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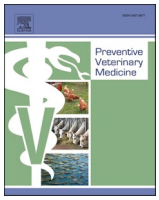

Antimicrobial resistance monitoring in commensal and clinical *Escherichia coli* from broiler chickens: Differences and similarities

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ABSTRACT

Background: In the Netherlands, antimicrobial resistance (AMR) is monitored in commensal indicator *Escherichia coli* from healthy broilers at slaughter as part of a European monitoring programme. In a separate programme for poultry health, AMR is monitored in veterinary pathogens from diseased broilers. So far, it is unknown how the outcomes of these two AMR monitoring approaches in the same animal population are associated.

Aims: This study aims to investigate the association between the outcomes of monitoring non-wildtype susceptibility (using epidemiological cut-off values, ECOFF, as prescribed by EU legislation) in commensal *E. coli* isolated from healthy broilers (i.e. active surveillance) with the outcomes of monitoring clinical resistance (using clinical breakpoints, to determine susceptibility for antibiotic treatment in veterinary practice) in *E. coli* isolated from diseased broilers (i.e. passive surveillance).

Methods: Data acquired by broth microdilution was analysed for commensal indicator *E. coli* and clinical *E. coli* from the Netherlands, 2014–2019. A generalized linear multivariable model (Poisson regression) was used to determine time trends and identify differences in mean resistant proportions.

Results: Observed resistant proportions of the monitored commensal *E. coli* and clinical *E. coli* were similar with overlapping confidence intervals for most time points for ampicillin, gentamicin, cefotaxime, tetracycline, colistin and trimethoprim/sulfonamide. The statistical analysis showed that only for cefotaxime and tetracycline, mean resistant proportions were different. In commensal *E. coli*, a decrease of resistant proportions over time was observed, except for gentamicin. In clinical *E. coli*, no time trend was detected in resistant proportions, except for cefotaxime and colistin.

Conclusions: Generally, the resistant proportions monitored in commensal and clinical *E. coli* were similar. However, some relevant differences were found, which can be explained by the type of monitoring approach, i.e. active or passive surveillance. The random sample of commensal *E. coli* isolated from healthy animals (active surveillance), was more suitable to monitor AMR time trends. The sample of clinical isolates from diseased animals (passive surveillance), resulted in a higher chance to detect low-prevalent resistance: i.e. cefotaxime and colistin. The clinical *E. coli* data showed more fluctuation over time, and data from a longer period of time would be needed to determine the association. This study shows the value of both an active and a passive surveillance component for AMR monitoring.

1. Introduction

Antimicrobial resistance (AMR) in livestock as public health hazard is monitored in commensal indicator organism *Escherichia coli (E. coli)*, and food-borne pathogens *(Salmonella, Campylobacter*). In the European Union, an active surveillance component is mandatory to sample food producing animals in slaughterhouses, isolate *E. coli* and determine their susceptibility for antimicrobials with a standardized susceptibility

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panel, as prescribed by EU legislation (European Commission, 2013). Results are reported yearly in Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals (MARAN) (Veldman et al., 2020) in the Netherlands and by the European Food Safety Authority (EFSA) on European level (EFSA (European Food Safety Authority) and ECDC (European Centre for DiseasePrevention and Control), 2018).

Several studies have shown that monitoring of AMR in commensal indicator organism *E. coli* is useful to detect AMR as a potential public health threat in food producing animals (Hesp et al., 2019; Dorado-Garcia et al., 2016; Hanon et al., 2015). The monitoring in commensal *E. coli* also has potential use for veterinary medicine. Veterinary prescription guidelines are based on AMR trends in commensal *E. coli* as well as on AMR trends in clinical isolates. However, commensal *E. coli* have no direct clinical significance, but are considered a potential source of resistance genes for pathogenic bacteria. Currently, it is mostly unknown how to interpret the meaning of non-wildtype susceptibility (NWT) in commensal *E. coli* from livestock for veterinary practitioners, i.e. how to relate this to clinical resistance. When bacterial isolates are collected from the same animal population in the same period of time, it could be hypothesized that the occurrence of AMR in these bacterial populations is similar. For a better understanding of AMR monitoring data in both commensal and clinical E. coli, this association should be investigated.

In the Netherlands, AMR monitoring in commensal and clinical *E. coli* is performed simultaneously in broilers. Apart from AMR monitoring in commensal *E. coli* isolates from slaughter animals, AMR is monitored in veterinary pathogens, acquired from clinical submissions and post-mortem examinations of livestock by Royal GD (GD) in Deventer (Royal GD, 2019; Wiegel et al., 2018), The Netherlands. These results are reported as part of the national farm animal health surveillance system, by order of the Dutch government and the animal industry. It is a passive surveillance component of clinical isolates from cases submitted by the industry to GD as part of the harmonized health monitoring. In diseased broiler chickens, among other bacterial species, *E. coli* are isolated and tested for antimicrobial susceptibility.

To investigate the association of the outputs of monitoring in commensal and clinical *E. coli*, this study compares monitored NWT susceptibility in commensal *E. coli* isolated from healthy animals at slaughter (i.e., active surveillance) with monitored clinical resistance in *E. coli* isolated from diseased broilers (i.e. passive surveillance). For that purpose, the antimicrobial susceptibility of bacterial isolates using broth microdilution was analysed of commensal indicator *E. coli* from MARAN and clinical *E. coli* from GD monitoring, the Netherlands, 2014–2019.

2. Methods

2.1. Sample collection

Sample collection in MARAN (2014–2019) was equal to described in Hesp et al. (2019). This consisted of a stratified random sampling strategy of caecal samples, each originating from a unique flock, collected at slaughterhouses. As defined by EFSA, this stratified sampling of caecal samples 'accounted for slaughterhouses processing at least 60% of the domestic annual production of the broiler population, with proportionate allocation to the slaughterhouse production' (Commission Implementing Decision (EU) 2013/652, Annex Technical Requirements 2.3). Data were stored in the laboratory database of WBVR and for the analyses transferred to MS Excel.

In GD monitoring (2014–2019), clinical *E. coli* isolates were obtained from lesions of diseased broilers submitted for pathology to GD, as well as a random selection of isolates cultured in private practice laboratories, also obtained from lesions of diseased broilers. Data were stored in the laboratory database of GD and for the analyses transferred to MS Excel. The clinical samples were mostly submitted by veterinarians for bacteriology before determining antibiotic treatment and 29% of the submitted samples were marked by veterinarians as 'no treatment'. Only 12% of the samples were marked as 'treatment'. In 59% of the submissions, the treatment marking was missing. However, it was assumed that all non-marked samples were untreated, as good veterinary practice prescribes to determine susceptibility before initiating an antibiotic treatment. A comparison was made between all clinical isolates and the dataset excluding the 12% of samples from treated animals (i.e. with 88% of the data). The resistant proportions and their confidence intervals were nearly identical (results not shown). Hence, all clinical *E. coli* data were included in the analysis.

2.2. Bacterial isolation and susceptibility testing

Isolation of *E. coli* in MARAN is described in Hesp et al. (2019). Commensal *E. coli* were isolated on MacConkey agar. Clinical *E. coli* isolates from post mortem examination were isolated on sheep blood agar. Samples were taken from bone marrow in the case of generalized infection or from affected tissues in case of localized infections.

Susceptibility testing in MARAN was performed by broth microdilution, determining minimum inhibitory concentrations (MIC) as prescribed by EU legislation (European Commission, 2013) and according to ISO 20776–1 using commercially available microtiter plates (Sensititre EUVSEC by Thermo Scientific, East Grinsted, United Kingdom). The testing panel in MARAN included 14 antimicrobials from 11 antimicrobial classes. GD performed broth microdilution with customized microtiter plates (Merlin Diagnostics, Bornheim-Hersel, Germany). This panel contained 20 antimicrobials of 11 antimicrobial classes. In both monitoring programmes, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker Daltonik GmbH, Bremen, Germany) was used to confirm that the isolates were *E. coli* (in MARAN this method was introduced in 2015, in the year 2014 isolates were biochemically identified).

MIC distributions were scrutinized of the antimicrobials present in both susceptibility testing panels (Supplementary Figs. S1-S7). This was to detect any methodological differences and to determine which antimicrobial classes could be compared. Regarding inoculum, microliter per well and incubation conditions no differences in methodology were detected, judged by the similarity in MIC distributions, showing consistency of both laboratories.

The antimicrobial classes present in the susceptibility testing panels of both monitoring laboratories were: ampicillin representing aminopenicillins, gentamicin representing aminoglycosides, cefotaxime as representative of cephalosporins, tetracycline representing tetracyclines, colistin representing polymyxins, ciprofloxacin and enrofloxacin representing fluoroquinolones. Non-wildtype susceptibility to ciprofloxacin in commensal isolates was compared to enrofloxacin resistance in clinical isolates. For the folate pathway inhibitors trimethoprim and sulfamethoxazole, the proportion of isolates resistant to both of these antimicrobials in commensal *E. coli* was compared to resistance to the combination trimethoprim/sulfamethoxazole in the clinical *E. coli* isolates. Susceptibility testing panels and concentrations ranges used in the two monitoring programmes are summarized in Supplementary Table S1. MIC data from both Dutch monitoring programmes were available from 2014 to 2019, for commensal *E. coli* isolates in MARAN (N = 1992) and clinical *E. coli* isolates in GD (N = 1253). The terms 'commensal' and 'clinical' are used in the rest of the paper to indicate the isolates from the two monitoring programmes.

2.3. Breakpoints

Table 1 presents the breakpoints used to calculate resistant proportions of the two populations. To determine proportions of NWT susceptibility in commensal indicator *E. coli,* internationally standardized epidemiological cut-off values (ECOFFs) were used (EUCAST, 2019). These were compared with proportions of clinical resistance determined with clinical breakpoints (CBP) used in clinical *E. coli* (EUCAST, 2020; Clinical and Laboratory Standards Institute, 2020).

Table 1
Breakpoints used to determine non-wildtype susceptibility (NWT) in commensal *E. coli* (n = 1992) and resistance (R) in clinical *E. coli* (n = 1253) [a].

Antimicrobial	Testing range commensal *E. coli* MARAN (mg/L)	Testing range clinical *E. coli* GD (mg/L)	ECOFF (mg/L)	Clinical Breakpoint EUCAST (mg/L)	Clinical breakpoint CLSI (mg/L)
			NWT (>)	**R (>)**	**R (>)**
Ampicillin	1–64	0.25–32	8	***8*** [a]	–
Gentamicin	0.5–32	2–8	2	***2***	–
Cefotaxime	0.25–4	1–4	0.25	***2***	–
Tetracycline	2–64	0.25–16	***8***	–	–
Colistin	1–16	0.5–16	2	***2***	–
Trimethoprim	0.25–32	0.5–16	4	***4***	–
Sulfamethoxazole	8–1024	64–256	64	–	–
Ciprofloxacin (commensal) and enrofloxacin (clinical)	0.015–8	0.25–2 *	0.064	0.5 (ciprofloxacin)	***1 (enrofloxacin)***

[a] Breakpoints in bold italic show criteria used for determining resistance in the clinical *E. coli* isolates

Fig. 1. Proportions with 95% confidence intervals of antimicrobial resistance in commensal *E. coli* from healthy broilers at slaughter (orange, dots) versus in clinical *E. coli* from diseased broilers (blue, triangles) for ampicillin, gentamicin, cefotaxime, tetracycline, colistin and trimethoprim/sulfonamide, the Netherlands, 2014–2019.

ECOFFs and CBPs defined by EUCAST were used, wherever available. For fluoroquinolones, the CBP for ciprofloxacin was applied in commensal *E. coli* in addition to the ECOFF to show the difference in resistant proportion between NWT susceptibility and clinical resistance for that specific example. For enrofloxacin no EUCAST CBP was available, hence a CLSI breakpoint for poultry was used (Clinical and Laboratory Standards Institute, 2020). For tetracycline the ECOFF was used for the clinical isolates in absence of a CBP (Table 1).

2.4. Statistical analysis

Trends were evaluated by plotting the observed resistant proportions with 95% confidence intervals (CI) of the two monitoring datasets, as well as with Poisson regression models. Using the MIC data and selected breakpoints, yearly resistant isolate proportions were calculated for each antimicrobial, and exact 95% CIs were calculated, using yearly resistant proportions and the total numbers of isolates tested (N). All statistical analyses were performed in R version 3.3.3 (R Foundation, Vienna, Austria) (R Core Team, 2017). Regression models were selected by comparison of lowest values for Akaike's Information Criterion (AIC), model fit was assessed by the scaled deviance.

In the analysis, a generalized linear multivariable model was used with Poisson distribution and a log link function (Poisson regression) for yearly resistance counts (n), with the log of the total number of strains per year (N) as offset. Two explanatory variables were included in the model: the first was a numeric variable for the years one to six (2014–2019), the second was a binary variable for the monitoring programme (0 for commensal *E. coli*, 1 for clinical *E. coli*). By using these explanatory variables in the model, time trends for both monitoring datasets were determined and quantified by the incidence rate ratio (IRR). Next to that, the model indicated whether the mean level of resistance differed between the two monitoring programmes.

3. Results

In this study, resistant counts were modelled for commensal *E. coli* from healthy broilers and for clinical *E. coli* isolated from diseased broilers. An overview of resistant counts and totals per year of both datasets is presented in Supplementary Table S2. MIC distributions showed that there were no methodological differences as a potential hurdle for analytical comparison (Figs. S1-S7). Observed resistant proportions of the commensal *E. coli* and clinical *E. coli* were similar with overlapping CI for many of the time points for ampicillin, gentamicin, cefotaxime, tetracycline, colistin and trimethoprim/sulfonamide (Fig. 1).

Model results for ampicillin resistance showed a decrease over time in the commensal *E. coli*: the IRR per year was 0.91 (CI 0.87–0.94) (Table 2). In contrast, clinical *E. coli* showed stable resistant proportions over time (Fig. 1-A) with no trend observed (Table 2). The means of ampicillin resistance were similar in both datasets (Table 2), also reflected by overlapping CI for most of the observed resistant proportions (Fig. 1-A).

Gentamicin resistance prevalence was low in both commensal and clinical *E. coli* (Fig. 1-B, Supplementary Table S2). In both programmes, no time trends were observed, and the means of the data did not differ (Table 2).

A decrease over time of already low prevalent cefotaxime resistance was observed in both commensal and clinical *E. coli* (Fig. 1-C, Table 2). However, the mean cefotaxime resistance in the clinical *E. coli* was estimated to be higher than in commensal *E. coli*, indicated by the IRR of 1.94 (CI 1.21–3.11, clinical relative to commensal *E. coli*, Table 2).

Resistance to tetracycline decreased over time in commensal *E. coli* but fluctuated in clinical *E. coli* (Table 2, Fig. 1-D). The clinical data had a slightly higher mean (IRR 1.14, CI 1.01–1.29, relative to commensal *E. coli*, Table 2).

Colistin resistance was not detected in commensal indicator *E. coli* but few resistant isolates were detected in clinical *E. coli* (Fig. 1-E, Supplementary table S2). The model estimated a decrease over time for the clinical data (IRR 0.67, CI 0.50–0.82, Table 2).

Findings for trimethoprim/sulfonamide resistance were similar to those for ampicillin. In commensal *E. coli* a decrease over time was detected, but not in clinical *E. coli*, and the means of both datasets were not different (Table 2). This was also observed in overlapping CI for resistant proportions per year (Fig. 1-F).

For fluoroquinolones, application of the EUCAST CBP instead of the ECOFF resulted in lower ciprofloxacin resistant proportions in commensal *E. coli*, which were comparable to enrofloxacin in clinical *E. coli* (Table 2, Fig. 2). For these related antimicrobials, a decrease of resistance over time was observed for ciprofloxacin in the commensal *E. coli*, but not for enrofloxacin in clinical *E. coli* isolates.

4. Discussion

This study compared antimicrobial susceptibility testing (AST) from an active surveillance component in commensal *E. coli* from healthy broiler chickens with a passive surveillance component in clinical *E. coli* from diseased broiler chickens. The results provide insight in the association between AST results in these bacterial populations, as well as an overview in the differences and similarities of both surveillance components, thereby improving the interpretation of AMR monitoring outcomes.

The methodology is different in the two monitoring systems as they

Table 2
Poisson regression estimates for time trends and difference between the mean prevalence of antimicrobial resistance in commensal *E. coli* (n = 1992) from healthy broilers at slaughter versus in clinical *E. coli* (n = 1253) from diseased broilers, the Netherlands, 2014–2019.

Antimicrobial	Time trend commensal *E. coli*		Time trend clinical *E. coli*		Difference in resistant proportions (commensal versus clinical *E. coli*)	
	Incidence rate ratio (95% CI)	P value[a]	Incidence rate ratio (95% CI)	P value	Incidence rate ratio (95% CI)	P value[b]
Ampicillin	**0.91 (0.87–0.94)**	**0.00**	1.02 (0.97–1.08)	0.39	0.98 (0.89–1.09)	0.72
Gentamicin	0.98 (0.88–1.10)	0.75	0.88 (0.74–1.04)	0.13	1.07 (0.78–1.45)	0.69
Cefotaxime	**0.68 (0.53–0.86)**	**0.00**	**0.65 (0.50–0.82)**	**0.00**	**1.94 (1.21–3.11)**	**0.01**
Tetracycline	**0.91 (0.88–0.95)**	**0.00**	0.98 (0.92–1.04)	0.53	**1.14 (1.01–1.29)**	**0.03**
Colistin	–	–	**0.67 (0.50–0.82)**	**0.04**	–	–
Trimethoprim/sulfonamide	**0.89 (0.85–0.93)**	**0.00**	0.99 (0.93–1.06)	0.79	0.96 (0.84–1.09)	0.50
Ciprofloxacin[c] and enrofloxacin	**0.89 (0.80–0.98)**	**0.02**	1.01 (0.88–1.16)	0.90	1.20 (0.92–1.57)	0.17

[a] Values in bold indicate significant time trends (the P value is <0.05)
[b] Values in bold indicate a significant difference in the mean resistance prevalence (the P value is <0.05)
[c] The EUCAST clinical breakpoint for ciprofloxacin (0.5 mg/L) was used for commensal *E. coli* to determine resistance and compare time trends, using the CLSI clinical breakpoint for enrofloxacin resistance (1.0 mg/L) in clinical *E. coli*

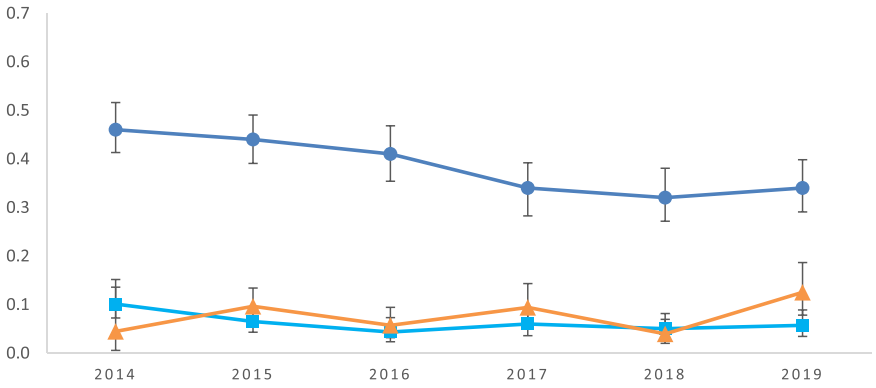

Fig. 2. Proportions with 95% confidence intervals of antimicrobial resistance in commensal *E. coli,* calculated with the EUCAST clinical breakpoint for ciprofloxacin of 0.5 mg/L (light blue, squares), and calculated with the epidemiological cut-off value of 0.064 mg/L (dark blue, dots), and for clinical *E. coli* calculated with the CLSI clinical breakpoint for enrofloxacin of 1.0 mg/L (orange, triangles), isolated from broilers, the Netherlands 2014–2019.

have different aims. To begin with, the sampling strategies differ. For the commensal isolates, a stratified random sample (active surveillance) from healthy animals at slaughter versus a selected sample from diseased animals (passive surveillance) from the same broiler population. The genetic background of the sampled *E. coli* populations is unknown and possible relatedness was not determined. Despite the differences in the monitoring, mean resistant proportions are similar for most antimicrobials in this study.

The test panel for monitoring in commensal *E. coli* includes antimicrobials relevant to human medicine, with wide concentration ranges to determine NWT susceptibility. The panel for monitoring in clinical *E. coli* consists of antimicrobials relevant for veterinary use in livestock species, with narrow ranges to determine clinical resistance.

The breakpoints used also differ between the two monitoring systems. In the active surveillance in commensal indicator *E. coli*, ECOFFs are used to early detect evolution of NWT susceptibility in gut bacteria (Frimodt-Moller, 2004). In the passive surveillance in clinical *E. coli* isolates, CBP are used, which are generally higher, and indicate clinical resistance for treatment strategy in veterinary practice. ECOFFs are internationally standardized (EUCAST, 2019), whereas standardized CBP are not available for all veterinary antimicrobials (Toutain et al., 2017). This did not harm our analysis, since the MIC distributions showed that the available internationally standardized CBP could be applied to the MIC of the clinical isolates. For fluoroquinolones and tetracyclines, standardized EUCAST veterinary CBP were absent, this was solved by using the CLSI CBP and ECOFF for the clinical isolates for these classes, respectively.

4.1. Similarities in AMR proportions

The mean resistant proportions were similar for the majority of the antimicrobials. Except for cefotaxime and tetracycline, for which the mean resistant proportion was higher in clinical isolates. Mesa-Varona et al. also found higher resistance to cefotaxime in clinical compared to commensal *E. coli* in German broilers 2014–2017 (Mesa-Varona et al., 2020). Contrarily to expectations, Mesa-Varona et al. (2021) found for ampicillin and tetracycline lower resistance levels for clinical isolates compared to non-clinical isolates in France and Germany. This does not correspond to our findings. This may be due to differences in sampling of clinical versus non-clinical isolates, or differences in antimicrobial use between countries (Joosten et al., 2019; Sarrazin et al., 2019).

Monitored resistant proportions are influenced by the breakpoints used (Schwarz et al., 2010). Especially when there is a gap between the CBP and the ECOFF, as shown in the example of fluoroquinolones. The ECOFF (0.064 mg/L) aims at detecting NWT susceptibility to monitor acquired resistance, these strains do not have to be clinically resistant. When the CBP for ciprofloxacin (0.5 mg/L) is applied to commensal *E. coli*, the resistant proportion is similar to the proportion for enrofloxacin in clinical *E. coli* (Fig. 2). Therefore, data from different AMR monitoring programmes has to be interpreted with caution. To enhance standardization of AMR monitoring, it is worth considering including both the ECOFF and the CBP in the concentration range of the susceptibility panel, for a complete view on the AMR situation.

4.2. AMR trend analysis

In the active surveillance in commensal *E. coli,* decreasing trends in time are detected for the majority of antimicrobials. Since 2009, as a result of antimicrobial use interventions, resistant proportions in Dutch animals have decreased for many antimicrobial classes (Dorado-Garcia et al., 2016; Netherlands Veterinary Medicines Institute SDa, 2021; Veldman et al., 2020) as was also observed in commensal *E. coli* in the present study.

In contrast, the observed resistant proportions in clinical isolates fluctuate more and no time trends are statistically significant for the majority of antimicrobials. However, in passive surveillance in clinical isolates resistant *E. coli* were detected for two low prevalent and relevant antimicrobials: colistin and cefotaxime. Colistin resistance decreased over time in the clinical *E. coli* and was not detected in commensal *E. coli.* The decrease over time for colistin resistance in the clinical isolates should be interpreted with care, since it concerns a limited number of resistant isolates (Supplementary table S2), and selection bias cannot be excluded. For cefotaxime decreasing trends were observed in both commensal and clinical *E. coli.* These decreasing trends correspond with a low or absent use of 3rd generation cephalosporins in broilers between 2014 and 2019 (Netherlands Veterinary Medicines Institute SDa, 2021; Veldman et al., 2020).

Apparently, the stratified sample of commensal *E. coli* isolated from healthy animals at slaughter is more suitable to monitor time trends in AMR in this animal population. Randomization of a sample in active surveillance helps to detect trends (Bisdorff et al., 2017). The selected sample of clinical isolates can be considered risk-based. Risk-based surveillance results in a higher chance to detect low prevalent incidents (Alban et al., 2016). Since the clinical *E. coli* data have shown more variation, more data from consecutive years would be needed to quantify the association with commensal *E. coli* over time. Especially, because the observed decrease over time was small. In conclusion, both active surveillance in commensal *E. coli* from healthy broilers and passive surveillance in clinical *E. coli* from diseased broilers have value for AMR monitoring. This study can be of aid in a joint interpretation of the monitoring outcomes.

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Declarations of interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2022.105663.

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