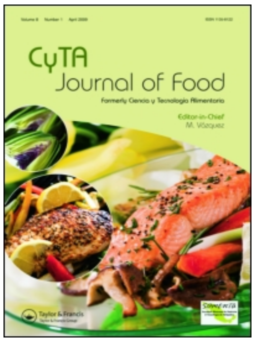

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Presencia y resistencia a antimicrobianos de *Escherichia coli* aislados a partir de alimentos en el estado de Hidalgo (México)

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The presence of *Escherichia coli* in foods taken from the grocery stores and the supermarkets in Hidalgo State (Mexico) was determined for 73 samples of poultry meat, 60 samples of pork, 86 samples of beef, and 66 samples of vegetables. A total of 352 *E. coli* strains were isolated, identified, and analyzed by an agar disk diffusion assay for their resistance to 10 antimicrobials. Poultry meat and vegetables taken from groceries showed significantly higher counts ($P = 0.0002$ and $P = 0.0461$, respectively) when compared with the samples taken from supermarkets. Compared with the isolates recovered from other foods, *E. coli* isolated from chicken meat had higher levels of antimicrobial resistance against all antimicrobials tested, with the exceptions of nitrofurantoin resistance of isolates from pork and streptomycin resistance in isolates from pork and beef. In addition, the *E. coli* isolates from samples taken from the groceries showed higher resistance rates than the isolates from samples taken from the supermarkets for the cases of pork isolates resistance to ampicillin ($P = 0.0497$), chloramphenicol ($P = 0.0075$), doxycycline ($P = 0.002$), and streptomycin ($P = 0.0094$) and beef isolates resistance against ampicillin ($P = 0.0048$), streptomycin ($P = 0.002$), and sulfisoxazole ($P = 0.003$). The present study revealed that the observed resistance rates correlated well with those reported in the national surveillance programmes of developed countries, with the exception of isolates from chicken meat, which have higher resistance rates. Also, from a microbiological safety point of view, samples taken from supermarkets were in a much better conditions than those obtained from the groceries.

Keywords: *E. coli*; antimicrobial; food-borne; resistance; México

La presencia de *Escherichia coli* fue investigada en 73 muestras de carne de pollo, 60 muestras de carne de cerdo, 86 muestras de carne de vacuno y 66 muestras de alimentos vegetales muestreados en pequeños ultramarinos y supermercados en el estado de Hidalgo (México). Un total de 352 aislamientos de *E. coli* fueron seleccionados, identificados y analizados mediante el método de difusión en disco para determinar su resistencia a 10 antimicrobianos. Las muestras de pollo y vegetales obtenidas en tiendas de alimentación mostraron recuentos de *E. coli* significativamente mayores ($P = 0{,}0002$ y $P = 0{,}0461$ respectivamente) que las obtenidas en supermercados. Comparados con los procedentes de los restantes alimentos, los *E. coli* obtenidos a partir de carne de pollo mostraron un mayor grado de resistencia a todos los antimicrobianos estudiados, excepto en lo referente a la nitrofurantoína en el caso de los aislados a partir de carne de cerdo y la estreptomicina respecto de los aislamientos de carne de cerdo y vacuno. Adicionalmente, en algunos casos, los *E. coli* obtenidos a partir de alimentos muestreados en pequeños ultramarinos mostraron mayores tasas de resistencia que los procedentes de alimentos muestreados en supermercados. Esto ocurrió en los aislamientos procedentes de carne de cerdo para la ampicilina ($P = 0{,}0497$), cloranfenicol ($P = 0{,}0075$), doxiciclina ($P = 0{,}002$) y estreptomicina ($P = 0{,}0094$), y en el caso de la carne de vacuno para la ampicilina ($P = 0{,}0048$), estreptomicina ($P = 0{,}002$) y sulfizoxazol ($P = 0{,}003$). El presente estudio demuestra que las tasas de resistencia observadas son compatibles con las publicadas en los programas nacionales de vigilancia de los países desarrollados, con la excepción de los aislamientos procedentes de carne de pollo, en los cuales las tasas de resistencia tienden a ser mayores. Además, desde el punto de vista de la seguridad microbiológica, las muestras obtenidas en supermercados mostraron condiciones significativamente mejores que las obtenidas en tiendas de alimentación.

Palabras clave: *E. coli*; antimicrobiano; origen alimentario; resistencia; México

Introduction

Resistance to antimicrobial agents has been recognized as a major concern in both human and veterinary medicine. The use of antimicrobial agents is considered the most important factor for the selection and dissemination of antimicrobial agent-resistant bacteria

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(White, 1998). Generally, these agents are used therapeutically in animals and humans for the control of bacterial infections, although in some countries (such as Mexico), antimicrobials may also be incorporated into livestock and poultry feed at sub-therapeutic doses as growth promoters. This practice is believed to enhance selection of resistant bacteria far more than the therapeutic use of antimicrobial agents in response to the clinical disease (Van den Bogaard, London, Driessen, & Stobberingh, 2001). As a result, this practice has been banned in the European Community (EC, 2003), but in most countries, it is still allowed.

In recent decades, there has been an increase in bacterial antibiotic resistance, especially in the developing countries (Nys, Okeke, Kariuki, Dinant, Driessen, & Stobberingh, 2004). Contrary to the developed nations, where most antimicrobial prescriptions are in the hands of the medical community (Phillips et al., 2003), in developing nations it is a common practice for the patients and the farmers to obtain the antibiotics directly from a pharmacist without a medical prescription. As a result, inadequate therapeutic protocols are often applied to humans and animals and contribute to the emergence and spread of antibiotic-resistant strains (Calva, Sifuentes-Osornio, & Ceron, 1996; Phillips et al., 2003). In addition, in situations typical to developing countries, increases in human and animal populations, in combination with a suboptimal management of fecal waste, also enable the dissemination of enteric resistant bacteria (selected during antimicrobial treatments) directly into the environment (Rosas et al., 2006). The antibiotic resistance of the bacterial isolates of animal origin can also present a potential hazard to the consumers via food-borne infections caused by the antibiotic-resistant bacteria, and can also contribute resistance genes to endogenous human bacteria (van den Bogaard et al., 2001).

Vegetables represent another way for transmission of antimicrobial resistant bacteria to food consumers. Antimicrobials are currently used to prevent bacterial diseases in plants and to protect fruit trees from fire blight (Vidaver, 2002). Additionally, contamination by human and veterinary sources of antimicrobials, their metabolites, and resistant bacteria can also occur via contact with sewage and waste water (Kemper, 2008; Phillips et al., 2003).

Monitoring the veterinary use of antimicrobial agents in animals destined for human consumption is considered as a risk-management option to prevent the development and spread of antimicrobial resistance in microorganisms present in food-producing animals (Vose et al., 2001). Programmes to monitor resistance are therefore essential. Many countries, such as Canada (CIPARS, 2005), Denmark (DANMAP, 2006), Spain (VAV Network, 2005), Sweden (SVARM, 2005), the Netherlands (MARAN, 2005), or the USA (NARMS, 2007) have established national surveillance programmes to monitor the antibiotic susceptibility of enteric bacteria isolated from animals and animal-origin foods. However, not much information is available about the monitoring of antimicrobial resistance in bacteria isolated from food in developing nations (such as Mexico).

Although a variety of different bacterial species have been used for these monitoring programmes, commensal bacteria are particularly important because their isolation and identification is relatively easy and allows the comparison of the resistance levels between different populations (Knezevic & Petrovic, 2008). When compared with the other microorganisms found mostly in foods, *Escherichia coli* has been a very useful biomarker for evaluating the development of antimicrobial resistance (Von Baum & Marre, 2005). In addition to its high frequency of mutation, which can favour the spontaneous development of antimicrobial resistance, *E. coli* also has the ability to transfer microbial resistance between individual bacteria (Kijima-Tanaka et al., 2003; Sáenz et al., 2001; Sayah, Kaneene, Johnson, & Miller, 2005; Von Baum & Marre, 2005). Thus, changes in the resistance of this species may serve as a good indicator of resistance in potentially pathogenic bacteria (Kijima-Tanaka et al., 2003; Von Baum & Marre, 2005).

The main goal of this study was to investigate the presence of antimicrobial resistance of *E. coli* strains derived from foods in Hidalgo State (Mexico). The implications of these results in terms of microbiological safety, especially those concerning the development and spread of the antimicrobial resistance up the food chain, are also discussed.

Materials and methods

Collection of food samples

A total of 285 raw, unprocessed, and unpackaged food samples were taken during 2008 from both the supermarkets and the grocery shops (including butcher and poultry shops). Among these, 73 were poultry meat samples (38 drumsticks taken from the supermarkets and 35 drumsticks taken from the groceries), 60 were pork samples (32 pork loin and chops taken from the supermarkets and 28 pork loin and chops taken from the groceries), 86 were the beef samples (42 beef fillets and chops taken from the supermarkets and 44 beef fillets and chops taken from the groceries), and 66 were the vegetable foods samples (11 samples of lettuce, 11 of spinach, and 11 of chards taken from both supermarkets and groceries). Not more than three samples from each group were taken from the same sale outlet, these samples were taken on different days. Samples were placed in sterile bags and transported to the laboratory in an ice chest in lesser than 1 h for immediate processing. All supermarkets and groceries were located in Hidalgo State (east-central México).

Microbiological analyses

Twenty-five gram portions were obtained for each sample, placed in a sterile masticator bag together with an appropriate volume (1/9) (w/v) of sterile 0.85% NaCl solution (Sigma, St Louis, MO), and homogenized in a stomacher (Seward, London, UK) for 1 min. After homogenization, samples were cultured for the presence of *E. coli*. One-hundred microliters ranging from 10^{-1} to 10^{-6} dilutions of food extracts were processed on plates of Eosin Methylene Blue (EMB) agar, prepared by following the manufacturer's instructions (Oxoid, Bakingstoke, UK). After 18–24 h of incubation at 37 °C, purple colonies with a dark centre and a metallic green sheen were considered as presumptive *E. coli* and counted.

Only plates containing less than 250 presumptive colonies were counted. The counts of *E. coli* obtained for each sample were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU^{-1} samples. When counts were below the detection limit (2 log CFU g^{-1} sample), a theoretical value of 1.9 log CFU g^{-1} was assigned prior to the determination of the average counts, which were determined for each food type taken from the groceries and the supermarkets.

Three colonies of presumptive *E. coli* were randomly picked from each sample, except for the case of samples in which only one of the two presumptive *E. coli* colonies were available; in these cases only one or two colonies were picked. Isolated presumptive *E. coli* were subsequently transferred onto Columbia agar supplemented with 5% sheep blood (BioMérieux, Plainview, NY), and incubated at 44 °C for 24 h to obtain pure cultures. These pure cultures were then analyzed for strain identification by colony and cell morphology, Gram stain, methyl red stain, oxidase and catalase activity, and indole production. Positive strains preliminarily identified as *E. coli* were confirmed by the API 20E miniaturized identification tests (BioMérieux). All identified *E. coli* were used for the determination of the antimicrobial susceptibility.

Aintimicrobial susceptibility testing of bacteria

Antimicrobial susceptibility testing was done for a total of 352 isolates of *E. coli*. Testing was carried out using agar disk diffusion on Müeller-Hinton agar plates (Oxoid), according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS, 2002) guidelines. Antimicrobial disks used were: ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), doxycycline (30 μg), ciprofloxacin (5 μg), fosfomycin (200 μg), gentamicin (10 μg), nitrofurantoin (300 μg), streptomycin (10 μg), and sulfisoxazole (300 μg) (Oxoid). Antibiotic resistance breakpoints considered were those recommended by CLSI for *Enterobacteriaceae* (NCCLS, 2002). *E. coli* ATCC 25922 was used as a reference strain for this study.

Antimicrobial agents were selected on the basis of their diverse chemical structures and mechanisms of action. *E. coli* isolates were classified as sensitive, intermediate, or resistant according to the criteria (inhibition diameter zones) established by the CLSI. Isolates showing resistance to at least three of the antimicrobial agents tested were considered to be multi-resistant strains.

Statistical analysis

The average log CFU g^{-1} values of *E. coli* in foods obtained from supermarkets and groceries were compared using a Student's *t*-test. The distributions of resistant strains between food type and origin, as well as multi-resistance patterns, were compared with the χ^2 test, and by the χ^2 test with the Yates' correction when only two categories were available. The differences were considered to be significant when P was lesser than 0.05. All statistical analyses were done with the Statgraphics version 5.0.1. software (SAS Institute, North Carolina).

Results and discussion

The average counts of presumptive *E. coli* (Figure 1) showed that poultry meat and vegetable foods had the greater levels of presumptive *E. coli* contamination out of all the tested foods. Furthermore, both poultry meat ($P = 0.0002$) and vegetables ($P = 0.0461$) taken from the groceries showed higher average counts of presumptive *E. coli* when compared with those obtained from their supermarket counterparts. No significant differences were found in the case of pork or beef. The higher levels of presumptive *E. coli* contamination in chicken meat and vegetables obtained from the groceries could be attributed to lower quality facilities and poor food hygiene practices in groceries compared to supermarkets. Contrary to similar products for sale in supermarkets, it is common practice for poultry meat and vegetables to be left without refrigeration in the Mexican groceries.

During recent years, in addition to official surveillance and antimicrobial monitoring programmes (CIPARS, 2006; DANMAP, 2006; MARAN, 2005; NARMS, 2007; SVARM, 2005; VAV Network, 2005), some studies have reported the antimicrobial resistance of *E. coli* isolated from meat samples and food-producing animals (Bywater et al., 2004; Guerra et al., 2003; Kijima-Tanaka et al., 2003; Knezevic & Petrovic, 2008; Miranda et al., 2008a; Sáenz et al., 2001; Van den Bogaard et al., 2001). However, these works involved samples taken directly from live or recently slaughtered animals, and do not take into consideration the contamination of foods during subsequent steps in the food chain. Also, despite the strong possibility of contamination of vegetable foods by resistant bacteria from veterinary antimicrobial

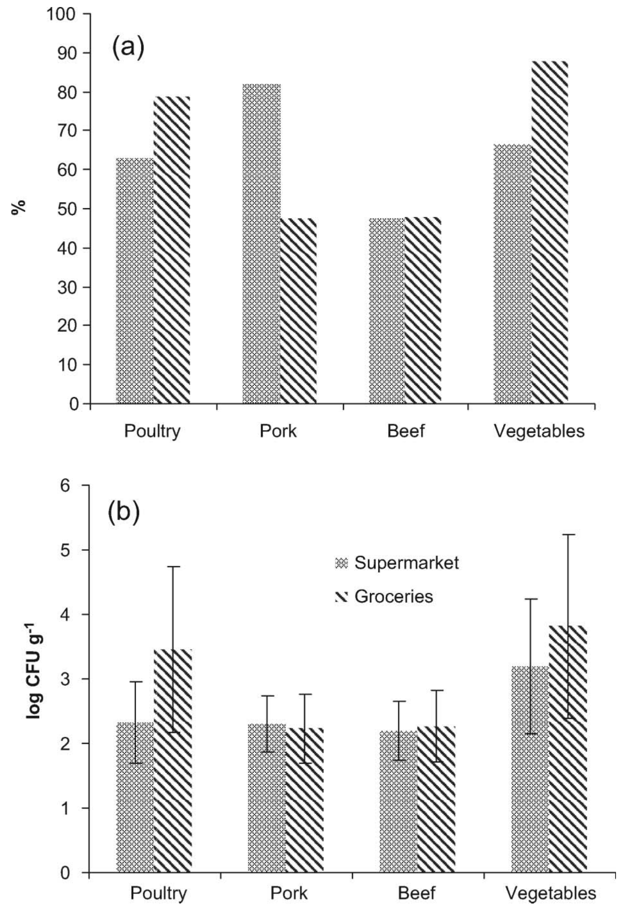

Figure 1. Percentages of positive samples (a) and average counts (b) of presumptive *Escherichia coli* isolated from poultry ($n = 73$), pork ($n = 60$), beef ($n = 86$), and vegetables ($n = 66$) obtained from supermarkets and groceries in Hidalgo State (Mexico).

Figura 1. Porcentajes de muestras positivas (a) y recuentos medios (b) de presuntos *Escherichia coli* aislados a partir de carne de pollo ($n = 73$), cerdo ($n = 60$), ternera ($n = 86$), y vegetales ($n = 66$) obtenidos en supermercados y pequeños ultramarinos en el estado de Hidalgo (México).

treatments via waste waters and sewage (Miranda et al., 2008b; Phillips et al., 2003; Sabaté et al., 2008), very little information has been reported about the resistance of *E. coli* isolated from vegetable foods.

Data provided in previous studies show that antimicrobial resistance varies widely among compounds, depending on the countries, host, or food from which the microorganisms have been isolated. The antimicrobial resistance rates obtained in our study (Table 1) for beef and pork are largely compatible with previously reported data for most of the antimicrobials tested. Interestingly, the resistance of isolates was remarkably higher for older compounds, with the exception of gentamicin, which is a relatively old compound that has had little use in animals. In agreement with the findings reported in other countries (Bywater et al., 2004; Knezevic & Petrovic, 2008; Miranda et al., 2008a), resistance to this antimicrobial of animal-origin strains is rare.

Table 1. Percentages of *Escherichia coli* isolated from poultry, pork, beef, and vegetables obtained from supermarkets (S) and groceries (G) in Hidalgo State (Mexico) that are resistant to antimicrobial agents by disk diffusion methods.

Tabla 1. Porcentajes de *Escherichia coli* aislados de carne de pollo, cerdo, vacuno, y vegetales obtenidos en supermercados (S) y pequeños ultramarinos (G) en el Estado de Hidalgo (México) resistentes a los agentes antimicrobianos mediante el método de difusión en disco.

	Poultry			Pork			Beef			Vegetables		
Antimicrobial agent (μg)	S ($n = 43$)	G ($n = 76$)	Total ($n = 119$)	S ($n = 43$)	G ($n = 35$)	Total ($n = 78$)	S ($n = 40$)	G ($n = 31$)	Total ($n = 71$)	S ($n = 36$)	G ($n = 48$)	Total ($n = 84$)
Ampicillin (10)	79.1	75	76.5^{a}	27.9	37.1	32.1^{b}	27.5	48.4	36.6^{b}	41.7	35.4	38.1^{b}
Cephalothin (30)	48.8	60.5	56.3^{a}	30.3	37.1	21.8^{b}	37.5	35.5	36.6^{b}	27.8	29.2	28.6^{b}
Chloramphenicol (30)	55.8	59.2	58^{a}	0	16.3	9^{b}	4.9	6.5	$5.6^{b,c}$	0	0	0^{c}
Ciprofloxacin (5)	51.2	78.9	77.3^{a}	11.6	5.7	$9^{b,c}$	0	3.2	1.4^{c}	14.6	22.2	17.9^{b}
Doxycycline (30)	55.8	56.6	56.3^{a}	16.3	71.4	41^{b}	25	48.4	35.2^{b}	0	0	0^{c}
Fosfomycin (200)	39.5	55.3	49.6^{a}	16.3	11.4	14.1^{b}	22.5	12.9	18.3^{b}	22.9	11.1	17.9^{b}
Gentamicin (10)	18.6	17.1	17.6^{a}	0	5.7	2.6^{b}	0	3.2	1.4^{c}	8.3	2.8	6^{c}
Nitrofurantoin (300)	23.3	11.8	16^{a}	13.9	8.6	11.5^{a}	15	12.9	14.1^{a}	8.3	10.4	9.5^{b}
Streptomycin (10)	25.6	39.5	34.5^{a}	14	37.1	24.4^{a}	20	38.7	28.2^{a}	2.8	10.4	7.1^{b}
Sulfisoxazole (200)	74.4	78.9	77.3^{a}	32.6	42.9	37.2^{c}	37.5	80.6	56.3^{b}	27.8	33.3	31^{c}

^{a,b,c}Values in the same total result row with different letters are significantly different by means of χ^2 test.

^{a,b,c}Los valores en la misma fila con letras diferentes presentan diferencias estadisticamente significativas mediante el test χ^2.

On the other hand, the resistance levels found for some antimicrobials in isolates obtained from the chicken meat tend to be higher than the levels reported in other countries (Bywater et al., 2004; Kijima-Tanaka et al., 2003; Miranda et al., 2008a; Sáenz et al., 2001; Van den Bogaard et al., 2001). It is remarkable that the high level of resistance observed in poultry-origin isolates for both antimicrobials are commonly used in poultry medicine, such as ampicillin (76.5%), doxycycline (56.3%), sulfisoxazole (77.3%), or ciprofloxacin (77.3%) (Miranda et al., 2008b; Van den Boogard et al., 2001) and also for antimicrobials that are rarely used in poultry medicine, such as chloramphenicol (58%). This high level of chloramphenicol-resistance of poultry-origin *E. coli* are in agreement with the chloramphenicol-resistance found in *E. coli* isolates from Mexico in the urban environments (33–53%) (Rosas et al., 2006) or in faecal samples of Mexican healthy volunteers (45–75%) (Nys et al., 2004).

It is also interesting that the isolates from the chicken meat showed higher resistance levels than those obtained from other type of foods, with the exception of nitrofurantoin resistance in isolates from pork and streptomycin resistance in isolates from pork and beef. On the other hand, resistance incidence in isolates obtained from pork and beef tend to be similar, except for ampicillin ($P = 0.0068$) and sulfisoxazole ($P = 0.0189$), which are significantly higher in beef-origin isolates.

However, isolates obtained from vegetable foods showed similar or lower resistance levels than isolates from all other foods tested, with the exception of ciprofloxacin resistance, which had higher resistance levels compared to isolates from beef ($P = 0.0033$). In this case, it is reasonable to think that ciprofloxacin-resistant *E. coli* could reach vegetable foods though human or poultry farm-influenced wastewater or sewage (Sabaté et al., 2008; Sayah et al., 2005).

The results obtained in the present work provide supporting evidence for the idea that the antimicrobial resistance of *E. coli* isolates can change depending on the sample source. Isolates from pork obtained from the groceries showed significantly higher resistance for the antimicrobials ampicillin ($P = 0.0497$), chloramphenicol ($P = 0.0075$), doxycycline ($P = 0.002$), and streptomycin ($P = 0.0094$) when compared with the resistance of isolates obtained from the samples bought from the supermarkets. In the case of isolates from beef, the same trend was found for ampicillin ($P = 0.0048$), streptomycin ($P = 0.002$), and sulfisoxazole ($P = 0.003$), whereas higher resistance rates for fosfomycin were found for isolates obtained from supermarkets ($P = 0.0013$) compared to isolates from groceries samples. This seems to suggest either a more frequent use of antimicrobials or less time taken for a withdrawal period in animals destined for the production of meat to be sold at the groceries than in animals destined to be sold in the supermarkets. No differences were found in the case of isolates from poultry meat and vegetables. However, it should be pointed out that the higher average counts found in samples taken from groceries could skew the results observed with regards to antimicrobial resistance.

The multi-drug resistance (to ≥ 3 antimicrobial agents, Table 2) exhibited by *E. coli* isolated from poultry (82.3%) was significantly higher ($P < 0.0001$) than those obtained for the case of strains isolated from pork (33.3%), beef (36.6%), or vegetables (26.2%). Additionally, multi-drug resistance was also higher for isolates from beef compared to isolates from vegetables ($P = 0.0259$). These results differ from those obtained by other authors such as Sayah et al. (2005) or Knezevic and Petrovic (2008) who obtained higher multidrug-resistance for *E. coli* isolated from pigs than for *E. coli* isolated from poultry and cattle. Multi-resistant strains could have been originated as the result of co-selection of resistance determinants, because exposing a bacterial population to one antimicrobial agent may result in resistance to other agents without any additional exposure (Sayah et al., 2005). Thus, it was reported that higher levels of multi-resistant strains in any group may also reflect more

Table 2. Multi-resistance patterns in *Escherichia coli* isolated from poultry, pork, beef, and vegetables in Hidalgo State (Mexico).

Tabla 2. Patrones de multiresistencia en *Escherichia coli* aislados a partir de carne de pollo, cerdo, vacuno, y vegetales en el Estado de Hidalgo (Mexico).

Number of resistant antimicrobials	Poultry ($n = 119$) No. (%)	Pork ($n = 78$) No. (%)	Beef ($n = 71$) No. (%)	Vegetables ($n = 84$) No. (%)
0	4 (3.4)	16 (20.5)	15 (21.1)	21 (25)
1	11 (9.2)	17 (21.8)	9 (12.7)	22 (26.2)
2	6 (5)	19 (24.4)	11 (15.5)	19 (22.6)
3	15 (12.6)	12 (15.4)	18 (25.4)	15 (17.9)
4	13 (10.9)	5 (6.4)	12 (16.9)	5 (6)
≥ 5	70 (58.8)	9 (11.5)	6 (8.5)	2 (2.4)
Multi-resistant strains (%)	98 (82.3)[a]	26 (33.3)[b,c]	26 (36.6)[b]	22 (26.2)[c]

[a,b,c]Values in the same total result row with different letters are significantly different by means of χ^2 test.

[a,b,c]Los valores en la misma fila con letras diferentes presentan diferencias estadísticamente significativas mediante el test χ^2.

recent antibiotic usage (Van den Bogaard et al., 2001). Because no information was available about data on the use of antimicrobial agents involved in the production of the foods tested in this study, no assessment was conducted foe the relation between administration of the antimicrobial agents to animals and the multi-resistance rates of *E. coli* isolated from the foods derived from these animals.

In conclusion, as previously reported in developed countries, the presence of antimicrobial resistance was much higher in isolates from poultry meat than from other foods tested and, the resistance rates against older drugs were higher than against the newer antimicrobials. Unfortunately, we could not correlate these antimicrobial resistance rates to antibiotic use because of the lack of official data on the use of antibiotics in México. In some cases, products taken from grocery shops had higher levels of *E. coli* contamination and antimicrobial resistance than products taken from supermarkets, indicating that sample source had an important effect on the microbiological safety of foods. Therefore, it is important to conduct surveillance studies annually repeated, and conduct them in other Mexican states to study temporal and allocation trends to be able to determine whether the generally lower levels of resistance to newer antibiotics can be maintained.

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