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Antimicrobial susceptibility of *Salmonella enterica* isolates from healthy breeder and broiler flocks in Portugal

Lurdes Clemente [a,*], Ivone Correia [a], Patrícia Themudo [a], Isabel Neto [b], Manuela Caniça [c,d], Fernando Bernardo [b]

[a] *INIAV – National Institute for Agrarian and Veterinary Research, Estrada de Benfica, 701, 1549-011 Lisboa, Portugal*
[b] *Faculty of Veterinary Science, Technical University of Lisbon, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal*
[c] *National Reference Laboratory of Antimicrobial Resistance, Department of Infectious Diseases, National Institute of Health Dr Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisbon, Portugal*
[d] *Centre for the Study of Animal Sciences (ICETA), University of Oporto, Rua D. Manuel II, Apartado 55412, 4051-401 Oporto, Portugal*

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ABSTRACT

Three hundred and thirty-three isolates representing 40 different serotypes of *Salmonella enterica*, recovered from environmental and faecal samples of breeder and broiler flocks from 2009 to 2011, were studied. Antimicrobial susceptibility was determined by measuring the minimal inhibitory concentration of 11 antimicrobials using the agar dilution method. *Salmonella* Havana, *S.* Enteritidis and *S.* Mbandaka were the most common serotypes isolated from broiler flocks, while *S.* Enteritidis was the common isolate from breeder flocks. The frequency of non-wild-type *Salmonella* isolates (those with decreased susceptibility to the different antimicrobials) varied according to serotype.

S. Mbandaka in broilers and *S.* Enteritidis in both breeders and broilers showed higher frequencies of reduced susceptibility to quinolones, but clinical resistance towards ciprofloxacin was not observed. Reduced susceptibility to sulfamethoxazole, tetracycline, ampicillin and streptomycin were common in *Salmonella* Typhimurium isolates. Two isolates of *S.* Havana from broilers were resistant to cefotaxime and phenotypically categorised as extended-spectrum β-lactamase producers. The results presented in this study provide useful data on the antimicrobial susceptibility of different *Salmonella* serotypes and highlight the high diversity of multi-drug resistance patterns present.

Introduction

Salmonella enterica is the second most important cause of foodborne disease in the European Union with a total of 99,020 confirmed cases in humans in 2010 (European Food Safety Authority, 2012a). Raw eggs are still the most frequent source of outbreaks, followed by fresh poultry meat, pork, fruit and vegetables. The continued increase in consumption of poultry products per capita[1] also increases the potential for human exposure to *Salmonella* via the food chain.

Salmonella enterica infection in humans usually results in a self-limiting gastroenteritis; however, young children, the elderly and immune-compromised people may experience enteric fever or an invasive form of the disease requiring antimicrobial treatment (Pui et al., 2011). In poultry, the clinical signs vary considerably depending on age of birds and/or infecting serotype. Infections caused by serotypes Enteritidis and Typhimurium are rarely responsible for severe illness and animals frequently become asymptomatic carriers except in young chicks and poults where acute outbreaks exhibiting clinical disease accompanied by high mortality rates may occur (Padron, 1990; Foley et al., 2008).

Salmonella can be introduced at all stages of the production cycle, though breeding flocks and hatcheries are critical sources and responsible for the quick spread of the infection (Foley et al., 2008). Several factors may affect the susceptibility of poultry to colonization, such as age, serotype, initial dose level, environmental stress, antimicrobial or anti-inflammatory treatments and competition with the enteric microbiota (Foley et al., 2008).

In addition to causing illness or death in both humans and poultry, there is a worldwide concern that the persistence of *Salmonella* serotypes that are resistant or show decreased susceptibility to several antimicrobials may reduce treatment options and, more importantly, lead to treatment failure (Newell et al., 2010). Fluoroquinolones such as ciprofloxacin are critically important antimicrobials in human and veterinary medicine. In animal isolates, the highest occurrence of decreased susceptibility to ciprofloxacin has been recorded in *Salmonella* spp. recovered from

* Corresponding author. Tel: +351217115343.
E-mail address: lurdes.clemente@iniav.pt (L. Clemente).

[1] See: http://www.thepoultrysite.com

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live chickens (*Gallus gallus*) and broiler meat (European Food Safety Authority, 2012a).

It was hypothesised that the withdrawal of growth promoters in Europe in 2006 would lead to decreased antimicrobial resistance in pathogenic microorganisms, but trade has resulted in the importation of poultry products from regions where the use of antimicrobials and growth promoters is not as well regulated as it is in the EU, resulting in the introduction of resistant organisms (Barrow et al., 2012).

We report here the results of a monitoring programme examining the antimicrobial susceptibility patterns of *Salmonella* serotypes isolated from breeder and broiler flocks in Portugal during 2009–2011. The ultimate aim of this programme is to contribute to a better understanding of the zoonotic potential of the circulating strains of *Salmonella* in a country where consumption of poultry meat is significant.

Materials and methods

Bacterial isolates

The National Veterinary Reference Laboratory (INIAV) received *Salmonella* isolates from the Salmonella National Control Programmes in food producing animals, and these were serotyped and susceptibility tested according to the guidelines of Commission Decision (CD) 2007/407/CE, concerning harmonised monitoring of antimicrobial resistance in *Salmonella* in poultry and pigs.[2] This analysis includes data from a total of 333 *Salmonella* isolates, from both breeders ($n = 58$) and broilers ($n = 275$). All samples were collected in the period of 2009–2011. The breeder and broiler farms were sampled and selected by the official authorities and were distributed throughout the country. The parent stock (breeders) had been imported from other European countries as day-old chicks while the broilers were born in Portugal. Birds were raised and managed in industrial units designed for a temperate climate. Faecal and environmental samples using sterile boots/sock swabs were collected in broiler flocks 3 weeks prior to slaughtering and, from breeder flocks three times during the production cycle.

All samples were examined according to ISO norm 6579: 2002 applied to *Salmonella* detection in food and animal feeding stuffs (Anon., 2002). Suspected colonies were further characterised by means of biochemical tests, using triple sugar iron agar slopes and API 20E strips (BioMérieux).

Salmonella serotyping

Salmonella isolates were biochemically confirmed and serotyped (Table 1), using the Kauffmann-White scheme (Grimont and Weill, 2007).

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) for 11 antimicrobials were determined by the agar dilution method (Clinical Laboratory and Standards Institute, 2008). Antimicrobials were tested in twofold concentration series over a range which was specific to each antibiotic: ampicillin (0.5–64 mg/L); cefotaxime (0.06–8 mg/L); chloramphenicol (2–256 mg/L); ciprofloxacin (0.008–8 mg/L); florfenicol (1–128 mg/L); gentamicin (0.25–32 mg/L); nalidixic acid (2–512 mg/L); streptomycin (2–512 mg/L); sulfamethoxazole (8–1024 mg/L); tetracycline (0.5–64 mg/L); and trimethoprim (0.25–32 mg/L). *E. coli* ATCC25922 strain was used as a control for MICs.

In order to assess decreased susceptibility of the isolates, epidemiological cut-off values from the European Committee for Antimicrobial Susceptibility Testing (EUCAST)[3] were used (Table 1) allowing the detection of any deviation in the susceptibility of the wild-type population (European Food Safety Authority, 2012b). MIC_{50} and MIC_{90} values, as well as rates of decreased susceptibility and resistance to critically important antimicrobials for humans (cefotaxime and ciprofloxacin), were calculated according to clinical breakpoints established by EUCAST for Enterobacteriaceae[4] (Table 1). Isolates were considered to be multi-drug resistant (MDR) if they presented reduced susceptibility to three or more structurally unrelated antimicrobials.

[2] See: http://eur-lex.europa.eu
[3] See: http://www.eucast.org
[4] See: http://www.eucast.org/clinical breakpoints

Phenotypic screening of extended-spectrum β-lactamases (ESBL)

Isolates exhibiting a non-wild-type MIC for cefotaxime (>0.5 mg/L) were tested phenotypically for the presence of ESBL by testing for synergy through disk combination (Mast Laboratories) including cefotaxime (30 μg), ceftazidime (30 μg) and cefpodoxime (10 μg), as single drugs and in combination with clavulanic acid (10 μg).

Statistical analysis

All statistical analyses were undertaken using SPSS v19.0 (IBM). The chi-square test was used to assess the association between *Salmonella* serotypes and antimicrobial susceptibility profiles. When the assumptions of the asymptotic method were not met, the exact significance was calculated by applying the Fisher exact test. Pairwise comparisons of different susceptibilities were undertaken using the Bonferroni correction.

Results

Antimicrobial susceptibility

Of the 333 *Salmonella* isolates selected and tested for antimicrobial susceptibility, 11 serotypes of *Salmonella enterica* were identified in breeders and 29 in broilers (Table 1). Of the serotypes recovered from broilers, *S.* Enteritidis and *S.* Mbandaka showed a higher frequency of reduced susceptibility to quinolones when compared with *S.* Havana and *S.* Typhimurium; the same comparison with other serotypes was observed in *S.* Enteritidis isolates recovered from breeders. Although no clinically-apparent resistance against ciprofloxacin was detected, 53.4% and 60.5% of the isolates recovered from breeders and broilers, respectively, exhibited a reduced susceptibility to this antimicrobial. *S.* Typhimurium and *S.* Enteritidis showed higher frequencies of decreased susceptibility to sulfamethoxazole and tetracycline.

Decreased susceptibility to ampicillin, chloramphenicol, florfenicol, streptomycin and gentamicin was either absent, or very low, in serovars Havana, Enteritidis and Mbandaka. Although few isolates of *S.* Virchow were tested, a high level of resistance to gentamicin and quinolones was detected.

Multiple resistance patterns

Thirty different patterns of decreased susceptibility were observed, of which half were classified as MDR (Table 2). In isolates recovered from broilers, MDR was most evident in isolates of *S.* Typhimurium (45.5%), followed by *S.* Enteritidis (31%), *S.* Havana (14.3%), *S.* Mbandaka (4.5%) and 13.7% in isolates in the group of other serotypes (Table 1). In breeders, MDR was only detected in isolates of *S.* Enteritidis (5.6%). Two *S.* Havana isolates recovered from broilers, with MICs for cefotaxime ≥8 mg/L and an ESBL phenotype, were also MDR.

Discussion

This study of *Salmonella* serotypes in Portugal supports previous studies (Papadopoulou et al., 2009), that infected breeding flocks and hatcheries, contaminated feed, environment and rearing sites are important potential sources for broiler contamination and, subsequently, human food poisoning.

S. Enteritidis, *S.* Havana and *S.* Mbandaka are all considered zoonotic or potentially pathogenic serovars for humans (Schiff and Saphra, 1941; Menon et al., 1994; Scheil et al., 1998; Backer et al., 2000; Boisrame-Gastrin et al., 2011), and were among the most prevalent serotypes recovered from Portuguese broiler flocks. It is likely that, at least for serovars Havana and Mbandaka, poultry feed containing cereal grain imported from non-European countries is one of the main sources for these serotypes in live birds.