PREVALENCE OF *HELICOBACTER*-LIKE BACTERIA IN THE GASTRIC MUCOSA OF PIGS SLAUGHTERED IN SOUTH-EAST SPAIN

Prevalencia de bacterias tipo *Helicobacter* en la mucosa gástrica de cerdos sacrificados en el sureste español

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ABSTRACT

We report an investigation of the prevalence of *Helicobacter*-like organisms morphologically similar to *Candidatus* Helicobacter suis (HLOs) detected by carbolfucsin in pig's gastric mucosa (cardiac, fundic and pyloric areas) and its relationship with mortality by gastric ulcer during fattening, the lesional stage at slaughter and the breed. Three studies were carried out in 1998 (study 1), 2000 (study 2) and 2005 (study 3) and animals in studies 1 and 3 were from the same farms of origin and common nursery. Study 1 included samples from 60 randomly selected stomachs of pigs from two batches showing very different mortality by acute gastric ulceration during finishing collected at slaughter. In study 2, samples were from Iberian, Duroc and Landrace animals. Study 3 included 20 stomachs from crossbreed animals. No relationship was found between the presence and density of bacteria with the mortality by gastric ulcer or with the lesional score or breed. However, a comparison of the samples collected in both studies pointed to a substantial increase in the prevalence and density of HLOs in gastric mucosa in study 2, showing an influence of the density in the cardiac area on the non-glandular mucosa lesional stage ($p=0.011$) There were differences between samples studied in 1998 and those collected in 2000 and 2005, being none significant between the latter. Regarding lesional severity, Iberian pigs mean lesional score was lower than in Landrace and Duroc pigs ($p<0.001$) and these had a similar score. The increase HLO infection overtime coincides with the ban in the use of antimicrobials as growth promoters in the European Union in 1998 and further studies should be carried out to confirm this important association.

Key words: esophagogastric ulcer, *Candidatus* Helicobacter suis, antimicrobials, growth promoters, zoonosis

RESUMEN

Al objeto de estudiar la prevalencia de bacterias morfológicamente similares a *Candidatus* Helicobacter suis (HLOs) mediante la tinción de carbolfucsina en mucosa gástrica porcina y clarificar la posible relación entre su prevalencia y el porcentaje de mortalidad durante el cebo, el estado lesional de la mucosa aglandular al sacrificio y la raza, se llevaron a acabo tres estudios en los años 1998, 2000 y 2005. En el estudio 1, se tomaron al sacrificio muestras de las regiones cardial, fúndica y pilórica de 60 estómagos de cerdos procedentes de dos lotes que habían tenido un porcentaje de mortalidad por úlcera gastroesofágica aguda muy dispar durante el cebo. En el estudio 2, llevado a cabo dos años después, se tomaron muestras al sacrificio de estómagos de animales de raza Ibérica, Duroc y Landrace. En el estudio 3, realizado en 2005 se tomaron muestras al sacrificio de 20 de cerdos híbridos comerciales. No se observó ninguna relación entre la presencia y densidad de bacterias ni con la mortalidad por úlcera aguda durante el cebo ni con el estado lesional al sacrificio ni con la raza de los animales. Sin embargo, al comparar las muestras de los tres estudios se observó un incremento sustancial en la prevalencia y densidad de HLOs en las tomadas en los estudios 2 y 3 con respecto a las tomadas en el estudio 1, observándose así mismo una influencia de la densidad de bacterias en la mucosa cardial con el grado de lesión en la mucosa aglandular en el estudio 2 ($p=0.011$). Hubo diferencias entre las muestras tomadas en el año 1998 comparadas con las tomadas en 2000 y 2005, sin que hubiera diferencias entre estos dos últimos grupos. Con respecto a la gravedad de las lesiones al sacrificio, éstas fueron menores en los animales de raza Ibérica que en los Landrace y Duroc ($p<0,01$) y similares entre estas dos últimas. Entre los hallazgos de este estudio destaca la coincidencia entre el incremento temporal de infección con HLOs y la prohibición del uso de antimicrobianos como promotores del crecimiento y éste aspecto debería ser investigado en futuros estudios.

Palabras clave: úlcera gastroesofágica, *Candidatus* Helicobacter suis, antibióticos, promotores de crecimiento, zoonosis.

INTRODUCTION

The discovery in 1984 of spiral-shaped bacteria related with gastric ulcer and some kinds of cancer in the human gastric mucosa (Marshall and Warren, 1984) represented a turning point in the knowledge of these diseases, despite the fact that neither organism fulfils the Koch's postulates (Marshall, 1995). These bacteria were taxonomically classified as *Helicobacter pylori* (Goodwin et al. 1989), and subsequently, *Helicobacter*-like organisms (HLOs) have been described in several species including dog (Jalava et al., 1998), cat (Lee et al., 1988; Jalava et al., 1998), cattle (De Groote et al., 1999a) and ferret (Fox et al., 1986). It has also been described in pigs, where it was first classified as *Gastrospirillum suis* (Mendes et al., 1991) due to its similarity with *G. hominis*. Later, this species was renamed as *H. heilmanii* type 1 and *Candidatus* Helicobacter suis was proposed for the strain observed in pigs (De Groote et al., 1999b), where its prevalence has been recorded to range from 9.5% (Grasso et al., 1995) to 86.6% (Cantet et al., 1999). As regards the responsibility of these bacteria in the onset or severity of oesophagogastric ulcer in pigs there is no consensus, some authors clearly support such a responsibility (Barbosa et al., 1995; Mendes et al., 1991; Queiroz et al., 1996; Roosendal et al., 2000; Choi et al., 2001) and others not (Krakowka et al., 1998; Kelly and Frienship, 2001, Melnichouck et al., 1999, Phillips et al, 2000).

The zoonotic character of these bacteria is still under debate, and it has been suggested that sheep (Papiez et al., 2003), dog, cat and pig (Jalava et al., 2001) could act as reservoirs of HLOs.

The ban in 1998 of the use of antimicrobials as growth promoters in the European Union (EU) has resulted in an increase in some enteric infectious diseases such as swine disentery or proliferative ileitis (Casewell et al., 2003;

Taylor, 2000), while little research has been carried out into influence of such a ban on the prevalence of HLOs in swine gastric mucosa.

MATERIAL AND METHODS

The studies were carried out during spring season of 1998 (study 1), 2000 (study 2) and 2005 (study 3). The schedule of samples collection was decided to avoid any kind of seasonal interaction in the results.

Study animals

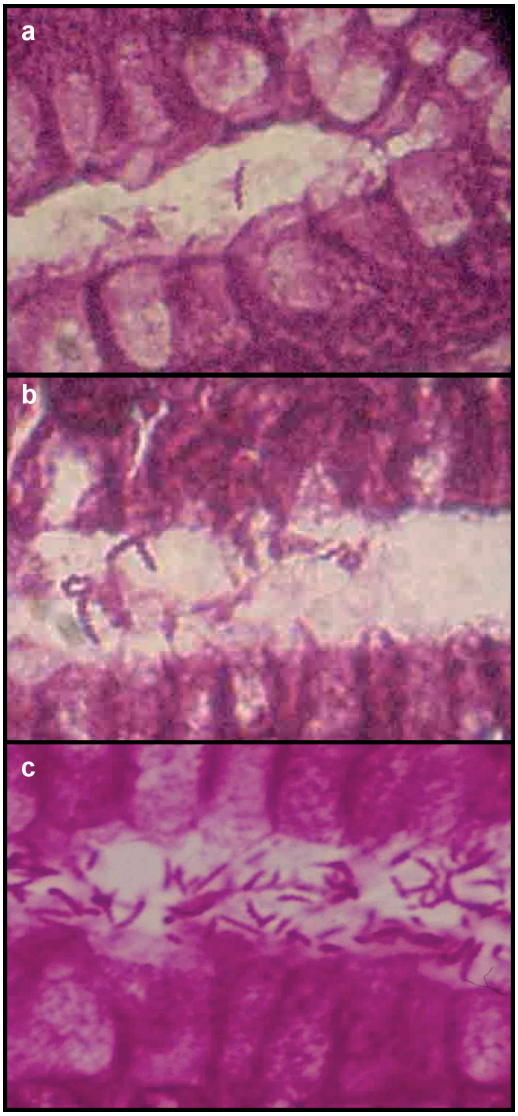
Study 1

Based on the mortality rates observed in the finishing in two groups of animals as a result of intragastric bleeding due to oesophagogastric ulcer during fattening, 60 stomachs were randomly selected and examined at the abattoir. Pigs were reared in two different buildings (group A and B) in the same finishing unit, under an all in-all out policy with cleaning and disinfection between batches, managed by the same farmer and reared under a three-sites-production system. All pigs, a commercial crossbred, were brought from the same origin farm and were positive to Porcine Reproductive and Respiratory Syndrome virus (PRRSv), *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, mange and Atrophic Rinhitis (AR). Animals were 6 months old at the moment of the slaughter.

Study 2

In the spring of 2000, 218 stomachs from batch A (Iberian pigs), 179 from batch B (Duroc) and 142 from batch C (Danish Landrace) were examined at the abattoir, being 75 stomachs randomly selected for microscopic examination (27 from batch A, 21 from batch B and 27 from batch C). Animals from batches A and B were brought from the same origin unit and were finished in different barns in the same fattening unit while batch C animals were brought from

Figure 1: **Score for the infectious density; a) Presence of isolated bacteria in a focal pattern; b) Presence of small bacteria groups in a focal pattern; c) Presence of small bacteria groups in a diffuse pattern or large groups under any pattern (CF).**



a different origin unit and fattened in a second finishing unit. The finishing units were managed under all in-all out by site policy with cleaning and disinfection between batches. The origin unit of A and B pigs had been subjected in 1999 to a mange, PRRSv, *M. hyopneumoniae* and *A. pleuropneumoniae* eradication plan using the modified Swiss Model (Barceló et al., 2001), based on a stop of farrows and the use of antimicrobials (enrofloxacin and tiamuline). At the moment of the study the animals remained free of the pathogens assessed through a sentinel program. The origin unit of C animals was free of PRRSv, *M. hyopneumoniae*, *A. pleuropneumoniae*, mange and AR. All three batches were reared under a two-site production system (farrowing and nursery at the same location). Animals were 6 months old at the moment of the slaughter, except the Iberian that were 10 months old.

Study 3

In the spring of 2005, samples from 20 stomachs were collected at abattoir. Animals were a commercial crossbred reared under three-sites-production system and were brought from the same common nursery that animals included in the study 1. Over the period between studies the origin farms suplying the common nursery were succesfully subjected to a mange eradication program. Animals were 6 months old at the moment of the slaughter.

Animal feeds

Generally, there were no quantitative or qualitative differences among the feeds used in the different batches, except for Iberian and Duroc animals (study 2) for wich the feed had, in terms of quality, a different content in fat to achieve an adequate to this production fatty-acids seroprofile.

During growing from 20 Kg to 50 Kg of live weight, the animals reared in 1998 were fed a standard pelleted feed containing 20 ppm of Tylosine phosphate (Trelacon 250®; ELANCO V., Spain) as growth promoter, and a standard feed without no growth promoter during finishing (from 50 kg to slaughter). In August 1998 of the use of Tylosine phosphate at growth promotion level was banned in the EU, so the animals reared in 2000 and 2005 were offered a standard pelleted feed with no additional antimicrobial as growth promoter during growing or finishing.

Management at the abattoir

All the animals were transported less than 40 km and, once at the abattoir, they were allowed a rest period lasting from 4 to 12 hours. After stunning in a chamber containing carbon dioxide the pigs were slaughtered by cutting the throat, and were bled out with a Rotastick® pump (Anitec, Sweden).

Stomachs were collected and moved to the sanitary facilities. Twenty minutes after slaughter they were opened along the greater curvature and gently washed with water for observation.

Macroscopic lesional score

All stomachs were examined grossly and scored using a standard lesional classification (Straw et al, 1993) modified as shown in Table 1. Furthermore, in study 1 all dead animals from both batches were necropsied in order to detect those that had died from gastric ulcer.

Histological analysis

Approximately 1 cm^3 tissue samples from the cardiac, fundic and pyloric areas of the stomach were taken using a sterile blade for each stomach. For histological examination, samples were fixed in 10% formalin, embedded in paraffin and cut to 5-μm sections.

The slides were stained with Warthin-Starry stain using as control a human gastric biopsy

Table 1: **Criterion for lesional scoring at slaughter, based on a previous macroscopic lesional description (Straw et al., 1992).**

Lesion	Score	Lesional stage
Normal keratinization	0	Nonglandular mucosa soft, white, smooth and glistening
Mild	1	Light yellow discoloration and keratinization of the superficial 1 or 2 cell layers, with a mild epithelial thickening involving less than 25% of the nonglandular gastric mucosa total surface
Moderate	2	Dark yellow discoloration and keratinization of the superficial epithelial layers, and development of wrinkling of the surface. Changes involving 25-50% of nonglandular mucosa surface.
Severe	3	Dark yellow discoloration and keratinization of the entire epithelial layer with a 2-fold increase in epithelial thickness, and rugged formation of the surface. Changes involving >50% of the surface area.
Erosion		
Mild	4	Filament-like erosions <2 cm in length with occasional areas of pin-point haemorrhage
Moderate	5	Linear erosions with areas of haemorrhage along the eroded area
Severe	6	Broad (>3 mm) erosions with haemorrhage along the entire length of affected areas
Ulcer	7	Complete epithelial loss with exposure of the underlying muscularis. Also healed ulcers

infected with *H. pylori*. Once the Warthin-Starry stain was validated in our laboratory as a useful technique for detecting HLOs in pigs, it was used to validate the carbolfucsin stain performed as described (Rocha et al., 1989). Finally, this technique was used as method of choice because it permitted a higher number of specimens to be managed simultaneously.

Stained tissue sections were examined by light microscope using a x100 oil-immersed objective to detect the presence of spiral-shaped microorganisms. Sections were scored according to the presence of bacteria and the density of infection, as is shown in Table 2 and ilustrated in Figure 1. This score gave the "Infection density" (ID) and was assessed in each mucosa portion from every stomach. The sum of the infection densities obtained for the 3 specimens from the same stomach was called "Infection index" (II) and ranked from 0 to 9. Both parameters were designed to simplify the comparision between animals and groups and to correlate infection density with the macroscopic lesional score.

Statistical analysis

Data from each group and year were included in a database (Excel®, Microsoft, USA) and subjected to statistical analysis using statistical software (Systat® v. 5.1, Systat, USA). The mean

Table 2: **Scoring criterion for infection density.**

Score	DESCRIPTION
0	Total absence of bacteria
1	Presence of isolated bacteria in a focal pattern
2	Presence of small bacteria groups in a focal pattern
3	Presence of small bacteria groups in a diffuse pattern or large groups in any pattern

lesional score of oesophagogastric mucosa was calculated by General Lineal Model, while to assess differences between breeds Tukey's test was used.

To asses the influence of HLOs on the severity of the lesions, the mean lesional score was calculated by ANOVA, the covariable being presence of HLOs regardless of the area stomach where HLOs were found. The statistical model used was:

$$X_{ijk} = ID_i + Inf_j + e_{ijk}$$

Where:

X = Lesional score at slaughter
ID = Infection density for each gastric area
Inf = presence of HLO regardless of gastric area
e = standard error

The mean ID and II was calculated by General Lineal Model, while to assess differences between years Tukey's test was used.

RESULTS

Study 1

Batches A and B showed different mortality rates from intragastric bleeding due to oesophagogastric ulcer during fattening: 2.33% (5.07% total mortality) vs. 0.24% (3.65% total mortality), respectively.

The percentage of stomachs showing an ulcer in the pars esophagea was higher in the group suffering a higher mortality by gastric ulcer during finishing (9.67% and 31.03%, respectively), even when there was no significant difference in the mean lesional score between batches (4.065±0.335 vs. 4.586±0.335, respectivelly). In the batch showing the lower percentage of ulcerated stomachs, the most prevalent lesional score was parakeratosis affecting the whole mucosa and the presence of slight erosions (35,48%).

Bacteria observed in the gastric mucosa were morphologically similar to *Candidatus* Helicobacter suis: around 7 μm of length and spiral shaped, allocated mainly in the lumen of the gastric foveolas.

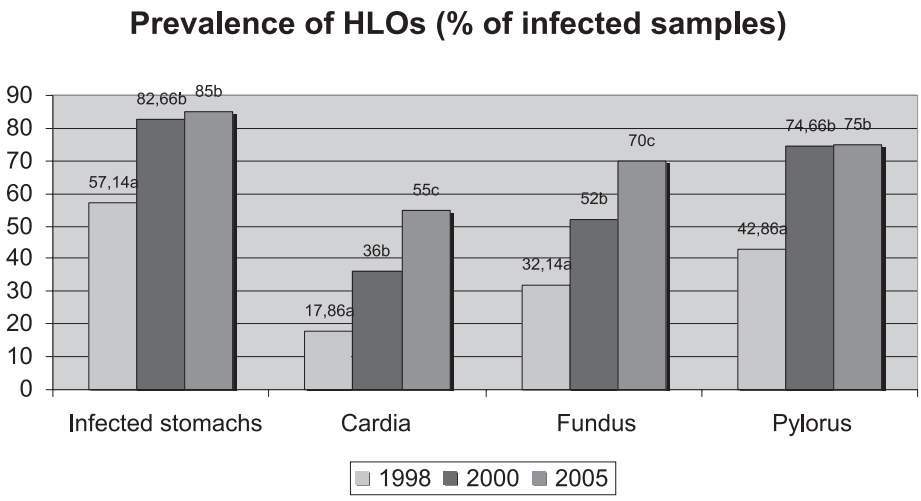
As regards the prevalence of HLOs there were no significant differences between batches in ID or II. Data are shown in Figures 2 and 3.

There was no observable influence of HLOs on the onset and severity of the lesions observed.

Study 2

There was a difference in prevalence of oesophagogastric mean lesions between breeds: 5.118±0.112 in Iberian, 6.022±0.124 in Landrace

Figure 2: **Prevalence of HLOs in samples collected in 1998, 2000 and 2005.**



and 6.196±0.139 in Duroc, respectively. There was no difference between Landrace and Duroc, being significant between Iberian and Landrace ($p<0.001$) and Iberian and Duroc ($p<0.001$).

With regards to the prevalence of HLOs, the ID and II showed no statistical relationship between breeds. Data are shown in Table 3.

No influence of the presence of HLOs regardless of gastric portion was observed, but there was a significant ($p = 0.011$) influence of the presence of HLOs in the cardiac mucosa on the severity of esophagogastric lesions.

None of the studied groups showed differences between them, so the data were grouped by year and compared.

Study 3

With regards to the prevalence of HLOs, the ID and II are shown in Figures 2 and 3, having a 85% of infected stomachs and a prevalence of infected samples of 55, 70 and 75% for cardiac, fundic and pyloric areas, respectively. The ID was 0.75, 1.56 and 0.85 for each gastric area with an II of 2.4.

Comparison of HLOs prevalence and bacteria density in studies 1, 2 and 3 samples

There was a noticeable difference between years as regards the percentage of stomachs infected: 57.14% showed HLOs in 1998, 82.66% in 2000, and 85% in 2005, being an increase of 25.52% and 27.86% compared with 1998, respectively. An increase of positive samples was detected comparing all three years in cardia and fundus, but there were no differences in pylorus, even when the percentage of infected stomachs (whatever the area) is not different comparing 2000 and 2005 studies.

As regards the ID, there were significant differences (Figure 3) between the gastric samples observed in 1998 and 2000 and 2005, for cardia and fundus, being no significant for pylorus. These differences results in an II of 1.143±0.249, 2.84±0.215 and 2.4±0.224 in 1998, 2000 and 2005, respectively, that represents a significant difference ($p<0.001$) between 1998 and 2000 and 2005 samples, being no difference between both last studies.

Figure 3**: Infection density and infection index in 1998, 2000 and 2005. Different suprescripts in the same categorie means significant differences (p<0,01)**

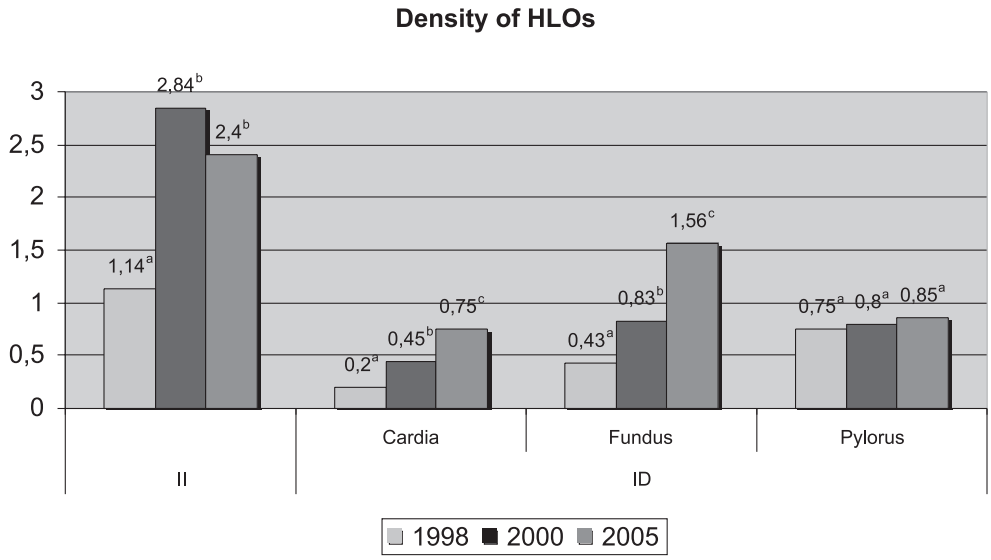


Table 3: HLOs prevalence and density for Iberian, Landrace and Duroc in Study 2**.**

			IBERIAN N = 27	**LANDRACE N = 21**	**DUROC N = 27**	**TOTAL N = 75**
Portions of gastric mucosa	Cardia	(%)	**15.38**	**47.36**	**51.85**	**36.00**
		ID*	**0.15**	**0.62**	**0.63**	**0.45**
	Fundus	(%)	**40.74**	**57.89**	**62.96**	**52.00**
		ID	**0.52**	**0.90**	**1.07**	**0.83**
	Pylorus	(%)	**74.07**	**89.47**	**70.37**	**74.66**
		ID	**1.74**	**1.38**	**1.52**	**1.56**
STOMACHS		(%)	**81.48**	**90.47**	**77.77**	**82.66**
		II†	**2.40**	**2.90**	**3.22**	**2.84**

* ID = Infectious density; † II = Infectious index

DISCUSSION

Comparing the results obtained in the batches observed in 1998, no correlation was found between mortality by gastric ulcer during fattening and the mean macroscopic lesional score, showing that macroscopic observation of stomach at slaughter is subjective, as are all macroscopic lesion-scoring systems, which only serve as a guide to the stage of the lesion (O'Brien, 1992). There was a difference in the severity of mean lesions at slaughter comparing the results from 1998 and 2000; but it should be taken into account that these changes in the mucosal lesional stage could be the result of the premortem management (fasting and length of rest period at the abattoir) as previously recorded (Muggenburg et al., 1967, Penny and Hill, 1973; Guise et al., 1995). Comparing the three different breeds involved in study 2, there was difference ($p<0.001$) in the mean lesional score observed for Iberian pigs, Landrace and Duroc, not being significant comparing Duroc and Landrace. Even when the age could influence the prevalence of lesions, in this study the oldest animals (iberian pigs) the lowest mean lesional score, suggesting a more strong influence from other factors than age of slaughter.

As regards the presence and density of HLOs, the most prevalent and highest density of HLOs was found in the pyloric area, followed by fundic and cardiac area. These findings agree with those previously recorded (Barbosa et al., 1995; Mendes et al., 1991). On the other hand, there was no relationship between mortality by gastric ulcer during finishing and the prevalence or infection density by HLOs. There are contradictory studies regarding the influence of HLOs on gastric ulcer and preulcerous lesions, some authors have recorded an influence (Barbosa et al., 1995, Querioz et al., 1996; Roosendal et al., 2000; Choi et al., 2001) but other have no found relationship between the presence of these bacteria and the lesions of the pars esophagea (Krakowka et al., 1998; Melnichouck et al., 1999; Phillips et al., 2000). However, some HLOs strains have been described as pathogenic for the pig (Krakowka et al., 2005a; Krakowka et al., 2005b).

The increase in the mean lesional score in stomachs collected between 1998 and 2000 was of note, even the lowest lesional value in the last year (Iberian pigs) being higher than the mean lesional score from 1998. When the data were analysed on a yearly basis there was a significant difference ($p<0.001$) in the mean lesional score, which suggests an increase in oesophagogastric lesions in the period between both surveys or an important influence of breed. On the other hand, the increase in HLOs prevalence and infection density during the period may have influenced the severity of the oesophagogastric lesional stage, a view supported by the finding that in 1998 this infection had no influence on lesional severity, while in 2000 infection density influenced the severity of non-glandular stomach lesions.

The data obtained in 2000 revealed no correlation between either ID or II and the breed, which suggests that there was no breed predisposition for HLOs infection, although more research under experimental conditions is necessary in this respect.

The 25.52% and 27.48% increase between years 1998 and 2000 and 2005 in the number of stomachs infected and significant differences in the ID in all detected gastric portions observed, except for pylorus, suggest that differences could be mainly due to use of Tylosine phosphate as growth promoter in 1998, since the feed composition remained constant over the period but it needs for further research.

The ban of most antimicrobials as growth promoters in 1998 following the "Precautionary Principle" of the EU has produced little effect on human health, except a decrease in the number of vancomycin-resistant enterococci strains isolated from human faecal carriers (Casewell et al., 2003). However, the consequences for animal health have been very different, with

a decrease in productivity and an increase in mortality being recorded in several countries (Wierup, 2001; Callesen, 2002; Wagener, 2002). The increase in mortality is a consequence of the prevalence of enteric infectious diseases, such as swine disentery, proliferative ileitis and colibacillary diarrhoea (Casewell et al., 2003). Our findings regarding the prevalence and infection density of HLOs suggest that HLOs prevalence in pig gastric mucosa may be influenced by this face, although more research is necessary to confirm this.

The role of different domestic species in HLOs transmission to humans is still under debate, although some authors have reported a higher seroprevalence of anti-*H. pylori* IgG in people working in pig and poultry abattoirs (Husson et al., 1991) and a higher prevalence of *H. pylori* infection in shepherds related with the contact with sheep and sheepherd dogs (Dore et al., 1999; Papiez et al., 2003). Even presence of the same HLOs in humans and pets living together has been described (Dietrich et al., 1998; Van Loon et al., 2003).

The influence of the ban of growth-promoters on the presence of HLOs in pigs and the possible effect on the transmission from animals to humans as a result of the ban should be carefully researched in the future. Even when it still not clear the responsibility of HLOs in the onset and severity of esophagogastric lesions, the increase in their prevalence could indicate the increase in the presence of pathogenic species such us HLOs strain 2662, but this term also needs for further research.

CONCLUSIONS

There was no relationship between the presence or infection density of HLOs and gastric ulceration of the pars esophagea in contrast, we recorded an increase in both parameters over time in pigs from the same origin farms and common nursery and overall, an increase in lesion severity in Landrace and Duroc breeds compared to the Iberian breed. The increase in HLOs infection over time coincides with the ban in use of antimicrobial growth promoters in the European Union in 1998 and this is an important issue that should be further investigated.

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