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Infection Dynamics and Risk Assessment of Bacterial Enteropathogens in Growing and Finishing Pigs in Eight Confinement Farms in Argentina

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Abstract

The purpose of this field study was to determine the dynamics of *Lawsonia intracellularis*, *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli* and *Salmonella enterica* infections and the variables associated with their detection risk, in eight intensive pig herds of Argentina. In diarrheic and non-diarrheic herds, a cross-sectioned fecal samples were obtained from pigs with and without diarrhea aged from 8 to 24 weeks old. Feces were scored visually in grades from 0 to 4. A multiplex PCR was performed to detect *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* and routine bacteriological procedure for the

isolation of *Salmonella enterica* and further serotyped in a reference laboratory. A multiple logistic regression model was applied to assess the risk factors associated with the agents under study such as type of farms, age of sampling, fecal score and single or mixed infection. *Lawsonia intracellularis* was the most frequently found in both diarrheic and non-diarrheic herds and in pigs with or without diarrhea. *Brachyspira hyodysenteriae* was found only in two diarrheic herds and only in diarrheic pigs. *Salmonella* sp. were found in five herds, in both non-diarrheic and diarrheic samples in the later only *S.* Typhimurium and *S.* Derby were detected. The risk of isolation *Salmonella* sp. and *L. intracellularis* increased with the age of the sampled pigs. In herds with or without diarrhea, more than one enteropathogen was identified. The infection dynamics in the farms evaluated was different from that observed in herds from countries with restrictions of antibiotic usage and highlights that antibiotics do not eliminate the infection but modify the dynamics of infection with evidence of subclinical infections.

Keywords: Swine; Diarrheic and Non-Diarrheic Herds; Growing And Finishing Pigs; Bacterial Enteropathogens; Risk Assessments

Introduction

In growing and fattening pigs, enteric infections are particularly of bacterial etiologies and can course as clinical and subclinical. Both presentations have a worldwide distribution, causing significant financial losses in indoor pig production systems due to a decrease in the average daily gain, and increases in the mortality rate, feed conversion rate and percentage of variation of the slaughter weight [27]. Bacterial enteric infections affecting pigs include porcine proliferative enteropathy, caused by *Lawsonia intracellularis* [29], swine dysentery, caused by *Brachyspira hyodysenteriae*, porcine colonic spirochetosis, caused by *Brachyspira pilosicoli* [11], and porcine enterocolitis, caused by *Salmonella enterica* [10]. Infections usually start at the end of the nursery stage, when piglets are susceptible due to the decrease of maternal derived antibodies (MDA) [4,25]. Nevertheless, both the infection and detection rates vary depending on the production system (all-in all-out [AIAO] *vs* continuous flow), the farm facilities (single *vs* multiple sites), the sampling methods (random *vs* directed), the diagnostic procedures (bacteriology, PCR or serology), and the use of antibiotics (prophylactic vs therapeutic doses, age) [7,22,32].

L. intracellularis and *B. hyodysenteriae* is mostly detected in herds and pigs with diarrhea [13,18]. However, both have also been isolated from pigs with subclinical infection [4, 11, 20]. Regarding *Salmonella* sp., pigs are the subclinical reservoir of numerous serotypes that might affect humans through contaminated pork products. However, few serotypes such as *S.* Typhimurium, *S.* Derby and *S.* Choleraesuis are pathogens for pigs [3,10].

In Argentina, studies about *L. intracellularis*, *B. hyodysenteriae*, *B. pilosicoli* and *Salmonella* sp. infections have been related to the single or multiple detection of these pathogens during diarrhea outbreaks, through longitudinal or cross-sectional serological studies and slaughterhouse monitoring [17,19,30,31]. However, references about their interaction in field conditions, clinical or subclinical presentation, type of diarrhea, and associated risk factors are scarce or null particularly in farms where the use of antibiotics in prevention or treatment doses are long lasting Thus, the aims of this study was to determine the dynamic of *L. intracellularis*, *B. hyodysenteriae*, *B. pilosicoli* and *Salmonella* sp. in eight diarrheic and non-diarrheic herds and to evaluate some risk assessment such as age of sampling, stool score and single or mixed infection.

Materials and Methods

Herd Selection and Sampling Procedures

A cross-sectional study was performed in eight farrow-to-finish farms with an average of 830 sows (range: from 70 to 2,500). The establishments were in main pigs producing provinces of Argentina such as Buenos Aires (4) La Pampa (1), Córdoba (1), Entre Ríos (1) and Santa Fe (1).

The farm selection criteria were made based on the acceptance, by the farm staff, of providing productive information at the time of the visit and the possibility, by the

personal involved in the study, to collect samples, perform necropsies of dead pigs and, eventually sacrifice animals with diarrhea.

Two of the farms (farms 6 and 7) used a continuous pig flow at the fattening facilities, whereas the remaining six (farms 1, 2, 3, 4, 5 and 8) were AIAO farms. Information about the vaccination schedule, vaccines used, and antibiotic administration (age, drug, frequency and duration of the treatment) was collected from each farm (table 1). All farms used PCV-2 and *Mycoplasma hyopneumoniae* vaccines at weaning.

A diarrheic herd was defined as one with clinical diarrhea requiring treatment.

Stool samples were collected directly from the rectum or from the floor when animals were defecating at the time of inspection at 8, 11, 15, 17, 20 and 24 weeks old. When diarrheic pigs in each age group were fewer than 10, a random sampling scheme was applied. Approximately 25 grams of feces were collected from each animal and placed in individual sterile plastic bags. Each sample was identified and assigned a score based on the characteristics mentioned below. The number of samples was established in order to identify at least one animal positive for any of the entities studied, considering an estimated prevalence of 20% and a confidence level of 95% and involved 10 diarrheic and 10 non-diarrheic pigs. This prevalence was calculated from previous studies in Argentina and elsewhere [1,16,24].

Feces were scored visually as follows: score 0 = well-formed feces with normal consistency, no diarrhea; score 1= semi-solid feces with no blood; score 2= watery feces with no blood; score 3= loose or formed feces with fresh blood; and score 4= watery feces with digested blood [31]. Furthermore, they were consider the color (blackish red; reddish; greenish yellow; yellow; grey and greenish) and the content (mucus; mucus and blood; fresh blood; digested blood; necrotic material; food without to digest; and without abnormal contents. Samples were refrigerated at 4ºC until reaching the laboratory.

Lawsonia intracellularis, Brachyspira hyodysenteriae and *Brachyspira pilosicoli* DNA detection

Samples were analyzed individually. A commercial kit for DNA extraction and purification (ZR Fecal DNA, MiniPrep, ZymoResearch, CA, USA) was used following the manufacturer's specifications. A multiplex PCR was applied for *L. intracellularis*, *B. hyodysenteriae* and *B. pilosicoli* detection and DNA visualization was performed with an ethidium bromide-stained agarose gel [15].

Salmonella spp. isolation and serotyping

Sample pool of five diarrheic feces and five non--diarrheic feces were made for each age group studied. Samples were processed according to Vigo *et al* [30]. *Salmonella* sp. isolates were identified by biochemical test and serotyped with the White-Kauffmann-Le Minor scheme by agglutination on slides (O antigen) and in tube (H antigen), using specific antisera (National Institute of Biological Production (INPB) - ANLIS "Dr. Carlos G. Malbrán", Argentina) according to Vigo *et .al* [31].

Statistical Analysis

A multiple logistic regression model was used to assess the risk factors associated with the agents under study. The categorical predictors were: a) farms (1 to 8), b) age of sampling (8, 11, 14, 17, 20, and 24 weeks old), c) fecal score (0-4), and d) single or mixed infection. The use of antibiotics was not considered as a variable because it was applied in all farms studied and in several age groups. For statistical analysis, R (The R Foundation, Vienna, Austria, 2012) was used. Associations with $P< 0.05$ values were considered statistically significant.

Results

Clinical diarrhea was observed in only four of the farms studied (farms 4, 6, 7 and 8) (table 1). All farms administered in–feed antibiotics at therapeutic doses at the growing-finishing stage. The duration of the treatment varied as follows: 4 weeks in farms 1 and 5; 5 weeks farm 4; 6 weeks in farms 2, 7 and 8; 7 weeks in farm 3 and 8 weeks, farm 6 (Table 1). It is noteworthy that farms categorized with clinical diarrhea received antibiotics for periods of 5 to

8 weeks. No antibiotics were used after 20 weeks of life. No vaccination against *Salmonella* sp. and *L. intracellularis* were applied in the monitored farms.

The table 1 shows the percentage of detection of the four bacterial agents studied in relation with the medication strategies and according with the age of pigs. Overall, *L. intracellularis* was detected in all herds, in 150 pigs (16.3%), *B. hyodysenteriae* in 13 pigs (1.4%) from two of the farms with clinical diarrhea (farms 6 and 7), and *B. pilosicoli* was not detected. It is important to point out that not all the age groups were available in farm 7, only 80 samples were collected from this farm.

Concerning *Salmonella* spp., 12 out of 184 fecal pool from five of the farms were positive for these bacteria (6.5%). The following *Salmonella enterica* serotypes were identified: *S.* Derby in farm 7 and *S.* subsp. I 4,12:i:- (S. Typhimurium monophasic variant) in farm 8, both of which were farms with clinical diarrhea. *Salmonella.* Tennessee in farm 2; *S.* Javiana in farm 1; and *S.* subsp. I 3,10:z10:- in farm 5; three farms where diarrhea was not relevant. In three out of the four farms with clinical diarrhea, more than one enteropathogen was detected (Tables 1 and 2).

Table 1: Proportion and absolute frequency (between parentheses) of identification of enteropathogens and antibiotic usage in each farm with (*) or without diarrhea

Farm	*L. intracellularis* Positive PCR	*B. hyodysenteriae* Positive PCR	*B. pilosicoli* Positive PCR	*Salmonella* sp. Positive pool	Weeks-old pigs /antibiotic
1	15.8% (19)	0	0	16.7% (4)	8, 9 = A16, 17=T/C
2	10.8% (13)	0	0	8.3% (2)	7, 10= A13, 14= T/C18,19= C/TY
3	7.5% (9)	0	0	0	5, 10, 12= A10, 11, 16, 17= T/C
4*	35% (42)	0	0	0	5=A12, 13, 19, 20= T
5	15% (18)	0	0	4.2% (1)	8, 9, 16, 17= A
6*	14.2% (17)	5.9% (7)	0	0	10,11, 14, 15, 18, 19, 22, 23=L
7*	3.8% (3)	7.4 % (6)	0	6.3% (1)	7, 8, 14, 16, 19, 20= T
8*	24.2% (29)	0	0	16.7% (4)	11, 12, 17, 18=T/C16, 19=N
Total	16.3% (150)	1.41% (13)	0	6.5% (12)	

T/C: tiamulin/chlortetracycline (220 ppm + 580 ppm); T: tiamulin (220 ppm);

L: lincomycin (140 ppm); A: amoxicillin (400 ppm); C/TY: chlortetracycline/tylosin (580 ppm +100 ppm) ; N: norfloxacin (400 ppm).

Table 2: Salmonella serovars isolated in each farm, number of positive pools by age of pigs with and without diarrhea

	Weeks-old pigs						
Farms #	**8**	**11**	**14**	**17**	**20**	**24**	**Serotype *Salmonella***
1					++*	++*	Javiana
2			++**				Tennessee
5						+**	Subsp I 3,10:z10:-
7				+**			Derby
8		+*		+**	++*		Typhimurium I 4,12:i:-

Table 3 shows the rate of detection of *L. intracellularis* and *B. hyodysenteriae* in relation to the stool score. *Lawsonia intracellularis* was identified in feces scored 0 (10.9%), 1 (17.7%), 2 (25.4%) and 4 (86.6%), whereas *B. hyodysenteriae* was detected only in feces scored 1 (1.5%); 2 (1.9 %) and 3 (42.8%). *Lawsonia intracellularis* was detected in 30.9 % of diarrheic samples and in 13.2% of non-diarrheic ones, whereas *B. hyodysenteriae* was identified only in a low percentage (4.9 %) of diarrheic samples. In relation to the age of detection, samples positive to *L. intracellularis* and *Salmonella* sp. were more frequently observed in 20- and 24- weeks old pigs, regardless of the fecal score or clinical diarrhea (table 4).

Table 3: Detection frequency of *Lawsonia intracellularis* (Li) and *Brachyspira hyodysenteriae* (Bh) and its relationship with stool score (*L. intracellularis*)

Farm	**Stool scores**																	
	0			**1**			**2**			**3**			**4**			**Total**		
	Li	**Bh**	**Total**	**Li**	**Bh**	**Total**	**Li**	**Bh**	**Total**	**Li**	**Bh**	**Total**	**Li**	**Bh**	**Total**	**Li**	**Bh**	**Total**
1	4	0	65	6	0	32	7	0	18	0	0	1	2	0	4	19	0	120
2	2	0	62	4	0	38	7	0	20	0	0	0	0	0	0	13	0	120
3	3	0	62	0	0	29	4	0	27	0	0	0	2	0	2	9	0	120
4	18	0	66	8	0	25	10	0	23	0	0	0	6	0	6	42	0	120
5	5	0	65	8	0	30	4	0	23	0	0	1	1	0	1	18	0	120
6	8	0	66	9	4	45	0	2	5	0	1	4	0	0	0	17	7	120
7	3	0	39	0	0	21	0	1	13	0	5	7	0	0	0	3	6	80
8	10	0	59	10	0	34	7	0	24	0	0	1	2	0	2	29	0	120
Total	53	0	484(10.9%)	45	4	254(17.7%)	39	3	153 (25.4%)	0	6	14	13	0	15 (86.6%)	150	13	920

Table 4: Results of logistic regression for *L. intracellularis* and *Salmonella sp.*

Lawsonia intracellularis detection		
Variable levels	OR	95% CI
Farm 1	4.5	17.4-1.1
Farm 4	14.8	54.2-4.0
Farm 5	3.7	14.3-1
Farm 6	4.0	15.3-1.0
Farm 8	8.8	32.8-2.3
14 weeks of age	7.4	26.1-2.1
17 weeks of age	12.3	43.1-3.5
20 weeks of age	30.8	106.1-8.9
24 weeks of age	47.4	163.1-13.8
Score 1	2.4	4.0-1.4
Score 2	4.7	8.2-2.7

Score 4	32.1	157.2-6.5
Salmonella sp. isolation		
	OR	95% CI
20 weeks of age	4.6	13.6-1.5
24 weeks of age	5.4	16.7-1.7

OR= risk of isolation; CI= confidence interval

Only statistically significant variables are shown.

The risk of isolation (OR) of *L. intracellularis* in pigs aged 24, 20, 17 and 14 weeks old was 47.4, 30.8, 12.3 and 7.4 times greater than in animals aged 8 weeks old and greater in farms 1, 4, 5, 6 and 8 (OR= 4.5, 14.8, 3.7, 4.0 and 8.8 respectively) than in farm 7. The risk of isolation of *Salmonella* spp. in pigs aged 24 and 20 weeks old was 5.4 and 4.6 times greater than in pigs aged 8 weeks old. Moreover, the detection risk in feces scored 4, 2 and 1 was 32.1, 4.7 and 2.4 times greater than in those scored 0 (Table 4). No other association was identified. The logistic regression for *B. hyodysenteriae* detection could not be conducted due to absence of positive pigs without diarrhea.

Discussion

Establishing the presence and frequency of enteric bacterial pathogens in pigs with clinical or subclinical infection at different ages allows determining the risk of occurrence of a potential outbreak and is an important step to determine internal biosecurity protocols in pig farms [7,16]. In this study, despite the high level of antibiotic use, the three bacterial enteropathogens were identified in pigs with and without diarrhea in diarrheic and non-diarrheic farms.

The most frequently detected pathogen was *L. intracellularis*, with the greatest detection rate observed in clinically affected farms. As reported previously, *L. intracellularis* infection is the main cause of diarrhea in growing-fattening pigs [7,18,25]. In farms with AIAO systems, *L. intracellularis* infection occurs in growers/finishers about 14-20 weeks old [29]. In our study, the highest detection rate (OR >30) was observed at 20-24 weeks old. These differences could be associated with the use of antibiotics. All the farms studied used in-feed lincomycin or tiamulin, which are first-choice antibiotics for the control of porcine proliferative enteropathy, in 10- to 20-weeks old pigs [29]. Afterwards antibiotic are banned in order to avoid finding waste at slaughter. It has been previously demonstrated that in--feed antibiotics affect the immune response and delay excretion [14,29].

The highest detection rate of *L. intracellularis* was observed in feces scored 0, 1 and 2, regardless of the presence of diarrhea at farm level. The subclinical infection of porcine proliferative enteropathy is characterized by loose-to-watery gray-green diarrhea (scores 1 and 2 in our study). In the present study, 10.9% of pigs with non-diarrheic feces were *L. intracellularis*-positive (table 3). The high percentage of positive PCR results in scores from 0 to 2 showed that the subclinical infection is a serious risk for production profitability [4,20,21] and can have a significant impact in the growth of pigs and can serve as source of infection of other pigs in the herd [19]. Watery bloody feces (score 4) positive to *L. intracellularis* were identified in 13 pigs, most of which were older than 17 weeks old. This finding is opposed to another study that demonstrated the reduction of susceptibility to *L. intracellularis* with the age associated with the increased of cellular immunity [6]. However, the presence of a low number of pigs with typical diarrhea of proliferative hemorrhagic enteropathy form could be considered a risk for a clinical outbreak, associated with a higher excretion rate of the agent [26].

Lawsonia intracellularis is frequently identified in Brazil, where it was reported that 50% of the farms are positive by the PCR technique, while 100% presented antibodies against *L. intracellularis* [23, 32]. In other parts of the world, detections at the farm level vary between 48.4% and 93.7%, being endemic in most pig-producing countries [7,25,26]. Variations in the percentages of positive animals may be due to the use of antibiotics, the short and intermittent elimination of *L. intracellularis* and the long duration

of antibodies in serum [7,23]. Furthermore, and in opposite to take place in our country, in those countries with a ban on the use of antibiotics, the reported prevalence has decreased due to the simultaneous implementation of hygiene and biosafety measures [13].

Salmonella enterica serovar Typhimurium has become a serious problem over the last two decades, especially in pig herds with a high health status [3,9]. Previous field studies in Argentina have shown that the most frequent *Salmonella* serovar was *S*. Typhimurium whereas cross-sectional intra-farm studies have revealed the coexistence of different serovars [18,30]. In this study, *Salmonella enterica* was identified in five herds. *Salmonella* Typhimurium monophasic variant, that lacks the second-phase flagellar antigens [26], and *S*. Derby, both serotypes that are pathogenic to pigs, were isolated only in two diarrheic herds (farms 7 and 8) in a low number of samples from pigs with and without diarrhea. *Salmonella* Typhimurium monophasic variant was identified only in farm 8 in 11,17 and 20-weeks-old pigs. In 1997, the emergence of a monophasic variant of *S*. Typhimurium (I,4,[5],12:i:-) was reported [10,26,33]. Single-phase *Salmonella* is considered an emerging human salmonellosis agent, whose main reservoirs are pork meat and its derived products. [7,9,26]. In 2020 in Argentina, two isolate of the monophasic variant of Salmonella Typhimurium (I 4,5,12:i:-) resistant to enrofloxacin and which also displayed multidrug resistance, was isolated from mesenteric lyphonodes of slaugther pigs [30]. Our study constitute the first report of this variant from live pigs.

This study constitutes the second report of the *Salmonella* Typhimurium monophasic variant in pigs in Argentina and highlights the role of pigs as *Salmonella* carriers for humans [30]. The detection risk for *Salmonella enterica* increased in pigs aged 20- and 24-weeks old and might also be due to the withdrawal of medicated feed at these weeks of age. Conversely, in antibiotic-free farms, natural infections with *Salmonella* mostly occur between weeks 8 and 14 [22]. The use of in-feed antimicrobials and AIAO systems can reduce the excretion, number and spread of bacterial infection at early stages of life [23]. During clinical disease, the organism inhabits a protected intracellular niche inaccessible to many common antibacterials [27]. The use of various antibiotics to treat enteric salmonellosis is widely used as prophylactic, because pigs with medicated feed have the antibiotic already present in the gastrointestinal tract to interact with the salmonella present in the herd, resulting in milder clinical signs due to decrease the inoculum [27].

The results of this study suggest that pig flow and antibiotic usage alter the infection dynamics of the farms evaluated, leading to a later infection. In the case of *Salmonella* infection, the high isolation in 20- to 24-weeks old pigs as well as the serotypes identified are in agreement with an extensive study carried out in Argentina and highlight the potential risk of *Salmonella* sp. contamination during slaughter [18,28,30,31].

Regarding *B. hyodysenteriae*, this bacterium was detected in diarrheic farms 6 and 7. It was only identified in feces with diarrhea and 46% of cases were associated with feces scored 3, mainly at the beginning of the fattening stage. Previous studies have reported that *B. hyodysenteriae* is more frequently detected in herds with hemorrhagic colitis and diarrhea with poor doing pigs [28]. Low *B. hyodysenteriae* detection (two out of eight herds) and absence of *B. pilosicoli* -as observed in this study- coincides with the low detection of these agents in intensive production systems worldwide due to technical and biosecurity improvements [7,15,16]. However, a current study carried out in Argentina on *Brachyspira* spp. in stool of finishing pigs collected from 53 farms found a regional prevalence of *Brachyspira* spp 75.5% which was lower in farms farms with >1001 sows. One hundred and twenty-eight isolates of *Brachyspira* were properly identified and the species found were *B. hyodysenteriae*, *B. pilosicoli*, *B. innocens,* and *B murdochii*. Both, *B. hyodysenteriae* and *B. pilosicoli* had low prevalence (1.9% and 7.5%, respectively), while *B. innocens* was isolated from 34% of the farms and *B. murdochii* was found in 39.6% [5].

In our study two positive herds used tiamulin and lincomycin for 8 and 6 weeks respectively (Table 1) to reduce the clinical outbreak. This could explain the low *B. hyodysenteriae* intra-herd detection rate (5.8% and 7.5%), in comparison to others report [2,5,9].

Similar to that found in other studies [13,18,26], more than one pathogen was identified in three out of the

four herds with diarrhea (farms 6, 7 and 8). Neither statistic association nor any particular combination tendency between pathogens was found [1,25,31]. Comparative studies of diarrheic and non-diarrheic herds have revealed that it is frequent to find more than one agent in herds with diarrhea and that the risk of clinical condition increases when two or more pathogens are present [13,26].

In conclusion, *L. intracellularis* was the most frequent pathogen both in diarrheic and non-diarrheic herds, whereas *B. hyodysenteriae* was detected only in diarrheic herds as well as in diarrheic stool. Subclinical conditions are important in the infection dynamics, as evidenced by the identification of *S.* Typhimurium and *S.* Derby in pigs without diarrhea and by the fact that one every 10.9 animals without diarrhea was *L. intracellularis*-positive. Moreover, coinfection was found in diarrheic herds. Finally, the infection dynamics was different from that observed in herds from countries with restrictions of antibiotic usage, a fact that highlights that antibiotics do not eliminate the infection but modify the dynamics of infection and delay the clinical signs and disease pattern.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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