Appendix II. Materials and Methods

Salmonella enterica

A total of 32,289 isolates have been tested for antimicrobial susceptibility between 1999-2014 (Table A1). Human isolates (N=18,454) concerned a selection from first isolates sent to the Dutch National Institute of Public Health (RIVM) by the regional public health laboratories. All strains were the first isolates recovered from patients with salmonellosis. The majority of the isolates from pigs (N=1976) and cattle, including calves, (N=997) were sent to the RIVM by the Animal Health Service from a diversity of surveillance programs and clinical *Salmonella* infections. Those from chickens (broilers, including poultry products, N=3709; layers, reproduction animals and eggs, N=1196) concerned mainly nonclinical *Salmonella* infections derived from a diversity of monitoring programs on the farm, slaughterhouses and at retail. Isolates from a diversity of other sources have been analysed as well (animal fodder and human food products; other animals from animal husbandry (e.g. horses, goats, sheep) and pets, samples from the environment, etcetera).

Table A1. Number of Salmonella isolates tested for susceptibility from 1999 – 2013 in the Netherlands.

	total number	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Human	18454	693	358	1095	892	1379	1316	1215	1324	1151	1498	1106	1494	1325	1414	1103	1091
Pig	1976	32	196	114	168	127	116	120	113	145	334	52	99	39	174	73	74
Cattle	997	18	29	56	33	23	106	90	156	94	76	73	41	68	37	52	45
Chicken (misc.)	1482	0	12	174	172	159	29	30	31	28	90	76	71	47	65	276	222
Broiler	2227	69	110	143	212	206	107	79	245	211	296	106	77	63	93	119	91
Layers/Repro/Eggs	1196	94	86	80	67	103	92	230	78	51	37	38	46	64	75	5	50
Other sources	5957	0	9	291	319	431	429	602	542	472	376	440	478	413	633	278	244
Total	32289	906	800	1953	1863	2428	2195	2366	2489	2152	2707	1891	2306	2019	2491	1906	1817

Representativeness of percentages of resistance for humans or animals over all types

In principal, if isolates are selected randomly from a source the percentage of resistant strains within a source can be computed straightforwardly. Standard statistical considerations would apply to indicate significant differences between years and between animal and human sources. Table A2 shows that quite substantial numbers are needed to indicate significant differences in resistance percentages less than 10%. However, resistance strongly depends on *Salmonella* type and many different types are involved; a cocktail of types that differ between sources and that may differ between years. Moreover, low numbers tested and incidentally missed, or selected types with rare antibiograms, may influence the resulting resistance percentages. Finally the source definition in itself may be biased, as the reason for sending-in isolates, especially from cattle and pigs, is often unknown. This explains many of the irregularities between years.

E. coli, E. faecium, E. faecalis and *Campylobacter* spp. isolated from slaughter pigs and broilers

E. coli and *E. faecium*, *E. faecalis* and *Campylobacter* spp. were isolated from faecal samples taken from healthy animals by the Food and Consumer Product Safety Authority as part of the national control programs. Samples were taken at slaughterhouses from pigs, poultry, veal calves (from 2012 onwards) and dairy cattle (in 2010 and 2011), or at farms from veal calves (before 2012) or dairy cattle (until 2010 and after 2011). For isolation of the above mentioned organisms one faecal sample was taken for each epidemiological unit (farm, flock or group of animals), or the caeca collected (broilers). At the laboratory the samples were 1:10 (w/v) diluted directly in buffered peptone solution with 20% glycerol and stored at –20°C. After arrival of the samples, isolation of *E. coli*, *Enterococcus faecium*, *E. faecalis* and *Campylobacter jejuni* and *C. coli* was performed without delay at CVI or the Food and Consumer Product Safety Authority.

For *E. coli* MacConkey agar and for the enterococci Slanetz and Bartley agar was inoculated with cotton swabs (*E. coli*), or 50 µl of a serial dilution (enterococci). A colony with typical morphology was subcultured to obtain a pure culture and stored at –80°C in buffered peptone water with 20% glycerol. *E. coli* was identified by Matrix assisted laser desorption/isonisation time-of-flight analyser (MaldiTof, Bruker). The final identification of the enterococci was done with MaldiTof. Only *E. faecalis* and *E. faecium* were tested for their susceptibility.

For isolation of *Campylobacter* CCDA-agar with 32 µg/ml cefoperazone and 10 µg/ml amphotericin B to inhibit growth of Gram-negative bacteria and fungi, was directly inoculated with a cotton swab. All campylobacters were typed with MaldiTof to the species level. Only *C. jejuni* and *C. coli* were tested for their susceptibility. All other spp. were excluded from the program.

Table A2. Power analysis to show the sample sizes needed to indicate significant differences in resistance percentages between groups (for example between years or between human and animal sources).

Level of significance = 0,05 and Power = 0,7			
R-group 1	R-group 2	Difference	N1=N2
40%	30%	10%	287
30%	20%	10%	251
20%	10%	10%	211
70%	50%	20%	111
60%	40%	20%	95
50%	30%	20%	84
40%	20%	20%	70
30%	10%	20%	59
60%	30%	30%	23

E. coli, E. faecium and *E. faecalis* isolated from raw meat products of food-animals

For isolation of all bacterial species raw meat products were rinsed with Buffered Peptone Water (BPW). For *E. coli* 10 ml BPW rinse was enriched in 90 MacConkey-, or Laurylsulphate broth. After overnight aerobic incubation at 44°C the broth was subcultured on Coli-ID agar (24 h at 44°C). For enterococci 10 ml BPW rinse was enriched in 90 ml Azide Dextrose broth. After overnight aerobic incubation at 44°C, the broth was subcultured on Slanetz and Bartley agar for 48 hrs at 44°C. Identification was done by PCR[1].

Shiga toxin producing *E. coli O157* (STEC)

For STEC only human strains were included. All sorbitol negative human strains from all medical microbiological laboratories in the Netherlands were sent to RIVM for serovar O157 confirmation and further typing.

Susceptibility tests

Susceptibility was tested quantitatively with the broth micro dilution test with cation-adjusted Mueller Hinton broth according to ISO standard 20776-1-2006 or CLSI guidelines M31-A3 for *Campylobacter* spp.. For broth microdilution, microtitre trays were used with dehydrated dilution ranges of custom made panels of antibiotics. Trek Diagnostic Systems, in the UK, manufactured these microtitre trays. ATCC strains *E. coli* 25922 and *E. faecalis* 29212 were used daily to monitor the quality of the results. For quality control of the results of campylobacters, *C. jejuni* ATCC 33560 was used as control strain.
The MICs were defined as the lowest concentration without visible growth. Strains with MICs higher than the epidemiological cut-off values were defined resistant.. Percentages of resistance were calculated. For *Salmonella*, *E. coli* O157, *Campylobacter* spp., and the indicator organisms *E. coli* and enterococci EUCAST epidemiological cut-off values were used as prescribed by EFSA[1,2] (Table A3).

Data interpretation needs to take into account that for some antibiotics the epidemiological cut-off values are substantially lower than the previously used clinical breakpoints, which may have affected the level of the resistance percentages. These percentages indicate the acquisition of resistance in intrinsically susceptible bacteria population as an effect of determinants like antibiotic usage. They cannot directly be translated in therapeutic failure, when antibiotics would be used to treat infection with those organisms.

Active surveillance of ESBLs

Since 2011, prevalence studies of ESBL/AmpC-producing *E. coli* were initiated in Dutch food-producing animals (veal calves, dairy cows and pigs). In 2011 till 2013 from Dutch slaughterhouses a faecal sample was taken from ten (apparently healthy) animals per slaughter batch of animals. Since 2014 faecal samples derived from the monitoring program were used. In addition raw meat samples were also included. Each faecal sample was analysed for the presence of ESBL/AmpC-producing *E. coli* using selective pre-enrichment in Tryptic Soya Broth with 1 mg/L cefotaxime followed by selective isolation on MacConkey agar with 1 mg/L cefotaxime. The meat samples were pre-enriched in Luria Broth with 1 mg/L cefotaxime followed by selective isolation on both MacConkey agar with 1 mg/L cefotaxime and Brilliance ESBL Agar (Oxoid, part of Thermo Fischer Scientific). From each plate colonies with the typical morphology of *E. coli* were selected. Identification of the bacterial species was done by MaldiTof. One positive *E. coli* per flock was screened for beta-lactamase gene families using the Check-Points CT101 miniaturised micro-array. Subsequently the genes were identified by dedicated PCR and sequence analysis. All isolates with a negative array result for ESBL or AmpC genes were examined for promoter mutants in the chromosomal *ampC*-genes.

[1]Report from the Task Force of Zoonoses Data Collection including a proposal for a harmonized monitoring scheme of antimicrobial resistance in *Salmonella* in fowl (*Gallus gallus*), turkeys, and pigs and *Campylobacter jejuni* and *C. coli* in broilers, *the EFSA Journal*.
www.efsa.europa.eu/en/efsajournal/pub/96r.htm

[2] Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal Escherichia coli and Enterococcus spp. from food animals.
www.efsa.europa.eu/en/efsajournal/pub/141r.htm

Active surveillance of carbapenem resistance

In 2014, all faecal samples sent to the Central Veterinary Institute (CVI) by the Dutch Food and Consumer Protection Authority (NVWA) for antimicrobial resistance surveillance in broilers, laying hens, slaughter pigs, veal calves and dairy cows were screened for the presence of carbapenem resistance. Of each faecal sample 1 gram was incubated overnight in 9 ml Tryptic Soy Broth containing 50 mg/L vancomycin. After incubation the culture was centrifuged and the pellet stored at -20°C. The RT-PCR was performed according to the manufacturer's description on the isolated DNA of the pellet. If the RT-PCR gave suspicious or positive results, three steps to confirm the results were made:

1. The DNA-lysate was used to run the CT102 micro array (Check-Points). This array detects the carbapenemase gene families NDM, KPC, VIM, IMP and OXA-48.
2. If the micro array was positive, the result was further confirmed by dedicated PCR and sequencing.
3. Moreover, for samples suspected to be positive the original faecal sample and the broth culture was inoculated on commercial selective plates (ChromID Carba and ChromID OXA (Biomerieux).

Table A3. Epidemiological cut-off values (mg/L) used for the classification of *Salmonella*, *E. coli* (indicator organism), *Campylobacter* spp. and enterococci. Isolates with MIC-values higher than those presented in this table are considered resistant.

	Salmonella	*E. coli*	*C. jejuni*	*C. coli*	*E. faecium*	*E. faecalis*
Ampicillin	8	8			4	4
Azithromycin*	16	16	-	-	-	-
Cefotaxime	0.5	0.25	-	-	-	-
Ceftazidime	2	0.5	-	-	-	-
Chloramphenicol	16	16			32	32
Ciprofloxacin	0.06	0.06	0.5	0.5	4	4
Colistin		2	-	-	-	-
Daptomycin	-	-	-	-	4	4
Erythromycin	-	-	4	8	4	4
Gentamicin	2	2	2	2	32	32
Linezolid	-	-	-	-	4	4
Meropenem	0.125	0.125	-	-	-	-
Nalidixic acid	16	16	16	16	-	-
Quinupristin-dalfopristin	-	-	-	-	n.a.	1
Streptomycin			4	4	-	-
Sulfamethoxazole	256	64			-	-
Teicoplanin	-	-	-	-	2	2
Tetracycline	8	8	1	2	4	4
Tigecycline	1	1	-	-	0.25	0.25
Trimethoprim	2	2	-	-	-	-
Vancomycin	-	-	-	-	4	4

*tentatively set ECOFF during the EURL AMR WP meeting on 25 April 2015 in Lyngby (DK)
n.a. not applicable