Risk factors for *Salmonella sp.* in pig lymph nodes in Portuguese abattoirs

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SUMMARY

Salmonella is one of the major causes of food borne disease in the European Union (EU). Some of the human cases are related to pork products. An EU baseline survey to assess the *Salmonella* pork prevalence was performed. Mesenteric lymph nodes were cultured and *Salmonella sp.* isolates were serotyped. Data concerning the animal and the slaughterhouse was also collected. The aim of the present study was to search for potential risk factors to the presence of *Salmonella sp.* in pigs lymph nodes in Portugal and to search for differences in the risk profile between groups of serotypes. The data was analysed using a Bayesian approach to incorporate the hierarchical structure of the data (samples nested in slaughterhouses). Two models were analysed: a binomial (presence/absence of *Salmonella sp.*) and categorical model (absence of *Salmonella sp.*, serotype Typhimurium or serotype 1,4,[5],12:i:-, other serotypes). A total number of 659 samples were tested, belonging to 36 slaughterhouses. Around 23.7% of the samples were positive for *Salmonella sp.*. In the binomial model a significant association was found for region of the slaughterhouse - Lisbon and Tagus Valley Region with lower risk compared to the Centre Region (OR=0.36). In the categorical model a significant association for category Typhimurium or 1,4,[5],12:i:- was found for the variable hour when the sample was taken – afternoon with lower risk compared to morning (OR=0.20). The association found for the slaughterhouse region should be a matter of furthers studies to evaluate the hygiene practices in the slaughterhouses of that region.

Keywords: *Salmonella,* lymph nodes, risk associations

RESUME

Facteurs de risque de *Salmonella sp.* dans les ganglions lymphatiques de porc dans les abattoirs portugais

La salmonelle est une des principales causes de maladies d'origine alimentaire dans l'Union Européenne. Certains cas sont liés à des produits du porc. Une enquête a été faite pour évaluer la prévalence de *Salmonella* chez le porc au Portugal. Les ganglions lymphatiques des carcasses ont été cultivés et si *Salmonella* était présent, cela était sérotypée. Alors, des données concernant l'animal et l'abattoir étaient également recueillis. L'objectif de cette étude était de rechercher des facteurs de risque pour la présence de *Salmonella* ganglions lymphatiques des carcasses. Ces données ont été analysées en utilisant un modèle linéaire généralisé mixte pour prendre en incorporer la structure hiérarchique des données. Deux modèles ont été réalisés: un modèle binomial (présence/absence de *Salmonella sp.*) et un modèle catégorique (absence de *Salmonella sp*, sérotype Typhimurium ou sérotype 1,4,[5],12:i:-, autres sérotypes). Un total de 659 échantillons ont été testés, ceux-ci provenant de 36 abattoirs. La prévalence de *Salmonella sp.* est de 23.7%. Un risque significatif a été trouvé pour la région des abattoirs - Lisbonne et dans la Vallée du Tage (OR = 0,38) avec moins de risque par rapport à la région du Centre. Dans le cas du modèle catégorique, les résultats significatifs furent obtenus uniquement pour la catégorie Typhimurium ou 1,4,[5],12:i:- pour la variable le temps de collecte d'échantillons - l'après-midi avec moins de risque que le matin (OR=0.20). Les résultats obtenus devraient initier de prochaines études sur les conditions d'hygiène dans les abattoirs des régions les plus fortement touchées.

Mots-clés: *Salmonella,* ganglions lymphatiques, les associations des risques

INTRODUCTION

Salmonella is one of the major causes of food borne disease in the world. Pork products are related with some of the human cases. Because of the health impact of this agent the industrialized countries are engaged in controlling this agent. For that the European Union (EU) approved legislation (EU Regulation No 2160/2003) that imposed a reduction on the prevalence of this agent in food production animals, such as pigs. To set the target of this reduction for each country, at EU level, it was decided to carry out baseline surveys to estimate the prevalence of *Salmonella sp.* in some food production animals. The objective of the surveys was to obtain comparable data for all Member States through harmonized sampling schemes. In this context, a baseline survey in pigs at the slaughterhouse was done. Slaughterhouses are an important control stage for this agent as in some cases when there is a poor hygiene at lairage and during the slaughter process the initial contamination of the infected pigs can be disseminated through the slaughterline. The risk factors known at this stage are: poor hygiene and stress during the transport [3], hygiene and time at lairage [2, 13], hygiene of slaughter equipment and of the slaughter process [4, 7, 11], season [11] and duration of the slaughter [11]. This dataset refer to the Baseline Survey on *Salmonella* Prevalence in Slaughter Pigs in Portugal. The aims of the study were: 1) to search for potential risk factors for the presence of *Salmonella sp.* in the lymph nodes of pigs slaughtered in this country, and 2) to identify differences in the risk profile between groups of serotypes.

MATERIAL AND METHODS

SAMPLING FRAME AND SAMPLE COLLECTION

The objectives, the sampling frame, the diagnostic testing methods, as well as the collection and reporting of data, and the timelines of the Baseline Survey in Slaughter Pigs were specified in the Commission Decision 2006/668/EC, Annex I. The sampling frame was the list of the slaughterhouses that together accounted for 80% of the pigs slaughtered within the Member State. The samples in Portugal were collected between January and September 2007. The sampling size was estimated by the Portuguese Veterinary Authorities (PVA) based in the Commission Decision 2006/668/EC, which took in consideration an estimated prevalence of 50%, considered an infinite population, a significance level of α=0.05 and 4% precision error. The minimum sample size for Portugal, according to this scheme, was 600 pigs, and an extra of 10% was taken to account for non-response. The number of pigs to sample was stratified by slaughterhouse and was proportional to slaughterhouse capacity. The sampling days for each slaughterhouse were selected at random. Written procedures were given to the local veterinary services to assure that sampling fulfilled the guidelines recommended by the Decision. Each sample was formed mainly by an aggregate of ileocaecal and sometimes jejunal lymph nodes to assure that it had at least 15 grams of lymph nodes. The collection of the lymph nodes was done in an aseptic way to avoid external contamination. The lymph node samples were sent to the laboratory for microbiological detection of *Salmonella* according to the procedure defined by Annex D of ISO 6579. Each *Salmonella* isolate was serotyped in the National Reference Laboratory for *Salmonella* according to Kaulfmann-White scheme.

DATA COLLECTION

Along with the sample collection, information concerning the pig and the slaughterhouse was also collected, to assess their potential influence to the presence of *Salmonella*. The variables collected were: transport of pigs from different herds to the slaughterhouse (yes or no); carcass approval for human consumption (totally versus partially); detection of lesions in the lymph nodes (yes or no); sample collection time; month the sample was collected; time since the animal arrived the slaughterhouse until it was killed; weight of the carcass; weight of the lymph node sample; region of the slaughterhouse and annual capacity of the slaughterhouse. Questions about hygiene at lairage and slaughter were not collected.

DATA ANALYSIS

Some quantitative variables were aggregated into categories, such as annual capacity of the slaughterhouse (less than 30.000 pigs slaughtered/year, between 30.000 and 100.000 pigs, and more than 100.000), sample collection time (8:01a.m to 12a.m, 12:01a.m. to 8p.m., and 8:01p.m to 8a.m), and time between arrival to slaughterhouse till slaughter of the animal (less than 12h, 12h to 24h, more than 24h). Descriptive statistics were calculated for the continuous variables that were not categorized. Other categorical variables were aggregated in few categories such as month when the sample was collected (January till March, April to June, and July till September).

The data has a "natural" multilevel structure; pigs which provide the lymph node samples (first level) were nested in slaughterhouses (second level). The data was analysed using a Bayesian hierarchical model. Monte Carlo Markov Chain (MCMC) was used for estimation and implemented in WinBUGS software (BUGS project, http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/), open source software.

The analysis consisted of two models with two different outcome variables: 1) a binomial model for the presence/absence of *Salmonella sp.*; 2) a categorical model for different groups of *Salmonella* serotypes. In this second model, besides the reference category "no *Salmonella*", the positive samples for *Salmonella* were divided in two groups: i) serotype Typhimurium or serotype 1,4,[5],12:i:-, ii) other *Salmonella* serotypes. These groups were formed because of the relevance of serotype Typhimurium to human cases [9]. Serotype 1,4,[5],12:i:- was added to Typhimurium group because they share similar characteristics in terms of genetic similarity, virulence and antimicrobial resistance [1]. The different serotypes were not analysed individually because of the low number of cases per serotype (Table 1). This approach intended to identify and explore differences in risk factors between the categories of serotypes and the "no *Salmonella*" category. In the binomial model a logit link function was used. In the categorical model it was used a logit conditional link function. The random effects were assessed for the slaughterhouse level. As not all slaughterhouses in the country were sampled having in the model a random slaughterhouse effect allows inferring information from the sample to all slaughterhouses population. The probability for each category of the categorical outcome is modelled using the same explanatory variables but different slope parameters to assess whether these variables affect each category in a different way. A preliminary univariable analysis to investigate the variables to be included in the multivariable model was performed. The variables with a $P<0.30$ were considered to enter into the multivariable model. A α=0.05 was considered in the final model.

The model was implemented in WinBUGS and it ran long enough with sufficient burn-in to ensure convergence to the posterior distribution of the parameters. Convergence was assessed by visual means (inspection of time-series plots) but also more formally using the Raftery and Lewis diagnostic, and the Gelman-Rubin R-hat diagnostic [10, 12]. R-hat should be arbitrarily close to 1 for convergence. The chains were thinned by only collecting 1 in 10 consecutive samples and this eliminated autocorrelation in posterior samples

Serotype	Number of samples typed	Percentage of samples typed
S. Typhimurium	57	36.5
*S.*Rissen	22	14.1
S. 1,4,[5],12:i:-	17	10.9
*S.*Derby	17	10.9
S. Enteritidis	9	5.8
*S.*Give	7	4.5
*S.*Newport	7	4.5
*S.*Anatum	6	3.8
*S.*Agona	5	3.2
*S.*Bovismorbificans	2	1.3
*S.*Gaminara	1	0.64
*S.*Havana	1	0.64
*S.*Mbandaka	1	0.64
*S.*Ohio	1	0.64
*S.*Eboko	1	0.64
*S.*Panama	1	0.64
*S.*Infantis	1	0.64
Total	156	100

Table I: Number and percentage of samples typed for each serotype

(using the R-CODA package [5], in R). Mixing in the chains was assessed by comparing the MC (Markov Chain) error with the standard deviation, for each parameter. Ideally, the MC error should be less than 5% of the standard deviations for good mixing [6] and this was true for all parameters here. The presence of confounding was investigated by analyzing the correlation matrix of the joint posterior distribution for all model parameters but especially the slope parameters.

Priors for fixed effects were expressed as a normal distribution with zero mean and 10^2 variance. For random effects the prior was expressed as a normal distribution with mean zero and τ variance. The τ variance was expressed as a gamma distribution (0.5,0.05). As the median is not affected by the asymmetry of the distributions we used it as central tendency measure. The posterior median results were then converted to odds ratio (OR) to easy interpretation and also the 95% OR posterior credible interval (CrI) was calculated.

RESULTS

A total number of 659 samples from 36 slaughterhouses were tested. *Salmonella sp.* was isolated from 156 samples (23.7% of prevalence). Table 1 shows the results for each serotype. Most of the positive samples were identified as *Salmonella* Typhimurium followed by *Salmonella* Rissen.

After grouping the serotypes for the categorical model, the group serotype Typhimurium or serotype 1,4,[5],12:i:- had 74 samples (47.4% of the positive samples) and the group other serotypes had 82 samples (52.6% of positive samples).

The descriptive analysis of categorical variables is shown in Table 2. The dataset presented some missing cases as reported in Table 2. Table 3 shows the descriptive statistic for the continuous variables. It is not evident any difference between the groups of serotypes and the group with no *Salmonella*.

Results of the binomial analysis of the data (no *Salmonella* versus *Salmonella* presence) are shown in Table 4. A significant association with the presence of *Salmonella* was found for the region of the slaughterhouse: Lisbon and Tagus Valley Region with lower risk (OR=0.38) compared with the Centre Region, adjusted for the month of sample collection, sample collection time and annual capacity of the slaughterhouse.

For the categorical model the following variables were selected to enter in the multivariable model, based in the results of the univariable analyses: region of the slaughterhouse, slaughterhouse annual slaughter volume, month when the sample was collected, and sample collection time. For the final multivariable mode two variables were selected to enter: region of the slaughterhouse and sample collection time. Table 5 shows the final adjusted model results. In this model a significant association with the presence of serotype Typhimurium or serotype 1,4,[5],12:i:- (compared to "no *Salmonella*") was found for sample collection time: collecting the sample at afternoon had a lower risk (OR=0.2) compared to morning, and adjusted for the region of the herd. This result has a wide 95% credible interval (0.03-0.77).

DISCUSSION

The detection of *Salmonella sp.* in the lymph nodes of slaughter pigs is an indicator of the infection status of pigs to *Salmonella sp.*. To define a reduction target for this agent and consequently a control programme it is important to have information concerning the country prevalence and risk factors present, hence justifying the present study.

Variables	Samples positive to *Salmonella sp.*			Negative samples
	Samples positive to S. Typhimurium or serotype 1,4,[5],12:i:-	Samples positive to other serotypes	Total positive	
Transport of pigs from different herds to the slaughterhouse				
No	58	67	125	392
Yes	16	14	30	104
Missing cases	1	1	2	8
Lesions in the lymph nodes				
No	64	69	133	434
Yes	11	12	23	70
Missing cases		1	1	
Partial rejection of the carcass				
No	74	82	156	500
Yes	1		1	4
Hours since the animal arrived the slaughterhouse and was killed				
< 12h	14	23	37	119
12-24h	52	50	102	320
>24h	8	9	17	61
Missing cases	1		1	4
Month when the sample was collected (2007)				
January to March	19	25	44	136
April to June	21	23	44	199
July to September	35	34	69	169
Sample collection time				
8:01a.m. to 12a.m.	61	68	129	397
12:01 to 8p.m.	2	6	8	42
8:01p.m. to 8a.m.	12	8	20	65
Region of the slaughterhouse				
Centre	12	12	24	45
North	23	23	26	166
Alentejo	2	2	4	11
Lisbon and Tagus Valley	38	45	83	282
Slaughterhouse annual slaughter volume				
< 30 000 carcass	2	2	4	24
30 000 – 100 000	29	39	68	219
> 100 000	44	41	85	261

Table II: Descriptive of explanatory variables concerning positive samples to *Salmonella* (S. Typhiumurium or serotype 1,4,[5],12:i:- versus other serotypes) and negative samples.

Variable	Presence of *Salmonella*	Mean	Minimum	Percentile 25	Median	Percentile 75	Maximum
Carcass weight (Kg)	No	80.5	57.6	74.2	80	85	167.7
	All samples positive	80.8	57	74	79.1	84.9	169.6
	Typhimurium or 1,4,[5],12:i:-	78.6	57	73.4	77.5	82	108.3
	Other serotype	82.9	64	75	80.3	87.1	169.6
Lymph nodes sample weight (g)	No	17	15	15	16	18	32.1
	All samples positive	17	14	15	16	18	28.4
	Typhimurium or 1,4,[5],12:i:-	17.4	14	15.1	16.4	18	28.4
	Other serotype	16.7	15	15	15.7	18	22.6

Table III: Descriptive measures of continuous variables for Salmonella presence by groups of serotypes

To improve the randomization and consistency of the samples collected the national authorities organized a training session for all the involved parties in the baseline study, before the beginning of the study. Also this data was validated by EFSA [8].

In this study we used hierarchical models that are naturally handled in the Bayesian framework because of the conditional independence assumed between each level in the hierarchy (lymph node samples in the first level and slaughterhouses in the second level). In conjunction with WinBUGS, freely available software, the methodology

Variable	Univariable		Multivariable adjusted model		
	Posterior median	Posterior SD	Posterior median	Posterior SD	OR (95%CrI)
Transport of pigs from different herds to the slaughterhouse					
No	0.0				
Yes	<-0.01	0.25			
Days in transport	-0.31	0.34			
Carcass weight					
Lesions in the lymph nodes					
No	0.0				
Yes	-0.20	0.33			
Partial rejection of the carcass					
No	0.0				
Yes	-0.44	1.22			
Hours since the animal arrive the slaughterhouse and is killed					
< 12h	0.0				
12-24h	0.12	0.24			
>24h	-0.03	0.36			
Month when the sample was collected* (in 2007)					
January to March	0.0		0.0		1.00
April to June	-0.36	0.25	-0.37	0.25	0.69 (0.42-1.12)
July to September	0.23	0.24	0.22	0.24	1.24 (0.77-1.99)
Sample collection time*					
8:01 a.m. to 12a.m.	0.0		0.0		1.00
12:01 to 8p.m.	-0.57	0.41	-0.60	0.43	0.55 (0.22-1.22)
8:01p.m. to 8a.m.	0.02	0.34	-0.27	0.37	0.76 (0.36-1.54)
Region of the slaughterhouse*					
Centre	0.0		0,0		1.00
North	-0.53	0.41	-0.72	0.42	0.48 (0.22-1.13)
Alentejo	-0.31	0.82	-0.77	0.83	0.46 (0.09-2.21)
Lisbon and Tagus Valley	-0.63	0.40	-1.01	0.41	0.36 (0.16-0.80)
Slaughterhouse annual slaughter volume*					
< 30 000 carcass	0.0		0.0		1.00
30 000 – 100 000	0.56	0.64	0.79	0.68	2.04 (0.66-9.64)
> 100 000	0.67	0.66	0.98	0.71	2.67 (0.76-12.34)

* variables that were selected to enter in the final multivariable mode

Table IV: Binomial multilevel model univariable and multivariable results showing posterior median, posterior standard deviation (SD), odds ratio (OR) and 95% credible interval (CrI).

presented in the paper provides a general modelling tool which allows to incorporate expert knowledge in the specification of the priors or to restrict the priors taking into account the lack of information in the response variable.

The results of the Baseline Survey to *Salmonella* in Slaughter Pigs in Portugal showed a high prevalence of *Salmonella sp.* (23.7%) in the country. The authors decided to do two different models (a binomial and categorical model) to explore and identify differences in risk factors for the infection of carcass between *Salmonella* serotypes. Control programmes will be implemented to control all serotypes of *Salmonella sp.*, but it is possible to have different risk profiles. The knowledge of differences in risk may help to improve the economics and efficiency of the control programmes. As the data has a relative small number of samples was not possible to perform an analysis for each one of the serotype found. Then it was decided to create three major groups: no *Salmonella sp.* (reference group), serotype Typhimurium or serotype 1,4,[5],12:i:-, and other serotypes. In the binomial model the possible risk association found was region of the slaughterhouse: Centre Region had a higher risk (OR=2.6) of presence of *Salmonella sp.* than in the Lisbon and Tagus Valley Region, when adjusted for the others variables in the model. This result should be a matter of further studies to evaluate if this association is due to slaughterhouse management practices, as is suggested by other studies [4,7,11], or due to infected herds.

In the categorical model the variable found to be significant at the multivariable model was the sample collection time (afternoon compared to morning) for the category of serotype Typhimurium or serotype 1,4,[5],12:i:- as a protective association, although it had a wide credible interval. A possible cause for this wide credible interval is that we have a relative

Variable	Multivariable model					
	Typhimurium or 1,4,[5],12:i:-			Other serotype		
	Posterior median	Posterior SD	OR (95%CrI)	Posterior median	Posterior SD	OR (95%CrI)
Sample collection time						
8:01 a.m. to 12a.m.	0.0		1.00	0.0		1.00
12:01 to 8p.m.	-1.63	0.86	0.20 (0.03-0.77)	-0.36	0.48	0.70 (0.26-1.69)
8:01p.m. to 8a.m.	0.05	0.44	1.05 (0.42-2.44)	-0.51	0.52	0.60 (0.20-1.55)
Region of the slaughterhouse						
Centre	0.0		1.00	0.0		1.00
North	-0.67	0.57	0.51 (0.17-1.61)	-0.68	0.51	0.51 (0.19-1.40)
Alentejo	-0.52	1.14	0.60 (0.05-5.19)	-0.68	1.09	0.50 (0.05-3.66)
Lisbon and Tagus Valley	-0.87	0.54	0.42 (0.15-1.25)	-0.73	0.49	0.48 (0.19-1.28)

Table V: Final categorical multilevel multivariable model results showing posterior median, posterior standard deviation (SD), odds ratio (OR) and 95% credible interval (95%CrI) for category 1 (Typhimurium or 1,4,[5],12:i:-) and for category 2 (other serotype).

small number of samples, although it is considered a protective factor. A biological explanation for this association could be that the animals slaughtered in the afternoon have spent less time at all in the lairage because they enter the slaughterhouse early morning to be culled in that same day . Because the transmission of this type of *Salmonella* is strongly associated to transmission between live animals, the reduction in the contact between pig from different origins at the lairage could play an important role in explaining this finding. This data are representative of Portuguese slaughter pigs and contribute with valuable information for assessing risk factors. However the data collected did not evaluate the hygiene of the transport and lairage, known risk factors in various studies which could enlighten the slaughterhouse risk factors in this country.

CONCLUSION

These results show an association between the region of the slaughterhouse and the lymph node *Salmonella sp.* positivity, which could be explained by different management practices in the slaughterhouses. As this study did not evaluate hygiene measures and management practices at each slaughterhouse it is necessary to perform such studies to enlighten these results. Also the results show a protective association for sample collection time for the group Typhimurium. The statistical methodology used in the study proved to be useful when we have small dataset and a multilevel structure of data, and it could be used in similar studies.

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