Veterinary Microbiology 258 (2021) 109120

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Prevalence, risk factors and genetic traits of *Salmonella* Infantis in Dutch broiler flocks

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ARTICLE INFO

Keywords:
Salmonella
Infantis
Epidemiology
Genomics
Broilers

ABSTRACT

Salmonella Infantis is a poultry-adapted *Salmonella enterica* serovar that is increasingly reported in broilers and is also regularly identified among human salmonellosis cases. An emerging *S*. Infantis mega-plasmid (pESI), carrying fitness, virulence and antimicrobial resistance genes, is also increasingly found. We investigated the prevalence, genetic characteristics and risk factors for (pESI-carrying) *S*. Infantis in broilers. Faecal samples from 379 broiler flocks (in 198 farms with ≥3000 birds) in the Netherlands were tested. A questionnaire about farm characteristics was also administered. Sampling was performed in July 2018-May 2019, three weeks before slaughter. Fourteen flocks (in 10 farms) were *S*. Infantis-positive, resulting in a 3.7 % flock-level and 5.1 % farm-level prevalence. Based on multi-locus sequence typing (MLST), all isolates belonged to sequence type 32. All but one isolate carried a pESI-like mega-plasmid. Core-genome MLST showed considerable heterogeneity among the isolates, even within the same farm, with a few small clusters detected. The typical pESI-borne multi-resistance pattern to aminoglycosides, sulphonamide and tetracycline (93 %), as well as trimethoprim (71 %), was found. Additionally, resistance to (fluoro)quinolones based on *gyrA* gene mutations was detected. *S*. Infantis was found more often in flocks using salinomycin as coccidiostat, where flock thinning was applied or litter quality was poor, whereas employing external cleaning companies, wheat in feed, and vaccination against infectious bronchitis, were protective. Suggestive evidence for vertical transmission from hatcheries was found. A heterogeneous (pESI-carrying) *S*. Infantis population has established itself in Dutch broiler flocks, calling for further monitoring of its spread and a comprehensive appraisal of control options.

1. Introduction

In recent years, the ranking of the most common *Salmonella enterica* subspecies *enterica* serovars isolated from broilers has changed in the Netherlands and in other industrialized countries. Specifically, serovar Paratyphi B variant Java was the dominant serovar in broilers until about the year 2010, when the serovar Infantis began to increase sharply (EFSA/ECDC, 2019). Since 2014, *S*. Infantis has been the most commonly isolated serovar in broilers in the Netherlands, accounting for 78 % of all *Salmonella* isolates from broilers reported in 2018 (Vlaanderen et al., 2019). Also Europe-wide, *S*. Infantis is currently the most common serovar in broilers (46–57 %) and broiler meat (37–51 %), and its prevalence has increased in breeding flocks as well (EFSA/ECDC, 2018, 2019). Moreover, *S*. Infantis ranked fourth among the serovars isolated from human salmonellosis cases in the Netherlands and in Europe in the past few years, after *S*. Enteritidis, *S*. Typhimurium and its monophasic variant (Vlaanderen et al., 2019).

S. Infantis is also increasingly associated with antimicrobial resistance. In 2014, a multi-drug resistant *S*. Infantis strain was identified in Israel carrying a mega-plasmid of approximately 300 Kb named pESI

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https://doi.org/10.1016/j.vetmic.2021.109120
Received 17 February 2021; Accepted 13 May 2021
Available online 15 May 2021

(plasmid of emerging *S*. Infantis) (Aviv et al., 2014); genes associated with antibiotic resistance, virulence and fitness were located on this plasmid. *S*. Infantis isolates carrying the pESI were shown to have increased ability to form biofilms and attach to host cells, leading to raised pathogenicity in mouse models (Aviv et al., 2016). After the first detection, pESI-carrying *S*. Infantis isolates have been reported worldwide, particularly in Europe and in the United States of America (USA) (Alba et al., 2020; Franco et al., 2015; Hindermann et al., 2017; Tate et al., 2017). Extended spectrum β-lactamase (ESBL) genes have also been found on the pESI of some *S*. Infantis isolates from Europe and the USA. Moreover, a *S*. Infantis clone was found in Italy that contained an *mcr-1* gene on a second plasmid, which causes resistance to the last-resort antibiotic colistin (Carfora et al., 2018). In a mouse model, it has also been shown that pESI can be transferred from *S*. Infantis to gut bacteria or to other *Salmonella* serovars through horizontal gene transfer during co-infection (Aviv et al., 2016), which entails that multi-resistance and increased virulence can be spread to other pathogens as well.

To provide baseline epidemiological data on (pESI-carrying) *S*. Infantis, this study investigated the prevalence, antibiotic resistance profiles and genetic characteristics of (pESI-carrying) *S*. Infantis in broiler flocks in the Netherlands, as well as the risk factors associated with its occurrence in those flocks.

2. Materials and methods

2.1. Sample collection and analysis

Fecal samples from 198 Dutch broiler farms with at least 3000 birds included in the annual surveillance system for zoonotic pathogens in farm animals in the Netherlands were collected for *Salmonella* testing. In parallel with sample collection, a comprehensive questionnaire about farm characteristics (e.g. farm infrastructure, husbandry practices, health management, etc.) was completed by the farmer. Sampling was performed between July 2018 and May 2019 within three weeks before slaughter. If available, two poultry houses (i.e. flocks) per farm were sampled. This was possible on 181 farms, whereas on 17 farms, only one poultry house was present, resulting in 379 flocks sampled. Per flock, three fecal samples (each consisting of 12 fresh droppings) were taken, i.e. at the front, central, and hindmost parts of the poultry house itself, and individually frozen with an equal amount of glycerol pending further investigation.

Subsequently, the three samples from each flock were pooled together by mixing 10 g of feces/glycerol material from each individual sample. This pooled sample of 30 g of feces/glycerol per flock was then suspended into 270 ml of Buffered Peptone Water (Biotrading) and incubated overnight at 37 °C. Afterwards, 100 µl of the suspension was transferred to Modified Semi-solid Rappaport-Vassiliadis (Difco) plates with novobiocin (Oxoid). After incubation for 24−48 hours at 41.5 °C, *Salmonella*-suspected colonies were re-incubated on Brilliance *Salmonella* Agar (BSA; Oxoid) plates for 18−22 hours at 37 °C. A maximum of three colonies per BSA plate were then grown on blood agar plates (Oxoid), after which they were serotyped using the xMAP *Salmonella* serotyping assay (Luminex). For isolates with negative or inconclusive reactions with O and H antisera, MALDI-TOF was used for confirmation of genus *Salmonella*. Minimum Inhibitory Concentrations (MIC) were determined by the dilution method using the EUVSEC panel for *Salmonella* and *E. coli* (Sensititre). This panel contains the following antibiotics: ampicillin (AMP), azithromycin (AZI), cefotaxime (CTX), ceftazidime (CAZ), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), gentamicin (GEN), meropenem (MER), nalidixic acid(NAL), sulphamethoxazole (SMX), tetracycline (TET), tigecycline (TIG), trimethoprim (TMP). The epidemiological cut-off values (ECOFFs) published by EUCAST (http://www.eucast.org/mic_distributions_and_ecoffs/) were applied.

2.2. Sequencing and bioinformatics

S. Infantis isolates were grown overnight in 9 ml Brain Heart Infusion tubes (Oxoid) at 37 °C. Cell pellets, obtained from 1.8 ml of the culture, were resuspended and stored in 450 µl DNA/RNA Shield (Zymo) at 4 °C. The DNA was extracted and sequenced by BaseClear (Leiden, the Netherlands). Paired-end 2 × 150bp short-reads were generated using a Nextera XT library preparation (Illumina) and were sequenced on a NovaSeq 6000 system (Illumina). The obtained reads were first trimmed and filtered with ERNE-filter (Del Fabbro et al., 2013) and *de novo* assembled with SPAdes 3.10.0 (Bankevich et al., 2012).

The sequence type (ST) was determined from the assembled genomes with *in silico* multi-locus sequence typing (MLST) using the traditional seven house-keeping gene scheme (Achtman et al., 2012). The *S*. Infantis isolates were also analyzed with the EnteroBase *Salmonella* core genome MLST scheme (Alikhan et al., 2018) using Ridom SeqSphere^{+} 6.0.2 (Ridom; Münster, Germany). The assembled genomes were screened for antimicrobial resistance genes (acquired as well as point mutations) based on the entries in the ResFinder database (Zankari et al., 2012) and for the presence of 233 *Salmonella* virulence factors (Kuijpers et al., 2019). To investigate whether the *S*. Infantis isolates harbored a pESI-like plasmid, the coding DNA sequences (CDS) of 283 genes were extracted from the pESI reference plasmid of *S*. Infantis strain 119944 (accession number: CP047882) and implemented as a scheme in SeqSphere^{+}. This included, for example, determinants specific for IncI1 plasmids and several toxin-antitoxin systems, K88 and other fimbrial factors, genes from the mercury resistance operon (*merTPCBDE*), but also the antimicrobial resistance genes *aadA1*, *dfrA14*, *sul1* and *tet*(A). These CDS also allowed to perform an *in silico* IncI1 plasmid MLST (pMLST) analysis (Carattoli et al., 2014). Nucleotide BLAST was used to confirm and investigate the pESI screening results of the broiler *S*. Infantis isolates in more detail. All the genomic sequences are available at the European Nucleotide Archive (ENA) within the study accession number PRJEB42821.

2.3. Risk factor analysis

Risk factors for the occurrence of *S*. Infantis in the flocks were assessed using logistic regression analysis in which the binary outcome variable was the presence or absence of *S*. Infantis in the flock. In total, 138 independent variables were obtained from the questionnaire and tested for association with the outcome variable. Flock age at sampling was always controlled in the analyses. A cluster-robust sandwich variance estimator was used to account for clustering of flocks at the farm level. Associations were expressed as odds ratios (OR) with 95 % confidence intervals (95 % CI). A complete-record analysis was performed. All models showed overall statistical significance (likelihood-ratio test, $p < 0.05$) and goodness-of-fit (Hosmer–Lemeshow test, $p > 0.05$). Pairwise correlations between independent variables were also assessed. Analyses were performed using Stata v. 16 (StataCorp, College Station, TX, USA). Overall, a $p < 0.05$ was considered statistically significant.

3. Results

3.1. Prevalence and resistance phenotype

There were 35 *Salmonella*-positive flocks (corresponding to 23 farms), resulting in a flock-level prevalence of 9.2 % (95 %CI 6.1–13.8 %) and a farm-level prevalence of 11.6 % (95 %CI 7.5–16.9 %) for *Salmonella* spp. Serotyping of these *Salmonella* isolates at the farm-level revealed the presence of *S*. Paratyphi B variant Java (6.1 %, 95 %CI 3.5–10.3), *S*. Infantis (5.1 %, 95 %CI 2.4–9.1 %) and *S*. Agona (0.5 %, 95 %CI 0.1–2.8 %). This corresponded to a flock-level prevalence of respectively 5.3 % (95 %CI 3.4–8.0 %), 3.7 % (95 %CI 1.9–7.0 %) and 0.3 % (95 %CI 0.0–1.5 %). For *S*. Infantis in particular, 14 flocks of ten farms were positive for this serovar.

The antimicrobial resistance phenotype of these 14 *S*. Infantis isolates revealed that one isolate was fully susceptible while the other 13 isolates were resistant to either four or five antimicrobials (Table 1). The most common phenotypic profile encountered among the 13 resistant isolates was CIP-NAL-SUL-TET-TMP (77 %). The other phenotype was CIP-NAL-SUL-TET (23 %).

3.2. Genetic traits of S. Infantis isolates

In silico MLST showed that all 14 *S*. Infantis isolates belonged to the same sequence type (ST), i.e. ST32. The cgMLST analysis of these isolates, including a pESI-positive reference (*S.* Infantis strain 119944; accession number: CP047881) showed considerable variation among the isolates included in this study. Even isolates from different flocks of the same farm (indicated with A and B in the similarly colored sphere of Fig. 1) were never 100 % identical, although they clustered together. Overall, three clusters and four isolates not belonging to any of these were identified (Fig. 1). The largest cluster (Cluster 1) contained six isolates originating from four different farms.

In silico screening of the assembled genomes showed a high prevalence of five antibiotic resistance genes. They encoded resistance against four different classes of antibiotics; aminoglycosides (*aadA1* and *aph* (3')-Ic), sulphonamide (*sul1*), tetracycline (*tet*(A)) and trimethoprim (*drfA14*). An indication for (fluoro)quinolone resistance was also observed among the *S*. Infantis isolates due to mutations at common positions in *gyrA*, which results in amino acid substitutions in *gyrA* (Table 1).

In silico screening for the presence of 233 *Salmonella* virulence factors revealed that all isolates included in this study harbored 157 of these genes (Supplementary Table S1). Most of these virulence gene sequences were identical (92.7 %, 144/157) to the virulence determinants in the control *S*. Infantis strain 119944, but 13 of them showed genetic differences in one or more isolates (Table 2). Five genes contained a single nucleotide polymorphism (SNP), which did not result in an amino acid substitution because they were synonymous SNPs. The other eight virulence genes contained non-synonymous SNPs. In the case of the outer membrane protein encoded by *sinI*, this SNP resulted in a premature stop codon (PMSC) that could attenuate virulence for the 11 isolates in which this was found. Only in the isolates from farms D and J this SNP was not found. As shown by the cgMLST-based MST (Fig. 1), the isolates from these farms were the most dissimilar when compared to the other isolates (Table 1).

In silico screening of the assembled genomes for the presence of a pESI-like mega-plasmid showed that only one of the 14 isolates did not contain this plasmid, i.e. 367SI-1 (farm J). In the other isolates, over 95 % of the 283 genes of the pESI reference genome were found (Supplementary Table S2). However, three isolates (from farms D and H) appeared to miss a class 2 integron that includes the trimethoprim resistance gene *drfA14* (Supplementary Table S2). This corresponds to the phenotype, as well as the screening results for antibiotic resistance genes (Table 1). Because of the use of short-read Illumina sequencing and due to working with *de novo* assemblies, some results regarding common IS family transposases were ambiguous. The *in silico* IncI1 pMLST analysis did not reveal the presence of *repI1*, which prevented determination of a ST. However, the other genes of the IncI1 pMLST scheme were identified and showed for all except one isolate the same allelic profile, i.e. *ardA*-2, *trbA*-21, *sogS*-9, and *pilL*-3. The isolate from farm E (130SI-1) differed in the pMLST results for the *trbA* gene: trbA-46.

3.3. Risk factors

Given the limited number of outcome events (i.e. 14 *S*. Infantis positive flocks), only univariable associations (adjusted for broiler age at sampling and clustering of flocks at the farm level) were assessed. Three factors were significantly associated with increased occurrence of *S*. Infantis in broiler flocks (Table 3): use of the coccidiostat drug salinomycin (OR 4.4), thinning of birds once (OR 2.2) or twice (OR 5.9) before sampling vs. no thinning at all, and litter quality being mostly 'wet and sticky' (OR 5.2) rather than 'dry and fresh'. Three factors were significantly associated with decreased occurrence of *S*. Infantis (Table 3): cleaning and disinfection operations performed by both farm personnel and external cleaning companies (OR 0.2) vs. farm personnel only, providing wheat in the feed (OR 0.2), and vaccinating birds against infectious bronchitis (OR 0.2).

Of these factors, only three were significantly and negatively associated with each other. These were 'cleaning and disinfection' and 'wheat feed provided' (phi-coefficient -0.14, $p = 0.006$) and 'cleaning and disinfection' and 'coccidiostat salinomycin used' (phi-coefficient -0.15, $p = 0.004$).

4. Discussion

Salmonella Infantis is one of the most common serovars causing human salmonellosis in Europe and is strongly associated with poultry (Alba et al., 2020). In this study, we determined the prevalence, risk factors and molecular characteristics of *S*. Infantis, including pESI-carrying *S*. Infantis, in broiler farms in the Netherlands.

A *S*. Infantis prevalence of 5.1 % at farm-level and of 3.7 % at flock-level was found. Moreover, a pESI-like mega-plasmid was present in 92.9 % of the obtained *S*. Infantis isolates. As the overall *Salmonella* prevalence was only twice as much as that of *S*. Infantis, it was confirmed that *S*. Infantis indeed is a dominant serovar in broilers. This

Table 1
Phenotypic resistance profile and *in silico* screening results for antibiotic resistance genes of the fourteen *S*. Infantis isolates from broiler flocks.

Isolate	Farm	Flock	Phenotypic profile [$]	*aadA1* [†]	*aph*(3')-Ic [†]	*dfrA14* [†]	*sul1* [†]	*tet*(A) [†]	GyrA position 83*	GyrA position 87*
15SI-1	A	A	CIP-NAL-SUL-TET-TMP	+	–	+	+	+	wt	G
16SI-1	A	B	CIP-NAL-SUL-TET-TMP	+	–	+	+	+	wt	G
38SI-1	B	A	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
39SI-1	B	B	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
46SI-1	C	A	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
86SI-1	D	A	CIP-NAL-SUL-TET	+	–	–	+	+	Y	wt
87SI-1	D	B	CIP-NAL-SUL-TET	+	–	–	+	+	Y	wt
130SI-1	E	A	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
144SI-1	F	A	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
234SI-1	G	A	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
235SI-1	G	B	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
275SI-1	H	A	CIP-NAL-SUL-TET	+	–	–	+	+	wt	G
302SI-1	I	A	CIP-NAL-SUL-TET-TMP	+	+	+	+	+	wt	G
367SI-1	J	A	Fully susceptible	–	–	–	–	–	wt	wt

Note: [$] CIP, ciprofloxacin; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim. [†]*aadA*1: aminoglycoside resistance gene, *aph*(3')-Ic: aminoglycoside resistance gene, *dfrA14*: trimethoprim resistance gene, *sul1*: sulfonamide resistance gene, tet(A): tetracycline resistance gene. *wt = wildtype (GyrA position 83 is a serine (S) while position 87 is aspartic acid (D), Y = tyrosine, G = glycine.

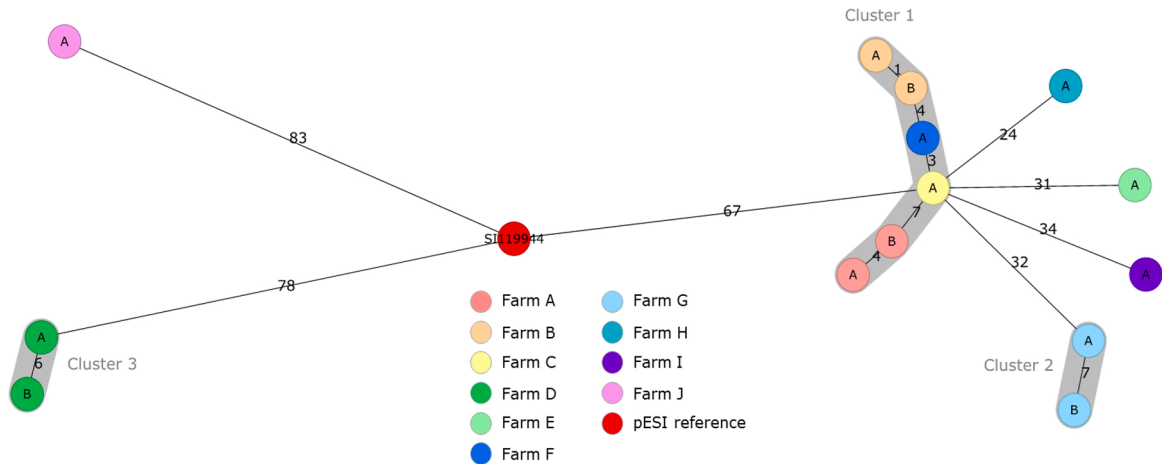

Fig. 1. Minimum Spanning Tree (MST) of the cgMLST analysis of fourteen *S*. Infantis isolates (ST32) from broilers. The MST is based on 2963 genes of the total of 3002 present in the official cgMLST scheme. Each sphere represents an isolate and the number on the connecting lines shows the number of genes different between the ST32 isolates. Clusters are indicated with a grey background color, with a cluster definition set to a maximum distance of seven genes. The different colors of the spheres display the ten positive farms, while the A and B in the spheres indicate from which flock of a farm the *S*. Infantis was isolated.

Table 2
Variants of virulence determinants in comparison to *S*. Infantis strain 119944.

Gene	Function	SNP[$]	AA[$] substitution	Isolate (Farm)
fimF	Type I fimbriae adaptor protein	T62C	V21A	367SI-1 (J)
hilD	Invasion regulatory protein	G468A	–	275SI-1 (H)
invA	Cell invasion protein	A1218G	–	367SI-1 (J)
invE	Cell Invasion protein	C456T	–	302SI-1 (I)
ratB	Outer membrane protein	T3898C	Y1300H	86SI-1 (D), 87SI-1 (D)
sifB	Secreted effector protein	G766A	V256I	6SI-1 (D), 87SI-1 (D)
sinI	Outer membrane protein	T589G	E197Stop	15SI-1 (A), 16SI-1 (A), 38SI-1 (B), 39SI-1 (B), 46SI-1 (C), 130SI-1 (E), 144SI-1 (F), 234SI-1 (G), 235SI-1 (G), 275SI-1 (H), 302SI-1 (I)
slrP	E3 ubiquitin-protein ligase	C178A	A60I	86SI-1 (D), 87SI-1 (D)
sscB	Secretion system chaperone	T238A	F80I	15SI-1 (A), 16SI-1 (A), 38SI-1 (B), 39SI-1 (B), 46SI-1 (C), 130SI-1 (E), 144SI-1 (F), 234SI-1 (G), 235SI-1 (G), 275SI-1 (H), 302SI-1 (I)
ssrA	Sensor kinase	C1894T	H632Y	130SI-1 (E)
stdB	Outer membrane usher protein	C438T	–	275SI-1 (H)
stfA	Fimbrial subunit	G192A	M64I	86SI-1 (D), 87SI-1 (D)
sthD	Fimbrial subunit	C420T	–	275SI-1 (H)

Note: [$] SNP = single nucleotide polymorphism; AA = amino acid.

also concurs with findings from poultry meat at retail (Vlaanderen et al., 2019) and reflects the current situation in several other European countries (Alba et al., 2020; EFSA/ECDC, 2018, 2019; Pate et al., 2019). Overall, these findings confirm that *S*. Infantis has successfully established itself in the Dutch broiler sector.

The rapid spread of *S*. Infantis in broilers has been related to the occurrence of the pESI mega-plasmid carrying genes associated with fitness, virulence and antimicrobial resistance (Aviv et al., 2016, 2014). In this study, pESI was found in all but one isolate. Yet, despite the ubiquity of pESI and the presence of only one ST among these isolates (i.e. ST32), the *S*. Infantis population was heterogeneous in terms of molecular relatedness at the cgMLST level, with some variation even between isolates from the same farm. This is consistent with the conclusions of a recent molecular analysis of *S*. Infantis isolates from all over Europe (Alba et al., 2020). Indeed, that study found the European *S*. Infantis population to be heterogeneous, with different genetic clusters defined at core genome level. However, the same study reported that pESI-like variants were present in 64 % of the isolates and were more genetically homogeneous and capable of infecting different clonal lineages in most of the countries. The same was observed here, as the identified genes of the IncI1 pMLST scheme revealed that all but one isolate had the same allelic profile. Therefore, pESI appears to be able to colonize different clonal lines of *S*. Infantis and to spread widely in poultry populations.

Of the three clusters of *S*. Infantis isolates found here, the largest included six isolates from four different farms. From the questionnaires completed by these farms, it appeared that they received chicks from the same two hatcheries. Although these hatcheries also provided chicks to flocks on other farms that tested negative, the observation is suggestive of possible vertical transmission. Together with Enteritidis, Typhimurium, Hadar and Virchow, Infantis is one of the target serovars included in the current control programs for *Salmonella* in breeding flocks of *Gallus gallus* (Commission Regulation (EU) No 200/2010), but not for broiler flocks (Commission Regulation (EU) No 200/2012) in the European Union.

The public health implications of *S*. Infantis in broiler flocks, particularly when the isolates carry pESI, are unclear. The proportion of *S*. Infantis infections among human salmonellosis patients in the Netherlands has stabilized in recent years and these infections do not appear to be associated with increased invasiveness in humans (Mughini-Gras et al., 2020). Moreover, in a mouse experiment, *S*. Infantis was found to be less virulent than *S*. Typhimurium (Aviv et al., 2019). However, *S*. Infantis without pESI was used in that experiment. In a different mouse experiment, it appeared that infection with *S*. Infantis causes a more severe inflammation when carrying pESI (Aviv et al., 2014). The consequences of infections with *S*. Infantis carrying pESI in humans, including the severity of infection and the implication of antibiotic resistance, have not yet been investigated.

It has been proven that pESI frequently harbors resistance genes

Table 3
Factors significantly associated with increased or decreased occurrence of *S.* Infantis in broiler flocks.

Risk factor	*S.* Infantis positive flocks	*S.* Infantis negative flocks	OR[1]	95 % CI[1]		p
Cleaning and disinfection						
Performed by farm personnel only	7 (50 %)	78 (22 %)	Reference			
Performed by farm personnel and professional cleaners	7 (50 %)	285 (78 %)	0.233	0.057	0.955	0.043
Wheat feed provided						
No	6 (43 %)	52 (14 %)	Reference			
Yes	8 (57 %)	313 (86 %)	0.235	0.055	0.999	0.049
Coccidiostat salinomycin used						
No	4 (29 %)	205 (56 %)	Reference			
Yes	10 (71 %)	160 (44 %)	4.372	1.101	17.363	0.036
Chickens vaccinated against infectious bronchitis						
No	2 (14 %)	11 (3%)	Reference			
Yes	12 (86 %)	352 (97 %)	0.170	0.034	0.855	0.031
Thinning of flock before sampling						
No	7 (50 %)	268 (75 %)	Reference			
Yes, once	4 (29 %)	78 (22 %)	2.182	0.382	12.451	0.380
Yes, twice	3 (21 %)	13 (3%)	5.883	1.025	33.760	0.047
Litter quality						
Mostly dry and fresh	10 (77 %)	334 (94 %)	Reference			
Mostly wet and sticky	3 (23 %)	21 (6%0	5.183	1.167	23.023	0.031

Note: [1]Estimates are adjusted for broilers' age at sampling and clustering of flocks at the holding level. OR = Odds Ratio. 95 % CI = 95 % Confidence Interval. Totals do not add up because of missing values.

against at least three antimicrobial classes (sulphonamides, tetracyclines and trimethoprim), with variable carriage of the aminoglycosides resistance gene *aadA1* and resistance to heavy metals and several toxin/anti-toxin systems (Alba et al., 2020; Aviv et al., 2014; Franco et al., 2015). The typical pESI-associated genes *aadA1*, *dfrA14*, *sul1*, and *tet(*A) were found in the isolates included in the present study, with resistance gene rates being particularly high (92.9 %) for aminoglycosides, sulphonamides and tetracycline, and somewhat lower (71.4 %) for trimethoprim. The presence of nearly all of these genes can be linked to the antimicrobial resistance phenotypes of the *S.* Infantis isolates (Table 1). Unfortunately, *aadA1* encodes for resistance to aminoglycoside antibiotics not included in the EUVSEC panel, i.e. streptomycin and spectinomycin. This is also the case for *aph*(3')-Ic (not part of pESI) which causes resistance to the aminoglycosides; kanamycin, neomycin and paromycin. Otherwise, a 100 % match between antimicrobial phenotype and genotype was found. The SUL-TET-TMP profile can be linked to the presence of *sul1*, *tet*(A), and *dfrA14*, respectively. Moreover, in all isolates with pESI and a CIP-NAL phenotype, resistance to these (fluoro)quinolones was the result of mutations in the chromosomal *gyrA* gene. The phenotypic and genotypic resistance profiles were consistent with the previously described pESI-carrying *S.* Infantis isolates from several countries (Alba et al., 2020; Franco et al., 2015; Hindermann et al., 2017). Resistance genes to macrolides, which have been described in 18 % of the isolates in an European study (Alba et al., 2020), were not found in the isolates investigated here. Interestingly, ESBL genes were not found either, whereas they have been reported in *S.* Infantis isolates from human patients in the Netherlands (Carfora et al., 2018). Moreover, the *mcr-1* gene causing resistance to colistin, which was reported previously (Carfora et al., 2018), was also not encountered in this study.

An increased risk for *S.* Infantis colonization was found for flocks in which salinomycin was used, as well as flocks where thinning was applied or where litter quality was poor, whereas the use of professional cleaners (i.e. employing external cleaning companies vs. own personnel only), providing wheat in the feed, and vaccinating against infectious bronchitis, had protective effects. Salinomycin is an ionophoric coccidiostat that is widely used as a supplement in poultry feed. Although it has been reported that salinomycin may reduce *Salmonella* prevalence in broilers (Bolder et al., 1999), other authors found no reduction in *Salmonella* prevalence or load when using this coccidiostat (Scalzo et al., 2004). On the contrary, they observed an increase of one log unit on *Salmonella* colony forming units between control and salinomycin-treated chickens. In addition, experimentally infected chickens (with *S.* Typhimurium) showed higher *Salmonella* isolation rates when treated with salinomycin vs. control group during the 3rd week post-dosing (Ford et al., 1981). Moreover, the latter study reported that salinomycin caused the decline of salmonellae to be more gradual, although at the end of the experiment the treatment and control groups were comparable. It is therefore difficult to explain why salinomycin treatment would be a risk factor for *S.* Infantis colonization, but it might be related to the overall farm management quality rather than the effect of the drug itself. Indeed, the fact that poor litter quality was also a risk factor and that employing professional cleaners was protective (as also found previously in Belgian broiler flocks as a risk factor for the presence of *Salmonella* spp. (Namata et al., 2009)) and negatively associated with salinomycin treatment itself, seems to point to (suboptimal) farm management practices as the main driver of *S.* Infantis colonization in broilers. The hypothesis is therefore that the use of coccidiostat drugs act as a proxy for lower quality farm management, such as lower biosecurity, as the need of using coccidiostat drugs implies the existence of that issue (i.e. coccidial infection risk) that cannot be controlled otherwise (e.g. with biosecurity) in the farm. However, it is also true that almost all (95 %) of the farms in this study used a coccidiostat (salinomycin or other drugs), so it cannot be excluded that the issue concerns specifically this component and that the use of it, alike other antimicrobials, might play a role (e.g. selective advantage) as to favor the establishment of (pESI-carrying) *S.* Infantis in the flocks.

Also flock thinning is by its nature a well-known 'breach of biosecurity', as well as a stressor for the birds, and a clear increase in *S.* Infantis colonization risk was observed as more thinning cycles were applied. The risks concern mainly the passive transfer of microorganisms, including *Salmonella* among others, from previously visited farms to the remaining flock during catching via, e.g., vehicles, crates, crews (boots, clothes, etc.) (FAO/WHO, 2009). Less straightforward is the interpretation of the protective effects associated with the use of wheat in the feed and vaccination against infectious bronchitis, which might, once again, mirror the overall farm management standards, as well as have genuine effects due to, e.g., improved health or welfare of the broilers resulting from the applied dietary and immunization schemes.

In conclusion, this study shows that a heterogenous (pESI-carrying) *S.* Infantis population has successfully established itself in broiler flocks in the Netherlands, with a prevalence of around 5% and some indications of potential vertical transmission. The isolates analyzed here were characterized by a high rate of resistance genes and phenotypical resistance to the typical pattern of multi-drug resistance to

aminoglycosides, (fluoro)quinolones, sulphonamides, tetracycline, and trimethoprim. Although the incidence of human *S*. Infantis infections in the Netherlands appears to be stable (Vlaanderen et al., 2019), the virulence and antimicrobial resistance genes harbored by pESI and the increasing trends observed in poultry worldwide call for continuous monitoring of the spread of *S*. Infantis and its large virulence plasmid, including a comprehensive appraisal of the options for control. These options should consider in particular biosecurity, as most of the risk factors identified seemed to be related, directly or indirectly, to this topic and less to other farm characteristics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the Dutch Ministry of Agriculture, Nature and Food Quality (grant number 9.2.24.B). The authors are thankful to Anjo Verbruggen and Sjoerd Kuiling for their contribution to the laboratory work.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2021.109120.

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