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Contrasting long-term dynamics of antimicrobial resistance and virulence plasmids in *Salmonella* Typhimurium from animals

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Abstract

Plasmids are mobile elements that can carry genes encoding traits of clinical concern, including antimicrobial resistance (AMR) and virulence. Population-level studies of *Enterobacterales*, including *Escherichia coli, Shigella* and *Klebsiella*, indicate that plasmids are important drivers of lineage expansions and dissemination of AMR genes. *Salmonella* Typhimurium is the second most common cause of salmonellosis in humans and livestock in the UK and Europe. The long-term dynamics of plasmids between *S.* Typhimurium were investigated using isolates collected through national surveillance of animals in England and Wales over a 25-year period. The population structure of *S.* Typhimurium and its virulence plasmid (where present) were inferred through phylogenetic analyses using whole-genome sequence data for 496 isolates. Antimicrobial resistance genes and plasmid markers were detected *in silico*. Phenotypic plasmid characterization, using the Kado and Liu method, was used to confirm the number and size of plasmids. The differences in AMR and plasmids between clades were striking, with livestock clades more likely to carry one or more AMR plasmid and be multi-drug-resistant compared to clades associated with wildlife and companion animals. Multiple small non-AMR plasmids were distributed across clades. However, all hybrid AMR–virulence plasmids and most AMR plasmids were highly clade-associated and persisted over decades, with minimal evidence of horizontal transfer between clades. This contrasts with the role of plasmids in the short-term dissemination of AMR between diverse strains in other *Enterobacterales* in high-antimicrobial-use settings, with implications for predicting plasmid dissemination amongst *S.* Typhimurium.

DATA SUMMARY

The authors confirm that all supporting data and protocols have been provided within the article or within supplementary data files.

(1) No whole-genome sequencing data were generated in this study. The accession numbers for publicly available sequences are detailed in Table S3, available with the online version of this article (Animal and Plant Health Agency isolates) and Table S1 (isolates selected to provide context to Animal and Plant Health Agency isolates).
(2) Metadata, AMRFinderPlus, PlasmidFinder results and sample accessions for Animal and Plant Health Agency isolates are provided in Table S3.
(3) Small plasmids (<7 kb) not identified using PlasmidFinder are summarized in Table S4.

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Abbreviations: AMR, antimicrobial resistance; APHA, Animal and Plant Health Agency; ARG, antimicrobial resistance gene; DT, definitive type; FPA, food-production animal; HGT, horizontal gene transfer; IS, insertion sequence; MDR, multidrug resistance; MLST, multilocus sequence typing; NFPA, non-food-production animal; pMLST, plasmid multi-locus sequence typing; SGI, Salmonella genomic Island; SNP, single nucleotide polymorphism; WHO, World Health Organisation.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Five supplementary figures and three supplementary tables are available with the online version of this article.

Impact Statement

Despite the fact that animals are often considered to be a possible source of antimicrobial resistance (AMR) in humans, our attention has been largely focused on AMR plasmids from human isolates. In fact, we know little about the nature and dynamic spread of plasmids in important zoonotic pathogens such as *Salmonella* Typhimurium, isolated directly from animals. In this study we characterize the long-term dynamics of plasmids in *S.* Typhimurium from food production animals, companion animals and wild birds collected in England and Wales. Our data span 25 years, enabling analysis of the persistence and dissemination of key plasmids linked with virulence and AMR. Unlike the other enteric bacteria highlighted as priority pathogens by the World Health Organization (WHO) for their role in disseminating AMR, in *S.* Typhimurium we show that AMR and virulence plasmids are typically highly clade-associated and persist over decades, with minimal evidence of horizontal transfer amongst clades. This has implications for how *S.* Typhimurium can be monitored most effectively and may inform the design of approaches to control AMR.

INTRODUCTION

Antimicrobial resistance (AMR) is a leading global public health threat. Plasmids are important vectors for the dissemination of antimicrobial resistance genes (ARGs) between bacteria, contributing to the emergence and dissemination of antimicrobial-resistant strains [1, 2]. In both animal and human populations, AMR can lead to treatment failure [3–6]. Additionally, antimicrobial-resistant strains and ARGs circulating in animals are a potential reservoir for human infections [7, 8]. Plasmid dynamics vary between bacterial hosts, plasmid types, environmental conditions and selective pressures, including antimicrobial use [9–12]. Multiple *Enterobacterales* species frequently colonize or infect the same host, providing opportunities for horizontal gene transfer (HGT) [13–15]. Population-level studies of *Enterobacterales*, including *Escherichia coli, Klebsiella* and *Shigella*, show that plasmids play a significant role in the dissemination of AMR genes in these hosts, and are indicated to have driven the expansion of some lineages [16–18]. Plasmid outbreaks across multiple clinically important bacterial hosts highlight the need to track plasmids independently [19–21]. Improving our understanding of the epidemiology of both antimicrobial-resistant bacteria and plasmids can inform effective surveillance and mitigation strategies.

Salmonella Typhimurium is consistently the second most common cause of salmonellosis identified in multiple species of food production animals and humans in the EU [22] and reporting of cases in UK animals is mandatory [23]. Historically, phage typing has been a valuable tool to classify *S.* Typhimurium isolates for surveillance purposes. More recently, multilocus sequence typing (MLST) has been used to differentiate *Salmonella*. Most *S.* Typhimurium are ST19, with some single and double locus variants [24]. The diversity of sequence types between *S.* Typhimurium is low and therefore frequently provides insufficient resolution for discerning relationships between strains [25], and the introduction of whole-genome sequencing has provided greater discriminatory power (26). *S.* Typhimurium can harbour multiple AMR plasmids, and isolates from clinically affected animals and humans are frequently multi-drug-resistant (MDR), with accumulation of resistance genes primarily through horizontal gene transfer (HGT) of mobile genetic elements (MGEs) [27–30]. *S.* Typhimurium can colonize the gut lumen and survive in tissues for extended time periods, providing opportunity for HGT with other *Enterobacterales* [15, 31–33]. Many *S.* Typhimurium strains carry a virulence plasmid (pSLT) that is specific to the serovar, and pSLT are indicated to be further restricted to *S.* Typhimurium lineages [34]. Previously, an AMR pSLT variant has been attributed to clonal replacement of a strain causing invasive disease in humans in sub-Saharan Africa [35, 36]. AMR plasmids have also been associated with clonal expansion of a *S.* Typhimurium strain in UK calves [37].

Although plasmids have been established to play a key role in ARG dissemination in *Enterobacterales* species, relatively little is known about the dynamics of plasmids amongst *S.* Typhimurium that circulate in food production and other animals. The transmissibility of many plasmids between *S.* Typhimurium has been studied *in vitro*, but the degree to which *in vitro* dynamics reflect natural populations is unclear [38, 39]. Many factors can influence the propensity of plasmids to transfer horizontally, including the presence of other MGEs [9, 40]. Ecological barriers, including antimicrobial use pressures, and animal host species may influence both the dissemination and maintenance of plasmids. Whether a plasmid will persist in the absence of selection for a plasmid-encoded trait is debated. In the absence of continued pressure from antimicrobial use, plasmids imposing a fitness cost may be lost from the host [41]. Further, fitness costs can increase with the breadth of resistance encoded by plasmids, which can result in loss of ARGs where not under selective pressure [42]. However, plasmid maintenance varies between bacteria, and host bacteria may evolve through compensatory mutations of the chromosome and/or plasmid which increase plasmid stability and can result in retention in the absence of antimicrobial use [43–47].The long-term persistence of plasmid variants at the population level has not previously been explored in the context of *S.* Typhimurium in animals in England and Wales. Understanding the contribution of plasmids to AMR maintenance and distribution could contribute to prediction of spread of AMR in *S.* Typhimurium populations.

Using national surveillance data, this study captures the dynamics of plasmid dissemination and long-term persistence in the context of *S.* Typhimurium in animals in England and Wales over a 25-year period, with the principal aim being to determine whether or not ARG plasmids are shared horizontally between diverse isolates. Whole-genome sequencing (WGS) data for 496 national surveillance *S.* Typhimurium isolates from animals in England and Wales were analysed to characterize virulence and AMR plasmid dynamics

between 1992–2016. The resolution afforded by WGS of these isolates enables determination of the propensity of plasmids to disseminate between phylogenetically diverse strains. Phenotypic plasmid profile data provide confirmatory evidence of plasmid presence and size. Contrasting with dynamics observed in other *Enterobacterales*, *S.* Typhimurium AMR and virulence plasmid carriage is highly clade associated, with minimal evidence of transfer of plasmids between *S.* Typhimurium clades.

METHODS

Bacterial isolates

Animal and Plant Health Agency (APHA) isolates

Salmonella infections of animals in England and Wales are reportable to the APHA, as required by the Zoonoses Order (1989) [23]. Isolates are submitted to the *Salmonella* Reference Laboratory at the APHA for confirmation and typing. *S.* Typhimurium isolates are classified using phage typing and undergo antimicrobial susceptibility testing against a panel of 16 antimicrobials using disc diffusion; classification as resistant or susceptible is based on zone sizes, as previously detailed [30]. Isolates for this study were obtained from the APHA collection representing *S.* Typhimurium isolates from animals or environmental sources between 1992 and 2016. Samples were chosen to capture the diversity of phenotypic AMR profiles, animal sources and years of sample collection. Ethical approval for characterization of the isolates was not required due to the surveillance mandate of APHA. Post-quality control (detailed below), whole-genome sequences of 496 *S.* Typhimurium isolates were used in the analyses (BioProject PRJEB10999).

Sequenced isolates originated from a wide range of host species, including cattle (27%, *n*=133), pigs (34.7%, *n*=171), poultry (4.9%, *n*=24), cats or dogs (9.5%, *n*=47), horses (5.8%, *n*=28), wild birds (5% *n*=25) and game birds (3.3% *n*=16). Information clarifying the purpose of sample collection was available for 265 isolates. The majority of these isolates (79.6%, *n*=211) were collected for diagnostic purposes and a further 57 (21.5%) isolates were collected for monitoring or non-statutory surveillance purposes. A minority of isolates (3.4%, *n*=9) were collected as part of a Zoonoses Order investigation and three were collected through the National Control Programme for active surveillance of *Salmonella* in chickens. Post-quality control, the number of isolates sequenced from each year ranged from 17 to 29 between 1992 and 2014. These data were supplemented by 14 isolates from 2015 and 3 isolates from 2016.

Contextual isolates

Publicly available *S.* Typhimurium isolates were included to provide additional context for the phylogeny and to determine whether patterns of plasmid dynamics were consistent between APHA isolates and wider data, focusing on isolates from the UK (summarized in Table S1). The reference genome D23580 (accession FN424405.1) was included, particularly due to interest in the dynamics of the virulence plasmid that encodes for MDR. The additional isolates include all available unique *S.* Typhimurium genomes (*n*=13) from the Murray collection, which enabled longer-term evaluation of plasmid dynamics, using samples collected between 1917 and 1941 [48]. Additional sequences from underrepresented groups of UK isolates were also included. The 11 isolates from wild passerines in England and Wales previously described by Mather *et al.* [49] were added to increase representation of isolates from wildlife. Phage type DT104 isolates from Scotland were included [50], the number of randomly selected isolates (*n*=10) was limited to avoid overrepresentation. Few monophasic variants of *S.* Typhimurium were sequenced for this study, therefore data were supplemented with monophasic *S.* Typhimurium genomic sequences of isolates (*n*=20) from animals in England and Wales, previously described by Petrovska *et al.* [51].

WGS, quality control, assembly and annotation

Isolates were grown in 1 ml Luria Bertani (LB) broth overnight and DNA extracted with ArchivePure (5 Prime, UK). DNA was quantified using Qubit (Thermo Fisher Scientific) and the 260/280 nm ratio determined using a NanoDrop. WGS of genomic DNA was performed on the Illumina HiSeq platform with 100 bp paired-end reads. Annotated assemblies were produced using the pipeline described previously [52]. Raw Illumina read data were used to generate multiple assemblies using VelvetOptimiser v2.2.5 [53] and Velvet (v1.2) [54] for each isolate and scaffolded using SSPACE [55] with sequence gaps filled using GapFiller (56:p.20). The resultant contigs were annotated using Prokka v1.5 [57] and the genus *Salmonella*-specific database from RefSeq [58].

Assembly statistics including total length, N50 and the number of contigs were evaluated. Isolates of total length <4 Mb (*n*=1) or >6 Mb (*n*=4) were excluded from further analysis. Kraken (v0.10.6) was used to assign taxonomic labels to sequences using a k-mer-based approach [59]. Analysis of heterozygous single-nucleotide polymorphisms (SNPs), indicating potential mixed samples of closely related strains, did not identify further isolates for exclusion.

Phylogenetic structure, single-nucleotide variant (SNV) analysis

To determine the evolutionary relationships between isolates, paired-end reads were mapped to the reference genome *S.* Typhimurium LT2 and pSLT sequences (accession AE006468 and AE006471) [60] using SMALT (v 0.7.4, http://www.sanger.ac.uk/science/tools/smalt-0). A *Salmonella enterica* serovar Heidelberg genome outgroup (accession ERR1251772) was included to root the phylogeny. Prior to generation of the chromosomal phylogeny, SNPs falling within plasmid and prophage regions were masked. Estimated recombination sites were identified on the basis of SNP density using Gubbins, and masked prior to calling SNP sites with Gubbins [61]. The alignment of informative SNP sites (*n*=31 065 for APHA plus UK context and Murray collection isolate

tree) were used to generate maximum-likelihood phylogenetic trees using RAxML (v 8.2.8) [62] with generalized time-reversible model and GAMMA correction for site rate variation with 100 bootstrap replications. The ggtree [63] and ggplot2 (v 2.0.0) R (v 3.5.3) packages were used to generate annotated phylogenies.

Classification of isolates into clades and sub-clades

A phylogeny-free population genetics approach was used to define clades and subclades through implementation of hierBAPS [64]. Singleton SNPs were removed from the core genome SNP site multiple sequence alignment prior to running hierBAPS with 100 iterations and estimated cluster sizes of 100, 300 and 500, the results of which were consistent. Hereafter, level 1 hierBAPS clusters are referred to as clades, and level 2 hierBAPS clusters as sub-clades.

Associations between traits and clades

Fisher's exact test with Bonferroni correction was used to test for associations of clades and source host species, geographical origin and ARGs using R (v 3.5.3). Where significant associations were identified, Fisher's exact tests were used to determine which clade(s) the trait was associated with and calculate the odds ratio relative to other clades. Only isolates from the APHA study were included in these analyses and those without metadata were excluded.

In silico detection of resistance genes and point mutations

Genotypic AMR, heavy metal resistance and biocide resistance were predicted using AMRFinderPlus (database version 3.6.7), which uses a BLAST approach to identify resistance genes and point mutations [65]. Resistance genes with ≥90% identity and 100% coverage of the reference sequence were recorded as present. Partial hits with ≥90% identity and ≥50% coverage, and with the ARG at the end of the contig, were assumed to be present, as assemblers can split genes over multiple contigs, although genes with partial hits that were internal to the contig were recorded as absent.

In silico plasmid identification

Incompatibility (Inc) groups are commonly used as a classification scheme for plasmids, based upon variation in loci encoding for plasmid replication [66]. Plasmid Inc types were identified by BLASTN query (at ≥95% identity and ≥90% coverage) of the contigs (≥800 bp) using PlasmidFinder [66]. Using *rep* genes to identify plasmids has limited sensitivity, therefore contigs were also queried against the curated *Enterobacterales* plasmid database (threshold at ≥90% identity, ≥90% query coverage and ≥90% reference sequence coverage) [67]. These methods are limited by the completeness of the databases.

Circularization of all contigs (≥800 bp) was attempted using the Circlator (v1.5.5) minimus2 function [68] and served to further increase the sensitivity of *in silico* plasmid detection. All circularizable contigs not previously identified were deduplicated using CD-HIT [69]. Each unique sequence was then compared using BLASTN against the National Center for Biotechnology Information (NCBI) database to determine whether the top hits (determined by bitscore) were from previously described plasmids [70, 71]. This approach is limited to fully assembled plasmids, omitting those with fragmented assemblies.

Putative plasmid contigs (with *rep* genes and/or high identity to a reference in the Orlek database) that did not circularize were further characterized. These linear putative plasmid contigs were queried against the NCBI RefSeq database to identify whether the top hit was chromosome or plasmid. If the top hit was a plasmid reference then this reference was queried against all contigs of isolate to determine if there was coverage of the remainder of the plasmid reference, identifying contigs putatively part of the same plasmid structure. Insertion sequences (ISs) commonly cause breaks in assembly, therefore the location of IS elements on all contigs in each isolate was determined using ISFinder [72] and used to infer the plasmid structures.

Classification of plasmid variants

Plasmids of the same Inc type were compared to identify variants, enabling tracking of plasmids across sub-clades. Subtyping schemes are available for some common Inc types termed plasmid multi-locus sequence typing (pMLST) [73–75]; . The pMLST scheme was used to classify IncF, IncHI1, IncI1 and IncN replicon types using ARIBA [76]. For Inc types with no pMLST scheme and for pMLST types observed across multiple subclades, further characterization of plasmids was necessary to determine whether highly similar plasmids were distributed across multiple sub-clades.

Circularizable contigs were opened at the replicon using the Circlator 'fixstart' function [68]. CD-HIT was then used to determine whether some sequences were identical in both length and identity [69] and unique sequences were analysed using BLASTN comparisons, manual annotation and visualization using ACT as appropriate.

pSLT virulence plasmid phylogeny

To determine the evolutionary relationship between the pSLT and *S.* Typhimurium host, a pSLT phylogeny was generated and then compared with the host chromosomal tree using a tanglegram, generated using the cophyloplot function from the ape package (v5.5) 77) in R, and visualization of host chromosomal hierBAPS clusters on the pSLT tree. Reads from each isolate were

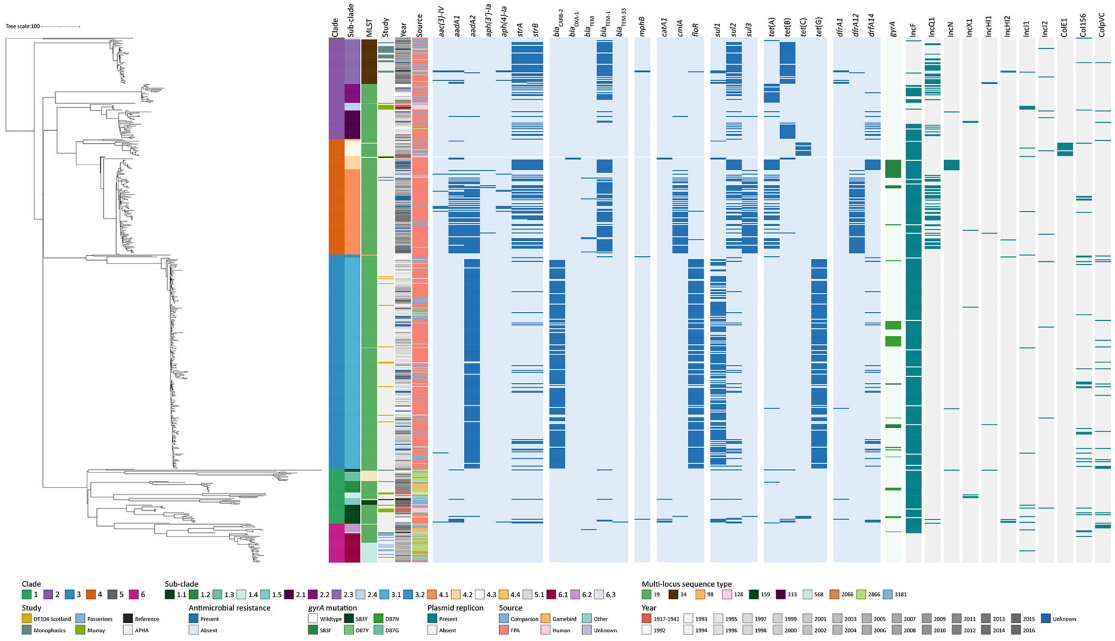

Fig. 1. Antimicrobial resistance and plasmid replicons. S. Typhimurium maximum-likelihood phylogeny of APHA isolates (*n*=496), contextual isolates (*n*=52) and references (LT2 and D2350) with heatmap of acquired antimicrobial resistance genes (grouped by antimicrobial class and shown where observed in >1 isolate), chromosomal gyrA mutations and plasmid replicon (Inc) types detected for each isolate.

competitively mapped against the LT2 chromosome and plasmid sequence (accession AE006468 and AE006471). Alignments of isolates with a minimum of 80% coverage of the pSLT LT2 reference plasmid were used to generate the phylogeny with RAxML (v 8.2.8) [62] with generalized time-reversible model and GAMMA correction for site rate variation with 100 bootstrap replications. Linear regression of root-to-tip genetic distance against sampling time was used to test for temporal signal in the pSLT phylogeny overall and for each sub-clade. The phylogenetic tree was visualized with chromosomal clade and sub-clade data using iToL v4 [78].

ASSESSMENT OF CO-EVOLUTION OF *S.* TYPHIMURIUM CHROMOSOME AND VIRULENCE PLASMID

If the virulence plasmid has co-evolved with the host bacteria it could be anticipated that pairwise host chromosome and virulence plasmid identity would increase linearly. For isolates with >80% read coverage of both the LT2 chromosome and virulence plasmid, pairwise SNP differences were calculated (https://github.com/simonrharris/pairwise_difference_count). Association between pairwise pSLT and chromosomal identities was tested for using a linear model [79]. TempEst (v1.5.1) was used to investigate whether there was a temporal signal for the pSLT phylogeny within each sub-clade [80].

Kado and Liu plasmid profiling

Laboratory-based identification of plasmid numbers and sizes was used to support *in silico* plasmid identification. At the APHA, the Kado and Liu method was used for plasmid detection [81]. In brief, alkaline conditions were used to denature chromosomal DNA at elevated temperatures, and proteins and cell debris were then removed using phenol–chloroform to reduce or eliminate chromosomal DNA. Electrophoretic analysis was then conducted on the product, and the size of the plasmid estimated. Kado and Liu plasmid profiling data were available for 414 isolates.

RESULTS

Clade associations with animal host species and phage types

The APHA isolates divide into six clades, defined using Bayesian hierarchical clustering. The 6 clades and 18 sub-clades identified are monophyletic except for 1 isolate at the sub-clade level (Fig. 1). Many clades were associated with one or more phage type, with some phage types being polyphyletic (DT8, DT2, 120, DT208, DT193, DT104 and U310). There were no significant associations between clade and the county-level geographical region from which the isolate was sampled. Isolates from food production animals were positively associated with clade 4 and negatively associated with clades 1 and 6. Isolates from cattle,

pigs, equids, game birds and wild birds were associated with one or more clades at the $P<0.05$ significance level post-Bonferroni correction (Table S2). The median year of isolate collection ranged between 2006 and 2009 for clades 1, 2, 4 and 6. The isolates from clade 3 were typically from earlier in the study period (median 1999). However, the range of years of isolation was similar amongst clades; from 1992 to 2015 or 2016 for clades 1, 2, 3 and 4 and from 1994 to 2015 for clade 6 (Fig. S1). There were only two isolates from clade 5, from 2010 and 2011.

MDR is more common in food production animal-associated clades

Resistance to three or more antimicrobial classes (MDR) was common to 65% of isolates. The number of ARGs per isolate (median=4) varied amongst sub-clades, being highest in sub-clade 4.1 (median=6, IQR 5–10), which was associated with phage type U288 and isolates from pigs. Isolates from clades (1 and 6) associated with non-food production animals (NFPAs) were typically phenotypically pan-susceptible to antimicrobials tested, with ARGs or point mutations conferring AMR detected in relatively few isolates (13%). In contrast, 92.4% of food production animal (FPA)-associated clade 4 isolates were resistant to one or more antimicrobial and the odds of being MDR were 7.88 times higher for isolates from FPA-associated clades than isolates from NFPA-associated clades [Fisher's exact test $P<5.2e\text{-}9$, OR 95% CI (4.31, 21.3)]. Interestingly, there was variation in predicted phenotypic resistance of isolates to antimicrobial classes within individual sub-clades and the proportion of isolates with the dominant AMR phenotype varying amongst sub-clades. Amongst most sub-clade 3.1 isolates (*n*=211/213), multiple ARGs were located within a chromosomally integrated *Salmonella* genomic island (SGI-1).

Isolates with heavy metal resistance genes all had one or more ARG, with 42/49 being MDR and 38/49 resistant to five or more antimicrobials. Most heavy metal resistance genes (*ars*, *sil* and *pco*) were only observed in sub-clade 2.3 isolates as part of *Salmonella* genomic island 4 (SGI4) (Fig. S2) as previously described [51]. A small number of isolates in other sub-clades also carried genes encoding for mercury and/or tellurium resistance genes. Two biocide resistance genes were observed; *qacEΔ1* was identified in all sub-clade 3.1 isolates and ≤4 isolates in four other sub-clades and *qacL* was encoded on SGI1 in sub-clade 3.1 isolates and was also common to 69.3% (*n*=61) of sub-clade 4.1 isolates.

Many AMR genes and plasmid Inc types are common to isolates in multiple sub-clades

Over half (8/15) of acquired ARGs and chromosomal mutations encoding for AMR (4/7) were shared amongst multiple sub-clades (Fig. 1). Of the ARGs unique to a single sub-clade, all were in ≤4 isolates except for $bla_{\text{CARB-2}}$ (*n*=189) and *tet* (G) (*n*=187). Mutations in *gyrA* conferring reduced susceptibility to fluoroquinolones were observed in isolates from six sub-clades with five variants.

The majority (92.7%) of isolates carried one or more plasmid replicon and some plasmid Inc types were observed across multiple clades (Fig. 1). IncF virulence plasmid replicons were the most common, though notably absent from half (*n*=15) of sub-clade 2.1 isolates [phage types 193 (*n*=8), U208 (*n*=2), U302 (*n*=1), 193 a (*n*=1), untypable (*n*=1), not recorded (*n*=2)]. IncF virulence plasmids were also absent from most (*n*=29 of 31) sub-clade 2.3 isolates [phage types 193 (*n*=14), 120 (*n*=7), 104b (*n*=2), untypable (*n*=1), 195 (*n*=1), 7 (*n*=1), U311 (*n*=1), not recorded (*n*=1)] and all sub-clade 6.1 isolates [phage types 56 variant (*n*=10), DT40 (*n*=4), RDNC (*n*=3), 193 (*n*=1), not recorded (*n*=2)]. IncQ1 replicons were observed in 76 isolates, spread across 5 sub-clades. IncN, IncHI1, IncHI2, IncI2, IncX1 and IncX4 plasmid Inc types were each observed in <15 isolates (Fig. 1). The temporal persistence of plasmid replicon combinations within sub-clades of ≥15 isolates is shown in Fig. 2; some replicon types were observed to persist over time in multiple sub-clades (e.g. IncF, IncQ1), whereas other replicon types were observed sporadically (e.g. IncI1, IncX1). Phage type classifications for isolates in each sub-clade are detailed in Table S3.

Virulence plasmids can carry ARGs but are not horizontally transferred amongst clades

There are three examples of sub-clade-specific IncF pSLT plasmids that had acquired one or more ARGs. Hybrid AMR–virulence pSLT plasmids with a class 1 integron containing *sul3*, *aadA1*, *aadA2*, *cmlA1*, *dfrA12* and bla_{TEM}, as described by Hooton *et al.* [82] [pSTU288-1 (CP004058)] in a phage type U288 isolate, were observed in 84/88 isolates in sub-clade 4.1. These pSLT had deletions of two genes (*spvR* and *spvA*) that are typically encoded in the pSLT virulence operon. All sub-clade 2.2 pSLT plasmids (*n*=15) contained a resistance region with *tet*(A), *arsR* and *arsP* encoding for tetracycline and arsenic compound resistance, respectively. In sub-clade 4.2, two isolates with a hybrid AMR–virulence plasmid were observed; these plasmids carried five ARGs [*tet*(B), *catA1*, $bla_{\text{OXA-1}}$, *aadA1*, *sul1*] and genes encoding for mercury resistance (*merC, merP, merT, merR*) (Fig. S2).

Interestingly, IncF plasmids including pSLT virulence plasmids appeared to be restricted to specific *S.* Typhimurium clades. The majority of IncF pMLST profiles (8/11) are unique to a sub-clade, although three pMLST profiles containing IncFIIS and/or IncFIB were observed across multiple clades (Fig. S3). The phylogeny of pSLT plasmids from IncFIIS and/or IncFIB replicon-positive isolates has a monophyletic association with clades and shows clear congruence with the chromosomal phylogeny as shown in Fig. 3. A tanglegram showing the congruence of chromosomal and pSLT phylogenies is shown in Fig. S4. The linear relationship between pairwise chromosomal identity and pairwise pSLT identity is also indicative

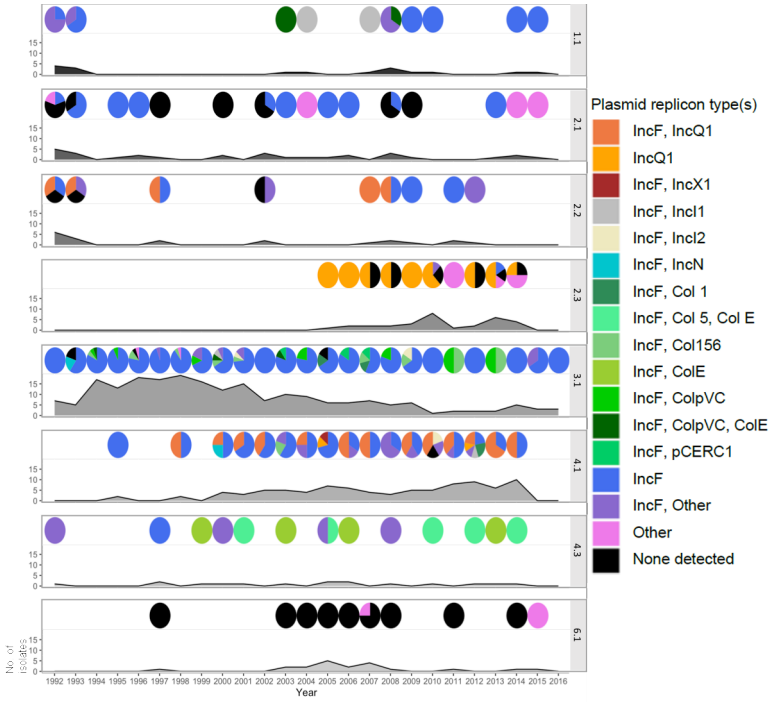

Fig. 2. Temporal patterns of plasmid replicon profiles within sub-clades. The frequency of isolates in each year is shown by the area plot for each sub-clade, with the frequency of isolates from the respective sub-clade on each *y*-axis. For each sub-clade, pie charts are coloured by the proportion of isolates with each plasmid Inc type profile for each year. The sub-clade number is shown on the right-hand side of each panel. Plasmid Inc type profiles are grouped into the 'Other' category where ≤3 isolates share the profile. Data for sub-clades with ≥15 isolates are shown.

of co-evolution (*P*-value <2^{e-16}, adjusted R-squared 0.961, Fig. S5). No temporal signal was identified for each sub-clade, therefore comparisons of the clock rate of the IncF plasmid and chromosome were not possible.

Most AMR plasmids are restricted to single sub-clades

Plasmid variants were defined by the combination of replicon(s), pMLST, AMR gene content and identity to other plasmids characterized in the isolate collection, as detailed below. The majority of AMR plasmid variants in the APHA collection are restricted to single sub-clades, with minimal evidence for *S.* Typhimurium widely sharing plasmids between sub-clades (Fig. 4). AMR ColE plasmids carrying *tet*(C) were only observed in sub-clade 4.3 (*n*=12). The five variants of IncX plasmids share ≥98% identity but <90% shared DNA sequence between clusters, and were also sub-clade-specific, with one variant carrying $bla_{\text{TEM-1D}}$.

IncQ1 plasmids were observed in three sub-clades, with plasmid variants specific to each subclade. IncQ1 plasmids (11069 bp) from sub-clade 4.1 (*n*=39) were highly similar (>99% identity and coverage) to a plasmid previously reported from a *S.* Typhimurium phage type U288 isolate from a pig in the UK [82] pSTU288-2 (CP004059), carrying *sul2, strA, strB* and *tet*(A) ARGs. A similar IncQ1 plasmid (11032 bp) was observed in a single isolate from subclade 1.1, differing by two small deletions, one of which was in the *tetR*(A) gene. Five isolates from sub-clade 2.1 carried an IncQ1 (9256 bp) plasmid with *sul2, strB* and *tet*(A), and an insertion of a gene cassette containing *dfrA14* in *strA,* which was presumed to disrupt expression of *strA* and result in loss of streptomycin resistance and gain of trimethoprim resistance. Additionally, the replicon IncQ1 and ARGs *sul2, strA* and *strB* (encoded on Tn*6029*) were chromosomally integrated in sub-clades 2.2 and 2.3, although deletions, likely caused by IS*26*, have resulted in loss of ARGs in many isolates (Fig. 1).

In contrast, an IncN plasmid encoding the resistance genes to *tet*(A), *dfrA14*, *sul2*, *strB* and $bla_{\text{TEM-1D}}$ was the exception, observed across 3 sub-clades in 12 isolates from pigs and 1 isolate from a dog. These IncN plasmids shared high identity and coverage (>99%) with a published plasmid (CP028173).

There was evidence of sporadic acquisition of plasmids without long-term carriage. There were multiple examples of IncHI1, IncHI2 and IncI variants unique to individual isolates with up to five ARGs. A 6.79 kb col plasmid similar to pCERC1 (NC_019070) [83] described in *E. coli* (>99.8% identity, >95% coverage) carrying *sul2, dfrA14* (inserted into *strA*) and *strB* conferring resistance to sulphonamides and trimethoprim was sporadically carried in the DT104-associated sub-clade 3.1 and in single isolates in four further subclades (Fig. 4).

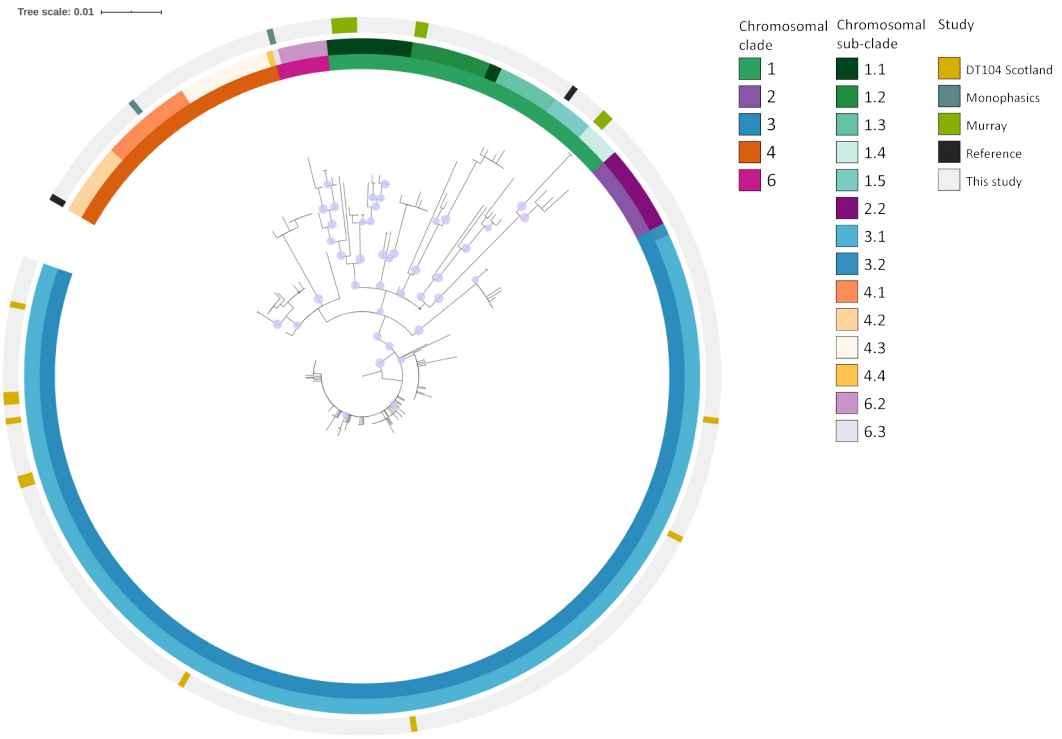

Fig. 3. Maximum-likelihood phylogeny of IncF virulence plasmid of isolates with IncFIIS and/ or IncFIB replicon. The inner ring is coloured by chromosomal clades (colours in legend). The middle ring is coloured by chromosomal sub-clades. The outer ring is coloured by the study of origin. Bootstrap values ≥80 are shown with grey circles. The phylogeny was based upon 644 non-private SNPs and the majority of SNPs (*n*=430) are in non-coding regions. Reads for each isolate were reference-mapped and included in the phylogeny where there was >80% coverage of the pSLT LT2 plasmid reference sequence (*n*=337).

Many small (<7kb) cryptic non-AMR plasmids are common to multiple sub-clades

In contrast to plasmids encoding AMR traits, cryptic plasmids without ARGs were typically observed across multiple clades and sub-clades (Table S4). ColpVC plasmids were identified across five clades (*n*=32 isolates), while Col1 plasmids were identified in two isolates in different clades, and neither plasmid type was associated with an ARG. Col5 (*n*=8 isolates) was observed in one sub-clade. The putative cryptic plasmids (circularizable contigs) not identified with PlasmidFinder or BLASTN against the Orlek plasmid database were grouped into 38 clusters based upon the closest reference sequence (NCBI BLASTN, ≥96% identity, ≥98% coverage). Over half (*n*=20) of the clusters were observed in more than 1 isolate, and 12 clusters were found across multiple clades. The contigs ranged in size from 1.6 to 6.9 kb and the majority carried *mob* and *rop* genes, with two carrying toxin–antitoxin encoding genes. The number of small non-AMR plasmids was not associated with the clade or FPA/NFPA status of isolates.

DISCUSSION

Characterizing the dynamics of plasmids amongst *S.* Typhimurium lineages has revealed multiple examples of stable associations of plasmid and host, with limited evidence of horizontal transfer of virulence and AMR plasmids between *S.* Typhimurium clades. These findings contrast with the highly dynamic nature of plasmid movement in other *Enterobacterales*, including *Shigella* spp. and *E. coli*, in which clinically significant plasmid outbreaks across multiple host lineages have been documented [16]. Understanding the relevant unit of transmission has implications for surveillance of *S.* Typhimurium, with the data indicating that pathogen, rather than plasmid, focused surveillance is justified. However, as previous studies in other *Enterobaterales* have indicated [16, 19], this is not necessarily the case for all bacteria of interest and thus a tailored approach may be required.

More intensive sampling may yet reveal more short-term dynamic flux of plasmids in *S.* Typhimurium as HGT of AMR plasmids that are transiently carried may not be observed in these national-level data. However, the APHA dataset provides an overview of an extended time period, which suggests long-term carriage of specific plasmids within sub-clades. The apparent stability of hybrid AMR-pSLT, IncQ1, IncN and ColE plasmid variants with their respective host clades is of concern due to the persistence of the encoded ARGs.

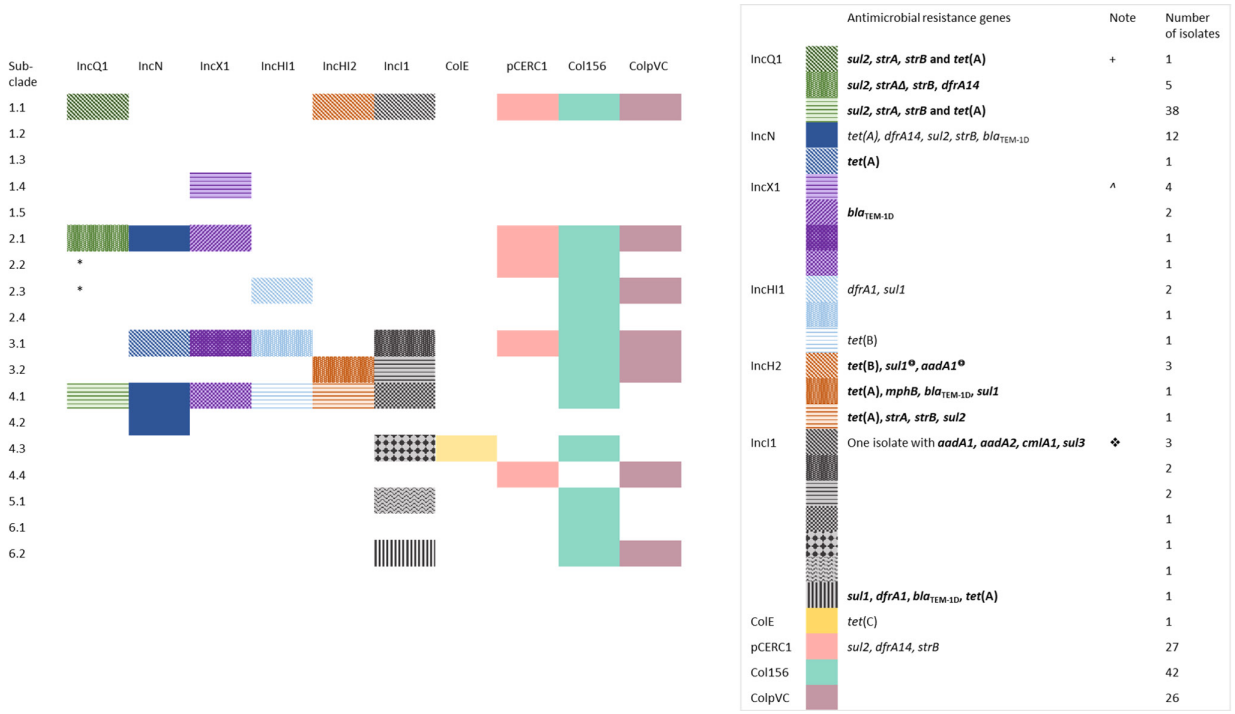

Fig. 4. Summary of plasmid variants and associated antimicrobial resistance genes (ARGs) observed in 496 *S.* Typhimurium isolates from the Animal and Plant Health Agency collection. ARGs are in bold where the ARG was observed on the same contig as the replicon. The remaining ARGs were inferred to be plasmid encoded through mapping to a reference plasmid. Solid colours were used to denote a plasmid observed across multiple sub-clades. * IncQ1 replicons were chromosomally integrated in sub-clades 2.2 and 2.3. + Differs from IncQ1 sub-clade 4.1 variant by two small deletions, including in tetR(A). ^ One isolate with a hybrid IncX, IncF plasmid (FIIS and FIB replicons) and three isolates with IncX plasmid. ◎ In sub-clade 1.1 one isolate had an IncHI2 replicon on the same contig as *tet*(B), *sul1* and *aadA1*. In the two further sub-clade 1.1 IncHI2-positive isolates only *tet*(B) was observed on the same contig, with *sul1* and *aadA1* inferred by mapping. ❖ Each sub-clade 1.1 IncI1 plasmid was a different plasmid multi-locus sequence type.

Multiple pSLT variants carried AMR genes, and similar convergence of AMR and virulence determinants on plasmids has also been reported in some strains of *Klebsiella pneumoniae* [18, 84] and *E. coli* [85]. Importantly, there were no apparent pSLT horizontal transfer events between clades, which concurs with a study of *S.* Typhimurium isolates from humans in Australia [34]. Multi-drug-resistant pSLT were also not observed to be shared between *S.* Typhimurium ST313 lineages that cause invasive disease in humans in sub-Saharan Africa [35, 36, 86]. These data suggest that pSLT variants have co-evolved with the bacterial host. The ostensible lack of horizontal transfer in natural populations is surprising given that pSLT are potentially self-transmissible, as observed in multiple *in vitro* studies, although the conjugation frequency is markedly reduced if the recipient cell already contains an F plasmid [38, 39]. The dynamics of pSLT appear to be consistent with non-conjugative virulence plasmids in multiple *Salmonella* serovars [87]. Limited sharing of IncF plasmids amongst clades has also been observed amongst ST131 *E. coli* clades, with depletion of *tra* genes in some clades [88]. The apparently fixed nature of pSLT has implications for ARG transfer, with the multiple pSLT variants carrying ARGs not anticipated to transfer to other strains of *S.* Typhimurium. However, this does not exclude the possibility of pSLT acting as a reservoir for ARGs for integration into other plasmids or as a recipient of additional ARGs. Horizontal transfer events of pSLT may be undetected due to a lack of fitness benefit to the recipient host preventing selection [89]. However, the ARGs carried by some pSLT variants would be expected to provide fitness benefit under antimicrobial selection pressure.

As anticipated, most isolates were MDR (65%), but there are striking differences in the carriage of ARGs and plasmid replicons between clades. Isolates from the FPA-associated lineages are more likely to be MDR and to harbour one or more AMR plasmids compared to an NFPA-associated clade, which may reflect selection pressure from husbandry and antimicrobial use upon strains circulating in FPA hosts. The low level of AMR amongst isolates from NFPAs is consistent with previous characterization of *S.* Typhimurium in wild birds [49]. The majority of samples from FPAs were from passive surveillance, likely for diagnostic purposes, therefore strains causing more severe clinical signs and strains not responding to initial antimicrobial therapeutics could be anticipated to be well represented in these data. MDR is a trait common to clonally expanded lineages observed in these data and the cyclical dominant *S.* Typhimurium waves previously reported [30]. The absence of chromosomally encoded AMR, such as within an SGI, or stable association with an AMR plasmid, may contribute to the lack of observed clonal expansion of NFPA lineages.

The majority of other AMR plasmids are also not widely shared amongst *S.* Typhimurium clades, with the exceptions being an IncN plasmid and a col plasmid. An MDR IncN plasmid was common to isolates from pigs (*n*=11) and one dog from three sub-clades, indicating either a common source or sharing of plasmids between sub-clades. Acquisition and dissemination of this plasmid in multiple sub-clades may reflect selective pressures of antimicrobial use (AMU) in this livestock sector. The pig industry had the highest AMU of livestock sectors in 2015, with the population correction unit in pigs being >4.6 times greater than the national cross-species use in the UK [90, 91]. High AMU in humans in both community and hospital settings has also been associated with HGT of plasmids between bacterial species and strains [16, 19]. IncN plasmids are typically self-transmissible and have a broad host range; such plasmids may therefore have a greater propensity to disseminate between clades compared to non-conjugative plasmids such as IncQ1 and ColE [20]. AMR pCERC1 col plasmids have previously been reported in both commensal and pathogenic *E. coli* [83, 85], and the presence/absence patterns in *S.* Typhimurium indicate multiple acquisitions without long-term maintenance.

Small plasmids are not conjugative [92] yet in contrast to AMR and virulence plasmids, many small (<7 kb) non-AMR plasmids were observed across multiple sub-clades. This could be due to larger conjugative plasmids having facilitated the transfer of small plasmids via a conjugative pilus, without either transfer or long-term maintenance of the larger conjugative plasmid [93]. Alternative mechanisms for horizontal transfer of the small plasmids include phage-mediated transfer [94]. Small col-like plasmids, including those with ARGs, have also been observed across diverse strains of *E. coli* [88, 95] and can exert minimal or no fitness cost, thereby contributing to long-term maintenance [47].

There are a multitude of potential ecological and mechanistic barriers to the transfer of plasmids amongst strains. Ecological barriers may also contribute to the observed plasmid distribution; the non-random distribution of clades between animal host species may limit opportunities for transmission of plasmids and other MGEs. A similar pattern has been observed elsewhere: between *Klebsiella* in Lombardy, Italy, where carbapenem-resistant clones were observed at high frequency in clinical settings but not in animals or in environmental settings, this separation was postulated to be due to the non-random distribution of *Klebsiella* species and strains between settings [96]. The long-term associations of AMR plasmids with sub-clades may be due to a combination of selection pressures, plasmid addiction/maintenance systems and compensatory mutations that can mitigate the fitness cost of plasmid carriage [42, 97–101]. Variation in compensatory mechanisms may have resulted in differences in the stability of various host–plasmid combinations between *S.* Typhimurium clades, contributing to the observation of multiple examples of AMR plasmids in a single clade or sub-clade.

The data suggest that plasmid transfer between *S.* Typhimurium is not a common phenomenon. The plasmid variants observed in a single isolate may represent short-term or localized host–plasmid combinations, or could be representatives of a widely disseminated strain, which could be evidenced by more extensive sequencing of isolates. This was most pronounced in one sub-clade associated with NFPAs in which all isolates from NFPAs were sensitive to the tested antimicrobials, but there were some FPA origin isolates that were resistant to antimicrobials, each of which had different ARGs and plasmid profiles. These isolates were more genetically divergent compared to isolates in clonally expanded clades and suggest separate ARG acquisition events. More intensive sampling may enable the identification of more of these 'rare' variants and may also identify further, hitherto undetected, dissemination of plasmids amongst clades or sub-clades. It is essential to have longitudinal data to determine whether any observed host–plasmid associations are stable and likely to contribute to clonal expansion and dissemination of AMR, or whether these host–plasmid combinations are highly transitory in nature and are not maintained in the long term.

In conclusion, long-term national surveillance data indicate that many AMR plasmids have stable associations with their *S.* Typhimurium host, and that there has been minimal sharing of virulence and AMR plasmids amongst those *S.* Typhimurium clades that have circulated in animals in England and Wales.

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Author contributions
Study design K.C.M., A.E.M., G.A.B., N.R.T., S.W.J., L.P.; data analysis K.C.M., G.A.B., A.E.M.; isolate acquisition, processing and phenotypic characterization L.P., N.E.M., S.A.C.; manuscript draft K.C.M. All authors contributed to manuscript editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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