OCCURRENCE OF B-LACTAM RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM THE BROILER PRODUCTION CHAIN

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Summary
In Italy, few studies have investigated the occurrence of β-lactamase producing *E. coli* in broilers. This paper aimed to determine the level of phenotypic β-lactam resistance, in particular to third-generation cephalosporins (3GCs), in commensal and clinical *E. coli* isolated from the whole broiler production pyramid. To this end, several samples were collected from three production chains (A, B, C), including breeders, growing broilers and carcasses; and they were analysed on both Eosin Methylene Blue agar (EMB) and EMB agar supplemented with 1mg/L cefotaxime (CTX-EMB).
A low occurrence of ESBL/AmpC-producing *E. coli* was found when the non-selective medium (EMB) was considered. In contrast, for 55.3% of samples, at least one ESBL/AmpC-producing commensal *E. coli* was isolated when the selective medium (CTX-EMB) was used. Resistant strains were present at all steps of the broiler production pyramid with a relative sample frequency of ESBL/AmpC-producing *E. coli* ranging from moderate (15%) to extremely-high ratios (85%). In contrast to commensal *E. coli*, only 6.6% of clinical strains were phenotypically resistant.
The high occurrence of ESBL/AmpC-producers is surprising considering that 3GCs have never been licenced for use in poultry in Italy. Nevertheless, the circulation of 3GC-resistant *E. coli* in all steps of the broiler production pyramid is worrying. Studying the genetic background of β-lactam resistance is crucial to elucidate the source and the transmission routes of ESBL/AmpC-producing *E. coli* along the broiler production chain.

INTRODUCTION

β-lactams are essential antimicrobials in the armament of poultry veterinarians. Narrow spectrum penicillins are the most common β-lactams used for the treatment of necrotic enteritis (Landoni & Albarellos, 2015). Amoxicillin and ampicillin are penicillins with a broader spectrum and are frequently employed in colibacillosis cases (Löhren *et al.*, 2009). Administration of extended-spectrum β-lactams is prohibited in poultry in Europe since 2012 (European Commission, 2015). In Italy, third- and fourth-generation cephalosporins have never been licensed for use in poultry (EFSA/ECDC, 2016). Bacterial resistance to both penicillins and cephalosporins in poultry has been reported from a multitude of studies globally (Ewers *et al.*, 2012; Olsen *et al.*, 2014). Recent publications have demonstrated that poultry could represent a reservoir of determinants for β-lactam resistance and that the risk of transmission to humans via the food chain is not negligible (Overdevest, 2011). Of particular concern are 3GCs, which are categorized as critically important antimicrobials for humans. For this reason, the levels of ESBL/AmpC resistance in food-producing animals are being monitored yearly

by the EU (EFSA/ECDC, 2018).
In Italy, few studies have focused on the occurrence of β-lactamase producing *E. coli* in the broiler industry and none has attempted to describe the problem with a comprehensive approach. In this paper, preliminary results of a monitoring aimed at determining the level of β-lactam resistance in commensal and pathogenic *E. coli* from the broiler production chain are presented.

MATERIALS AND METHODS

Sample collection

In total, three production chains (chains A, B and C) were monitored from January 2017 to January 2018. Each chain corresponded to a breeder flock and for each breeder flock, four broiler farms and their carcasses were sampled. From each production chain, the following samples were collected: a) cloacal swabs from broiler breeders at the age of one-day-old and during the laying period at approximately 30 weeks of age (20 swabs/flock/sampling); b) cloacal swabs from broilers in commercial farms at the age of one-day-old and before slaughter at the age of approximately 30 days (20 swabs/flock/sampling); c) tissue samples (air sacs, lungs, brain, liver, pericardium) from chickens showing pathological lesions suggestive of colibacillosis (at least 10 dead birds); d) broiler carcasses after chilling at the slaughterhouse (20 carcasses/flock).

Isolation, identification and detection of β-lactamase-producing E. coli

Samples from farms were directly streaked on Eosin Methylene Blue agar (EMB) and EMB agar supplemented with 1mg/L cefotaxime (CTX-EMB) and incubated at 37 °C for 24 h. Carcasses were analysed by both a qualitative and quantitative method. For the qualitative method, carcasses were rinsed with Buffer Peptone Water (BPW), rinsates were incubated (37 °C for 24 h) and streaked on EMB and CTX-EMB. For the quantitative method, rinsates and three serial dilutions (10^{-1} to 10^{-3}) were streaked on EMB and CTX-EMB. Tissue samples were streaked only on EMB. A minimum of five suspect *E. coli* colonies was isolated from each sample and subjected to species confirmation by a combination of biochemical tests and PCRs targeting genus-specific housekeeping genes (McDaniels *et al.,* 1998). At least three confirmed *E. coli* isolates (minimum 2 isolated from EMB and 1 isolated from CTX-EMB, if positive) were screened for ESBL/AmpC production by combination disk diffusion test using cefotaxime and ceftazidime discs, with and without clavulanic acid and according to CLSI guidelines (CLSI, 2018). Additionally, a cefoxitin disc (30 µg) was used to detect potential AmpC producers. Moreover, 62 strains (60 commensal and 2 clinical) isolated from chain B and showing phenotypic resistance or intermediate resistance to at least one cephalosporin were screened for narrow-spectrum β-lactam (ampicillin and amoxicillin/clavulanic acid) resistance according to CLSI guidelines (CLSI, 2018).

Estimation of relative sample frequency of ESBL/AmpC-producing E. coli

The relative sample frequency of ESBL/AmpC-producing *E. coli* was established by identifying a sample as positive if at least one ESBL/AmpC-producing *E. coli* was isolated from it and was calculated for both isolation media

RESULTS

Relative sample frequency of ESBL/AmpC-producing E. coli for CTX-EMB isolated strains

At the top of the production pyramid (one-day-old PS chickens), extremely-high levels of ESBL/AmpC-producing *E. coli* were found with 85% positive samples for chain A and all (100%) samples positive for chain C. Due to time limitations, one-day-old PS chickens of chain B were not sampled. During the laying period, percentages dropped significantly (15% for both chain A and C), whereas sample relative frequency for chain B breeders was 30%. For broilers sampled in commercial farms, ESBL/AmpC-producing *E. coli* were found in high percentages on average (Table 1). Conversely, extremely-high sample prevalence was found for chain C at the age of one-day, which dropped significantly before slaughter (Table 1). At the bottom of the production pyramid, the prevalence of ESBL/AmpC-producing *E. coli* in carcasses ranged from high to very high percentages (Table 1).

Relative sample frequency of ESBL/AmpC-producing E. coli for EMB isolated strains

Significantly fewer positive samples were found when strains isolated from EMB were considered, except for the one-day-old PS chickens of chain A (Table 1). Moreover, for 14 out of 42 sampled flocks, the relative sample frequency was 0% (data not shown). For example, samples from PS chickens of chain B and C during the laying period were found negative on EMB, whereas on CTX-EMB the percentage of positive samples was 30% and 15%, respectively. Overall, when both media resulted in at least one positive sample per flock, the percentage of positive samples was seven times lower for EMB, on average.

Prevalence of clinical ESBL/AmpC-producing E. coli

Out of 152 strains subjected to disk-diffusion test, 6.6% (n = 10) were found positive for ESBL (n = 5) and AmpC (n = 5) production. These isolates derived mainly from chain C (n = 7), breeders (n = 3) and broilers (n = 4).

E. coli resistant to narrow-spectrum β-lactams

Sixty-one out of 62 strains were found resistant to ampicillin with only one commensal *E. coli* being susceptible. In contrast, only two commensal *E. coli* strains showed resistance to the amoxicillin/clavulanic acid combination. Both clinical strains were resistant to ampicillin but susceptible to amoxicillin/clavulanic acid.

DISCUSSION

A substantial difference between the two isolation methods was found with regard to the estimation of relative sample frequency of ESBL/AmpC-producing *E. coli*. To note, the absence of 3GC-resistant *E. coli* would have been found for 14 flocks, if only EMB without the addition of cefotaxime had been used. This finding is in line with the results from the EFSA/ECDC report (2016). Analysis of samples with the CTX-EMB isolation method revealed an overall high prevalence (55.3%) of ESBL/AmpC-producing *E. coli* in the broiler production chain. ESBL/AmpC-producing isolates were present at all steps of the broiler production pyramid for all the examined production chains, a finding described also in similar studies in Eu-

rope (Dierikx *et al.*, 2013; Nilsson *et al.*, 2014). Strikingly, extremely-high prevalence was identified in day-old breeders, suggesting a contamination of chicks at the hatchery. The significant reduction of ESBL/AmpC-producers during the laying period supports this; however, additional information is needed to verify this hypothesis. Prevalence in broilers ranged from high (37.5%) to extremely-high (76.3%) percentages indicating a well-established presence of 3GC-resistant *E. coli* in commercial farms. At the bottom of the production pyramid, carcasses were found highly contaminated with resistant *E. coli*, representing a risk of transmission to humans via the food chain (Overdevest, 2011). The median level of *E. coli* load in carcasses was approximately 3 Log CFU/ml for each chain considering the EMB counts, while the median level of contamination detected on CTX-EMB was near to the limit of detection (1 Log CFU/ml). However, some samples showed a load of *E. coli* enumerated on EMB+CTX over 3.5-4 Log CFU/ml.

The overall high occurrence of commensal 3GC-resistant *E. coli* in the broiler production pyramid becomes difficult to explain considering the prohibition of 3GC use in poultry in Italy. One possible explanation could be the persistent presence of resistant *E. coli* in the poultry environment (e.g. water, dust, flies), which results in the gut colonisation of chickens at the farm (Blaak *et al.*, 2015). A more probable explanation is the selection pressure exerted by the use of narrow-spectrum β-lactams leading to co-selection of 3GC-resistance (Dierikx *et al.*, 2013; EFSA/ECDC, 2016). The occurrence of penicillin resistance among cephalosporin-resistant *E. coli* found in our study supports this hypothesis and suggests that these strains may carry plasmids that harbour multiple resistance determinants.

Dissemination of 3GC-resistance determinants among clinical *E. coli* strains was detected but resistance remained at low levels, which is in agreement with the study of Niero *et al.* (2018) in Italy, while other studies reported higher resistance rates (Chalmers *et al.*, 2017; Ozaki *et al.*, 2017). However, the use of non-selective media could have negatively influenced the isolation rate of 3GC-resistant *E. coli*. Nevertheless, these strains could represent a potential risk not only for poultry but for public health as well, since a link between pathogenic lineages of human and avian *E. coli* has been proposed (Manges, 2016).

CONCLUSION

This study demonstrated a high occurrence of 3GC-resistant commensal *E. coli* in the examined broiler production chains with extremely-high percentages at the top of the production pyramid. In contrast, dissemination of resistance determinants in clinical *E. coli* was low.

Phylo-typing of strains and genotyping of their resistance determinants is crucial to shed light on the transmission routes and reveal whether clonal (vertical) or horizontal transmission of ESBL/AmpC-producing *E. coli* occurs in the broiler production pyramid.

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Table 1. Relative sample frequency of ESBL/AmpC-producing *E. coli*

Production stage	Chain	% Relative frequency of isolation from EMB (range[1])	% Relative frequency of isolation from CTX-EMB (range)
PS chickens (1-day)	A	85	85
	B	N.D.[2]	N.D.
	C	55	100
PS chickens (30-weeks)	A	5	15
	B	0	30
	C	0	15
Broilers (1-day)	A	8.8 (0 - 20)	58.8 (35 - 75)
	B	2.5 (0 - 5)	57.5 (25 -95)
	C	23.8 (0 - 60)	76.3 (60 - 100)
Broilers (30-days)	A	3.8 (0 - 5)	37.5 (15 - 60)
	B	6.3 (0 - 20)	51.3 (35 - 95)
	C	3.8 (0 - 10)	40 (45 - 70)
Carcasses	A	5 (0 -10)	61.3 (40 - 75)
	B	6.3 (5 - 10)	36.3 (15 - 50)
	C	5 (0 - 15)	56.3 (45 - 70)

[1]The mean prevalence from four sampled flocks was calculated for broilers and carcasses.
[2]Not determined.

ELEVATA MORTALITÀ IN RIPRODUTTORI DI GALLINA FARAONA ASCRIVIBILE AD INTOSSICAZIONE DA ALOFUGINONE

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Summary
A high mortality event has been reported in a guinea fowl breeders premise in Northern Italy. Dead animals were necropsied in order to find out the aetiology of the phenomenon. Among the various findings, urate salt precipitates have been observed in kidney parenchyma later confirmed by the histopathological investigation. No pathogenic microorganisms were detected after bacteriological, virological, and parasitologic testing were performed. On the contrary, halofuginone traces were detected in the feed given to the animals, raising the hypothesis of a possible intoxication as the cause of the high mortality event on the farm.

INTRODUZIONE

Gli episodi di elevata mortalità costituiscono un importante problema sanitario e gestionale negli allevamenti avicoli. Le cause possono essere molteplici: di natura infettiva, di natura ambientale o di natura tossica. L'alofuginone è un analogo dell'alcaloide febrifugina, isolato per la prima volta dalla pianta *Dichroa febrifuga*[1], che viene comunemente utilizzato per il controllo della coccidiosi nell'industria avicola in tutto il mondo[2]. Sebbene esistano studi sulla tollerabilità della molecola nelle specie pollo e tacchino[3], sono purtroppo scarsi i dati riguardo la tolleranza di questa nella gallina faraona, nonostante sia comprovata la sua tossicità sia nella pernice[4] che nell'anatra[5].
Nel presente contributo si riporta un caso di elevata mortalità a eziologia non confermata in un allevamento di riproduttori di gallina faraona concomitante al rinvenimento di alofuginone nel mangime.

Descrizione del caso:
Nel Settembre del 2017 viene segnalato un evento di elevata mortalità (20% dei capi totali) presso un'azienda di riproduttori di gallina faraona, in cui erano presenti circa 15000 animali. L'anamnesi raccolta riferiva un improvviso calo dell'ovodeposizione concomitante ad una diminuzione dell'ingestione di alimento. I test per influenza aviare sono risultati essere negativi. Al fine di meglio comprendere le possibili cause della mortalità ed escludere, in particolare, eventuali forme infettive, si è proceduto con gli approfondimenti diagnostici del caso.

MATERIALI E METODI

Esame post-mortem e campionamento:
Cinque carcasse di animali adulti, di ambo i sessi, sono state conferite presso la sezione di Verona dell'Istituto Zooprofilattico delle Venezie (IZSVe) per essere sottoposte a indagine necroscopica e a eventuali successivi approfondimenti diagnostici al fine di

escludere la presenza di agenti eziologici responsabili di malattia infettiva. Sulla base delle notizie cliniche e delle lesioni rilevate in corso di necroscopia, da due carcasse sono state prelevate porzioni rappresentative del rene, dello stomaco muscolare, dell'ovidotto, fissate con formalina tamponata al 10% e inviate presso il laboratorio di diagnostica specialistica e istopatologia dell'IZSVe per le indagini istopatologiche del caso. Sono stati inoltre prelevati campioni da ovidotto, cervello e rene, in seguito sottoposti a esami batteriologici in sede. Dagli stessi organi sono stati prelevati campioni da inviare presso il laboratorio di virologia speciale e sperimentazione dell'IZSVe per eseguire indagini virologiche. Infine, il contenuto intestinale degli animali è stato utilizzato per eseguire esami parassitologici in sede.

Esami batteriologici:
Gli esami batteriologici sono stati effettuati seminando i campioni di organi in *Heart Infusion Broth* (HIB). Aliquote di HIB sono state successivamente seminate su agar sangue (AS), MacConkey agar (MCA) e *Bile-Esculin Agar* (BEA). Le piastre sono state incubate aerobicamente (AS e MCA) e in microaerofilia con CO_2 al 5% (AS e BEA) a 37 ± 1°C per poi essere esaminate dopo 24 e 48 ore d'incubazione. L'identificazione dei batteri cresciuti in piastra è stata effettuata sulla base delle caratteristiche morfologiche delle colonie, colorazione di Gram, test della catalasi e dell'ossidasi, e caratteristiche biochimiche.

Esame parassitologici:
Sono stati eseguiti esami coprologici qualitativi a fresco e mediante flottazione. Il primo esame prevede il posizionamento di una minima quantità di contenuto intestinale stemperato con una goccia d'acqua di fonte su di un vetrino portaoggetto, in seguito coperto da un vetrino coprioggetto. Il secondo esame prevede un passaggio preliminare di arricchimento mediante flottazione. Entrambi i tipi di preparazione sono stati osservati al microscopio utilizzando un obiettivo ad ingrandimento 20x.

RISULTATI
I soggetti conferiti presentavano un buono stato di nutrizione. All'esame esterno, gli animali mostravano imbrattamento pericloacale con materiale di colore verde-biancastro, di grado variabile nei vari soggetti. All'apertura della cavità celomatica è stata riscontrata involuzione dell'ovidotto in tutti i soggetti. I quadri patologici osservati nei reni degli animali erano variabili; in particolare due soggetti presentavano lesioni puntiformi biancastre riferibili ad urati a carico del solo parenchima renale; in altri due soggetti, a carico dei reni è stato possibile apprezzare aree iperemico/emorragiche frammiste ad aree di aspetto rosa pallido. Nell'ultimo animale è stato rilevato un pallore renale diffuso. In tutti i soggetti, ad eccezione dell'ultimo soggetto descritto, è stato possibile rilevare la presenza di urati sulla superficie degli organi interni. Rare lesioni emorragiche focali e non erosive, di forma lineare od ovalare, erano visibili sulla mucosa del ventriglio di due soggetti. In tutti i soggetti analizzati il contenuto dei ciechi era molto denso. E' stato riscontrato lieve pallore diffuso sul parenchima epatico in due soggetti insieme alla presenza di marcata neovascolarizzazione dei sacchi aerei addominali.
All'indagine istopatologica dei due campioni di tessuto renale conferito, è emersa fibrosi interstiziale con ispessimento della capsula glomerulare in presenza di focolai

multipli di congestione in un campione. In un secondo campione si sono apprezzati focolai multipli di necrosi dell'epitelio dei tubuli renali con aspetti di mineralizzazione degli stessi e presenza multifocale di cristalli aghiformi compatibili con urati.
Dagli esami batteriologici, virologici e parassitologici non è stata rilevata alcuna presenza di agenti patogeni che potessero giustificare tale quadro sintomatologico ed anatomopatologico.

DISCUSSIONE e CONCLUSIONI

Il quadro sintomatologico, congiuntamente ai riscontri patologici e alla negatività emersa dalle analisi effettuate, ha indirizzato il sospetto eziologico verso una causa di natura non infettiva. Un graduale miglioramento della situazione clinica, con arresto della mortalità, è stato ottenuto mediante somministrazione di una diversa partita di mangime. Il riscontro di tracce di alofuginone nell'alimento permette di ipotizzare un suo possibile ruolo nella patogenesi della *défaillance* renale acuta osservata in questi animali adulti. Dati gli esigui studi presenti in bibliografia, non si conoscono ancora il grado di tossicità e gli effetti avversi di questa molecola per la gallina faraona, sebbene, in soggetti giovani, sembri responsabile di una diminuzione dell'assunzione d'alimento e dell'accrescimento in assenza di mortalità[6]. Tuttavia, nel caso da noi descritto, si può ipotizzare che il particolare stato funzionale degli animali adulti (fase di riproduzione) abbia potuto influire sulla gravità dei risvolti clinici e patologici. Ci proponiamo, quindi, di approfondire il caso al fine di meglio chiarire il grado di tossicità di tale sostanza in questa categoria produttiva.

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