Risk factors for ceftiofur resistance in *Escherichia coli* from Belgian broilers

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SUMMARY

A cross-sectional study on 32 different Belgian broiler farms was performed in 2007 and 2008 to identify risk factors for ceftiofur resistance in *Escherichia coli*. On each farm, one *E. coli* colony was isolated from 30 random birds. Following susceptibility testing of 14 antimicrobials, an on-farm questionnaire was used to obtain information on risk factors. Using a multilevel logistic regression model two factors were identified at the animal level: resistance to amoxicillin and to trimethoprim–sulfonamide. On the farm level, besides antimicrobial use, seven management factors were found to be associated with the occurrence of ceftiofur resistance in *E. coli* from broilers: poor hygienic condition of the medicinal treatment reservoir, no acidification of drinking water, more than three feed changes during the production cycle, hatchery of origin, breed, litter material used, and treatment with amoxicillin. This study confirms that not only on-farm antimicrobial therapy, but also management- and hatchery-related factors influence the occurrence of antimicrobial resistance.

Key words: Antimicrobial drugs, antimicrobial resistance in agricultural settings, emerging infections, *Escherichia coli*.

INTRODUCTION

Ceftiofur is a third-generation cephalosporin antibiotic which is solely used in veterinary medicine and currently only registered for swine and cattle in the European Union. Up to 2000, ceftiofur was also authorized in Belgium as a subcutaneously injectable antimicrobial in 1-day-old chicks. This was subsequently banned because no maximum residue level (MRL) was established for this target animal species. No single compound of the cephalosporin group is currently registered for use in poultry in the European Union [1].

In a large-scale Belgian survey in 2007–2008 on faecal *E. coli* from broiler chickens, a remarkably high average level of 37% ceftiofur resistance was found [2]. Comparison with older, smaller-scale

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Belgian studies in *E. coli* from poultry suggests a gradual but substantial increase in resistance towards this important antimicrobial compound. The reported ceftiofur resistance percentages were 6% and 28% in the period 2001–2003 [3] and 2006 [4], respectively.

As a consequence of worldwide reports of high and increasing levels of cephalosporin resistance in both veterinary [5–7] and human [8–11] medicine, the World Health Organization advisory panel ranked third- and fourth-generation cephalosporins in the top three of most important antimicrobials for treatment in humans. This prioritizes this subgroup of β-lactam antimicrobials as a class for which risk-management strategies are most urgently needed [12].

The public health concern of cephalosporin resistance in commensal *E. coli* from animals lies in the fact that this resistance might be transferred to animal-associated pathogenic bacteria [13] or to human commensal and/or pathogenic strains via the food chain or through direct contact [14–16]. Commensal bacteria play a crucial role in the acquisition and transfer of resistance genes because of their high reproduction capacity and large population [17]. Pathogenic *E. coli* strains are still one of the most common causes of bacterial infection in humans [18, 19].

To stop emerging – and ultimately reduce – resistance levels it is first necessary to identify the factors that influence the presence of this resistance. Therefore the aim of this study was to identify factors, both at animal and flock level that may influence the occurrence of ceftiofur resistance in faecal *E. coli* in Belgian broilers.

MATERIAL AND METHODS

Thirty-two broiler farms were selected randomly from all commercial Belgian broiler farms with a minimum capacity of 10 000 chickens. All farms were sampled twice leaving one production round in between unsampled. The visits all took place during 2007–2008 and each visit coincided when the birds were in their fifth week of production, i.e. the week prior to slaughter. At each sampling, individual faecal swabs from 30 randomly selected broiler chickens were collected, as well as one questionnaire for each visited farm. In the first five flocks, more samples were taken (89–100) for the additional purpose of later detection of extended spectrum β-lactamases (ESBLs).

For the isolation of *E. coli*, the swabs were cultured on MacConkey agar plates (Oxoid, France) and incubated aerobically for 24 h at 37 °C. From each sample, one random colony matching the morphology consistent with *E. coli* was purified on MacConkey agar. Suspected *E. coli* colonies were confirmed by means of positive glucose/lactose fermentation, gas production, and absence of H_2S production on Kligler iron agar (Oxoid), indole production (Indole spot on; Becton Dickinson, USA) and absence of aesculine hydrolysis (Bile aesculin agar; Oxoid). The Kirby–Bauer disk diffusion method was used for susceptibility testing (NeoSensitabs, Rosco, Denmark) of 14 antimicrobials (amoxicillin–clavulanic acid, ampicillin, apramycin, ceftiofur, chloramphenicol, enrofloxacin, florphenicol, flumequine, gentamicin, neomycin, nalidixic acid, streptomycin, tetracycline, trimethoprim–sulfonamide). The guidelines of the Clinical Laboratory Standards Institute (CLSI) were followed for standardization of inoculums, incubation conditions and internal quality control organisms (CLSI M31-A3). After 18 h of incubation, inhibition zones were read and interpreted according to the veterinary manufacturer's guidelines according to CLSI [2]. Susceptibility test results were converted to a binary outcome: sensitive vs. non-sensitive (resistant + intermediate) [2]. The susceptibility test results (sensitive or resistant) of the 13 antimicrobials excluding ceftiofur were tested at the bacterium level as potential covariables for ceftiofur resistance.

The samples obtained in the first five flocks were additionally plated on MacConkey plates enriched with 8 μg/ml ceftiofur. Isolates growing on this medium were further examined for the presence of ESBLs [20].

The questionnaire was completed on-farm by means of a personal interview with the farmer. Hygiene scores (three categories: visibly clean, some contamination, dirty) were awarded by the interviewer without the farmer's involvement. The same questionnaire was used for all farms and on both sampling occasions, the interviewer was also the same on all occasions. Information was gathered on 31 different potential farm-level risk factors: on-farm presence of other animals, pets, rodents, season, type of drinking water, quality checks of drinking water, use of disposable clothing for visitors, use of footbaths at entrance of stable and hygienic condition, hygienic condition of the stable, cleaning procedure, temperature of cleaning, disinfection procedure, sanitary transition period, application system for medicines and hygienic condition of the reservoir for medication application, rinsing of the reservoir after

treatment, acidification of drinking water, feed mill, feed changes, use of anticoccidials, stocking density, hatchery, breed, litter material, humidity of the litter, temperature regimen, depopulation regimen, *Salmonella* status, mortality, and treatments applied.

A multilevel logistic regression model with ceftiofur resistance as a binomial outcome variable was fit to the data. All covariables and all 31 factors were tested univariably. The shape of the relationship with the outcome variable was assessed for all continuous variables by plotting the log odds of the outcome *vs.* the continuous variable [21]. If there was a non-linear relationship, the continuous variable was categorized. The variables with a P value of $\leqslant 0.2$ (odds ratio different from 1) were withheld as input in the multivariable multilevel logistic regression model. Pearson and Spearman correlation coefficients were calculated to explore the relationship between all selected independent variables. If correlation between two variables was >0.6, only the most significant variable was retained in the model. The model was built in MLW in (University of Bristol, Bristol, UK), with the factor 'farm' included as a random effect to account for the repeated observations per farm. The model was built backwards, gradually excluding the non-significant factors to finally only retain significant factors. To check for the presence of confounding, a Mantel–Haenszel analysis was performed. Confounding was considered present if changes in the odds ratio of $>20\%$ could be observed. Interaction effects were checked for all significant main factors in the model.

RESULTS

E. coli was recovered in 92·3 % of all samples, resulting in 2076 isolates. The overall prevalence of ceftiofur resistance found in *E. coli* was 34 % in the first sampling of the farms and 42 % at the second sampling. A large between-farm variation was seen; at the first sampling round, farm levels of ceftiofur resistance ranged between 8 % and 62 %, and for the second round between 9 % and 73 %. The isolation on ceftiofur-supplemented plates for the samples from flocks 1–5 resulted in a growth percentage of 63 %. The results of the detection of ESBLs have been published in Smet *et al.* [20].

In the univariable analysis of bacterium-level co-variables, antimicrobial resistance against four agents was seen to be significantly associated with ceftiofur resistance: resistance to amoxicillin–clavulanic acid ($P<0.01$), to nalidixic acid ($P=0.12$), to neomycin ($P<0.01$), and to trimethoprim–sulfomethoxazole ($P<0.01$). On the farm level, 14 risk factors were withheld following univariable analysis: drinking-water quality checking interval ($P<0.01$), absence of footbaths at the entrance of the stable ($P<0.01$), hygienic condition of the stable ($P<0.01$), hygienic condition of the medicinal treatment reservoir ($P<0.01$), no drinking water acidification ($P<0.05$), more than three feed changes per production cycle ($P<0.01$), anticoccidial drug administered ($P<0.01$), stocking density ($P=0.18$), hatchery ($P<0.01$), breed ($P<0.05$), litter material ($P=0.06$), applying two phased depopulation regimens ($P=0.16$), amoxicillin treatment ($P<0.01$) and enrofloxacin treatment ($P<0.01$). Checking of the correlations showed that no relevant Pearson or Spearman correlation coefficients were present in these variables.

In the multilevel multivariable model, with farm included as a random effect, the factors presented in Table 1 remained significantly associated with ceftiofur resistance.

No significant confounding was found to be present and no interaction effects were found to be significant for the variables in the model.

DISCUSSION

Although since 2000 onwards, ceftiofur has been withdrawn from use in poultry because of the lack of establishment of MRLs [1] a large increase in resistance has been observed in broilers in recent years [2]. In the present study, high ceftiofur resistance prevalences in broiler *E. coli* could be observed, with large between-farm variations. Selective plating showed that in 63 % of the samples from the first five flocks, ceftiofur-resistant *E. coli* were present, indicating vast spread of ESBLs in Belgian broilers. This is in contrast with the evolution of ceftiofur resistance in pigs and cattle where the use of ceftiofur is still permitted and where no important increases in resistance have been observed [4]. These species are also less exposed to mass medication than broilers, with the exception of finishing pigs and veal calves. If cephalosporin resistance in broilers keeps increasing, the use of cephalosporins in veterinary and human medicine is likely to become heavily jeopardized [22]. In a recent publication, a very high correlation ($r = 0.9$, $P<0.01$) was found between ceftiofur-resistant *Salmonella enterica* serovar Heidelberg isolated from retail chicken and the incidence of ceftiofur-resistant

Table 1. *Results of the multilevel multivariable analysis of covariables and risk factors for ceftiofur resistance in 32 Belgian broiler farms*

Factor	Category	Frequency of occurrence	OR	95 % CI	*P* value
Bacterium-level covariables					
AMC susceptibility test result	Sensitive	87·7	Ref.		
	Resistant	12·3	7·74	3·00–19·94	<0·01
TMP-S susceptibility test result	Sensitive	43·8	Ref.		
	Resistant	56·2	1·95	1·26–3·03	<0·01
Farm-level risk factors					
Clean hygienic condition of the	No	78·6	Ref.		
treatment reservoir	Yes	21·4	5·18	1·55–17·29	<0·01
Acidification of drinking water	Yes	18·1	Ref.		
	No	81·9	3·47	1·05–11·50	<0·05
>3 feed changes/cycle	No	20·0	Ref.		
	Yes	80·0	8·25	1·39–48·80	<0·01
Hatchery	A	23·8	Ref.		
	B	44·0	15·60	0·82–297·33	0·08
	C	8·4	14·79	2·27–96·33	<0·01
	D	8·8	50·60	5·55–461·68	<0·01
	E	10·6	1·02	0·17–6·03	0·09
	F	1·4	2·40	0·29–19·88	0·35
	G	3·0	655·89	50·12–8582·84	<0·01
Breed	Cobb	12·2	Ref.		
	Ross	87·8	9·14	2·30–36·41	<0·01
Litter material	Wood curls	47·5	Ref.		
	Straw	25·7	5·08	1·76–14·63	<0·01
	Flax	26·8	8·04	2·00–32·41	<0·01
Amoxicillin treatment	No	58·4	Ref.		
	Yes	41·6	4·76	2·16–10·50	<0·01

OR, Odds ratio; CI, confidence interval; AMC, amoxicillin-clavulanic acid; TMP-S, trimethoprim–sulfonamide.

Salmonella enterica serovar Heidelberg infections in humans [23]. This illustrates the importance for public health of ceftiofur resistance in the broiler ecosystem.

The results of the current study indicate that many factors are associated with ceftiofur resistance in faecal *E. coli*. Some are biologically explainable whereas others are unexpected and more difficult to interpret. The single most expected risk factor, namely the use of ceftiofur, is not present due to the absence of any record of ceftiofur use in Belgian broilers as a result of the lack of a MRL for ceftiofur in poultry since 2000. However, possible off-label use cannot be ruled out. In Canada, increases or decreases in ceftiofur resistance in both retail chicken *E. coli* and *Salmonella* isolates were found to follow a similar trend to the use of ceftiofur in hatcheries [23].

The observation in our study that hatchery of origin has a large significant effect on ceftiofur resistance level suggests that hatchery-related factors influence the occurrence of ceftiofur resistance. This might be a sequel to the historically permitted use of ceftiofur, often applied in combination with vaccination in newly hatched chicks and still having an effect due to the persistence of resistance, or due to a (continued) off-label use of ceftiofur in young chicks. Further research should be conducted to elucidate this relationship; however, the strong relationship between the use of ceftiofur in hatcheries and changing levels of ceftiofur resistance in both *Salmonella* and *E. coli* in the Canadian study allows us to rely more on the second hypothesis [23]. Mentions of unnecessary or off-label use of ceftiofur in the poultry industry occur worldwide and are linked to cephalosporin resistance [24, 25].

Moreover, the use of other antimicrobials might, through cross- or co-resistance, act as covariables and select for ceftiofur resistance. In the current study this was observed for the use of amoxicillin and resistance against amoxicillin–clavulanic acid. This is in line with molecularly confirmed cross-resistance across these compounds, i.e. amoxicillin and amoxicillin–clavulanic acid may select for genes that

confer resistance to extended spectrum cephalo-sporins like the ESBLs, CTX-M or TEM, and AmpC β-lactamases like CMY-2 [20]. The study of Smet et al. [20] indicated the presence of these genes in ceftiofur-resistant E. coli originating from the same samples used in the present study. Resistance to trimethoprim–sulfonamide was also significantly as-sociated with ceftiofur resistance, probably through genetic linkage between trimethoprim–sulfonamide and cephalosporin resistance determinants as has already been observed in Enterobacteriaceae. Sal-monella enterica serovar Virchow, can harbour dfrA1 (encoding trimethoprim resistance) and sul1 encoding sulphonamide resistance) that are physically linked to CTX-M-2 [1].

Besides amoxicillin treatment and hatchery of origin, other flock-level management factors also proved to be significant risk factors. In disease con-trol, hygiene and sanitation are very important and modifiable assets to prevent disease introduction and spread in a herd or flock. With regard to resistance, an inverse effect seems to be present. As an example in this study it was found that a clean treatment reser-voir is a risk factor for ceftiofur resistance. This might be the result of a dilution effect by susceptible bacteria due to a soiled ('dirty') environment, resulting in a more diverse intestinal microbiota. Comparable re-sults were found in tetracycline-resistant lactose-positive coliforms originating from fattening pigs in Belgium [26], where better sanitation measures were also identified as adding to the risk.

Acidification of drinking water also had a con-siderable effect on the intestinal flora. Acidifying drinking water induces a shift in intestinal flora be-cause of the low acid resistance of Enterobacteriaceae. This will have a general effect on the E. coli popu-lation [27, 28]. Our results suggest that the ceftiofur-resistant subpopulation is more sensitive to the acid, resulting in a larger adverse effect on that sub-population thereby reducing the chance of isolation. This might be the consequence of the possible loss of vitality of bacteria that often goes together with acquiring drug resistance [29]. Changing the feed more than three times during the production cycle also affected the level of ceftiofur resistance. Feed changes inevitably cause stress for chickens, and can cause an increase in the prevalence of resistant bac-teria, as stress is a factor that has been reported as an increasing factor for the prevalence of resistant bacteria in pigs, not linked to antimicrobial use [30]. Moreover, increased prevalences of antimicrobial

resistance linked to changes in the microbial popu-lation may be caused by the occurrence of other stress-induced genes that possibly occupy the same genetic elements of the bacteria as those that harbour resistance determinants [31].

The litter material on which the broilers were kept was also identified as a risk factor. Compared to wood curls, straw and flax unfavourably affected the level of ceftiofur resistance. Litter material has been described as influencing the composition of gut flora. Aktan & Sagdic [32] observed that the litter material used can indeed affect the composition of the microbiota, as did Torok et al. [33], while Fries et al. [34] were not convinced of this finding and conversely allocated no effect of litter material to gut composition. How this would differently affect the resistant vs. non-resistant subpopulations of the same genera is not clear, but different litters may provide different bacterial growth conditions, e.g. different pH or humidity. According to our study this may also influence the magnitude of the ceftiofur-resistant E. coli population. A similar effect is seen for breed, e.g. where the Cobb breed would be favourable for the acquisition of ceftiofur resistance. This again is not readily explainable. Mentions of antimicrobial resistance being dependent on a species' breed, with comparable management, have, to our knowledge, not been made. This would then again have to be linked to different colonization conditions imposed by the breed, leading to different compositions of microbiota, which can also effect the resistant population, or it could be related to origin of the birds, e.g. parent of grandparent lines. Since no definite link between the latter two factors and the occurrence of antimicrobial resistance has been estab-lished before, these factors should be further studied in order to detect the mechanism by which they affect ceftiofur resistance in E. coli. However, it should be borne in mind that in observational studies one can never fully exclude the possibility of type I errors.

It is not yet fully understood by which mechanism all identified risk factors influence the acquisition of ceftiofur resistance in E. coli from broilers, and this warrants further research. Yet the results clearly indicate that a variety of factors are involved and that ceftiofur resistance is not solely attributable to the use or abuse of the antimicrobial or related com-pounds. Including these factors can also allow the control of any confounding that might exist between the association of antimicrobial use and resistance. Even though several of the working mechanisms are not yet fully understood, the observed increase in

resistance merits full attention. Since many factors are modifiable through management changes, broiler production should consider adaptations that avoid the aforementioned risk factors for ceftiofur resistance from both an animal and public health point of view.

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DECLARATION OF INTEREST

None.

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