Longitudinal study of an Australian pig farm infected with monophasic *Salmonella* Typhimurium-like PT193 (1,4,[5],12:i:- PT193) using MLVA

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Abstract

An Australian weaner-to-finish pig herd with a history of entrenched *Salmonella* infection (>10 months in duration) was sampled on 5 occasions over a 15 month period commencing in 2011. On each occasion, either 12 or 18 individual pen faecal samples were collected from pigs between 6 weeks and 22 weeks of age (n=71). From the *Salmonella* positive samples (n=42), a total of 346 isolates were further processed (up to 10 colony picks per sample). All of the 123 isolates typed were identified as monophasic *S*. Typhimurium-like PT193 (mSTm193). Results of antibiotic sensitivity testing found 336 of 346 isolates were resistant to ampicillin, streptomycin, sulphathiozole and tetracycline (ASSuT). In addition, all 346 isolates were examined by MLVA and 13 different closely-related MLVA profiles were identified. Only 2 MLVA profiles persisted throughout the 15 month study. This study is the first report of monophasic *S*. Typhimurium-like PT193, its persistence and associated clinical disease in an Australian pig herd.

Introduction

Although traditional serotyping differentiates *Salmonella* into more than 2,000 serovars, a relatively small subset of serovars is frequently associated with human infection and the contamination of food. Phage typing provides an additional level of discrimination for serovars such as S. Typhimurium (STm), but even more discriminatory DNA techniques are required to accurately ascertain the origin of a particular isolate. Consequently, regulatory authorities are increasingly turning to molecular techniques to more accurately identify the source of human outbreaks of food borne disease.

Traditionally, Australian pigs have been considered to have low rates of infection with *Salmonella* spp., despite the absence of an official control program. For example, the carcase *E. coli* and *Salmonella* monitoring (ESAM) detection rate of *Salmonella* is between 1 and 2%: relatively low by international standards. Further, *S*. Typhimurium is isolated from only 5% of positive ESAM samples and was likewise isolated infrequently from on farm sampling of pig herds, except in the rare cases of clinical salmonellosis, a condition almost invariably associated with *S*. Typhimurium in Australia (Hamilton *et al*, 2000; 2002; 2004; 2006; 2007). None-the-less, when pork is associated with foodborne salmonellosis outbreaks, it is the serotype *S*. Typhimurium that is usually implicated (Pointon, 2015).

In the last 4 to 5 years there appears to have been a change in the *Salmonella* landscape in Australia. Anecdotally pig veterinarians began reporting more clinical cases and public health authorities have become increasingly concerned with the emergence of mSTm193 in human cases and its possible link to pigs (Pointon, 2015). Therefore, the aim of this study, therefore, was to find a herd with an established STm (preferably PT193) infection and follow it over time using molecular methods to assess on-farm persistence and to add to our understanding of the inherent stability and relatedness between *Salmonella* isolates.

Materials and Methods

An “eco shelter” based deep litter weaner to finisher farm was identified with 5% mortality and 15% morbidity

in 4-10 week old pigs. Weaners arrived in batches of 600 and were placed in shelters holding 300 pigs, with cohorts being moved every 6 weeks to larger shelters. Bedding (barley straw) management was good with liming between batches. *Salmonella* Typhimurium had been isolated from lung and spiral colon lesions at post mortem several times over a 10 month period prior to this study; the diagnosis being *Salmonella* septicaemia.

Pre-study samples

Fortuitously, 3 isolates from 3 post mortems conducted on 4-8 week old pigs 2 months prior to this study commencing (i.e. in April 2011), were able to be obtained from the investigating laboratory. They were serotyped, phage typed, and confirmed as mSTM193.

Farm study samples

For the following 13 months (June 2011 to July 2012), individual pen faecal samples (i.e. from a single pig) were opportunistically collected by the consulting veterinarian during routine farm visits on 5 separate occasions, with either 12 or 18 samples collected each time from pigs aged between 6 and 22 weeks. Three age groups were targeted (6-10 weeks, 12-13 weeks and 17-22 weeks), with 6 samples taken from each age group available during the visit. For each sample, a minimum of 25g material was collected from a fresh, undisturbed faecal pat. For logistic reasons (e.g. the farm was relatively isolated and more than 2000km from the laboratory), the period from collection to laboratory testing varied from 4 to 18 days, with samples being kept at 4°C.

Weaner source farm samples

The weaner source farm was hundreds of kilometres distant from the study farm, did not have regular veterinary visits and had not agreed to participate in the study. Eventually, 3 months after the farm study was completed, an opportunity arose to collect 4 faecal samples on this source farm – from a single pen of 80 weaners.

Laboratory methods

Once received, the entire fecal sample was weighed then added to Buffered Peptone Water (BPW) at a ratio of 1:10 for pre-enrichment and incubated overnight at 37°C. Aliquots of this pre-enrichment (3 x 33µL) were inoculated onto the surface of a modified Rappaport-Vassiliadis medium (MSRV) plate (van de Giesson et al, 2003). These plates were then incubated upright for up to 18-24 hours at 42°C +/- 1°C and observed for typical halos of motile bacteria (ISO 6579). Sub-cultures were taken from the outside edge of the halo and purity confirmed by streaking onto CLED with Andrade's agar and genus confirmed by latex agglutination using Serobact™ Salmonella. Suspicious colonies that were latex agglutination negative were checked by biochemistry (MICROBACT™ 24E). Where available, 10 colony picks were taken from all confirmed positive cultures and isolates sent to the Microbiological Diagnostic Unit Public Health Laboratory (MDUPHL), Melbourne, overnight for serotyping, phage typing and molecular characterization using a multi-locus variable tandem repeat analysis (MLVA) approach. In addition MDUPHL performed antimicrobial susceptibility testing on all isolates by an agar dilution method. MLVA was conducted on all 10 colony picks (except Batch 5 where for logistic reasons only 5 picks were tested). To contain costs, serotyping and phage typing was limited to a subset of colony picks from each positive faecal sample. DNA was extracted using the Wizard Genomic DNA Purification Kit and a multiplex PCR targeting variable number tandem repeat (VNTR) loci STTR 5, STTR 9 and STTR10 and another targeting VNTR loci STTR 3 and STTR6 were conducted as per Torpdahl *et al*. (2007). MLVA of *Salmonella* isolates was performed as per Lindstedt *et al* (2004).

Results

The results are summarised in Table 1.

Table 1. MLVA profiles (n=13) identified at different sampling periods

Sampling	Sample date	# positive faecal samples	# isolates phage & serotyped	# isolates MLVA tested	# MLVA profiles identified	Profiles identified
Pre-study	April 2011		3	3	1	C
Batch 1	2 June 2011	7/18	43	43	4	ABCE
Batch 2	1 July 2011	11/12	15	110	7	ACDEFGI
Batch 3	25 August 2011	7/12	32	70	6	ACDEGH
Batch 4	7 October 2011	7/12	18	70	6	ACDJKL
Batch 5	27 July 2012	10/17	12	50	4	ACHM
Weaner farm	23 October 2012	1/4	2	10	2	CH
Total		43/75	125	356	13	A to M

Pre-study

The 3 laboratory *Salmonella* isolates provided, taken at post mortem some 2 months prior to the farm study, were identified as mSTm193, were resistant to ampicillin, streptomycin, sulphathiozole and tetracycline (ASSuT). The 3 isolates had the same MLVA profile (notated as C in Table 1), which persisted throughout the farm study and, in addition, was identified in isolates from the weaner source farm samples taken 18 months later.

Farm Study

In total, 42/71 individual pen faecal samples collected over the 13 month farm study were positive for Salmonella spp. Positive samples were found in the three age groups targeted, however positivity appeared to reduce with age from 6/6 and 5/6 samples positive at 6-8 weeks to 0/6 and 2/6 at 17-22 weeks. From the 42 positive samples, 343 colony picks were sent for further characterization. Overall, 333/343 isolates were resistant to ampicillin, streptomycin, sulphathiozole and tetracycline (ASSuT). Of the 10 remaining isolates, 2/10 colony picks from Batch2/sample4 and 7/10 picks from Batch3/sample1 were resistant to Tetracycline only and 1/10 colony picks from Batch3/sample4 was fully sensitive to ASSuT. All 120/120 isolates selected for phage and serotyping from the 5 batches of the farm study were identified as mSTM193 (Table 1). There were 13 different MLVA profiles identified from the 356 isolates tested (3 pre-study, 343 farm study and 10 weaner source farm), notated as Profiles A to M in Table 1. Profiles A and C persisted throughout the farm study while Profiles B, F, I, J, K, L and M were each only identified in a single batch. From the 10 colony picks per sample examined by MLVA, the number of different profiles identified varied between one and four (i.e. 1/42 Salmonella positive faecal samples had at least four different profiles present and 18/42 samples had at least two).

Weaner source farm

Of the 4 weaner farm faecal samples, only one was positive. All 10 colony picks from this sample displayed ASSuT resistance. The isolates were identified as mSTM193 represented by 2/13 MLVA profiles (C and H) seen in the farm study (Table 1).

Discussion

This longitudinal study provides the first direct evidence of the emergence and persistence of a new multi-drug resistant *Salmonella* strain (mSTM193) in an Australian pig herd, with clinical significance to both pigs and humans. Further, the identical MLVA profiles (A, B, C, E, and F) have been seen in an increasing number of human infections, with the result that pigs cannot be excluded as a possible source (Anon 2014a; Anon 2014b). Previous *Salmonella* farm studies conducted in Australia have largely been cross sectional and have generally isolated a variety of serovars, particularly when multiple colony picks are tested. A surprising finding in this study was the absence of isolates other than mSTM193 over the 13 months, despite the increased rigor of characterising 10 colonies picks per positive sample, in comparison to the common investigating laboratory practice of characterising only 1. The emergence of mSTM193 in Australia, a decade after its rise to prominence in Europe, raises questions as to its mode of entry, particularly considering Australia's strict quarantine restrictions and the failure of STm DT104 to appear at all. The resistance profile, ASSuT, reflects that seen in Europe (Hopkins *et al.,* 2010). MLVA is a powerful and sensitive technique for strain differentiation in this setting. How long do they persist within a herd to inform human illness attribution investigations? What is clear from the study so far is that in order to answer the question 'when are different MLVA profiles different?, possibly related? closely related? or the same?' it is critical to gain both experience in the technique and an understanding of the context in which it is used.

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