Evaluation of Antimicrobial Resistance Using Indicator Bacteria Isolated From Pigs and Poultry in Chile

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ABSTRACT

Antimicrobial resistance is a phenomenon of increasing importance, as demonstrated by the emergence of different international antimicrobial resistance surveillance programs. The aim of this research was to perform an antimicrobial resistance evaluation in indicator bacteria isolated from pigs and poultry in Chile. One hundred *Escherichia coli* and 80 *Enterococcus* species strains were isolated from fecal samples from pigs, and 99 *E coli* and 80 *Enterococcus* species strains were isolated from cloacal samples from poultry. The samples were analyzed using the dilution plates method. *E coli* strains showed a high percentage of antimicrobial resistance (>87%) in both animal species, with resistance to tetracycline and streptomycin being the most commonly observed patterns. Poultry strains showed higher resistance levels (20%–59%) than strains obtained from pigs (6%–12%) when they were tested against quinolones. Ninety percent of *Enterococcus* species strains isolated from both animal species showed resistance, most commonly to tetracycline and erythromycin. In both animal species, multiresistance occurred in more than 50% of isolated strains. These results indicate a high level of multi-resistance in indicator bacteria in Chile, suggesting that an antimicrobial resistance surveillance program is needed in Chile in order to detect bacterial resistance.

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INTRODUCTION

Antimicrobial agents are the main therapeutic tools used against human and animal bacterial infections. However, since their first use in the early 1940s it has been known that bacteria carry mechanisms that allow them to resist antimicrobials. In the 1980s, experts believed that the solution to resistance was to search for new antimicrobial agents, spurring the development of cephalosporins and fluoroquinolones, among others. However, bacterial resistance increased, becoming a worldwide human and veterinary medicine concern.[1] The World Health Organization (WHO) has concluded that antimicrobial resistance is a serious and complex worldwide problem requiring the creation of a global surveillance system in veterinary and human medicine.[2] Accordingly the United States of America and the European Union have permanent bacterial resistance monitoring programs, and their findings are periodically provided to veterinarians.[3-5]

These programs involve 3 groups of bacteria in poultry, pigs, and cattle: pathogenic, zoonotic, and indicator bacteria.[3-6] Indicator bacteria are part of human intestinal flora and in other mammals, birds, and insects. They are capable of surviving in poor media such as water, floors, and inanimate surfaces. The importance of these bacteria is their ability to acquire and disseminate resistance that could be transmitted to pathogenic or zoonotic bacteria. In Spain, *Escherichia coli* is used as indicator bacteria in pigs and poultry[6] and in Denmark and other European countries *Enterococcus* species is included, especially *E faecium* and *E faecalis*.[7,8]

In Chile, as in the rest of the world, antimicrobial agents are the main therapeutic tool for treatment of bacterial diseases in human and veterinary medicine. According to the Veterinary Laboratory Association (Santiago, Chile), this group of drugs is the main therapeutic arsenal in the nation. However, even though it is known that pathogenic bacteria is being isolated from dairy cattle that is resistant and multidrug resistant (MDR) against different antimicrobials agents,[9-11] there is no information about such risk in indicator bacteria isolated from pigs and poultry. Therefore, the present study considered the use of *E coli* and *Enterococcus* species to evaluate antimicrobial resistance in pigs and poultry.

MATERIALS AND METHOD

Samples

Cloacal samples from poultry in 35 different flocks from central Chile were collected from June to December 2003. Three cloacal samples were obtained from each bird using sterile swabs.

Pig fecal samples were obtained from 2 slaughterhouses in the metropolitan region, taking only 3 samples from each of 35 swine farms. Samples (2 ± 0.5 g) were collected at the evisceration section. All samples were transported at 5˚C ± 1 in Cary-Blair medium (BBL, Becton, Dickinson, Franklin Lakes, NJ, USA) and processed during the next 24 hours.

Isolation and Identification

One hundred ninety-nine *E coli* strains and 80 and 96 *Enterococcus* strains were isolated from pigs and poultry, respectively. *E coli* samples were cultured in McConckey agar (BBL) and a rapid diagnostic test (API20E, bioMerieux, Durham, NC, USA) was used for its identification. *Enterococcus* species were cultured in M-*Enterococcus* agar (BBL) and the identification was performed by conventional methods according to guidelines of the *Manual of Systematic Bacteriology*.[12] Once identified, 1 strain of *E coli* or *Enterococcus* was taken from each sample. The selected strains were kept at 5˚C ± 1 until susceptibility testing was performed.

Antimicrobial Susceptibility Test

All isolates were tested for susceptibility to different antimicrobials using the dilution plates method as described by the National Committee for Clinical Laboratory Standard Guidelines.[13,14] *E coli* ATCC 25922 and *E faecalis* ATCC 29212 were used as quality control organisms. The antimicrobials tested were:

- for *E coli*: nalidixic acid (98% purity), cefazolin (98% purity), ciprofloxacin (100% purity), enrofloxacin (100% purity), streptomycin (98% purity), gentamicin (63.8% purity), oxytetracycline (96% purity), sulfamethoxazole/trimethoprim (100% purity), chloramphenicol (98% purity).
- for *Enterococcus*: amoxicillin/ clavulanic acid (55.8% purity), penicillin (1,658 UI/mg), cloramphenicol (98% purity),

erythromycin (99.9% purity), streptomycin (98% purity), gentamicin (63.8% purity), oxytetracycline (96% purity), and vancomycin (100% purity).

RESULTS

Table 1 shows the percentages of *E coli* resistance, minimum inhibitory concentrations required to inhibit the growth of 50% of strains tested and 90% of strains tested, respectively (MIC_{50}, MIC_{90}), and MIC ranges observed against the tested antimicrobials in both animal species. Higher frequencies of resistance were observed for oxytetracycline and streptomycin in pigs and poultry isolates. For quinolones (nalidixic acid) and fluoroquinolones (enrofloxacin and ciprofloxacin), the highest percentages of resistance were found in poultry strains (59.1% against nalidixic acid and 28.5% against enrofloxacin), while resistance percentages for pig strains were 12% to quinolones and 6% to fluoroquinolones.

Resistance to sulfamethoxazole/-trimethoprim was 16.3% and 21.0% for poultry and pigs, respectively. Gentamicin resistance was low in both animal species. All isolates were sensitive to cephalosporins (cefotaxime and cefazoline). Table 2 shows the percentage of resistant strains against *Enterococcus* species, MIC_{50}, $MIC_{90,}$ and MIC ranges observed against the tested antimicrobials in both animal species. These strains showed high degrees of resistance to oxytetracycline and erythromycin. Lower levels of resistance to streptomycin (close to 45% in pig isolates and 25% in poultry isolates) were observed. Resistance to penicillin in poultry isolates was higher (17.7%) than in pig isolates (6.0%), with all the isolates being susceptible to teicoplanin, amoxicillin/clavulanic acid, and vancomycin. Less than 6% of the poultry strains were resistant to gentamicin and cloramphenicol.

More than 60% of the strains were resistant to 2 or more antimicrobials (Table 3). Complete patterns of *E coli* and *Enterococcus* species multiple resistant isolates are shown in Tables 4 and 5, respec-

Table 1. Percentages and Minimum Inhibitory Concentration (MIC) Values of Resistant *Escherichia coli* Strains Isolated from Pigs and Poultry in Chile*

Antimicrobial Agent	Pigs: Percentage of Resistant Strains (%)	Pigs: MIC Ranges	Pigs: MIC_{50} ($\mu g/mL$)	Pigs: MIC_{90} ($\mu g/mL$)	Poultry: Percentage of Resistant Strains (%)	Poultry: MIC Ranges	Poultry: MIC_{50} ($\mu g/mL$)	Poultry: MIC_{90} ($\mu g/mL$)
Enrofloxacin	6.0	0.125–64	0.125	0.5	28.5	0.125–64	≤ 0.125	8
Sulfamethoxazole/ Trimethoprim	21	0	1	16	16.3	0.125–2	≤ 0.125	2
Cefoxatime	0.0	0.125	0.125	0.125	0.0	0.125	≤ 0.125	≤ 0.125
Oxytetracycline	96	0.125–128	128	128	80.6	0.125–128	64	128
Gentamicin	2.0	0.125–64	0.5	1	1.02	0.125–32	≤ 0.125	≤ 0.125
Ciprofloxacin	6.0	0.125–8	0.25	0.5	20.4	0.125–8	≤ 0.125	4
Streptomycin	84.0	0.25–128	128	128	57.1	0.125–128	32	≥ 128
Cefazolin	0.0	0.125	0.125	0.125	0.0	0.125	≤ 0.125	≤ 0.125
Nalidixic acid	12.0	0.125–128	1	64	59.1	0.125–128	≤ 128	≥ 128

*MIC_{50}, MIC_{90} indicates minimum inhibitory concentrations required to inhibit the growth of 50% of strains tested and 90% of strains tested, respectively.

Table 2. Percentages and Minimum Inhibitory Concentrations (MIC) Values of Resistant *Enterococcus* Species Strains Isolated from Pigs and Poultry in Chile

Antimicrobial Agent	Pigs				Poultry strains			
	Percentage of Resistant Strains (%)	MIC Ranges	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Percentage of Resistant Strains (%)	MIC Ranges	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Penicillin	6.0	0.125-64	0.25	4	17.7	0.125–32	≤ 0.125	16
Vancomycin	0.0	0.125	0.125	0.125	0.0	0.125	≤ 0.125	≤ 0.125
Tetracycline	82.3	0.125-128	128	128	81.2	0.125–128	64	≥128
Amoxicillin/clavulanic acid	0	0.125	0.125	0.125	0.0	0.125	≤ 0.125	≤ 0.125
Erythromycin	49.9	0.125-128	64	128	64.5	0.125–128	16	≥ 128
Teicoplanin	0.0	0.125	0.125	0.125	0.0	0.125	≤ 0.125	≤ 0.125
Gentamicin	2.0	0.125-128	0.125	0.125	5.2	0.125–128	≤ 0.125	≤ 0.125
Streptomycin	44.1	0.125-128	0.125	128	22.9	0.125–128	≤ 0.125	≥ 128
Chloramphenicol	2.0	1-64	1	1	0.0	0.125–8	≤ 0.125	≤ 0.125

*MIC50, MIC90 indicates minimum inhibitory concentrations required to inhibit the growth of 50% of strains tested and 90% of strains tested, respectively.

tively. For *E coli* isolates the tetracycline/streptomycin resistance pattern was the most frequent in pigs and poultry (51% and 13.26%, respectively).

Resistance in avian *Enterococcus* species to tetracycline/streptomycin was most frequent (33.3%), while resistance to tetracycline/streptomycin/erythromycin was most frequent in pig isolates (24.5%).

DISCUSSION

In this study we demonstrated the widespread occurrence of antimicrobial resistance to oxytetracycline. Similar information has been reported by national and international studies. The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme[3] reports *Enterococcus faecalis* resistance levels of 45% and 83% for broilers and pigs, respectively. Aarestrup et al[15] pointed out that it is common to find resistance to tetracyclines in pathogenic, zoonotic, and indicator bacteria such as *E coli*, *E faecium,* and *E faecalis*, probably a consequence of selection pressure by the massive use of these drugs. This fact was proven by Sunde et al,[16] who pointed out that the highest resistance levels to tetracycline were found in strains isolated from pig farms where the use of antimicrobials was considered high. In Chile, there is no formal guideline about the total use of tetracycline in animal production. This antimicrobial agent has been used for decades in our country as a therapeutic agent and growth promoter. This could explain the high resistance levels seen in *E coli* to streptomycin, and in *Enterococcus* species to erythromycin.

Our results demonstrate that *E coli* strains isolated from pigs showed low resistance to quinolones and fluoroquinolones, supporting the conclusion that there is good antimicrobial management on pig farms in Chile. The situation is different in poultry production because high antimicrobial resistance levels (between 20% and 59%) were observed. The decrease in susceptibility in poultry isolates could be due to *Salmonella* being declared endemic during the last

Table 3. Percentages of Mono- or Multi-resistant *Escherichia coli* and *Enterococcus* Species Strains Isolated from Pigs and Poultry in Chile

	Escherichia coli		*Enterococcus species*	
Number of Antimicrobials Agents	**Percentage of Resistant Strains (Poultry) (%)**	**Percentage of Resistant Strains (Pigs) (%)**	**Percentage of Resistant Strains (Poultry) (%)**	**Percentage of Resistant Strains (Pigs) (%)**
1	12.2	12.0	7.2	25.5
2	13.2	53.0	6.2	29.4
3	12.2	22.0	21.8	28.4
4 or more	52.06	11.0	58.3	3.9

Table 4. Patterns of Multi-resistance in *Escherichia coli* Strains Isolated from Pigs and Poultry in Chile

Antimicrobial Agents' Multi-resistance Patterns*	Percentage of Resistant Strains (Pigs) (%)	Percentage of Resistant Strains (Poultry) (%)
Enr-Tm-Cip-S-N	—	10.20
Tm-S-N	—	13.26
Tm-S	51	13.26
Enr-Sxt-Tm-Cip-S-N	—	4.08
Enr-Tm-Cip-N	—	4.08
Enr-Sxt-Tm -S-N	—	3.06
Enr-Tm-S-N	—	3.06
Tm-N	3	10.2
Sxt-Tm-S-N	2	2.04
Tm-S-N	3	1.02
Sxt-Tm-S	17	1.02
S-N	—	1.02

*Enr indicates enrofloxacin; Tm, oxytetracycline; Cip, ciprofloxacin; S, streptomycin; N, nalidixic acid; Sxt, trimethoprim/sulfamethoxazole.

decade in Chile.[17] This called for the implementation of fast control and prevention measures, such as antimicrobial use. Our results are similar to that found in the international literature. For example, surveillance studies done in Spain have demonstrated that in both healthy and sick animals the percentage of *E coli* strains resistant to quinolones were 44% in poultry.[18,19] We suggest that this high resistance could be due to the massive use of antimicrobial drugs, mainly as prophylactics.

Quinolone resistance is important in human and veterinary medicine, since the situation becomes more serious when it is demonstrated that a bacteria resistant to one quinolone can be resistant to all antimicrobials of the same family. Van den Bogaard et al[20] related quinolone use in turkeys with the development of resistance to ciprofloxacin in *E coli* strains isolated from turkeys and from people associated with turkey production. Van den Bogaards' study concluded that the resistant strains are disseminated from animals to humans, and also that their resistance genes can recognize antimicrobial agents used only in human medicine. In veterinary medicine, the authorized quinolones are enrofloxacin, oxolinic acid, and flumequine, ciprofloxacin being used exclusively for human use. In our research, more than 95% of the enrofloxacin-resistant strains were also resistant to ciprofloxacin, a predictable situation according to a study by Jacobs-Reistma et al,[21] which describes an increase

Table 5. Patterns of Multi-resistance of *Enterococcus* Species Strains Isolated from Pigs and Poultry in Chile

Antimicrobial Agents' Multi-resistance Patterns*	Percentage of Resistant Strains (Pigs) (%)	Percentage of Resistant Strains (Poultry) (%)
Tm-E	20.5	33.3
Tm-E-S	24.5	10.4
P –Tm	—	5.2
P-Tm-E-S	1.9	4.16
P-Tm –E	—	3.12
Tm-E-G-S	—	4.16
P–Tm –S	1.9	2.08
Tm –S	8.8	1.04

*P indicates penicillin; E, erythromycin; S, streptomycin; Tm, tetracycline; G, gentamicin.

of *Campylobacter* strains resistant to ciprofloxacin after enrofloxacin treatments.

This situation has started a worldwide controversy about the use of the fluoroquinolones in veterinary medicine. New restrictive measures concerning the use of fluoroquinolones in animals have been suggested, since ciprofloxacin and new fluoroquinolones, such as levofloxacin, trovafloxacin, and clinofloxacin, are highly effective in the treatment of critical diseases produced by multi-resistant bacteria in humans. In addition, the Japanese government implemented 3 restrictions against the use of fluoroquinolones in veterinary therapeutics: (1) these drugs can be prescribed only when resistance to other antimicrobials is detected; (2) they can be administrated only under veterinarian surveillance; and (3) therapy with these drugs must be limited to 5 days or less.[22]

In Chilean veterinary medicine, no such measures have been considered, even though high levels of ciprofloxacin resistance have been reported in human medicine. A study by Pinto[23] analyzing trends in antimicrobial resistance of important bacteria between 2000 and 2001, showed a progressive increase in resistance to ciprofloxacin in communitarian *E coli* (15%) and *Enterococcus faecium* (83%), and nosocomial *E. faecalis* strains (47%).

The high susceptibility to cephalosporins found in *E coli* strains and to amoxicillin/clavulanic acid in *Enterococcus* strains leads us to suggest that these antimicrobial agents could be used against important pathogenic bacteria for veterinary medicine in Chile if they are used responsibly to avoid the emergence of resistant strains.

This study included tests of vancomycin against strains of *Enterococcus* species because reports[24] have shown that some antimicrobial agents used in veterinary medicine as growth promoters are associated with vancomycin resistance in human medicine, a situation that was not observed in this study.

The high percentage of resistance to two or more antimicrobials suggests that Chile is part of the worldwide multiresistance problem. *Salmonella typhimurium* DT104 is one of the most representative examples in public health.[25] According to our results, an important number of strains (over 61%) were resistant to more than one antimicrobial agent, the most common being tetracycline/streptomycin in both animal species against *E coli* strains. In *Enterococcus* strains the most common pattern of resistance is streptomycin/tetracycline/erythromycin in pigs and tetracycline/erythromycin in poultry. In Germany, Guerra et al[26] studied *E coli* susceptibility isolated from pigs, cattle, and poultry. Thirty-two percent were multiresistant, and of the 75 resistant phenotypes, resistance to streptomycin/sulfamethoxazole/tetracycline patterns was the most frequent.

The results of our research and that of other studies done nationally[9–11] suggest that Chile must follow the recommendations of WHO (2000–2001) on the fair use of antimicrobial agents in animal production. Among other important points, it suggests the obligatory use of veterinary prescription in animal production systems. However, one of the most important recommendations concerning bacterial resistance is the creation of resistance surveillance systems through permanent monitoring programs to identify the emergency of pathogenic, indicator, and zoonotic resistant bacteria in order to take corrective actions to safeguard animal and human health.

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