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## **Antibiotic Resistance and Chromosomal Variation in Equine Faecal *Salmonella* spp.**

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### **Authors' contributions**

This work was carried out in collaboration between all authors. MOA designed the study, carried the experiments, the analysis, and wrote the first draft of the manuscript. NJW, PDC and MB contributed to the study design, the analysis of the results and writing the manuscript. All authors read and approved the final manuscript.

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### **ABSTRACT**

**Aims:** Equine hospital *Salmonella* spp. were investigated retrospectively using antibiotic resistance typing and macro-restriction pulsed field gel electrophoresis techniques.

**Study Design:** Retrospective study.

**Place and Duration of Study:** Department of Comparative Molecular Medicine and department of Animal and Population Health, School of Veterinary Science, University of Liverpool.

**Methodology:** Twenty four achieved *Salmonella* isolates of equine faecal origin, previously collected from a UK equine hospital, were serotyped, phagetyped and tested both for antimicrobial susceptibility, by disc diffusion (BSAC) and for genetic relatedness by XbaI I-PFGE.

**Results:** Most isolates were *Salmonella typhimurium* (n=21), including five DT104; two were *Salmonella enteritidis*, and one was untypeable. Sixteen isolates, including five *Salmonella typhimurium* DT104 isolates exhibiting the classic penta-resistance

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phenotype (ACSSuT), were characterized as multidrug resistant (MDR). Fourteen MDR isolates showed additional resistance to florfenicol, although no resistance to ciprofloxacin was detected. MDR isolates showed two dominant resistance phenotypes: ACTSSuFlo (N=8) resistant to ampicillin, chloramphenicol, tetracycline, trimethoprim, streptomycin, sulfamethoxazole and florfenicol and ACTTrSSuFlo (N=6) which is also resistant to trimethoprim. Dendrogram analysis identified eleven distinct genetic groups showing an overall similarity of 84%. The dominant resistance phenotypes were located mainly in two genetic groups: ACTSSuFlo isolates were all collected in the same year and were restricted to one clonal PFGE group showing >99.5% genetic similarity; ACTTrSSuFlo isolates were genetically more diverse with 4/6 clustering in a closely-related group showing 94% similarity.

**Conclusion:** These findings suggest that although some *Salmonella* infections were introduced to the hospital from outside, infections spreading within the equine hospital could also be a reservoir of MDR *Salmonella* zoonotic infections and play a role in the development and dissemination of antibiotic resistance.

**Keywords:** Zoonotic enteric bacteria; antimicrobial resistance; DT104; multidrug resistant *Salmonella*; PFGE.

## 1. INTRODUCTION

*Salmonella* is a zoonotic organism, and a serious public and animal health concern (Singh et al., 2009). *Salmonella* is also an important nosocomial pathogen of hospitalized horses (Dargatz and Traub-Dargatz, 2004; Ekiri et al., 2009), responsible for enteric gastrointestinal diseases (Ernst et al., 2004). Although *Salmonella* is rarely detected in healthy horses (Pichner et al., 2005), *Salmonellae* remain major contributors to acute enteric disease, responsible for many cases of diarrhoea in horses (Kim et al., 2001). Furthermore, horses can be asymptomatic carriers, shedding *Salmonella* in their faeces (Sanchez et al., 2002). Horses admitted to hospitals are likely to be more at risk of *Salmonella* infection due to stressors, such as surgery, antibiotic therapy (Ernst et al., 2004) or a combination of these factors, (Ekiri et al., 2009) making them more likely to shed bacteria. Horses can be a source of infection with *Salmonella* and several studies have documented zoonotic transmission to humans (Hoelzer et al., 2011).

Emergence of multi-drug-resistance (MDR) is a global problem, particularly in pathogens causing nosocomial infections (Ahmed et al., 2012; Singh et al., 2009) including several *Salmonella* serovars (Zhao et al., 2007). Antimicrobial therapy has been shown to influence the excretion and transmission of multidrug resistance (MDR) both in humans and animals (Ahmed et al., 2012). In humans, *Salmonella enterica typhimurium* and *Salmonella enteritidis* are the most common serovars associated with gastroenteritis and *Salmonella enterica* serovar *typhimurium* definitive phage type 104 (DT104) is frequently isolated from food animals (Lawson et al., 2004). Recently, the increase in MDR-*Salmonella* has been especially important owing to the emergence of the multi-resistant *Salmonella typhimurium* DT104 in both humans and animals. Worldwide human outbreaks of different *Salmonella* species exhibiting an MDR phenotype are reported to have zoonotic origins (Rayamajhi et al., 2008), related to the consumption of animals products including horse meat (Espie et al., 2005; Doorduyn et al., 2008).

Molecular and antibiotic resistance studies of *Salmonella* from horses are limited in comparison to human and other animal studies (Ahmed et al., 2009; Zhao et al., 2007). The aim of this retrospective analysis is to investigate the possible source of *Salmonella* infections in an equine hospital and investigating the antibiotic resistance and genetic relatedness amongst archived *Salmonella* isolates.

## 2. MATERIALS AND METHODS

Twenty four isolates of *Salmonella* spp. of faecal origin, were obtained from the Philip Leverhulme Equine Hospital (PLEH) archive for analysis. Isolates were originally derived from faecal samples, collected between 1995-2000, at the PLEH, University of Liverpool. These had already been isolated and confirmed using selective laboratory methods and further confirmed as *Salmonella* spp. by agglutination tests and Api20E, using *Salmonella typhimurium* DWC 2578 as a positive control. Isolates were serotyped and phagetyped by the Department of Medical Microbiology, University of Liverpool and the Veterinary Laboratory Agency (VLA-UK). Antibiotic susceptibility was determined by disc diffusion testing in accordance with BSAC guidelines as described (Andrews, 2008). Florfenicol resistance in chloramphenicol resistant isolates we characterized using a criterion of  $R \leq 18\text{mm}$ , following personal communication with J.M. Andrews. Isolates that exhibited resistance to at least four drugs were characterized as multidrug resistant MDR. Antibiotics tested were - ampicillin (10 $\mu\text{g}$ ), apramycin (30 $\mu\text{g}$ ), chloramphenicol (30 $\mu\text{g}$ ), florfenicol (30 $\mu\text{g}$ ), tetracycline (10 $\mu\text{g}$ ), trimethoprim (2.5 $\mu\text{g}$ ), streptomycin (10 $\mu\text{g}$ ), Sulphamethoxazole (100 $\mu\text{g}$ ), gentamicin (10 $\mu\text{g}$ ), nalidixic acid (30 $\mu\text{g}$ ) and ciprofloxacin (1 $\mu\text{g}$ ).

Phylogenetic relatedness of the 24 isolates was investigated using macrorestriction PFGE analysis: DNA extraction, XbaI digestion and PFGE were all performed according to (Schott et al., 2001). Data were obtained from the digital gel image using Quantity One and Diversity Database software (Bio-Rad). Genetic distance calculations were based on band sharing and relationships calculated using the unweighted pair group method with averages (UPGMA), with Nei's coefficient. UPGMA data was then used to construct dendrogram showing the genetic relationship between the different isolates.

## 3. RESULTS AND DISCUSSION

Of the 24 isolates, 21 were *Salmonella enterica* serotype *typhimurium*, of which five were *Salmonella typhimurium* DT104; two were *Salmonella enteritidis* and one was non-typable (Table 1).

All isolates were resistant to at least one antimicrobial agent (tetracycline). 75% (16/24) were MDR, i.e. resistant to at least four antibiotics; of these, all except one were *Salmonella typhimurium*. 88% of the MDR isolates shared the penta phenotype ACSSuT, Among the 16 MDR isolates, two phenotypes predominated, ACTTrSSuFLO (N=6) and ACTSSuFLO (N=8), with five of the latter confirmed as phagetype DT104. Two further MDR isolates had the phenotype TTrSSuN (Table 1).

**Table 1. Serotype, phage type and antibiotic resistance phenotypes of archived *Salmonella* from horses at the PLEH equine hospital**

No.	Year	Phagetype	Serotype	AMs resistant profile (ph)
1	1995	104	<i>typhimurium</i>	ACTTrSSuNFlo <sup>MDR</sup>
2	1995	4	<i>enteritidis</i>	TSu
3	1995	-	<i>typhimurium</i>	ATSu
4	1995	-	untypable	TTrSSuN <sup>MDR</sup>
5	1995	193	<i>typhimurium</i>	TTrSSuN <sup>MDR</sup>
6	1995	-	<i>typhimurium</i>	TSu
7	1996	-	<i>typhimurium</i>	TSu
8	1996	- -	<i>typhimurium</i>	ACTTrSSuFlo <sup>MDR</sup>
9	1996	6	<i>typhimurium</i>	ACTTrSSuFlo <sup>MDR</sup>
10	1996	104	<i>typhimurium</i>	ACTTrSSuFlo <sup>MDR</sup>
11	1996	-	<i>typhimurium</i>	ACTTrSSuFlo <sup>MDR</sup>
12	1997	104	<i>typhimurium.typ</i>	ACTSSuFlo <sup>MDR</sup>
13	1997	-	<i>himurium</i>	ACTSSuFlo <sup>MDR</sup>
14	1997	-	<i>.typhimurium</i>	ACTSSuFlo <sup>MDR</sup>
15	1997	-	<i>typhimurium</i>	ACTSSuFlo <sup>MDR</sup>
16	1997	-	<i>typhimurium</i>	ACTSSuFlo <sup>MDR</sup>
17	1997	-	<i>.typhimurium</i>	ACTSSuNFlo <sup>MDR</sup>
18	1997	-	<i>.typhimurium</i>	ACTSSuFlo <sup>MDR</sup>
19	1997	-	<i>.typhimurium</i>	T
20	1998	104	<i>.typhimurium</i>	ACTSSuFlo <sup>MDR</sup>
21	1998	195	<i>.typhimurium</i>	T
22	1999	-	<i>.enteritidis</i>	T
23	2000	-	<i>.typhimurium</i>	T
24	2000	104	<i>typhimurium</i>	ACTTrSSuFlo <sup>MDR</sup>

\*Abbreviations: - = (non), AMs = (Antimicrobials), A= (Ampicillin ), C = (Chloramphenicol ), T = (Tetracycline), S = (Streptomycin ), Su = (Sulfamethoxazole ), Tr = (Trimethoprim ), N = (Nalidixic acid), Flo = (Florfenicol ), <sup>MDR</sup> = (Multidrug resistant)

Antibiotics tested were - ampicillin (A) (10µg), apramycin (Apr) (30µg), chloramphenicol (C) (30µg), florfenicol (Flo) (30µg), tetracycline (T) (10µg), trimethoprim (Tr) (2.5µg), streptomycin (S) (10µg), Sulphamethoxazole (SuTX) (100µg), gentamicin (Gen) (10µg), nalidixic acid (Nal) (30µg) and ciprofloxacin (Cip) (1µg).

PFGE analysis showed several different genotypes, although one genotype predominates. Dendrogram analysis of XbaI banding patterns indicates that the 24 isolates shared >84 % identity overall and could be divided into eleven clades (PFGE groups) according to the similarity of the banding pattern.

Fig.1 shows dendrogram analysis and antibiogram profiles of *Salmonella enterica* isolates: genetic similarity calculations used to construct the dendrogram were based on band sharing, determined from PFGE of XbaI fragments. Antibiotic resistance patterns were derived from MIC determinations; grey bars represent resistance. Abbreviations: A, ampicillin; C, chloramphenicol; T, tetracycline; Tr, trimethoprim; S, streptomycin; Su, sulphamethoxazole; N, nalidixic acid; F, florfenicol.

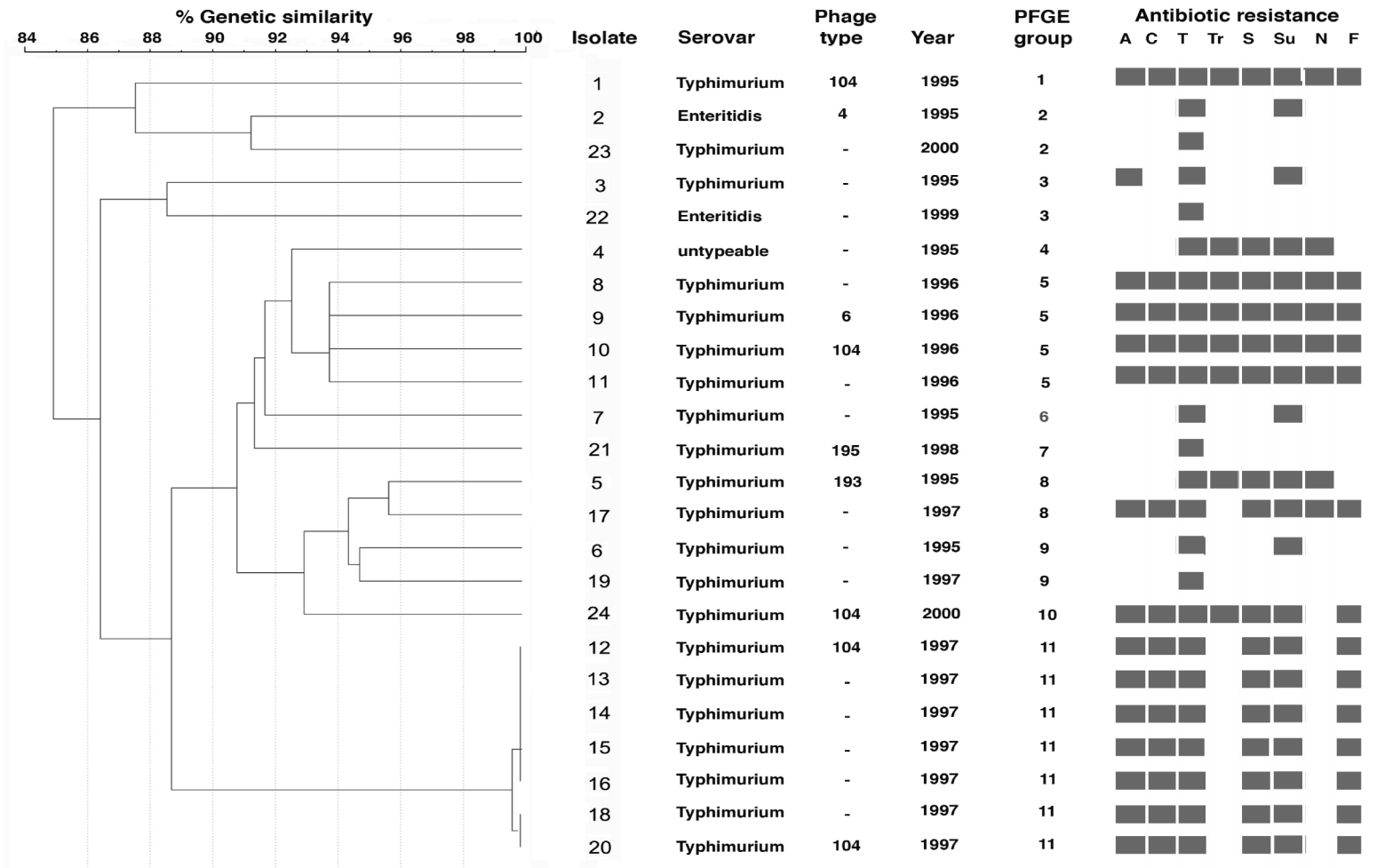


Fig.1. Dendrogram analysis and antibiogram profiles of *Salmonella enterica* isolates

5/11 patterns were unique, a further 5/11 were each represented by two isolates. However, 30% (7/24) of the isolates fell into one clade, PFGE group 11. These isolates in PFGE group 11 were all collected in 1997 and showed a genetic similarity of >99.5%. All these isolates were MDR and showed identical antibiotic resistance profiles. The clonal nature of these isolates suggests that they may represent an endemic strain circulating in the equine hospital at that time. A further cluster of four isolates (PFGE group 5) was identified amongst isolates recovered in 1996. This group of isolates is more varied genetically, showing 94% similarity and having three different phage types, nevertheless, all are MDR and share identical antibiotic resistance patterns. When this is combined with the susceptibility testing results, this would suggest that isolates recovered in 1996 may represent an epidemic of MDR *Salmonella*, including DT104.

Most of the isolates were *Salmonella typhimurium*, the serovar most commonly reported in horses, in which MRD is increasingly prevalent (Van Duijkeren et al., 2002) and which is responsible for both human and animal outbreaks in Europe (Horby et al., 2003; Van Duijkeren et al., 2002). The use of PFGE for identifying genetic relatedness and possible clustering of MDR *Salmonella typhimurium* DT104 in animals is well-established (Zeng et al., 2011) and susceptibility testing is recognized as an important component of any preliminary identification of MDR *Salmonella* strains especially *Salmonella typhimurium* DT104 (Thorsteinsdottir et al., 2007). The resistance patterns identified in our isolates are similar to other studies of MDR *Salmonella* DT104 in horses, where the resistance pattern ACSSuT is the most widely reported (Van Duijkeren et al., 2002).

Many studies have shown the increasing prevalence of antibiotic resistance in *Salmonella typhimurium* in both farm animals and humans (Dargatz and Traub-Dargatz, 2004; Zeng et al., 2011). All the *Salmonella* in this study were resistant to at least one antibiotic and many were multidrug resistant. Two distinctive resistance phenotypes were noticed among the multidrug resistant isolates: ACTTrSuFlo and ACTSuFlo. These were similar to the resistance profiles of *Salmonella typhimurium* from two human outbreaks in England in 2000 (Horby et al., 2003) and also were attributed to the consumption of contaminated food of equine origin in Netherlands (Van Duijkeren et al., 2002). Recent increases in MDR-*Salmonella* have been of particular importance owing to the emergence of the multi-resistant *Salmonella typhimurium* DT104 in both humans and animals (Lawson et al., 2004; Rayamajhi et al., 2008). Although MDR-*Salmonella* types, especially DT104, have been associated with meat consumption and contact with infected animals (Espie et al., 2005) (Doorduyn et al., 2008), our findings also suggest that horse faeces could be a potential source of human MDR *Salmonella* infections.

Trimethoprim resistance in *salmonellas* has been attributed to the use of this agent to combat the emergence of DT104 in cattle in the UK, so finding trimethoprim resistance among equine *salmonellae* may suggest that these isolates originated in food animals, or might have acquired this resistance type through genetic elements originating from food animal bacteria. Similarly, florfenicol is not licensed for use in horses, but it is licensed for cattle with respiratory infections. *Salmonella typhimurium* DT104 with *floSt* have been isolated from many different animals including horses (Hall, 2010). In this study, florfenicol resistance was detected using disk diffusion method in MDR *salmonellas*, suggesting that this resistance might be encoded within MDR system, which probably, again, originated in food animals. It is well documented that MDR is most likely to be related to the carriage of integrons, as in the case of DT104, and horizontal transfer of these genes might have occurred. The possibility of transfer of plasmids amongst *salmonellae* in the intestinal tract of

humans has been reported as has transfer of resistance between different organisms such as *Salmonella* and *E. coli* (Winokur et al., 2001).

The *Salmonella* isolates we describe had been collected from horses intermittently over the years to produce the archive used in this study. Aside from clusters of genetically related strains isolated in 1996 and 1997, the variation in resistance phenotypes and in PFGE profiles suggests that salmonellosis was probably introduced episodically by individual horses. On the other hand, the cluster of four isolates sharing 94% genetic similarity and having identical antibiotic resistance profiles suggests that in that year (1996) there may have been an 'epidemic' of equine *salmonellas* coming into the hospital, probably as the result of spill-over of salmonellosis from other species. Samples recovered in 1997, also show identical antibiotic resistance profiles, but are distinct from the 1996 cluster. In the 1997 cluster, the very tight genetic similarity (>99.5% identity) of the isolates in this clade, suggests that these may represent an endemic outbreak in the hospital.

#### **4. CONCLUSION**

Overall, this study shows that the resistance phenotype of *Salmonella* can be a useful epidemiological tool, especially when combined with molecular techniques. The MDR isolates in this study appeared to be closely related and probably represent spillover into horses of an epidemic of MDR *salmonellas*, including DT104, in other domestic animals and humans. The MDR phenotypes in equine fecal samples complicates the use and selection of antibiotic drugs for treatment of clinical equine *Salmonella* infections and indicates that horses in a hospital setting may be a source of zoonotic MDR *Salmonella* infection for humans.

#### **CONSENT**

Authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this paper.

#### **ETHICAL APPROVAL**

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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## COMPETING INTERESTS

The authors declare no relationship (commercial or otherwise) that may constitute a dual or conflicting interest.

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