly viraemic pigs entering the slaughter line as those posing the greater risk for consumers. Our study suggests that prevention of HEV infection through consumption of pork meat at pre-harvest/harvest stages should focus on reducing the flow of highly viraemic pigs into the food chain.

1. INTRODUCTION

Hepatitis E virus (HEV) is an emerging foodborne zoonotic pathogen; the total number of new cases per year, globally, is estimated at 20 million, resulting in 3.4 million symptomatic cases of hepatitis E (WHO, 2018). The World Health Organisation (WHO) estimates that hepatitis E caused approximately

44,000 deaths in 2015, accounting for 3.3% of the mortality due to viral hepatitis (WHO, 2017). Eight different HEV genotypes have been identified so far (Nimgaonkar et al., 2018) and routes of transmission differ by genotype. G1 and G2 are obligate human pathogens and are currently circulating in low- and middle-income countries in Asia, Africa and Central America in epidemic waves linked to the consumption of contaminated water and poor sanitation. In contrast, G3 and G4 have a wider host range, are zoonotic and usually responsible for autochthonous human cases in high-income countries (Ankcorn & Tedder, 2017). G3, which is the main genotype circulating in Europe, can infect both wild and domestic pig populations in addition to other animals such as deer or chamois (Di Bartolo et al., 2017; Spahr et al., 2018; Trogu et al., 2020). In humans, most HEV infections have been associated with consumption of contaminated water and undercooked pork products (EFSA, 2017). Several studies reported a rise in the incidence of hepatitis E in some parts of Europe (Adlhoch et al., 2016) and consumption of raw/undercooked pork products has been identified as the possible source of foodborne HEV transmission that could explain the increase of autochthonous human cases in high income countries (Chan et al., 2017; Cossaboom et al., 2016; Said et al., 2014,2017).

HEV infection in pigs does not lead to any clinical signs or production losses, consequently the incentive of the pig industry to control HEV is to ensure the safety of pork products for final consumers. As for other foodborne hazards, decision-making along the pork chain to mitigate the risk of consumers being exposed to HEV through pork and pork products should ideally be informed by probabilistic risk assessment (FAO, 2007). Three probabilistic models of HEV at different stages of the pork chain have been published so far. One of them investigated the effects of on-farm dynamics and animal host-pathogen interactions on the proportion of infected pigs at slaughter (Crotta et al., 2018) whilst the other two estimated the risk of human exposure to HEV through consumption of specific pork products (Müller et al., 2017; Sarno et al., 2016).

However, none of these studies attempted to model the fate of the virus along the slaughtering line and explore the effects of the different phases of the slaughtering process on the overall risk of the carcass and meat being contaminated; reasons probably lie in the substantial lack of HEV-specific data that may preclude quantitative modelling.

Understanding the relationships between events at slaughtering and how they modulate the fate and the level of contamination of HEV in the final product intended for human consumption, is however pivotal for the evaluation of control options to mitigate risk by means of full farm-to-fork quantitative microbial risk assessment (QMRA) models. Detailed farm-to-fork probabilistic models have been recently developed for *Salmonella* spp., which is an important enteric pathogen of interest for the pig industry and of public health importance (Hill et al., 2016; Simons et al., 2016; Swart et al., 2016). It is

worth highlighting that despite *Salmonella spp*. being a well-known foodborne pathogen, subject to detailed studies for many years, modellers had to make a large number of assumptions and overcome numerous data gaps to tackle quantitatively the dynamics of the system.

Given the relatively early stages of knowledge of HEV epidemiology, its transmission pathways through the pork chain and absence of HEV-specific data describing the fate of HEV in pig carcasses during slaughtering, attempting the parameterisation of a full quantitative probabilistic model would be, at this stage, premature.

In this context, a qualitative risk assessment framework providing a descriptive or categorical characterization of the risk is an ideal option to: (i) systematically synthesize through a reasoned and logical discussion the available knowledge and uncertainties to provide a preliminary qualitative risk estimate and (ii) screening the risk pathways to determine whether any of them merit further quantitative investigation (FAO, 2009). Therefore, objectives of this study were to: (i) evaluate, using a qualitative and, where possible quantitative risk assessment frameworks, the risk of HEV being present on carcass and in meat at the end of the slaughtering process and (ii) identify, by means of sensitivity analysis, the uncertainties and data gaps that should be addressed to allow full quantitative assessment of the risk.

2. MATERIAL AND METHODS

Our approach consisted in a detailed assessment of the biological events occurring during the different steps of the slaughtering of pigs and subsequent evaluation of the extent to which these can favour or prevent HEV contamination of the carcass. The model considers the lairage (where the animals are housed when entering the abattoir) as the starting point of the slaughtering process and the post-mortem inspection after evisceration as the endpoint. A brief description of the typical slaughter line is outlined in section 2.1.

Four risk pathways were identified:

1. Presence of HEV on carcass after processing due to faecal contamination of the skin from environment and contacts with contaminated faeces during transport and lairage.
2. Presence of HEV on carcass after processing due to contact with viraemic blood at bleeding
3. Presence of HEV on carcass after processing due to faecal/bile cross-contamination during evisceration.
4. Presence of HEV in meat due to residual blood in muscular tissue after bleeding.

For each pathway, the sequence of sufficient and necessary events leading to the virus being present on a carcass were identified and the qualitative likelihoods presented in Table 1 were assigned to

each event upon reasoned and logical discussion of the available scientific evidence for pathways 1,2, and 3. For pathway 4, availability of necessary data in scientific literature allowed quantitative estimation of the risk.

Table 1. *Definition of the qualitative terms used to describe the likelihoods of the necessary events leading to the presence of HEV in the final product after slaughtering.*

Likelihood	Description
High (H)	Expected to occur
Moderate (M)	Occurrence less than 50% probability
Low (L)	Unlikely to occur
Very low (VL)	Rarely occur
Extremely low (EL)	Very rarely occur
Negligible (N)	Chance of occurrence so small that can be ignored

For the pathways assessed qualitatively, the likelihoods assigned to each event were combined to derive the overall estimate of the risk. The final risk estimates were therefore expressed as cumulative likelihoods obtained combining the qualitative estimates of the inputs according to the matrix of conditional probabilities presented in Table 2 and previously adopted by European Food Safety Authority (EFSA) and other qualitative risk assessments (Crotta et al., 2016; EFSA, 2008a; Peeler & Thrush, 2009).

Table 2. *Combination matrix used for the estimation of the conditional likelihoods. The product of two probabilities is always less than the lowest probability and is sometimes given as a range (e.g. N-EL). However, as explained in the EFSA report, since qualitative term covers a wide range of likelihoods the combined estimate is in some case equal to the lower estimate (e.g. a step 'n' with an estimate of VL with a step 'n+1' with an estimate of EL produces and an overall estimate of N-EL).*

	Conditional likelihood step *'n'*					
Likelihood step '*n+1*'	*N*	*EL*	*VL*	*L*	*M*	*H*
H	N	EL	VL	L	M	M
M	N	EL	VL	VL	L	M
L	N	EL	EL	VL	VL	L
VL	N	N-VL	EL	EL	VL	VL
EL	N	N	N-EL	EL	EL	EL
N	N	N	N	N	N	N

Within this qualitative risk assessment, we describe the likelihood of HEV passing from one stage of the slaughtering line to the next in qualitative terms; and given that some viral particles do make the transition, within each step we also assess the likely amount of HEV being transferred. The expected amount of virus is described by the ordinal ranges defined in Table 3.

Table 3. *Definition of the qualitative ranges used to describe the likely amount of HEV RNA in the different events; gc = genome copies.*

Likelihood	Description
R0	0 gc/g or gc/mL or gc/cm^2
R1	1-10 gc/g or gc/mL or gc/cm^2
R2	10-10^2 gc/g or gc/mL or gc/cm^2
R3	10^2-10^3 gc/g or gc/mL or gc/cm^2
R4	>10^3 gc/g or gc/mL or gc/cm^2

In order to avoid overconfidence on the outcomes and prevent misinterpretation, an assessment of the uncertainty surrounding each estimation was included (Table 4), and expressed as: High, (H) Moderate (M) or Low (L).

Table 4. *Definition of the qualitative terms used to define the levels of uncertainty surrounding the likelihoods assigned to the steps along the qualitative pathways.*

Uncertainty	Interpretation
Low (L)	The estimation is strongly supported by data-evidence. Agreement by different authors
Moderate (M)	The estimation is supported by few or Incomplete data. Some authors report conclusions slightly different from some other
High (H)	The estimation is supported only by scarce data or it is based on Hypothesis/Assumptions. Strong disagreement from different authors

With respect to the uncertainties, the worst estimate among the risk factors and along the steps of the pathways was conservatively considered; this way, a high uncertainty in one step is enough to lead to a high uncertainty in the overall outcome. An exception was made if the occurrence of the event in step '*n* + 1' is **Negligible** with **Low** uncertainty. In addition, arrows on top of the uncertainty labels are used to specify the expected *direction* of the uncertainty. As an example, a likelihood estimate of **Medium** associated to a level of uncertainty equal to $\overleftarrow{\boldsymbol{M}}$ indicates that while the likelihood was judged to be "Medium" the available evidence suggests there is a certain degree of uncertainty around this estimate. The likelihood estimate could therefore be different from Medium, but different toward the lower rather than higher levels of the likelihood scale.

For each qualitative pathway, a sensitivity/uncertainty analysis was carried out. This was done by evaluating what the cumulative likelihood would be if the uncertain likelihood estimates were different, according to the direction indicated by the uncertainty.

2.1. Outline of the slaughtering line

The model considers the lairage (where the animals are housed when entering the abattoir) as the starting point of the slaughtering process and the post-mortem inspection after evisceration as the endpoint.

Lairage and stunning. Upon entry to the slaughterhouse, animals are usually kept in lairage (holding pens) for a period before being slaughtered. This is to provide a buffer to supply the processing line and allow the animals to recover from the stress of transport to avoid negative effects on meat quality (Warriss, 2003). Prior to slaughter, pigs are stunned through electronarcosis or gas. During the slaughtering process, pig carcasses often come into physical contact with each other and cross-contamination of the skin may occur.

Bleeding. After stunning, pigs are hoisted by one leg and killed by severing the major blood vessels from which carotid arteries and the jugular veins arise at the inlet of the chest. In this procedure, the knives are routinely disinfected in hot water at not less than 82°C (EC, 2004) and only touch a small area (that is trimmed during carcass dressing); the potential contribution of residuals of viraemic blood transferred from the blade to the following pig is considered to be negligible.

Scalding. After the blood is drained, the carcass is moved to the scalding bath. At scalding, pigs are submerged into a scalding bath containing hot water at around 62-63°C for 6-7 minutes. This procedure loosens the hair in the follicle; if lower temperatures are used, the hair will not be loosened while at very high temperatures, the skin will be cooked and the hair will become difficult to remove. After scalding, the carcasses are de-haired.

Dehairing-singeing-polishing. Dehairing can either be done manually or in the dehairing machine consisting of a rotating drum with flexible extensions. At this stage, the bulk of the hairs are removed, and the vigorous mechanical action of the dehairing machine might lead to a small amount of faeces being extruded from the anus. After dehairing, the carcasses go through a singeing oven where the skin is exposed to very high temperatures (~1000°C) for about 25 seconds. After singeing, a machine with hard-rotating brushes removes the burnt hair left on the carcass. At this stage, there is chance for a small amount of faecal material quantified in 1g in a previous study by means of expert opinion (Swart et al., 2016) to be mechanically released from the anus. Plugging of the anus is a practical option

to prevent release of faeces during processing (Purnell et al., 2010). After polishing, carcasses go to the evisceration station.

Evisceration. Evisceration is recognized within the Hazard Analysis of Critical Control Point as a Critical Control Point for management of foodborne hazards due to the high risk of carcass contamination with faecal matter and bile at this step of the processing. Guts or other internal organs such as the stomach or the gall bladder can be accidentally punctured/ruptured during manual evisceration leading to a leak of faeces or bile on and into the carcasses. If any rupture occurs, the carcass is diverted to a separate line where any visible contamination is trimmed.

Trimming. Trimming is done manually, with a sterilized knife; in this operation, a large portion around the contamination is removed. At this stage, even if all the visible contamination is removed, microscopic contamination cannot be excluded.

2.2. Pathway 1: Presence of HEV on carcass after processing due to faecal contamination of the skin from environment and contacts with contaminated faeces during transport and lairage.

The overall likelihood for the presence of HEV on carcasses due to fecal contamination of the skin depends on:

(i) The likelihood of HEV being present on the skin of animals entering the slaughtering line;

(ii) The likelihood of this -or a fraction of this- initial contamination persisting on the carcass and successfully making the transition across the slaughtering steps of: scalding, dehairing and singeing

(iii) The likelihood of the carcass being contaminated or re-contaminated at polishing.

The risk pathway is outlined in Figure 1. While relevant for the risk of HEV contamination due to faecal and bile-cross contamination, what happens during evisceration is assumed to be independent with respect to the likelihood of HEV contamination due to initial level of faecal contamination on the skin. Similarly, while relevant for the likelihood of HEV contamination due to contact with viraemic blood during bleeding, what happens during bleeding is independent from the likelihood of HEV contamination considered within this specific pathway.

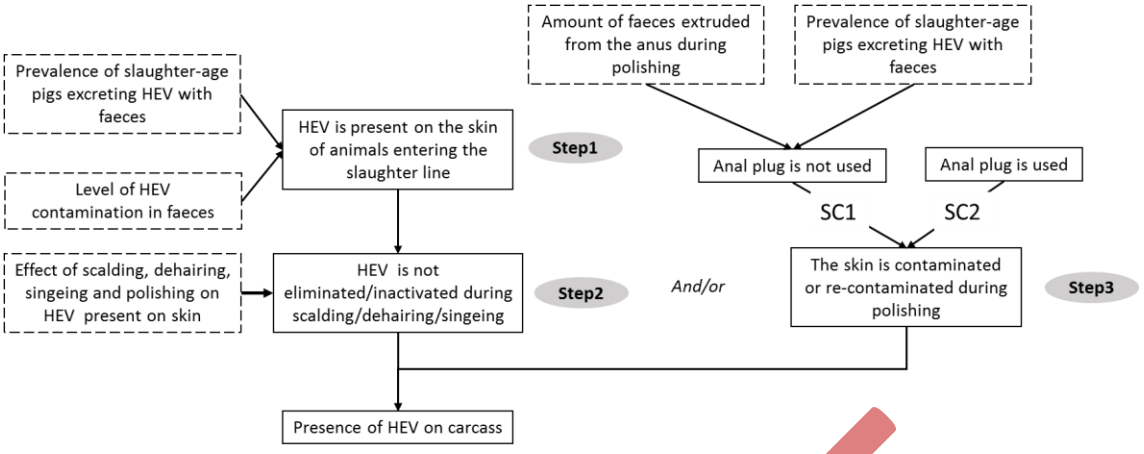

Figure 1. Pathway nr.1; flowcharts outlining the required steps for HEV to be present on a carcass after processing because of faecal contamination of the skin from environment and contacts with contaminated faeces during transport and lairage.

Step 1: Likelihood of HEV being present on the skin of animals entering the slaughter line.

The presence and level of HEV contamination on the skin of pigs has not yet being investigated. The initial level of HEV on the skin of the animals entering the slaughtering line is dependent upon a number of biological and management factors including: proportion of animals excreting HEV with faeces, level of HEV in contaminated faeces, contact rate during transport and lairage, amount of faeces transferred amongst animals and between the animals and the lairage environment. In addition, the process of cross-contamination at this stage involves many events that are interdependent and highly uncertain. This is because environmental conditions are highly variable and will probably affect both the likelihood of faecal material being transferred from animal to animal and survival of the virus. Several studies have investigated the presence of HEV RNA in faeces of naturally infected pigs at slaughter reporting observed prevalence estimates of 33.3% in Italy, (Di Bartolo et al., 2011), 3%, 41% and 38% in Czech Republic, Spain and Italy respectively (Di Bartolo et al., 2012) and 14% and 17% in UK (Berto et al., 2012; Grierson et al., 2015). The proportion of pigs excreting infectious HEV with faeces at the end of the production cycle was estimated to range from 25.3% to 30.8% by a transmission model fitted from longitudinal data of naturally infected pigs (Crotta et al., 2018). From these data, and assuming homogeneous mixing of animals at lairage, the likelihood of HEV being present on the skin of a random pig at beginning of the slaughter line is cautiously considered as **Medium** at a **Moderate** and bidirectional level of uncertainty**.** This means that while the most representative likelihood estimate for this event is evaluated to be “Medium”, because of the uncertainty, it is possible for the real estimate to be “Low” or “High”.

Level of HEV contamination on skin from contaminated faeces.

The level of HEV in contaminated faeces has been observed to range approximately between 10^4 to 10^6 genome copies (gc)/g (Kanai et al., 2010; Leblanc et al., 2010); hence, for this assessment, the level of HEV contamination on the skin of contaminated animals is assumed to lie within the range described by **R2** (see Table 3) with **Moderate** uncertainty. Assuming a level of skin contamination ranging between 10 to 10^2 gc/cm^2 implies that there is the equivalent of 0.01 to 1 g of faeces uniformly distributed for every 100 cm^2 of carcass' surface. To reflect hat this is probably an overestimation, the uncertainty is assigned a left pointer.

Step 2: Effect of scalding, dehairing and singeing on the level of HEV contamination on the skin and likelihood of HEV not being eliminate/inactivated.

Although HEV-specific data are not available, experimental studies comparing the level of bacteria from bleeding to end of the singeing process reached similar conclusions: after singeing, the number of bacteria on the skin can be expected to be from 99.96% to 99.99% lower than that observed immediately after bleeding (Bolton et al., 2002; Pearce et al., 2004; Wheatley et al., 2014). Hence, considering the time-temperature combinations at which carcasses are exposed during scalding (i.e. 62-63°C for 6-7 minutes) and singeing (i.e. ~1000°C for about 25 seconds) and the recent data demonstrating inactivation of the HEV G3 when exposed to temperatures of at least 65°C for 5 minutes (Imagawa et al., 2018), it is biologically reasonable to assume this heat treatments have a substantial impact on the reduction of the level of HEV contamination on the skin. For this reason, the likelihood of HEV on the skin surviving both the scalding and singeing stages is assumed to be **Negligible** at **Low** uncertainty.

Step 3: Likelihood of skin being contaminated or re-contaminated at polishing.

After singeing, a machine with hard-rotating brushes removes the burnt hair left on the carcass. At this stage, it is again possible that a small amount of faecal material is mechanically released from the anus. Assuming homogeneous dispersion on the carcass of a small amount of faeces previously quantified in 1g (Swart et al., 2016), it is assumed that the likelihood of HEV being present on the skin as a result of polishing is **Medium** (i.e. equal to the likelihood of HEV being present in faeces) with a level of contamination described by the range **R1** at **Low** uncertainty. This estimation is justified by the extremely low amount of faeces that would be uniformly distributed on the carcass surface during polishing. As an example, considering the carcass surface can be estimated as: $S_c = 734 * m^{0.656}$ (Kelley et al., 1973), with *m* being the body mass, if we consider the median value for carcass weight in UK (79Kg) as reported by EFSA baseline study (EFSA, 2008b), this results in 1g of faeces uniformly dispersed over a surface of ~12800cm^2. Plugging of the anus is a practical option to prevent escape of

faeces during processing (Purnell et al., 2010); under this scenario (***SC2***), the likelihood of HEV being present on skin due to polishing becomes **Negligible** at **Low** uncertainty.

2.3. Pathway 2: Presence of HEV on carcass after processing due to contact with viraemic blood at bleeding.

The presence of HEV on carcass due to contamination from blood leaking on the skin during bleeding (Figure 2) depends on:

(i) Likelihood of HEV being present in bloodstream of animals entering the slaughter line;

(ii) Likelihood of HEV contaminating the external skin as a consequence of contact with viraemic blood during bleeding;

(iii) Viraemic titre of HEV in blood of viraemic pigs;

(iv) Effect of scalding, dehairing, singeing and polishing on the level of HEV contamination on the skin.

While relevant for the HEV contamination from faecal and bile-cross contamination, what happens during polishing and evisceration is assumed not to be relevant with respect to the likelihood of HEV contamination of meat due to blood leaking on carcass during bleeding.

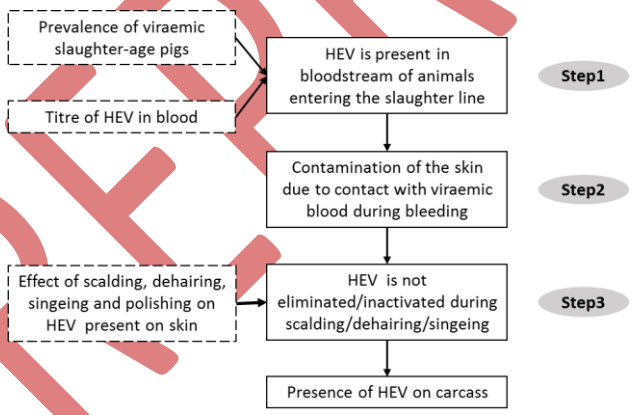

Figure 2. Pathway nr.2; flowcharts outlining the required steps for HEV to be present on a carcass after processing because of contact with viraemic blood during bleeding.

Step 1: Likelihood of pigs being viraemic at the time of slaughter.

Studies investigating the prevalence of slaughter-age viraemic pigs reported observed prevalence of 3% and 44,4% in two different studies in UK (Crossan et al., 2015; Grierson et al., 2015) and 6.3% in US (Sooryanarain et al., 2020). In two longitudinal studies investigating the presence of HEV in naturally infected swine herds, presence of HEV RNA in blood of slaughter-age pigs was observed in 11.8% and 12.5% of the subjects respectively (de Deus et al., 2008; Leblanc et al., 2007). Evidence seems to suggest that likelihood or a random pig being viraemic at slaughter should be lower than the likelihood for HEV to be present in faeces. The likelihood for this event is therefore estimated to be

Low but with **High** and right pointing uncertainty. This indicates that it is possible that the real prevalence of viraemic pigs at slaughter is higher than "Low".

Step 2: Likelihood of HEV contaminating the skin at bleeding.

During bleeding, blood drains from the vessels of the neck coming in contact with the external surface of the carcass, mainly in the area of the head considering pigs are hoisted during bleeding to facilitate quick and complete drain of blood. At this stage, HEV contaminating the external skin is considered as a certain event if the animal is viraemic, therefore, the likelihood of HEV contaminating the skin through the blood leaking on the carcass is assumed as equal to the likelihood for step 1: **Low** but with **High** and right-pointing uncertainty.

Viraemic titre of HEV in blood of viraemic pigs.

Viraemic titre of HEV RNA in blood of viraemic pigs at slaughter is highly variable and observed to range from <100 to 10^6 HEV RNA genome copies/mL (Grierson et al., 2015; Sooryanarain et al., 2020). This high variability is probably explained by the variability in the stage of the individual course of HEV infection different pigs have reached at the time of slaughter. For this reason, viraemic titre in blood of viraemic pigs and the level of contamination on skin in contact with leaking blood is conservatively considered to lie within the range **R3** with **High** and left-pointing uncertainty to indicate this is a worst scenario and the actual titre of HEV in viraemic pigs shall be lower.

Step 4: Effect of scalding, dehairing and singeing on the level of HEV contamination on the skin and likelihood of HEV not being eliminate/inactivated.

At this stage, as discussed for in pathway 1, the overall likelihood of HEV on skin successfully surviving both the scalding and singeing stages is assumed to be **Negligible** at **Low** uncertainty.

2.4. Pathway 3: Presence of HEV on carcass after processing due to faecal and bile cross-contamination during evisceration

Within this pathway, only the events occurring during evisceration are assumed to be relevant for the overall likelihood of HEV being present on meat as a result of cross-contamination with faeces and bile during evisceration (Figure 3). Within the evisceration step of the slaughter line, the following events are taken into account:

(i) The likelihood of HEV being present in faeces and bile,

(ii) The likelihood of internal organs being ruptured during evisceration,

(iii) Amount of faeces or bile content leaking on carcass as a result of ruptured gut or gallbladder,

(iv) Level of HEV contamination in bile and faeces,

(v) Proportion of non-visible contamination remaining onto/into the carcass following trimming.

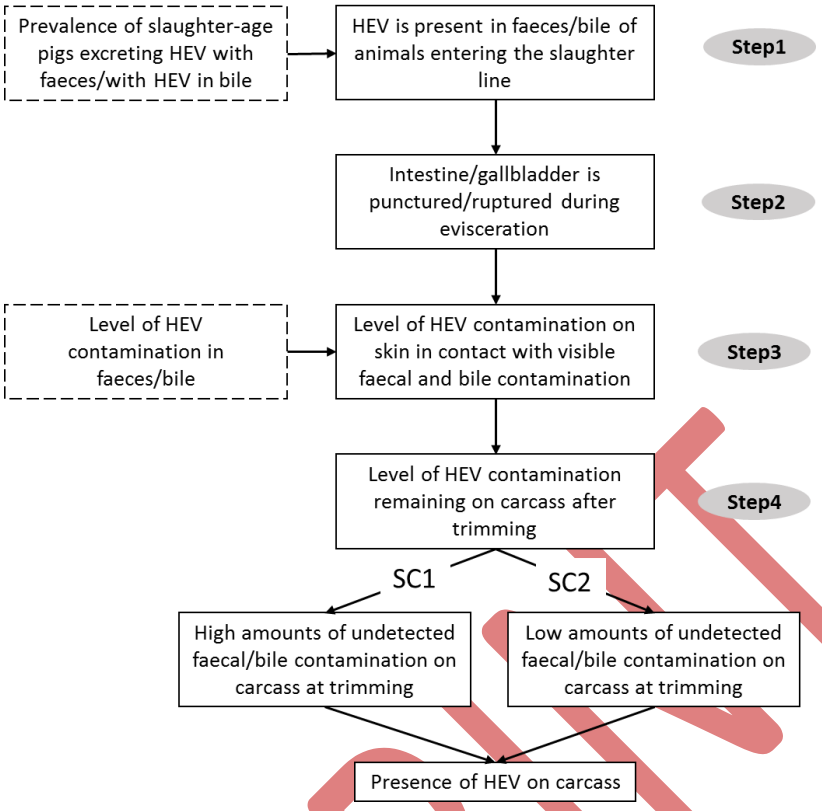

Figure 3. Pathway nr.3; flowcharts outlining the required steps for HEV to be present on a carcass after processing because of faecal/bile contamination during evisceration.

Step1: Likelihood of HEV being present in faeces and bile.

The probabilities of bile and faeces being contaminated are assumed to be related to the infectious status of the animal entering the slaughtering line. The likelihood of HEV being present in faeces, considering the evidence presented in step 1 of pathway 1, is evaluated as **Medium** with a **Moderate** and left-pointing level of uncertainty.

For the occurrence of slaughter-age animals with contaminated bile, again, high variability in the observed prevalence is reported in literature with estimates ranging from below 1% up to 51.1% (Salines et al., 2017). Within this qualitative risk assessment, the likelihood of pigs having HEV in bile is considered to be **Medium** with **High** left-pointing uncertainty.

Step 2: Likelihood of internal organs being punctured/ruptured during evisceration.

Data to estimate the likelihood of gut and gallbladder being punctured or ruptured during evisceration are only available from a recent study where estimates for the occurrence of these events were quantified by means of expert opinion elicitation (Crotta et al., 2019). Results of that study estimated the median probability of rupturing the gut and the gallbladder during evisceration around 2% in high and middle throughput abattoirs. However, results of that study also indicate a certain degree of uncertainty between estimates of different experts, this was due to the different perceptions,

professional experiences and confidence the interviewees had around the variability in the number of events (i.e. ruptures). For these reasons, the likelihood of the gut or gallbladder being ruptured during evisceration is considered **Very Low** at a **Moderate** right-pointing level of uncertainty.

Step 3: Level of HEV contamination on skin in contact with faecal and bile contamination following accidental rupture of gut or gallbladder.

The level of HEV in contaminated faeces and bile is typically high, up to 10^6 cg/g; therefore, it can be easily assumed that the level of HEV contamination on the skin in contact with the leaked faeces or bile lies within the range **R3** with **Low** uncertainty.

Step 4: Level of HEV contamination remaining on carcass after trimming.

Considering that faecal and bile-contaminated carcasses are detected by visual inspection by abattoir staff and retained for trimming where contaminated areas are removed; the overall amount of HEV contamination eventually remaining on carcass as result of ruptured gut and gallbladder is therefore to be attributed to microscopic "spots" of contamination that remained undetected during inspection and trimming. The amount and dispersion of non-visible contamination that remains on a carcass are challenging to evaluate even in qualitative terms and experimental studies to gather quantitative data would be probably complex. Considering these are likely to remain unknown parameters, two possible scenarios are evaluated at this stage:

SC1: high likelihoods of **High** amounts of undetected faecal/bile contamination on a carcass at trimming

SC2: high likelihoods of **Low** amounts of undetected faecal/bile contamination on a carcass at trimming

2.5. Pathway 4: Presence of HEV in meat due to residual blood in muscular tissue after bleeding.

Considering that the virus does not replicate in the cells of the muscular tissue, it is reasonable to hypothesize that the presence of HEV RNA in meat is in fact due to the residual blood remaining in muscles of viraemic pigs after bleeding. To explore the extent to which this pathway could indeed be relevant, the expected level of HEV RNA in meat (HEV_{MEAT}) is quantified as:

$$HEV_{MEAT} = R_{BLOOD} * C_V$$

Where R_{BLOOD} is the residual blood remaining into the muscular tissues after bleeding in mL/Kg and C_V is the viral load in the bloodstream in genome copies/mL. The volume of blood present in the carcass of a slaughtered pig is related to the initial blood volume and the efficiency of exsanguination but limited research on this subject has been conducted. The volume of blood released from slaughtered pigs during exsanguination has been estimated to be in the range of 40–60% of total blood volume or

approximately 4–5% of their live weight (Warriss, 1984) while the residual blood content in muscle post-slaughter has been estimated at 2–9 ml/kg of muscle (Warriss & Wilkins, 1987). Accordingly, R_{BLOOD} (expressed in mL/g rather than mL/Kg) is described as:

$$R_{BLOOD} \sim Uniform(2; 9) * 10^{-3} mL/g$$

The empirical distribution describing the level of HEV contamination in blood of viraemic pigs (CV), was parameterized from data reported by (Grierson et al., 2015). In that study, HEV RNA was detected in 36 plasma samples with a viral load ranging from detectable but below the limit of quantification (i.e. 22 International Unit (IU)/mL) to a maximum of 10^6 IU/mL. The correction factor of 2.25 was used to convert the results to genome copies (ARUP, 2020) and the resulting values were used to parameterize an empirical cumulative distribution describing the variability in the observed viral load in blood of viraemic pigs as follows:

$$C_V \sim Cumulative(min; max; \{p\}; \{x\})\ gc/mL$$

where min and max are the Minimum (49.5 gc/mL) and Maximum ($1.3x10^6$ gc/mL) observed values while x and p are the vectors of the values of CV {49.5, $6.1x10^2$, $1.3x10^3$, $6.3x10^3$, $9.9x10^3$, $4.3x10^3$, $1.3x10^6$}(gc/mL) at the percentiles {0.83,0.86,0.89,0.92,0.94,0.97} respectively.

Simulation and sensitivity analysis.

Output of the stochastic model is presented as cumulative probability distribution describing the simulated overall level of HEV contamination in meat (HEV_{MEAT}). In order to evaluate the relative impact of the inputs, a sensitivity analysis was performed and a tornado chart was used to rank the inputs by their influence on the outputs' mean. Results were obtained as a mean of 500.000 Monte Carlo iterations using the software @Risk (version 7.0.1 for Excel, Palisade Corporation, Newfield, NY).

3. RESULTS

The estimated likelihoods and uncertainties for each step combined as per the combination matrix (Table 2) provided the cumulative likelihoods; these are reported for pathway 1, 2 and 3 in Tables 5-8 with the estimated likelihoods and uncertainties in brackets.

Table 5. *Summary of risk estimates for pathway 1: 'Presence of HEV on carcass after processing due to faecal contamination of the skin from environment and contacts with contaminated faeces during transport and lairage.' (H = High, M = Medium, L = low, VL = Very Low, EL = Extremely Low N = Negligible, U = Uncertainty).*

Step	Description	Likelihood (conditional)	U	Contamination	U
1	Likelihood of HEV being present on the skin of animals entering the slaughter line	M	$\vec{M}$	$R2$	$\overleftarrow{M}$
2	Likelihood of HEV not being eliminated/inactivated during scalding, dehairing and singeing	$N(N)$	L	$R0(R0)$	L
3-SC1	Likelihood of the skin being faecal contaminated during polishing (no plug of anus)	$M(M)$	$\vec{M}$	$R0$	$L(M)$
3-SC2	Likelihood of the skin being faecal contaminated during polishing (plug of anus)	$N(N)$	$L($	$R0(R0)$	$L(M)$

Table 6. *Summary of risk estimates for pathway 2: 'Presence of HEV on carcass after processing due to contact with viraemic blood at bleeding' (H = High, M = Medium, L = low, VL = Very Low, EL = Extremely Low, N = Negligible, U = Uncertainty).*

Step	Description	Likelihood (conditional)	U	Contamination	U
1	Likelihood of HEV being present in bloodstream	L	$\vec{H}$	$R3$	$\overleftarrow{H}$
2	Likelihood of HEV contaminating the skin during bleeding	$certain\ (L)$	$/(\vec{H})$	$R3$	$\overleftarrow{H}$
3	Likelihood of HEV not being eliminated/inactivated during scalding, dehairing and singeing	$N(N)$	$L(L)$	$R0(R0)$	L

Table 7. *Summary of risk estimates for pathway 3(faeces): 'Presence of HEV in meat due to faecal cross-contamination during evisceration' (H = High, M = Medium, L = low, VL = Very Low, EL = Extremely Low, N = Negligible, U = Uncertainty).*

Step	Description	Likelihood (conditional)	U	Contamination	U
1	Likelihood of HEV being present in faeces	M	$\overleftarrow{M}$	$R3$	L
2	Likelihood of internal organs being ruptured during evisceration	$VL(VL)$	$\overleftarrow{M}(\overleftarrow{M})$	$R3$	$\overleftarrow{H}$
3	Level of HEV contamination on skin in contact with faecal contamination following accidental rupture of the gut	/	/	$R3$	L
4-SC1	High amounts of non-visible contamination on carcass	$H(VL)$	$\mathrm{L}(\overleftarrow{M})$	$R3$	L
4-SC2	Low amounts of non-visible contamination on carcass	$H(VL)$	$\mathrm{L}(\overleftarrow{M})$	$R0$	L

Table 8. *Summary of risk estimates for pathway 3(bile): 'Presence of HEV in meat due to bile cross-contamination during evisceration' (H = High, M = Medium, L = low, VL = Very Low, EL = Extremely Low, N = Negligible, U = Uncertainty).*

Step	Description	Likelihood (conditional)	U	Contamination	U
1	Likelihood of HEV being present in bile	M	$\overleftarrow{H}$	$R3$	L
2	Likelihood of internal organs being ruptured during evisceration	$VL(VL)$	$\overleftarrow{M}(\overleftarrow{H})$	$R3$	$\overleftarrow{H}$
3	Level of HEV contamination on skin in contact with bile contamination following accidental rupture of the gallbladder	/	/	$R3$	L
4-SC1	High amounts of non-visible contamination on carcass	$H(VL)$	$\mathrm{L}(\overleftarrow{H})$	$R3$	L
4-SC2	Low amounts of non-visible contamination on carcass	$H(VL)$	$\mathrm{L}(\overleftarrow{H})$	$R0$	L

Results of qualitative sensitivity analysis for pathway 1 indicate that reducing the uncertainty in step 1 and 3 is unlikely to make any difference on the final estimate. In fact, even if under the worst scenario, the likelihood of step 1 was High instead of Medium, the Negligible chances for HEV to survive scalding and singeing in step 2 (with low uncertainty) makes the actual value of step 1 irrelevant. Similarly, even if the likelihood of the skin being faecal-contaminated during polishing is high, the amount of uniformly distributed faeces on a carcass would be so low to generate a level of HEV contamination per cm^2 of little/no concern in terms of risk of human exposure. Again, results of qualitative sensitivity analysis for pathway 2 indicate that reducing the uncertainty in step 1 is unlikely to make any difference on the final estimate. Even if under the worst scenario, the likelihood of step 1 was High instead of Low, the Negligible chances for HEV to survive scalding and singeing in step 2 (with low uncertainty) makes the actual value of step 1 irrelevant for the final estimate.

From the sensitivity analysis on both the pathways, it can be observed how the likelihood estimates for step 1 and step 2 are already considering, according to the available evidence, the worst scenarios for these events. For both the steps, according to the direction of the uncertainty, different but lower likelihoods are possible. This means that with the most likely cumulative likelihood being Very Low, if considering the uncertainty, the final estimate can only be lower than Very Low. Estimations are however very different for the final level of undetected contamination at trimming, within the range R3 in scenario 1 and R0 if considering scenario 2. The level of contamination in meat (HEV_{MEAT}) is estimated by two parameters only: the level of HEV in viraemic pigs (C_V), and the amount of residual blood per Kg of muscle tissue (R_{BLOOD}). In viraemic pigs, the minimum and maximum simulated values were 0.10 and $1.1x10^4$ gc/g respectively with $3x10^{-1}$, $1.6x10^1$, $4.8x10^2$ and $5.3x10^3$ gc/g at 50th, 90th, 95th and 99th percentile respectively (Figure 4).

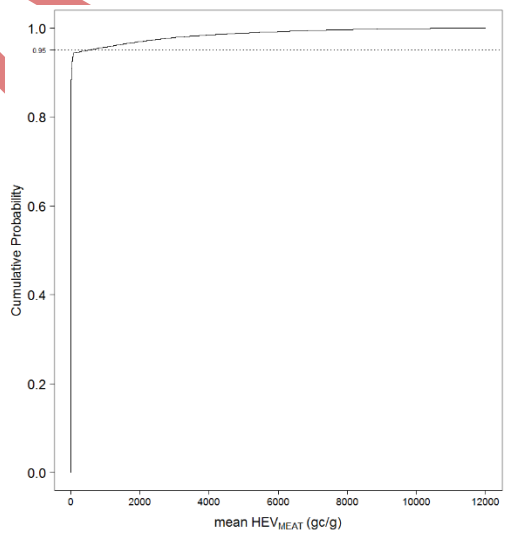

Figure 4. Cumulative probability describing the level of HEV in meat of viraemic pigs.

Results of sensitivity analysis in Figure 5 clearly indicate how HEV_{MEAT} is more affected by the viraemic titre in blood of infected animals than the amount of residual blood in tissues after bleeding.

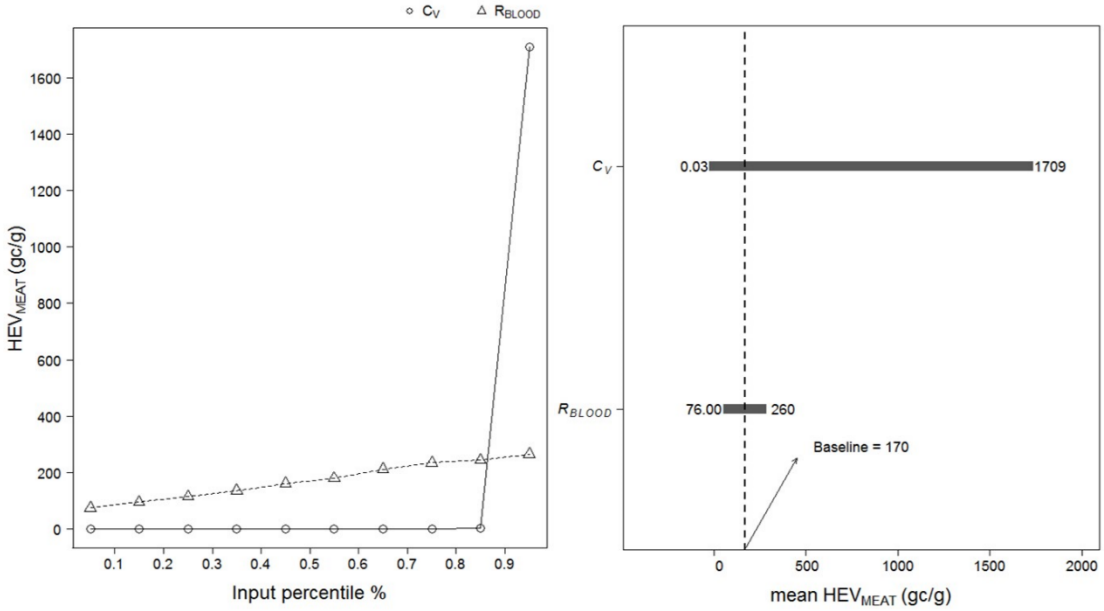

Figure 5. Sensitivity analysis. Following simulation, the spider plot (left) shows the mean values of HEV_{MEAT} obtained as a function of different percentiles values of the input variables. The tornado chart (right) shows the values of the lowest and highest means values for HEV_{MEAT} that are be obtained as a function of each individual variable when the other was held at its baseline value. C_v= Viral load in blood of viraemic pigs; R_{BLOOD}= Residual blood content in muscle.

4. DISCUSSION

In this work, we evaluated qualitatively and, when possible, quantitatively, the main risk pathways describing the dynamics governing the fate of HEV along the slaughtering line. As previously noted, considering the current important knowledge gaps in the epidemiology and host-pathogen interactions of HEV in pigs, the objective of this work was not to provide an accurate estimation of actual level of contamination to be expected in the meat or skin of a random pig. This would be unrealistic considering the existing knowledge gaps and the assumptions that would be required to describe probabilistically complex events such as the initial level of contamination on the skin or the cross-contamination due to animal-animal and animal-environment contacts/interactions. Probabilistic models have extensively been used in the context of food safety, supporting evidence-based control strategies for different food-borne hazards (EFSA, 2020; FDA, 2003) and highlighting

fundamental knowledge gaps that prevent accurate quantification of risk (Crotta et al., 2017). Indeed, our approach, although qualitative for most of the pathways, allowed not only to explicitly describe the dynamics that are hypothesized to affect the fate of HEV along the slaughtering process but also to evaluate the expected relevance of the risk pathways themselves and the potential effects of the inputs/events for which data are lacking. This is particularly useful in perspective of the development of comprehensive quantitative risk assessment models.

Our results suggests that a better characterisation of the initial level of skin contamination of animals entering the slaughterhouse or of the spread of viraemic blood on carcass during bleeding, which are both complex processes, would be uninformative. Because of the overall reduction effect exerted by steps until and including singeing, the final level of HEV contamination eventually present on the carcass after processing is totally driven by the contamination resulting from the uniform dispersion of contaminated faeces during polishing and/or accidental rupture of the intestines/gallbladders during evisceration. The amount of faecal contamination due to polishing is related to the amount of faeces mechanically extruded from the anus at that stage (if the anus is not plugged); this is expected to be so low as to generate a uniformly distributed HEV contamination that is probably irrelevant.

As for the events at evisceration, the joint likelihood "HEV is present in faeces/bile ∩ gut/gallbladder are accidentally ruptured" is indicative of a Very Low probability for contaminated faeces/bile to contaminate the carcass and even when it happens, the actual level of contamination on carcass is determined by the amount of contamination remaining undetected at trimming. The amount and spread of this contamination is not, in our opinion realistically predictable. However, from our scenario analysis it can be concluded that high amount of non-visible contamination because of ruptured gut/gallbladder at evisceration are possible but very rare events. For this reason, extensive studies aimed at getting accurate insight of the parameters regulating the extent of undetected contamination are poorly justified and doing a simple microbiological survey comparing the viral load between trimmed/non trimmed carcasses is probably the best option.

Several studies have investigated the occurrence and abundance of HEV in pork products so far (Berto et al., 2012; Boxman ILA et al., 2019; Giannini et al., 2018; Hao et al., 2018; Intharasongkroh et al., 2017; Leblanc et al., 2010; Moor et al., 2018; Szabo et al., 2015; Wilhelm et al., 2014). However, while the presence of HEV RNA was common in liver and liver-products, HEV has rarely been detected in meat, not even when a high viral load was detected in paired liver samples from the same animal (Feurer et al., 2018). This is consistent with results of our simulations (Figure 5) on the level of contamination in meat of viraemic pigs where values higher than $1x10^2$ gc/g were observed only above the 94^{th} percentile.

In conclusion, our risk assessment captures the biological risk pathways describing the main factors we hypothesize can explain the presence of HEV on processed carcass (via faecal and bile cross-contamination) and in meat (via viraemic blood). Considering that the skin is an edible part of slaughtered pigs, our results suggest that although rare, the presence of HEV on skin of processed carcasses cannot be excluded and for this reason, this should be targeted by microbiological surveys to better quantify the overall risk of human exposure to HEV through consumption of pork products. However, in contrast to other enteric bacterial pathogens such as *Salmonella spp.*, which pose a risk for consumers through external contamination of meat, the main risk posed by HEV seems to be the presence of the virus in meat as a result of residual viraemic blood. This risk pathway, described quantitatively in our study, represents to our knowledge the first modelling attempt to explaining the presence of HEV in meat. Results suggests highly viraemic pigs as the animals posing the greater risk for the presence of HEV in meat, with the risk being directly related to the presence of residual blood in muscular tissues after bleeding and the level of HEV contamination in the bloodstream of viraemic pigs.

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