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Longitudinal characterization of monophasic *Salmonella* Typhimurium throughout the pig's life cycle

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ABSTRACT

Swine have been described as an important reservoir of multidrug resistant monophasic *Salmonella* Typhimurium, though information on its ecology is scarce. A longitudinal study was performed in order to elucidate the *Salmonella* 4,[5],12:i:- dynamics throughout the pig's production cycle. A total of 209 faecal samples were collected from 10 sows and in six sampling times during the life of 70 pigs from a Portuguese industrial farm, and 43 isolates of *S.* 4,[5],12:i:- were identified and characterized regarding clonality and antimicrobial resistance phenotype and genotype. Most isolates (n = 42) exhibited resistance to at least ampicillin, kanamycin, neomycin, streptomycin, tetracycline and sulfonamides (encoded by bla_{TEM}, *aphAI-IAB*, *strA*, *strB*, *tetB* and *sul2*, respectively). Isolates obtained during the finishing phase showed additional resistance to chloramphenicol and florfenicol (*floR*), gentamicin and netilmicin (*aac(3′)-IV*). To our knowledge, this study is the first description of *aphAI-IAB* in *S.* 4,[5],12:i:-. PFGE analysis showed uneven distribution of isolates into three clusters, A (n = 34), B (n = 8) and C (n = 1). PFGE cluster A was predominant in sows (n = 5) and piglets in the farrowing phase (n = 17) and in pigs in the early finishing phase (n = 11) suggesting a carryover from birth to adult age. The introduction of PFGE cluster B isolates in adulthood could have had an external source, reinforcing the relevance of environmental transmission in the farm ecosystem. This study reveals a dynamic interaction between monophasic *S.* Typhimurium and the pressures exerted under an intensive swine production setting.

1. Introduction

Salmonella is a worldwide spread pathogen, and salmonellosis remains one of the most important foodborne zoonotic diseases today. Since 2007 there has been a continuous decrease in human salmonellosis cases notified in Europe (EFSA and ECDC, 2014). Nevertheless, it remains one of the most frequent zoonosis affecting humans and the most important regarding swine-related transmission (EFSA and ECDC, 2014). Although the most commonly reported *Salmonella* serovar is *S.* Typhimurium, a monophasic variant of this serovar, *S.* 4,[5],12:i:- has spread worldwide, since its emergence in the mid-1990's (EFSA BIOHAZ, 2010). The monophasic variant *S.* 4,[5],12:i:- has been increasingly responsible for *Salmonella* outbreaks., being the third most commonly reported serovar in the EU in 2012 (EFSA BIOHAZ, 2010; EFSA and ECDC, 2014; ECDC, 2015), and frequently reported across the world (Soyer et al., 2009; Ido et al., 2010; Tennant et al., 2010).

Another reason for concern is the presence of multidrug resistant serovar *S.* 4,[5],12:i:- isolates (Hopkins et al., 2010; EFSA BIOHAZ, 2010; ECDC, 2015). There are two prominent lineages within the *S.* 4,[5],12:i:- serovar in Europe: the Spanish and the European. Associated with phage type U302, the Spanish lineage consists of highly similar pulse-field gel electrophoresis (PFGE) clusters bearing plasmid-mediated resistance to ampicillin (A), phenicols (chloramphenicol, C), aminoglycosides (gentamicin, G and streptomycin, S), sulfonamides (Su), diaminopyrimidines (trimethoprim, Tp) and tetracyclines (T) (ACGSSuTTp type) (Antunes et al., 2011; García et al., 2013). The European strains on the other hand form a group of different phage and PFGE clusters that typically share a chromosomally encoded resistance to ampicillin, streptomycin, sulfonamides and tetracyclines (ASSuT type) (Hauser et al., 2010; Hopkins et al., 2010; Antunes et al., 2011;

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Rodríguez et al., 2012). Additional resistance to other antimicrobials such as fluoroquinolones has been detected in several strains from the European lineage, as well as resistance to third and fourth generation cephalosporins caused by plasmid-mediated extended spectrum β-lactamases (Rodríguez et al., 2012).

Prevalence studies have been carried out to evaluate the epidemiology of *Salmonella* across the swine production cycle (Funk et al., 2001; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005; Dorr et al., 2009; Keelara et al., 2013). Moreover, in a previous study (Fernandes et al., 2012), we have found evidence of a relationship between sow and a newly born litter that points to the existence of a transmission route of Salmonella. However, to our knowledge none of the previous studies were focused on the progression of monophasic *Salmonella* Typhimurium from birth to slaughter nor at the individual pig level. This study aims to characterize the dynamics of *S.* 4,[5],12:i:- in the pig reservoir throughout its productive life.

2. Materials and methods

2.1. Salmonella isolates

The *Salmonella* isolates included in this study were obtained from a longitudinal study conducted from November 2011 to July 2012 on an industrial swine farm in Portugal. Ten sows were randomly chosen and seven newborn piglets from each corresponding litter were ear-tagged with a code for litter (letter A–J) and piglet (number 1–7) identification. The farm had an ongoing antimicrobial protocol: a single dose of ceftiofur (Naxcel® 20 mg/piglet IM) was administered to all newborns as a preventive measure against neonatal infections such as navel infections, arthritis and colibacilosis diarrhea; after weaning, apramycin, colistin and zync were administered for the following two weeks, and a similar regimen was followed in the finishing phase, excluding zync (Table 1).

2.2. Sampling

Each pig involved in the study was sampled six times throughout its life: faecal samples were collected with sterile swabs at birth (before ceftiofur administration), after weaning, at the nursery unit, after entering the finishing unit and one day before transportation to the abattoir. After slaughter, samples were obtained by swabbing 100 cm^2 of the surface of the carcass at four locations (leg, belly, loin and blade) using a sterile sponge moistened with buffered peptone water (BPW) specific for this purpose (Table 1). The first samples collected at birth and samples from 10 sows were included in this study, although they have been partially characterized before (Fernandes et al., 2012), since we have new data regarding the isolates' antimicrobial resistance and clonal relationship with the newly isolated strains.

2.3. Laboratory testing

2.3.1. Isolation and identification of monophasic Salmonella Typhimurium

Each faecal sample was processed as previously published (Fernandes et al., 2012). Briefly, recommendations from the ISO 6579:2002 Annex D guidelines were followed, and one presumptive *Salmonella* colony was selected from XLD—Xylose Lysine Deoxycholate agar (Biokar Diagnostics, Beauvais, France) for serotyping according to the Kaufmann-White-LeMinor scheme and subjected to a species-specific PCR assay for the confirmation of *Salmonella* identification. The monophasic Typhimurium serotype was identified as recommended by the European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ, 2010), with a multiplex PCR following a negative phase inversion (Tennant et al., 2010).

2.3.2. Antimicrobial susceptibility testing

Susceptibility testing for 23 different antimicrobials was executed using the broth microdilution method (VetMIC Stördjur, National Veterinary Institute, Uppsala, Sweden) and the disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI, 2013a). The antimicrobials tested were amikacin, ampicillin (A), amoxicillin/clavulanic acid (Ac), cefotaxime, cefoxitin, ceftazidime, ceftiofur, chloramphenicol (C), ciprofloxacin, danofloxacin, enrofloxacin, florfenicol (F), gentamicin (G), kanamycin (K), nalidixic acid (Na), neomycin (N), netilmicin (Ne), streptomycin (S), sulfonamides (Su), sulfamethoxazole/trimethoprim (Sxt), tetracycline (T), tobramycin (To) and trimethoprim. For the sow and piglet samples, chloramphenicol, ciprofloxacin, danofloxacin, kanamycin, netilmicin and tobramycin were tested to complement the previous screening panel (Fernandes et al., 2012). Results were interpreted according to the veterinary guideline CLSI Vet01-S2 when applicable, which included breakpoints for amoxicillin/clavulanic acid, chloramphenicol, sulfonamides and tetracycline (CLSI, 2013a). Recommendations from the veterinary working party of the Antibiogram Committee of the French Society for Microbiology were followed in regard to the clinical breakpoints of ampicillin, ceftiofur, cefoxitin, danofloxacin, enrofloxacin, florfenicol, gentamicin, nalidixic acid, neomycin and streptomycin (CA-SFM, 2013). CLSI M100-S23 human susceptibility criteria were used for cefotaxime, ceftazidime, ciprofloxacin, netilmicin, trimethoprim and tobramycin (CLSI, 2013b).

2.3.3. Detection of antimicrobial resistance genes using PCR

Salmonella 4,[5],12:i:- isolates were screened for the following antimicrobial resistance genes *strA* (designed for this study: forward primer 5′ TGACTGGTTGCCTGTCAGAG 3′ and reverse primer 5′ CGGTAAGAAGTCGGGATTGA 3′) and bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{CTX-M}, *sul1*, *sul2*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(3′)-*

Table 1
Sources used and antimicrobial exposure of all sampling phases.

Sampling Phase Number	Unit	Time of life	Age Group	Sample Source		Antimicrobial exposure
				Sows	Pigs	
1	Farrowing	24 h after farrowing/birth	Sows/piglets	Pen	Rectal	Piglets – ceftiofur 20 mg IM;
2	Weaning – 1st stage[a]	4 weeks after birth	Pigs	–	Rectal	Apramycin + Colistin + Zinc for 2 weeks after weaning
3	Weaning – 2nd stage	10 weeks after birth	Pigs	–	Rectal	
4	Finishing[b]	14 weeks after birth	Pigs	–	Rectal	Apramycin + Colistin for 2 weeks during finishing phase
5	Finishing[c]	25 weeks after birth	Pigs	–	Rectal	
6	Slaughterhouse	25 weeks after birth	Pigs	–	Carcass	

[a] Samples collected 24 h after weaning.
[b] Samples collected 24 h after transfer to the finishing unit.
[c] Samples collected at the finishing unit 24 h before being shipped to the slaughterhouse.