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Factors associated with the serological prevalence of *Salmonella enterica* in Greek finishing swineherds

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SUMMARY

Blood samples were taken from 50 finishing pigs at 90–105 kg in each of 59 randomly selected farrow-to-finish herds. The sera were tested for antibodies to *Salmonella enterica* by the Danish mix-ELISA. Samples with an optical density of $>10\%$ were considered to be positive. Associations between the odds of seropositivity of pigs and possible risk factors were evaluated in multivariable logistic regression models. The results of the analysis indicated that pigs fed non-pelleted dry or wet ration had 11 ($P=0{\cdot}0004$) or 9 ($P=0{\cdot}02$) times, respectively, lower odds of seropositivity than those fed pelleted ration. The risk of seropositivity was 4 ($P=0{\cdot}0006$) times higher in pigs fed a combination of chlortetracycline, procaine penicillin and sulphamethazine during fattening than in those fed an approved growth promotor or a probiotic.

INTRODUCTION

Consumption of contaminated pork and its products has been estimated to account for about 10–15% of the total human incidents of salmonellosis in Denmark, 14–19% in The Netherlands and 18–23% in Germany [1]. Although salmonellae are ubiquitous in nature the most frequent sources of contamination of pork and its products are the sub-clinically on-farm infected pigs [2]. Pigs that shed salmonellae have increased probability of producing contaminated carcasses and are the likely sources of cross-contamination for uninfected pork [3]. Prevention of human salmonellosis has been attempted by the implementation of nation-wide programmes either for control [4] or for eradication of pig salmonellosis [5]. In Denmark, after 7 years of operation of the national control programme, the number of human cases attributed to the consumption of contaminated pork declined from approximately 1100 in 1993 to 166 in 2000 [6].

The national control programmes incorporate generally two elements, namely monitoring and surveillance. Their primary goal is to identify and control herds whose pigs have high risk of shedding salmonella at slaughter and thus contaminate the subsequent levels of production of pork and its products. The methods to control the spread of salmonellae in pigs and also among pigs that are reared in the high-risk herds should be effective and should not disrupt current management. It is likely, that, because of the multifactorial nature of pig salmonellosis and because of its ability to maintain strong on-farm cycles [2, 7], some variation in the intervention strategies may be required.

Thus, in April 1996, we initiated a multi-national project [8, 9], supported by the European Union (EU), to investigate several aspects of the epidemiology of *Salmonella enterica* in European swine. In this paper, we present the results of the analysis of the

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risk factors for seropositivity of pigs in Greek finishing herds.

MATERIALS AND METHODS

Herds

Fifty-nine farrow-to-finish herds were selected by simple random sampling from the country's national registry, after excluding those herds with less than 20 sows and those that were located on Greek islands. Thus, the sampling frame included approximately 90% of the total Greek herds. The geographical distribution of the herds across the country and the regional populations of sows were reported in a previous article [10].

Sample size

The calculations of the sample size of herds and of finishing pigs within the herds have been presented [11]. Briefly, the number of herds was calculated by standard formulae [12] assuming that the prevalence of seropositive herds was 50%, the allowable error 13% and the level of confidence 95%. The minimum number of finishing pigs to be sampled within the selected herds was calculated by the standard formula for calculation of the minimum sample for detection of an infected animal in an infected population [13]. Assuming an average population size of 750 finishing pigs, diagnostic sensitivity of the serological test equal to 95% [14] and confidence of 95%, 50 blood samples were sufficient to detect at least one infected pig when the expected within-herd minimum seroprevalence was 6% or more. This sample size is also sufficient to estimate a within-herd seroprevalence of 50% with 95% confidence and an accepted error of $\pm 14\%$ [13].

Blood sampling and serological testing

From each finishing section of the farrow-to-finish herds, 50 pigs were blood sampled; in 55 of the herds the samples were taken at slaughter, and in the other 4 they were taken on the farm 2–4 days before the pigs were sent to the slaughterhouse. The liveweight of all sampled pigs was 90–105 kg.

The sera were tested for antibodies to *Salmonella enterica* by the Danish mix-ELISA [14] in the Danish Veterinary Laboratory. The mix-ELISA contains the O-antigens 1, 4, 5, 6, 7 and 12 derived from group B and group C1 isolates and measures an optical density (OD) as a percentage of a known positive control. Samples with an OD of $>10\%$ were considered to be positive for *S. enterica*.

Data collection and factors examined

Herd-level data were gathered by a standard questionnaire that had been pilot tested (available in English on request). The farmers were personally interviewed by two of us (LL and EG). Many of the variables recorded concerned general management of the herd and management and feeding of finishing pigs. We also recorded a number of health and productivity parameters (Table 1).

Statistical analysis

The herd was the unit of concern. The dependent variable was a binomial proportion with the number of pigs with OD $>10\%$ in the numerator and the number of pigs sampled in each herd in the denominator. Because of the contagious nature of pig salmonellosis and therefore, the positive correlation in the serologic responses of pigs from the same herd there was *a priori* concern for variability in the observed proportions of seropositive pigs in excess of the binomial variance. Thus, we estimated the intra-herd correlation coefficient (ICC) and evaluated its significance at the 5% level as proposed by Fleiss [15]. We estimated, subsequently, a variance inflation factor (VIF), based on the significant ICC, with the following formula $\mathrm{VIF} = 1 + \mathrm{ICC}(m-1)$, where m is the mean within-herd sample size, and multiplied the VIF with the simple binomial variance to obtain the design-based variance [16]. This variance was used in the construction of the 95% confidence interval (CI) of the mean within-herd seroprevalence of infection.

Each of the herd factors considered was initially regressed on the number of seropositive over the number of pigs tested in each herd, in logistic regression models (SAS PROC GENMOD, 17). During this screening of the data each bivariable model contained the variable FEEDTYPE in addition to the factor examined, because this variable was identified as an important risk factor in several studies [11, 18, 19]. The significant overdispersion in the proportion of seropositive pigs was accounted for by specifying the PSCALE option in the model statement of all models. This option calculates a scale parameter from the square root of the Pearson's Chi-square statistic divided by its degrees of freedom and adjusts with it the standard errors of the parameter estimates and the

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Table 1. *Description of factors examined for possible association with the risk of* salmonella enterica *seropositivity of finishing pigs from 59 Greek farrow-to-finish herds*

Management factors and health parameters (coding)	
HERDSIZE – Sows and gilts on the premises; a gilt was a female pig over 6 months of age that was selected or purchased for breeding, was bred at least once, but had not farrowed (number)	AGE – the age in days at which pigs were transferred into the finishing sections (number)
GILTPURCH – Female pigs over 6 months of age that were purchased for breeding in the last year (number)	TRANSFIN – Average number of pigs per transfer into the finishing sections (number)
GILTHERDS – Multiplying herds that supplied the herd with gilts (number)	WGT – Average weight of pigs at transfer into the finishing sections (number)
BOARPURCH – Male pigs over 6 months of age that were purchased for breeding in the last year (number)	BATCH – All-in all-out management of the finishing sections (yes/no)
BOARHERDS – Multiplying herds that supplied the herd with boars (number)	MANURE – Pens were cleaned to a manure-free stage between successive batches of finishers (yes/no)
QUARFAC – The herd had quarantine facilities for the incoming breeding stock? (yes/no)	DISINF – Cleaned pens were disinfected between successive batches of finishers (yes/no)
QUARUSE – The herd used the quarantine facilities consistently? (yes/no)	PENEMPTY – Average time in days finishing pens remain empty between successive batches (number)
DAYSQUAR – Average time in days the incoming stock were quarantined (number)	PLACEBACK – Runt pigs were placed back into pens where there were other pigs (yes/no)
CLOTHCHAN – Visitors were allowed entrance only after changing into clothes provided by the farmer (yes/no)	ALRES – Pigs were fed ad libitum or restricted diets
FOOTCHAN – Visitors were allowed entrance only after changing into footwear provided by the farmer (yes/no)	HOMEMIX – Finishing feed was mixed on the farm (yes/no)
HANDWASH – Visitors were allowed entrance only after washing their hands (yes/no)	FEEDTYPE – Finishing feed was dry and non-pelleted, pelleted or wet
SENUVIT – There were several separate units/sections for finishing pigs (yes/no)	CHANGEFEE – The feed type had changed in the last six months (yes/no)
M2TOTAL – The total surface in square meters of the finishing sections (number)	ACID – Was any acid added into the finishing feed or water? (yes/no)
SLATFL – The finishing sections had slatted floors (yes/no)	CATGROWTH – The feed contained antibiotics or approved growth promoters or probiotics
PARTSLAT – the proportion of slatted over the total surface of the finishing sections (number)	WHEY – Was whey added into the finishing feed? (yes/no)
PENSEP – Pens in the finishing sections were separated by concrete walls (yes/no)	TREATGROU – Were finishers group-treated in the last three months? (yes/no)
SCON – Finishing pigs of adjacent pens had snout contact (yes/no)	ABIOT – Which were the antibiotics prescribed? (bacteriostatic, bactericidal and combinations of both)
FITFIN – Concrete pen separations were fitted to the ground surface of the section (yes/no)	DIA – Did the finishers exhibit signs of diarrhea in the last three months? (yes/no)
	FREQSLIGHT – How many times per month were finishers delivered to the slaughterhouse? (number)
	SLAUGHPG – How many pigs were delivered per batch? (number)

likelihood ratio statistics. This adjustment is good when herd sample sizes are large and relatively equal as were in this analysis (range 46–50, 90% quantile 50). All factors with a level of significance $\leqslant 0{\cdot}25$, as evaluated by the likelihood ratio χ^2 statistic, were further considered in a multivariable logistic model. This model was reduced in a stepwise approach (backward elimination of a variable followed by a test for forward selection of variables eliminated at previous steps) by employing a likelihood ratio test at each step. Model reduction was terminated when all variables in the model were significant at $P<0{\cdot}05$. Two-factor interaction terms between the variables in the reduced model were not created. Selection of the final

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model was based on the examination of regression diagnostics, goodness-of-fit criteria [20] and biologic plausibility. Adjusted odds ratios with accessory 95% CIs were calculated by exponentiation of the final model coefficients and their 95% profile likelihood CIs.

RESULTS

Serological prevalence

The distribution of the within herd seroprevalence is shown in Figure 1. At least one seropositive pig was found in 52/59 (88%; 95% CI: 80–96%) herds. Fifty per cent of the herds had from 0–5 seropositive pigs. The mean seroprevalence was 15·6% (95% CI: 11–20%). Serological results of pigs from the same herd were similar ($P<0{\cdot}0001$). The intra-herd correlation coefficient was 0·22.

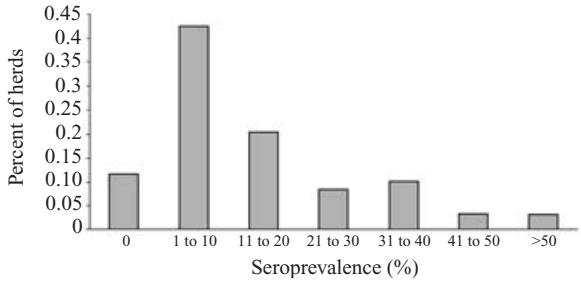

Fig. 1. Frequency distribution of *Salmonella enterica* seroprevalence in finishing pigs from 59 farrow-to-finish Greek herds (Danish mix-ELISA positive cut-off OD >10%).

Logistic regression analysis

Of the variables evaluated in the bivariable models for association with the odds of a pig being seropositive, six (DIA, TREATGROU, HOMEMIX, PENSEP, SENUVIT, CATGROWTH) were found significant at $P<0{\cdot}25$. After the stepwise reduction, only one, CATGROWTH, was significant at the 5% level. The two-factor interaction between this variable and FEEDTYPE, although biologically plausible, was not evaluated for significance because feeding pelleted feed was recorded in only one herd.

Pigs that were fed non-pelleted dry or wet ration had 11 or 9 times lower risk of testing seropositive than those that were fed pelleted ration. There was no significant ($P=0{\cdot}35$) difference in the risk of seropositivity between the pigs that were fed non-pelleted dry and wet ration. Pigs that were fed a combination (CYFAC®) of procaine penicillin (36·6 mg/g), chlortetracycline (73·2 mg/g) and sulphamethazine (73·2 mg/g) as growth promoter had four times higher odds of testing seropositive than those that were fed a ration containing an approved growth promoter or a probiotic (Table 2).

DISCUSSION

The use of the ELISA to detect pigs that were infected with *Salmonella enterica* has several advantages over the traditional isolation from faecal or swab samples: the most important being the greatly improved sensitivity, the low cost per sample and the ability to test large numbers of samples. On the other hand, the ELISA may not detect pigs infected with salmonella not belonging to the serological groups B, C1 and D1 or pigs infected shortly (i.e. 1–2 weeks) prior to sampling [14, 21]. The members of the serological groups against which the ELISA detects antibodies comprise at least 90% of the salmonellae isolated in Danish and in Dutch finishing pig-herds [22, 23]. Of the isolates from samples collected from the environment and from pig-carcasses in two Greek slaughterhouses over a period of 2 years at least 85% had O-antigens that will be detected by the serological method [9]. Culturing of final feed samples and of faecal samples from pens with finishers in serologically tested farrow-to-finish herds revealed contamination with serotypes that, with the exception of *S. london*, may elicit serological responses that will be detected by the ELISA [10]. Moreover, the *S. london* contaminated herd was serologically positive [9].

The interpretation of the ELISA test results at the OD >10% as cut-off improves its ability to detect moderate serological responses elicited by non-typhimurium serotypes [14] possibly at the expense of a proportion of its specificity especially in non-endemically infected populations [24]. Nielsen et al. [26] calculated that the ELISA had a sensitivity of 95% at the OD >10% as cut-off. However there was no field evaluation of the performance of the test in blood samples from Danish pigs. Recently, Enoe et al. [25] calculated, in latent class models, the sensitivity and the specificity of the ELISA in meat juice from Danish pigs at 60 and 100%, respectively. The sensitivity of the test in meat juice had been experimentally shown to be inferior to that in blood samples [26]. Lo Fo Wong [11] demonstrated that increases in the specificity of the test affect the strength of the calculated associations more than increases in the sensitivity. Five of the tested herds had only a single

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Table 2. *Factors associated with the risk of* salmonella enterica *seropositive finishing pigs. Samples with optical density percentage >10% in the Danish-mix ELISA were considered to be positive*

Factor	Number of herds	Odds ratio	95% CI*	*P*-value†
Type of feed				0·02
Pelleted	1	1‡		
Non-pelleted and dry	52	0·08§	0·01–0·45	
Wet	6	0·1§	0·01–0·67	
Combination of tetracycline, procaine penicillin and sulphamethazine in feed for growth promotion				0·001
No	54	1		
Yes	5	4·1	1·8–9·2.	

Scale parameter 2·85.
* 95% profile likelihood CI.
† *P*-value of the likelihood ratio test.
‡ The comparison group.
§ No significant difference ($P=0·35$).

sero-reactor, thereby; they were more likely to have been miss-classified. Re-analysis of the data with these herds assumed completely seronegative resulted in the same final model as the one presented in the results, however, with slightly larger odds ratios.

The type of feed as well as the size of the feed particles seems to significantly affect the ability of the pig's gut to resist colonization and multiplication of salmonella. In this study, we detected a highly significant difference between the serological results of a herd that was being fed pelleted feed compared to those of other herds being fed non-pelleted feed. The herd that was being fed pelleted feed had 31/50 seropositive pigs which was the second highest within herd seroprevalence. Also we isolated *S. typhimurium* from faecal samples that were collected from finishing pens at the day of blood sampling [10]. Nevertheless, we cannot rule out the possibility that the association between feeding of pelleted feed and seropositivity of finishers was confounded by another non-measured risk factor. However, the questionnaire used to collect the data in Greece was very detailed and was applied to all participating in the SALINPORK project countries. The comparable re-analysis of the combined data from all the participating countries also identified the feeding of pelleted feed as an important risk factor [9, 11]. Therefore, evidence from this and other studies shows that the proportion of seropositive pigs was lower in herds that finishers were given dry non-pelleted [11, 27] or wet feed [18, 19, 28] than in those herds where pelleted feed was offered. Although the exact mechanism has not been clarified, it has been suggested that coarsely ground grain may not be digested as well as more finely ground pelleted feed. Thus, some part of the undigested nutrients could be fermented into the large intestine resulting in the formation of volatile fatty acids and creating a hostile environment for salmonella [29, 30]. In this study we could not show a significant protective effect of wet over dry non-pelleted feed, likely because of the small number of herds giving wet feed to finishers. Other studies have found that finishers on liquid feed, fermented or not, were less likely to test seropositive than those on dry feed [18, 19]. This effect is probably due to a synergism of the following factors: (1) the lower pH of the wet feed (before consumption and while in the stomach of the pig) enhances the antimicrobial activity of lactic and acetic acid on salmonella, (2) the lower number of *Enterobacteriaceae* and salmonella in the gastric content, and (3) the failure of the few salmonella that survive the gastric content to compete for adherence sites because of the high numbers of *Lactobacillus plantarum* and of indigenous microflora [31].

The use of a combination of bacteriostatic and bacteriacidal antibiotics for extended periods in finishers feed, which is intended to promote the growth of pigs, increased the risk of seropositivity compared to herds that were feeding growth promoters or probiotics. The latter group included two herds fed tylosin at a

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level (20 ppm) approved for growth promotion. The herds feeding non-approved for growth-promotion antibiotics were most likely using the antibiotics as a substitute for failures in their management in conflict with recommendations on prudent use. Recent reports on the resistance patterns of *S. enterica* in finishing pigs in the USA indicate that resistance to chlortetracycline, procaine penicillin and sulphamethazine is widespread [32, 33]. Resistance to penicillin is significantly more frequent in isolates from the ileo-caecal lymph nodes than from the caecum, possibly pointing to an increased invasiveness of the former. This bactericidal chemotherapeutic is probably effective against the indigenous Gram-positive flora of the intestine, resulting in decreased colonization resistance [19, 34]. Therefore, the herds that use prophylactically not approved for growth-promotion antibiotics, may adversely affect not only the resistance patterns of salmonella but also the frequency of infection in the pigs. This may be true even for some of the approved for growth-promotion antibiotics since Van der Wolf et al. [23] found that finishing pigs, fed tylosin as growth promoter, were more frequently seropositive than those fed other growth promoters. Since this bacteriostatic antibiotic is known to be effective against Gram-positive bacteria but not against Gram-negative bacteria like salmonella, they also explained the association as being '… the damaging effect of tylosin on the endogenous flora resulting in a decreased colonization resistance'. Efficient proliferation of resistant microorganisms only occurs when selective pressure exists; that is only when the prevalence of resistance traits against a drug coincide with sub-therapeutic long-time use in the animal population [35]. The complete ban of some or even of all chemotherapeutic growth promoters in finishing feeds has to be considered, in the future, as an option to reduce the development of resistant salmonellae and to reduce the frequency of pig salmonellosis. In the meantime, credible systems for monitoring the use of antibiotics in pig herds should be developed and instituted by all EU countries [36].

Salmonella are able to maintain strong on-farm transmission cycles in some farms but not in others [7]. Thus, the distribution of the within herd seroprevalences is expected to result in there being many herds with no or few sero-reactors (lower-end cluster) and a number of herds with many sero-reactors (upper-end cluster), e.g., exhibit variation in excess of that assumed by the binomial variance. Other sources that increase the variability of the seroprevalence estimator are the segregation of pigs in pens and/or finishing compartments [19]. Unfortunately, we did not collect data about the pen or the finishing compartment where sampled pigs were raised and thus we could not appropriately control these sources of variability by multi-level modeling. This fact as well as the lower number of sampled herds may have affected our ability to identify some risk factors that appeared significant in other risk factor studies such as the snout-contact among pigs of adjacent pens or the continuous flow of pigs through the finishing herd [11] or the herd size of the finishing herds [23].

In conclusion, salmonella seroprevalence in 59 Greek finishing herds was positively associated with the use of pelleted feed and the prolonged inclusion of an antibiotic containing procaine penicillin, chlortetracycline and sulphamethazine as a growth promoter. The same effect of the type of feed on seropositivity is consistently identified in similar risk factor studies reported from other countries. The underline pathogenic mechanism should be further elucidated. In the meantime, Greek herds that feed pelleted feed to finishers should be identified, monitored for salmonella seroprevalence and obliged to change their feeding system. The prolonged feeding of antibiotics for growth promotion appears not only to influence the resistance patterns of salmonella but also to adversely affect the resistance of the pig's intestine to colonization.

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