

Antimicrobial susceptibility of *Salmonella* Gallinarum and *Salmonella* Pullorum isolated from ill poultry in Brazil

Suscetibilidade a antimicrobianos de *Salmonella* Gallinarum e *Salmonella* Pullorum isolados de aves doentes no Brasil

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ABSTRACT

Salmonella Gallinarum (*S. Gallinarum*) and *Salmonella* Pullorum (*S. Pullorum*) are poultry host-specific, agents of fowl typhoid and pullorum disease, respectively. These biovars cause septicemic infections, resulting in high mortality. Outbreaks are frequently reported worldwide, causing losses due to the elimination of infected flocks and treatments. The use of antimicrobial agents is frequent in poultry farms to prevent or treat gastrointestinal infections. In the present research it was evaluated the antimicrobial susceptibility of 50 *S. Gallinarum* and *S. Pullorum* isolates, from outbreaks that occurred between 1987 to 1991 and 2006 to 2013. The comparison of the susceptibility profiles showed that all isolates were susceptible to β -lactams. All isolates from 1987-1991 were susceptible to all antibiotics tested except NAL and CIP (78%). The susceptibility profile of *S. Gallinarum* (2006 - 2013 period) was the following NAL (58%), CIP (63%), ENR (67%), TET (92%), FFC (96%) and SXT (96%). *S. Pullorum* isolates (2006 - 2013 period) showed the following susceptibility rates to NAL (65%), CIP (71%), ENR (94%) and TET (94%). All isolates were susceptible to β -lactams tested, however, resistance to quinolones and fluoroquinolones increased over time. Furthermore, low levels of resistance to other antibiotics were found in recent isolates, such as tetracyclines.

Key words: quinolones, chicken, fowl typhoid, antimicrobial susceptibility, *Salmonella*.

RESUMO

Salmonella Gallinarum (*S. Gallinarum*) e *Salmonella* Pullorum (*S. Pullorum*) são patógenos hospedeiro-específico de aves, agentes do tifo aviário e pulorose, respectivamente. Estes biovars causam infecções septicêmicas, resultando em alta mortalidade. Surtos são frequentemente relatados em diversos países, causando prejuízos devido à eliminação de lotes

infectados e tratamentos. Agentes antimicrobianos são utilizados frequentemente em granjas avícolas para prevenir ou tratar infecções gastrointestinais. No presente trabalho, foi avaliada a susceptibilidade antimicrobiana de 50 isolados de *S. Gallinarum* e *S. Pullorum*, obtidos durante surtos que ocorreram entre 1987 a 1991 e entre 2006 a 2013. A comparação dos perfis de sensibilidade mostrou que todas as amostras são sensíveis aos β -lactâmicos. Todos os isolados de 1987-1991 foram sensíveis a todos os antibióticos testados, exceto NAL e CIP (78%). O perfil de susceptibilidade de *S. Gallinarum* (surtos de 2006 a 2013) foi NAL (58%), CIP (63%), ENR (67%), TET (92%), FFC (96%) e SXT (96%). Isolados de *S. Pullorum* (surtos de 2006 a 2013) apresentaram as seguintes taxas de sensibilidade: NAL (65%), CIP (71%), ENR (94%) e TET (94%). Todas as amostras foram sensíveis aos β -lactâmicos testados, no entanto, a resistência às quinolonas e fluoroquinolonas aumentou ao longo do tempo. Além disso, baixos níveis de resistência a outros antibióticos foram encontrados em isolados recentes, tais como as tetraciclina.

Palavras-chave: quinolonas, galinhas, tifo aviário, susceptibilidade antimicrobiana, *Salmonella*.

INTRODUCTION

Salmonella enterica serovar Gallinarum biovar Gallinarum (*S. Gallinarum*) causes fowl typhoid in commercial poultry, a disease characterized by acute systemic infection, with mortality rates reaching 80% of affected poultry flocks at any age. *S. enterica* serovar Gallinarum biovar Pullorum (*S. Pullorum*) causes pullorum disease, which is a severe

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septicemic disease that causes high morbidity and mortality in young birds, specially newly hatched chicks, causing high losses to the poultry producers (SHIVAPRASAD 2000).

Both biovars cause host-specific infections in birds. The incidence of these bacteria in hatcheries and farms is controlled by biosecurity, cleaning and disinfection of the facilities (SHIVAPRASAD 2000). Vaccination is also used to prevent these diseases (LEE et al. 2005). However, due to failures in biosafety measures or ineffective immunization, outbreaks in commercial flocks are often reported worldwide (PULIDO-LANDINEZ et al. 2014; O.I.E. 2015).

The antibiotic treatment for fowl typhoid and pullorum disease in Brazil is only allowed for commercial egg layer-hens and meat producing broilers and not accepted for breeder lines, being necessary to eliminate infected flocks (BRASIL 2003). The preventive use of antimicrobial drugs such as β -lactams, aminoglycosides and fluoroquinolones has been largely applied in the poultry industry to control intestinal infections or to treat systemic bacterial infections, including those ones caused by *S. Gallinarum* and *S. Pullorum* in commercial chickens. Both biovars can persist in the host, with no signs of the disease in surviving birds or genetically resistant chickens and also survive in the environment for long periods. Thus, these bacteria are able to cause recurrent infections in flocks, even after therapy (SHIVAPRASAD 2000; WIGLEY et al. 2001; LEE et al. 2005).

The continuous use of antimicrobial drugs in poultry production has contributed to the emergence and maintenance of resistance genes and resistant bacteria in the poultry environment (TOLLEFSON and MILLER 2000; LIEBANA et al. 2013). Many studies have reported resistance to antibiotics in paratyphoid serovars of *Salmonella* spp. isolated from food producing animals (CLEMENTE et al. 2013; DE JONG et al. 2014). Although, *S. Gallinarum* isolated from chickens in Tunisia, were recently reported to be susceptible to all drugs tested (TURKI et al. 2014), other studies about the antimicrobial susceptibility profile of animal host-specific *Salmonella* serovars are still scarce.

Evaluation of susceptibility to fluoroquinolones of *S. Gallinarum* from outbreaks in Korea, showed no resistance to this class of antibiotics in isolates from 1995, however isolates from 2001 showed reduced susceptibility to enrofloxacin, ciprofloxacin, norfloxacin and ofloxacin (LEE et al. 2004).

Resistance to quinolones can be based on chromosomal mutations besides plasmids carrying resistance genes (MARTINEZ-MARTINEZ et al. 1998; RUIZ 2003; JACOBY 2005). Plasmid-mediated quinolone resistance (PMQR) can favor the selection of resistant bacterial strains in environments with antibiotic use. First PMQR reports occurred during the 1990s (MARTINEZ-MARTINEZ et al. 1998) and were described as accessory mechanisms that could increase the levels of quinolone resistance when associated to chromosomal mutations in bacteria (ROBICSEK et al. 2006). The main PMQR genes circulating are *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*, which produce pentapeptide proteins capable to cause resistance to quinolones by protecting the antimicrobial target protein, type II DNA topoisomerases (RODRIGUEZ-MARTINEZ et al. 2011). Expression of these genes alone, usually confers low levels of quinolone resistance, but it may increase the selection of resistance mechanisms, leading to the emergence of high-level quinolone-resistant bacteria (STRAHILEVITZ et al. 2009; TAMANG et al. 2011).

In the present research, it was evaluated the susceptibility of *S. Gallinarum* and *S. Pullorum* isolates collected from infected chickens in Brazil to different classes of antimicrobial drugs, including β -lactams and quinolones, used currently or in the past in animal production. Furthermore, the results were evaluated, comparing the susceptibility profiles among isolates from the period of 1987 to 1991 with isolates from 2006 to 2013. Additionally, the presence of genes associated with PMQR were investigated by PCR in quinolone resistant isolates.

MATERIALS AND METHODS

Bacterial isolates

A total of 32 *S. Gallinarum* and 18 *S. Pullorum* were used in the present study. These bacteria were isolated from poultry showing clinical symptoms of these diseases, from South, Southeast and Midwest regions. The isolates were kept frozen at -80C, in the bacterial stocks at the School of Agricultural and Veterinary Sciences – UNESP (Jaboticabal, SP) and at the Instituto Biológico (Descalvado, SP). Isolation and identification were performed as described by (WIGLEY et al. 2005), briefly liver and spleen samples were collected from ill chickens with sterile swabs and incubated at 37C for 24h in selenite broth. After incubation the enriched swabs were plated on McConkey and XLT4 agar and incubated at 37C for 24h. Suggestive *Salmonella* spp.

colonies were sent to the Instituto Adolfo Lutz, SP, for serotyping according to the White-Kauffmann-Le Minor scheme (POPOFF 2001) and stored at -80°C in LB broth containing 50% Glycerol. The confirmed isolates were divided in three groups for analysis, the first group consisted of 9 isolates (8 *S. Gallinarum* and 1 *S. Pullorum*) collected during the period of 1987 to 1991, the second consisted of 24 *S. Gallinarum* isolates and the third group consisted of 17 *S. Pullorum* isolates. The last two groups of samples were collected from 2006 to 2013.

Antimicrobial susceptibility

The *S. Gallinarum* and *S. Pullorum* isolates were spread on the surface of Muller Hinton agar plates. The susceptibility test was performed by agar disk diffusion method using beta-lactams amoxicillin/clavulanic acid (AMC 20/10µg⁻¹), cefotaxime (CTX 30µg), ceftazidime (CAZ 30µg), ceftiofur (CTF 30µg), cefepime (FEP 30µg), aztreonam (ATM 30µg), ertapenem (ETP 10µg) and non-beta-lactams, nalidixic acid (NAL 30µg), ciprofloxacin (CIP 5µg), enrofloxacin (ENR 5µg), tetracycline (TET 30µg), trimethoprim-sulfamethoxazole (SXT 1.25/23.75µg), chloramphenicol (CHL 30µg) and florfenicol (FFC 30µg). The results were evaluated according to the Clinical Laboratory Standards Institute recommendations (CLSI 2002; CLSI 2013) considering the intermediate as resistant. *Escherichia coli* ATCC 25922 was used as quality control.

Detection of PMQR determinants

Isolates exhibiting breakpoint indicative of resistance to NAL or CIP were screened by PCR for detection of the following PMQR genes, *qnrA*, *qnrB*, *qnrS*, and *acc(6')-Ib-cr* genes. Screening of PMQR determinants was carried out by multiplex PCR with primers for *qnrA* gene, F (5'-AGAGGATTTCTCACGCCAGG-3') and R (5'-TGCCAGGCACAGATC-TTGAC-3') amplifying a 580bp product; primers for *qnrB* gene, F (5'-GGMATHGAAATTCGCCACTG-3') and R (5'-TTTGCGYGYCGCCAGT-CGAA-3') to amplify 264 bp and primers for *qnrS* gene, F (5'-GCAAGTTCATTGAACAGGGT-3') and R (5'-TCTAAACCGTCGAGTTCG-GCG-3') to amplify 428 bp. The PCR reaction was performed using the following cycling profile: 10 minutes at 95°C and 30 cycles of 1 minute at 95°C, 1 minute at 54°C, 1 minute at 72°C and 10 minutes at 72°C for the final extension (CATTOIR et al. 2007). Another PCR was done to search *aac(6')-Ib-cr* gene using the primers F (5'-TTGCGATGCTCTATGAGTGGCTA-3') and R

(5'-CTCGAATGC-CTGGCGTGT-3'), to amplify 482 bp (MINARINI et al. 2008).

Statistical analysis

Statistical difference for resistance levels to each antimicrobial was compared between the recent isolates (2006-2013) with isolates from the period of 1987-1991. Data from the antimicrobial susceptibility tests were analysed by the nonparametric chi-square method using 5% significance level.

RESULTS

All *S. Gallinarum* (8/8) and *S. Pullorum* (1/1) isolated between 1987 and 1991 were susceptible to beta-lactams AMC, CTX, CAZ, FEP, ATM, ETP, CTF and to the non-beta-lactams ENR, TET, CHL, FFC and SXT. Among these isolates, the susceptibility to the quinolone NAL and to the fluoroquinolone CIP was 78% (7/9), being only 1 *S. Pullorum* isolate and 1 *S. Gallinarum* isolate resistant to those antibiotics.

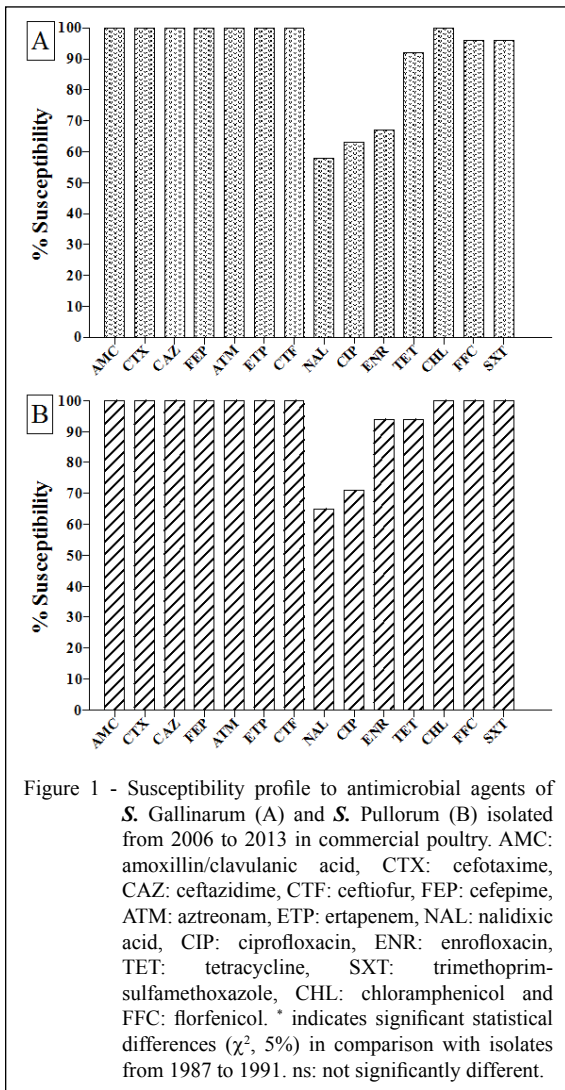
All *S. Gallinarum* isolates from 2006 to 2013 (24/24) were susceptible to beta-lactams AMC, CTX, CAZ, FEP, ATM, ETP, CTF, although, the percentage of isolates susceptible to the non-beta-lactams were noticed as follows: NAL (58%; 14/24), CIP (63%; 15/24), ENR (67%; 16/24), TET (92%; 22/24), CHL (100%; 24/24), FFC (96%; 23/24) and SXT (96%; 23/24) (Figure 1).

The results of the susceptibility test of *S. Pullorum* isolates collected from 2006 to 2013 are shown in figure 1. All isolates (17/17) demonstrated susceptibility to the beta-lactams AMC, CTX, CAZ, FEP, ATM, ETP, CTF and to CHL, FFC and SXT. However, among these isolates, susceptibility to other non-beta-lactams were the following: NAL (65%; 11/17), CIP (71%; 12/17), ENR (94%; 16/17) and TET (94%; 16/17).

The PCR screening for the PMQR genes *qnrA*, *qnrB*, *qnrS*, and *acc(6')-Ib-cr* showed that none of these resistance genes was present in any *S. Gallinarum* or *S. Pullorum* isolates.

DISCUSSION

Fowl typhoid and pullorum disease are controlled by biosecurity and vaccination by the poultry industry, however, these bacteria are still present in the poultry environment and outbreaks are often reported worldwide (O.I.E. 2015). Considering the epidemiology involved in *S. Gallinarum* and *S. Pullorum* infections, characterized by the spread of



these bacteria within different flocks and the possible carrier state in chickens (SHIVAPRASAD 2000), the use of prophylactic doses may cause selection of drug-resistant strains (SILLEY et al. 2011; LIEBANA et al. 2013; DE JONG et al. 2014).

The present study assessed and compared the susceptibility to critically important classes of antibiotics such as quinolones, fluoroquinolones and beta-lactams of isolates collected during 1987 to 2013. All *S. Gallinarum* and *S. Pullorum* isolated from 1987 to 1991 were susceptible to 12 out of 14 antimicrobial drugs, including the beta-lactams tested in this study. However, 78% were susceptible to the quinolone NAL and to the fluoroquinolone CIP. The quinolones and fluoroquinolones are frequently elected drugs for treatment and prevention of *Salmonella* infections. The increased resistance to nalidixic acid has been

attributed before to the use of enrofloxacin (DAVIES et al. 1999). However, in the present study the isolates from 1987 to 1991 that were nalidixic acid and ciprofloxacin resistant, showed susceptibility to enrofloxacin. These results show lower resistance levels for the older isolates. In comparison with older isolates, *S. Gallinarum* and *S. Pullorum* from 2006 to 2013 showed diverse phenotypes, with resistance to the antimicrobial agents, NAL, CIP, ENR, TET, FFC and SXT (Figure 1). The increasing resistance in enterobacteria could be attributed to the presence of conjugative plasmids carrying resistance genes in the environment (LIEBANA et al. 2013). However, the search for PMQR genes in these isolates did not show the presence in any isolate. Thus, the resistance to quinolone noticed in these isolates might be caused by mutations in the drug target enzymes or increased expression of efflux system (JACOBY 2005).

In South Korea, no resistance to fluoroquinolones was detected in *S. Gallinarum* isolates from chickens from 1995 (LEE et al. 2004), however a number of isolates from 2001 were reported resistant to fluoroquinolones showing an increased MIC that ranged from 2 to 8 $\mu\text{g/ml}$ (KANG et al. 2010). Our results also suggest an increasing resistance to this class of antibiotics, especially in *S. Gallinarum* which was statistically different from the levels detected in older isolates ($P < 0.05$). Despite the resistance noticed in *S. Pullorum* from the period of 2006-2013 was not statistically different from older isolates ($P > 0.05$) it is important to notice that novel resistance was detected in the recent isolates that were not present in the bacteria from 1987-1991, such as ENR and TET (Figure 1).

All isolates of both biovars *S. Gallinarum* and *S. Pullorum* evaluated in the present study were susceptible to the β -lactam class of antibiotics, even those more recently isolated, from 2006 to 2013. Even though the isolates may show susceptibility to first and second generation cephalosporins, aminoglycosides or cephamycins *in vitro*, these drugs have no activity against *Salmonella* sp *in vivo* (CLSI 2013).

Antibiotics are usually incorporated with feed or water and administered to animals to prevent gastrointestinal infections that interfere with nutrient absorption and weight gain in animals (DE JONG et al. 2014). Many studies report an increasing resistance to β -lactams in paratyphoid *Salmonella* enterica and *E. coli* isolated from humans and animals (MARANO et al. 2000; CLEMENTE et al. 2013; LIEBANA et al. 2013; FERREIRA et al. 2014). *S. Gallinarum* and *S. Pullorum* are host-specific biovars that invade quickly through the

intestinal mucosa colonizing the internal organs, such as spleen and liver, the elected sites for bacterial survival and replication (WIGLEY et al. 2001). This characteristic differentiates the typhoid biovars from the paratyphoid and other non-invasive enterobacteria. The invasive nature of these biovars limits its contact with enterobacteria, which may hamper or decrease the chances of horizontal transfer of resistance genes and plasmids in the intestinal lumen environment.

The administration of enrofloxacin was shown to select resistant enterobacteria and is inefficient to eliminate *Salmonella* from internal organs (HUMBERT et al. 1997; BARROW et al. 1998). In many countries the control of salmonellosis in poultry is not performed with antibiotics because of the poor history of these drugs in eliminating *Salmonella* colonization and due to the risks by promoting and selecting resistant strains in the fields (CASTANON 2007).

Overall, this research has evaluated the susceptibility profile of *S.* Gallinarum and *S.* Pullorum isolated from 1987 to 1991 and 2006 to 2013 in Brazil. The comparison of the profiles showed no resistance to β -lactams. However, isolates from 2006 to 2013 presented higher rate of resistance to quinolones (NAL) and fluoroquinolones (CIP, ENR) and resistance to other classes of antibiotics that were not noticed in older isolates (1987-1991), such as TET, FFC and SXT. Moreover, no PMQR gene was found in these isolates.

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